Membrane Location of Apocytochrome *c* and Cytochrome *c* Determined from Lipid-Protein Spin Exchange Interactions by Continuous Wave Saturation Electron Spin Resonance

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ABSTRACT Apocytochrome *c* derived from horse heart cytochrome *c* was spin-labeled on the cysteine residue at position 14 or 17 in the N-terminal region of the primary sequence, and cytochrome *c* from yeast was spin-labeled on the single cysteine residue at sequence position 102 in the C-terminal region. The spin-labeled apocytochrome *c* and cytochrome *c* were bound to fluid bilayers composed of different negatively charged phospholipids that also contained phospholipid probes that were spin-labeled either in the headgroup or at different positions in the *sn*-2 acyl chain. The location of the spin-labeled cysteine residues on the lipid-bound proteins was determined relative to the spin-label positions in the different spin-labeled phospholipids by the influence of spin-spin interactions on the microwave saturation properties of the spin-label electron spin resonance spectra. The enhanced spin relaxation observed in the doubly labeled systems arises from Heisenberg spin exchange, which is determined by the accessibility of the spin-label group on the protein to that on the lipid. It is found that the labeled cysteine groups in horse heart apocytochrome *c* are located closest to the 14-C atom of the lipid acyl chain when the protein is bound to dimyristoyl- or dioleoyl-phosphatidylglycerol, and to that of the 5-C atom when the protein is bound to a dimyristoylphosphatidylglycerol/dimyristoylphosphatidylcholine (15:85 mol/mol) mixture. On binding to dioleoylphosphatidylglycerol, the labeled cysteine residue in yeast cytochrome *c* is located closest to the phospholipid headgroups but possibly between the polar group region and the 5-C atom of the acyl chains. These data determine the extent to which the different regions of the proteins are able to penetrate negatively charged phospholipid bilayers.

INTRODUCTION

Apocytochrome *c* is the heme-free precursor of the mitochondrial redox carrier protein cytochrome *c*. The apoprotein is synthesized on cytoplasmic ribosomes and is then imported into the mitochondrion where the heme group is attached by the action of the enzyme heme lyase, which is located in the space between the outer and inner membranes (for a review see Stuart and Neupert, 1990). The precursor protein does not bear a presequence, nor has a receptor protein been found for apocytochrome *c*, which could mediate its passage across the outer membrane. As a result, considerable interest has focused on the role of the lipids of the outer mitochondrial membrane in the import process.

Apocytochrome *c* binds strongly to bilayer membranes composed of negatively charged lipids (Rietveld et al., 1983), and the latter therefore constitute an extremely useful model system for investigation of the translocation process. Experiments on proteolytic cleavage by trypsin trapped within anionic lipid vesicles have demonstrated that the apoprotein but not the holoprotein is at least partially translocated across the lipid bilayer (Rietveld et al., 1986; Jordi et al., 1989). Additionally, electron spin resonance (ESR) studies with spin-labeled lipids have revealed a direct interaction of the bound apoprotein with the lipid chains, indicating a partial penetration of the protein beyond the surface of the bilayer (Görrissen et al., 1986). Furthermore, a combination of fluorescence, FTIR, and NMR studies suggested a location for apocytochrome *c* that is partially at the surface of the membrane and partially penetrated into the bilayer interior (Berkhout et al., 1987; Vincent and Gallay, 1991; Muga et al., 1991a; De Jongh et al., 1992).

To delineate the mechanism by which apocytochrome *c* penetrates and is translocated across negatively charged lipid bilayers, it is necessary to define the locations of the various segments of the protein in association with the lipid membranes. In the present work, this has been done by measuring the spin-spin interactions between a spin label on the protein and a spin label attached either to the polar headgroup or at positions in the acyl chain of the phospholipid molecules (cf. Fig. 1). For this purpose, horse heart apocytochrome *c* was spin labeled on one of the cysteine residues in the N-terminal region to which the heme group is attached in the holoprotein, or yeast cytochrome *c* was spin labeled at the single free cysteine residue in the C-terminal region. The attachment of

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Abbreviations used in this article: ESR, electron spin resonance; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; CW, continuous wave; DSC, differential scanning calorimetry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; 5-MSL, 3-maleimido-2,2,5,5-tetramethyl-pyrrolidine-*N*-oxyl; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; *n*-PGSL, 1-acyl-2-[*n*-(4, 4-dimethyl-oxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; *n*-PCSL, 1-acyl-2-[*n*-(4, 4-dimethyl-oxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; T-PASL, 4-(1, 2-dipalmitoyl-*sn*-glycero-3-phospho)-2,2,6,6-tetramethyl-piperidine-*N*-oxyl; NEM, *N*-ethylmaleimide.

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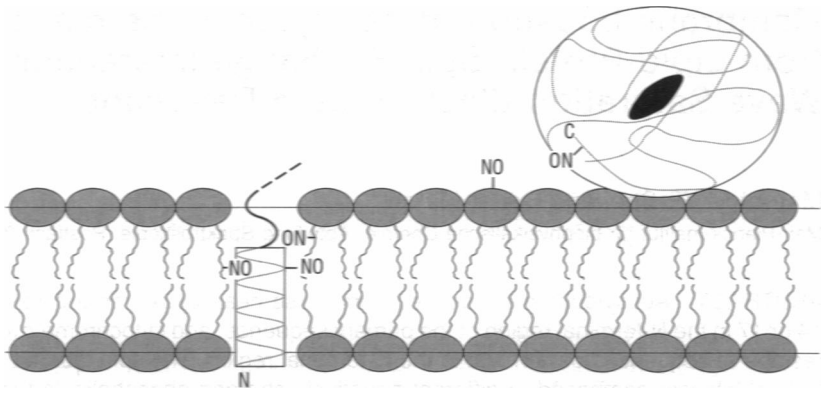

FIGURE 1 Diagrammatic illustration of relative locations of spin-labeled (-NO) lipids and proteins that could give rise to mutual Heisenberg spin exchange between the two labeled species. Left side: membrane insertion of the spin-labeled N-terminal segment of apocytochrome *c*. Right side: membrane surface location of holocytochrome *c* spin labeled at the C-terminal. The highest (collisional) spin exchange frequency is obtained for the spin label group on the lipid that is positioned at the same depth in the membrane as that on the protein.

the spin labels has been shown not to disturb the properties of these proteins (Drott et al., 1970; Zuniga and Nall, 1983). Experiments have been performed with bilayers composed solely of negatively charged lipids to focus on the role of the anionic lipid component, and also with bilayers of zwitterionic lipids that contain 15% of negatively charged lipids to model the lipid composition of the outer mitochondrial membrane (Hovius et al., 1990).

The method used for detecting spin-spin interactions of labels on the protein with those on the lipid was determination of the progressive saturation with increasing microwave power of the integrated intensity of the ESR spectrum from the combined spin-labeled system. Heisenberg exchange between the spin labels is determined by their mutual accessibility and gives rise to an enhanced relaxation of both spin label populations relative to that which would be observed in the absence of spin exchange. Thus an alleviation of saturation in the doubly labeled system relative to that which would be predicted from the saturation behavior of the two singly labeled systems is an indication of accessibility of the label groups on protein and lipid. The necessary theory is presented in terms of the spin exchange frequency between the two labeled species.

METHODS

Proteins

Cytochrome *c* from horse heart (type VI) and from *Saccharomyces cerevisiae* (baker's yeast) were obtained from Sigma Chemical Co. (St. Louis, MO). Apocytochrome *c* was prepared by removal of the heme group from horse heart cytochrome *c* (Fisher et al., 1973) and subsequently subjected to the renaturation procedure described by Hennig and Neupert (1983). The renatured apoprotein was stored in 0.5- and 1.0-mg aliquots at a concentration of ~1.7 mg/ml in 10 mM HEPES, 50 mM NaCl, pH 7.0, 0.01% v/v 2-mercaptoethanol in liquid nitrogen and used only once, immediately after thawing on ice. Spin-labeled apocytochrome *c* and yeast cytochrome *c* were prepared by reacting the cysteine residues of the proteins with 3-maleimido-2,2,5,5-tetramethyl-pyrrolidine-*N*-oxyl (5-MSL, Aldrich, Milwaukee, WI). Typically, a 5-mM ethanolic 5-MSL solution was added to a solution of 1.5 mg/ml apocytochrome *c* in 10 mM ammonium acetate, pH 5.0, at a ratio of 0.7 mol 5-MSL/mol apocytochrome *c* or to a solution of 6 mg/ml yeast cytochrome *c* in 10 mM sodium phosphate, pH 7.0, at a ratio of 1.5 mol 5-MSL/mol cytochrome *c*. After removal of the unreacted 5-MSL by passing apocytochrome *c* over a Sephadex G-25 column and cytochrome *c* over a carboxymethyl cellulose column, the spin-labeled apocytochrome *c* was lyophilized and the spin-labeled yeast cytochrome *c* was dialyzed against 10 mM HEPES, 150 mM NaCl, pH 7.0. Subsequently, spin-labeled apocytochrome *c* was subjected to the renaturation procedure described in the reference above and stored as for unlabeled apocytochrome *c*. These procedures yielded a labeling level of ~0.2 mol 5-MSL/mol apocytochrome *c* with the spin label attached to the cysteine residue either at position 14 or 17 and of ~0.15 mol 5-MSL/mol yeast cytochrome *c* with the spin label attached to the cysteine residue at position 102. Because of indications of a possible partial reduction of the headgroup spin label (T-PASL) by the free sulfhydryl groups of the cysteine residues at positions 14 and 17 in apocytochrome *c*, these were alkylated with *N*-ethylmaleimide (NEM), essentially according to the method described above. Instead of the spin label solution a 2.3-fold molar excess of 0.1 M NEM solution in ethanol was added to the protein solution.

Phospholipids

Spin-labeled phosphatidylcholines (*n*-PCSL) and from these the corresponding phosphatidylglycerols (5- and 14-PGSL), spin labeled at either position 5 or 14 in the *sn*-2 chain, were synthesized by B. Angerstein, essentially as described in Marsh and Watts (1982). Phosphatidic acid spin labeled at the phosphate of the lipid headgroup (T-PASL) was prepared by H. Eibl and A. Watts at this institute, essentially according to the method of Eibl (1978). DOPG was obtained from Avanti (Birmingham, AL). DMPG was synthesized from DMPC (Fluka, Buchs, Switzerland) by a headgroup exchange reaction catalyzed by phospholipase D according to the method of Comfurius and Zwaal (1977).

Sample preparation

Samples were prepared in a buffer consisting of 10 mM HEPES, 50 mM NaCl, pH 7.0. To obtain samples in which (spin-labeled) yeast cytochrome *c* is bound at lipid to protein ratios that are comparable to those of the other lipid-protein samples, a buffer containing 10 mM HEPES, 150 mM NaCl, pH 7.0 was used. All experiments were performed in the absence of oxygen; for this reason the buffers were saturated with argon, and care was taken to keep the samples in an argonated environment by flushing the tubes and the ESR capillaries with argon (Snel and Marsh, 1993).

For preparation of doubly labeled samples of spin-labeled proteins bound to spin-labeled lipid dispersions and of the corresponding singly labeled samples, dry lipid films, consisting of either 1 mg DMPG/DMPC or 0.5 mg DOPG with 1 mol % spin-labeled phospholipid, were hydrated with the appropriate buffer. Additionally, the DOPG films contained 0.1 mol % butylated hydroxytoluene to prevent lipid peroxidation. The lipid dispersions were frozen and thawed five times, after which an argonated protein solution was added containing an amount of protein equal in mass to lipid. The samples were incubated for 30 to 40 min at 37°C, and the resulting lipid-protein complexes were collected by centrifugation (1000–1400*g*, 10 min), transferred to ESR capillaries, and concentrated further by centrifugation. The concentrations of the protein solutions were 1.7 mg/ml renatured apocytochrome *c* (unlabeled, NEM-labeled, or spin-labeled), 5.7 mg/ml spin-labeled yeast cytochrome *c*, and 5 mg/ml cytochrome *c*. For preparation of

samples with horse heart cytochrome *c*, 1 mg of the protein was added to a lipid dispersion that contained 0.5 mg DOPG.

After the ESR measurements, the lipid-protein complexes were dissolved in 25 μl 1-M NaOH for determining the lipid and protein contents. The phospholipid concentration was determined by the method of Eibl and Lands (1969). Protein assays were performed according to the modified method of Lowry (Peterson, 1977) with bovine serum albumin as standard. The mean lipid/protein mole ratios of the various lipid-protein complexes were DMPG/apocytochrome *c* ~13, DOPG/apocytochrome *c* ~11, DMPC+DMPG/apocytochrome *c* ~60, and DOPG/cytochrome *c* ~14. For the complexes with negatively charged lipids alone, the amount of protein added was considerably less than that to produce saturation binding; therefore close to 100% of the protein would be bound. For the complexes containing DMPC, the amount of protein added was more than that to produce saturation binding. However, in all cases the complexes were resuspended in buffer and recentrifuged, and excess supernatant was removed. Therefore, the amount of any free protein was likely to be small.

ESR spectroscopy

CW power-saturation ESR experiments were performed on a Varian (Palo Alto, CA) E-12 Century Line 9 GHz ESR spectrometer. A standardized sample configuration was used in all CW saturation experiments (Fajer and Marsh, 1982; Hemminga et al., 1984), and all measurements were performed under critical coupling conditions. Before the ESR capillaries were sealed, excess supernatant and sample were removed from the pellets in the capillaries to obtain samples ≤5 mm in length to avoid inhomogeneities of the microwave and modulation fields in the ESR cavity. The microwave field was calibrated by measuring the CW saturation properties of deoxygenated aqueous peroxylamine disulphonate, as described by Kooser et al. (1969). The microwave cavity Q was measured as described in Fajer and Marsh (1982) and corresponding corrections made in calculating the root mean square microwave field, $\langle H_1^2 \rangle^{1/2}$ at the sample. Conventional in-phase absorption spectra were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.25 Gauss p-p at various decreasing microwave powers corresponding to values of $\langle H_1^2 \rangle^{1/2}$ ranging from ~600 to 6 mGauss. The total static field scan width was 100 Gauss. The temperature was regulated with a pure nitrogen gas flow system and sample capillaries were centered in a standard 4-mm quartz tube containing light silicone oil for thermal stability. Spectral subtractions were performed as described by Marsh (1982) and analysis of the CW saturation experiments as described below.

THEORY

Analysis of CW saturation experiments

Assuming Lorentzian spin packets, the saturation of the double-integrated intensity, S_{ESR}, of the first derivative ESR spectrum from a single spin label species, i, is given by the following equation (see, e.g., Portis, 1953):

$$S_{ESR} = S_{ESR}^{\circ}/(1 + \sigma_i^{eff})^{1/2} \tag{1}$$

where S_{ESR}° is the value that S_{ESR} would have in the absence of saturation, and $\sigma_i^{eff} = \gamma^2 H_1^2 T_{1,i} T_{2,i}$ is the effective saturation parameter of species i, with $T_{1,i}$ and $T_{2,i}$ being the effective spin-lattice and spin-spin relaxation times, respectively. This equation is valid independent of the degree of inhomogeneous broadening in the (powder) ESR spectrum (Fajer et al., 1992; Páli et al., 1993).

Because the spectral intensities are additive, the saturation of the double-integrated intensity of the ESR spectrum from a system containing two spin label species, P and L, is given correspondingly by

$$S_{ESR} = S_{ESR}^{\circ}\left[\frac{f}{(1 + \sigma_P^{eff})^{1/2}} + \frac{1 - f}{(1 + \sigma_L^{eff})^{1/2}}\right] \tag{2}$$

where f is the fraction of the total integrated intensity contributed by spin label species P in the absence of saturation. Fitting the saturation curve of the two-spin label system by a single-component model corresponds to taking $S_{ESR} = S_{ESR}^{\circ}/(1+\sigma_{PL}^{eff})^{1/2}$, where σ_{PL}^{eff} is the effective saturation parameter for the combined system (cf. Eq. 1). With this assumption, Eq. 2 may be rewritten as

$$\frac{S_{ESR}}{S_{ESR}^{\circ}} = \frac{f}{[1 + \sigma_{PL}^{eff}(T_1T_2)_P^{eff}/(T_1T_2)_{PL}^{eff}]^{1/2}} + \frac{1 - f}{[1 + \sigma_{PL}^{eff}(T_1T_2)_L^{eff}/(T_1T_2)_{PL}^{eff}]^{1/2}}. \tag{3}$$

Clearly this latter equation is an approximation that does not describe the entire saturation curve exactly but may be used to relate the saturation properties of the combined system to those of the single systems at a particular microwave H_1 field, e.g., at the half-saturation point where $S_{ESR}/S_{ESR}^{\circ} = ½$ and therefore $\sigma_{PL}^{eff} = 3$. Such a procedure is necessary when the saturation parameters of the two species are not sufficiently well separated to be able to distinguish the two components in the saturation curves (cf. Páli et al., 1993).

If there is no Heisenberg spin exchange between the two different spin label species in the combined system, then Eq. 3 can be used to predict the $(T_1T_2)_{PL}^{eff}$ product from the values, $(T_1T_2)_P^{\circ}$ and $(T_1T_2)_L^{\circ}$, obtained from the corresponding single spin-labeled systems by use of Eq. 1. If Heisenberg spin exchange does take place between the two spin label species, then the values of $(T_1T_2)_P^{eff}$ and $(T_1T_2)_L^{eff}$ appearing in Eq. 3 for the combined system will differ from those corresponding to no spin exchange that are determined in the singly labeled systems. This difference is related to the spin exchange frequency in the following section.

CW saturation of two spin label species undergoing mutual Heisenberg spin exchange

The effects on the CW ESR saturation properties of Heisenberg spin exchange between the two spin label species, P and L, can be analyzed by using the rate equations for the population differences, n_P and n_L, between the $M_S = \pm ½$ levels of the P and L spin labels, respectively. If transitions are induced between the levels of spin label P by the H_1 microwave field, i.e., it is the saturation of spin label P that is considered, the steady state condition for the population difference is (cf. Marsh, 1992)

$$\frac{dn_P}{dt} = -2Wn_P + \frac{n_P^{\circ} - n_P}{T_{1,P}^{\circ}} - [(1 - f)n_P - fn_L]\tau_{ex}^{-1} = 0 \tag{4}$$

where W is the rate at which transitions are induced by the H_1 field, n_P° is the Boltzmann equilibrium value of n_P, and

$T_{1,P}^{\circ}$ is the spin-lattice relaxation time of spin label P in the absence of Heisenberg exchange (the value of which is taken also to approximate the effects of spin diffusion processes within the single species, P). Here τ_{ex}^{-1} is the Heisenberg spin exchange frequency which is linearly related, by the bimolecular rate constant for spin exchange, to the total spin label concentration (Marsh, 1992). The corresponding steady-state condition for the spin labels L that are not irradiated by the H_1 field is

$$\frac{dn_L}{dt} = \frac{n_L^{\circ} - n_L}{T_{1,L}^{\circ}} - [fn_L - (1-f)n_P]\tau_{ex}^{-1} = 0 \qquad (5)$$

where n_L° is the Boltzmann equilibrium value of n_L, and $T_{1,L}^{\circ}$ is the spin-lattice relaxation time of spin label L in the absence of spin exchange. Solution of Eqs. 4 and 5, together with the equilibrium condition that $n_L^{\circ}/n_P^{\circ} = (1-f)/f$, yields the standard expression (cf. Slichter, 1980) for the saturation of the spin label species P

$$n_P = n_P^{\circ}/(1 + 2WT_{1,P}^{eff}) \qquad (6)$$

where the effective spin-lattice relaxation rate, $1/T_{1,P}^{eff}$, of spin label P in the presence of Heisenberg exchange with the spin label L is given by

$$\frac{T_{1,P}^{\circ}}{T_{1,P}^{eff}} = 1 + \frac{(1-f)T_{1,P}^{\circ}\tau_{ex}^{-1}}{1 + fT_{1,L}^{\circ}\tau_{ex}^{-1}}. \qquad (7)$$

Similarly, it is found that when the spin labels L are irradiated by the H_1 field their saturation is characterized by the effective spin-lattice relaxation rate, $1/T_{1,L}^{eff}$, that is given by

$$\frac{T_{1,L}^{\circ}}{T_{1,L}^{eff}} = 1 + \frac{fT_{1,L}^{\circ}\tau_{ex}^{-1}}{1 + (1-f)T_{1,P}^{\circ}\tau_{ex}^{-1}}. \qquad (8)$$

The right side of these latter two equations can be expressed in terms of a single normalized exchange frequency, $T_{1,P}^{\circ}\tau_{ex}^{-1}$, together with the ratio, $T_{1,P}^{\circ}/T_{1,L}^{\circ}$, of the spin-lattice relaxation times of the two species in the absence of spin exchange.

The changes in $T_{1,i}^{eff}$ caused by spin exchange are much greater than the corresponding changes in $T_{2,i}^{eff}$, because the former is far more sensitive to weak Heisenberg spin exchange (cf. Marsh and Horváth, 1992). Therefore, $T_{2,i}^{eff}$ can be considered to remain constant when calculating the effect of the mutual spin exchange on the $(T_{1,i}T_{2,i})^{eff}$ products of the two spin labels in the combined system. Eq. 3 for the saturation of the combined system in the presence of mutual spin exchange can then be written in the following form:

$$\frac{S_{ESR}}{S_{ESR}^{\circ}} = \frac{f}{[1 + \sigma_{PL}^{eff}(T_1T_2)_P^{\circ}/(T_1T_2)_{PL}^{eff}(T_{1,P}^{eff}/T_{1,P}^{\circ})]^{1/2}} + \frac{1-f}{[1 + \sigma_{PL}^{eff}(T_1T_2)_L^{\circ}/(T_1T_2)_{PL}^{eff}(T_{1,L}^{eff}/T_{1,L}^{\circ})]^{1/2}} \qquad (9)$$

where substitution can be made from Eqs. 7 and 8 to give the explicit dependence on the normalized spin exchange frequency, $T_{1,P}^{\circ}\tau_{ex}^{-1}$.

The above formulation attempts to approximate the effects of spin-diffusion processes such as nuclear relaxation and rotational motion that take place within a single species (P or L) by an effective T_1°-relaxation time. The error involved in this approximation will depend to some extent on the nuclear relaxation and rotational diffusion rates and on the degree of spectral anisotropy and the restricted selection rules for these processes relative to the unrestricted spin exchange (cf. Marsh, 1992). That such an approximation is acceptable is demonstrated by similar determinations of the rates for the analogous two-site physical exchange, in which both the overall exchange rates and their relative values were in reasonable agreement with those obtained by other means (Horváth et al., 1993). This latter work also shows that undue complications do not arise from the simultaneous saturation of the two species in the CW experiment, presumably because only a very restricted population relative to the total is irradiated at any one time.

RESULTS AND DISCUSSION

The location of the N-terminal section of apocytochrome *c* bound to lipid membranes has been determined from the accessibility of the spin label on the protein to spin labels at different positions on the phospholipid molecule. For comparison and further calibration of the location, similar experiments were performed with the holoprotein, which is expected to have a surface location. In order to concentrate directly on the role of negatively charged lipids, experiments were performed with bilayers composed of these lipids alone. Then experiments were performed on lipid mixtures with a negatively charged lipid content resembling that of the outer mitochondrial membrane to check whether the effects of negatively charged lipids alone could be detected in a physiologically relevant model. In this section, the CW saturation experiments used to detect mutual spin-spin interactions will be described first, followed by use of the spin-exchange frequencies to determine mutual accessibilities; finally, the implications of the results will be discussed.

Spectral features and analysis of CW saturation experiments

The ESR spectra of the 14-PGSL spin label incorporated in DMPG bilayers in the presence of unlabeled or spin-labeled apocytochrome *c* and of the spin-labeled apoprotein bound to DMPG are given in Fig. 2. The ESR spectrum of the doubly labeled system (Fig. 2 *c*) is composed of a superposition of the individual component spectra from the spin-labeled phospholipid and the spin-labeled protein in the samples. Either overlaying the spectra of the individual components (Figs. 2 *a,b*) on those of the doubly labeled sample (Fig. 2 *c*), or spectral subtraction, show that the line shapes and line widths of the individual components are not appreciably different in the doubly labeled sample from those in the singly labeled samples. This indicates that any effects of spin exchange between the two components on the T_2 relaxation (i.e., line widths) in the doubly labeled sample are

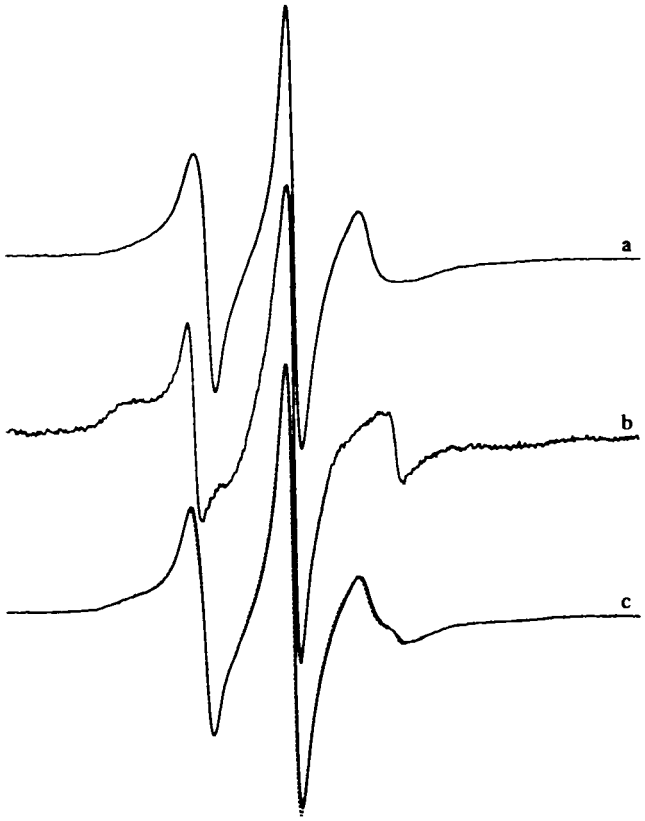

FIGURE 2 ESR spectra of singly and doubly spin-labeled complexes of apocytochrome *c* bound to DMPG bilayers containing 1 mol % 14-PGSL (*a*, *c*) recorded at 30°C, with a microwave field of ~58 mGauss. ESR spectra from a sample (*a*) with spin-labeled phospholipid and unlabeled apocytochrome *c* at a lipid/protein ratio (L/P) of 12 mol/mol, (*b*) with spin-labeled apocytochrome *c* and unlabeled lipid at an *L/P* of 13.5 mol/mol, and (*c*) with both spin-labeled phospholipid and spin-labeled apocytochrome *c* at an *L/P* of 13 mol/mol. The dotted line in the spectrum (*c*) is obtained by addition of the spectra of the singly labeled components, weighted according to the values of *f* given in Table 1. Total scan width is 100 Gauss.

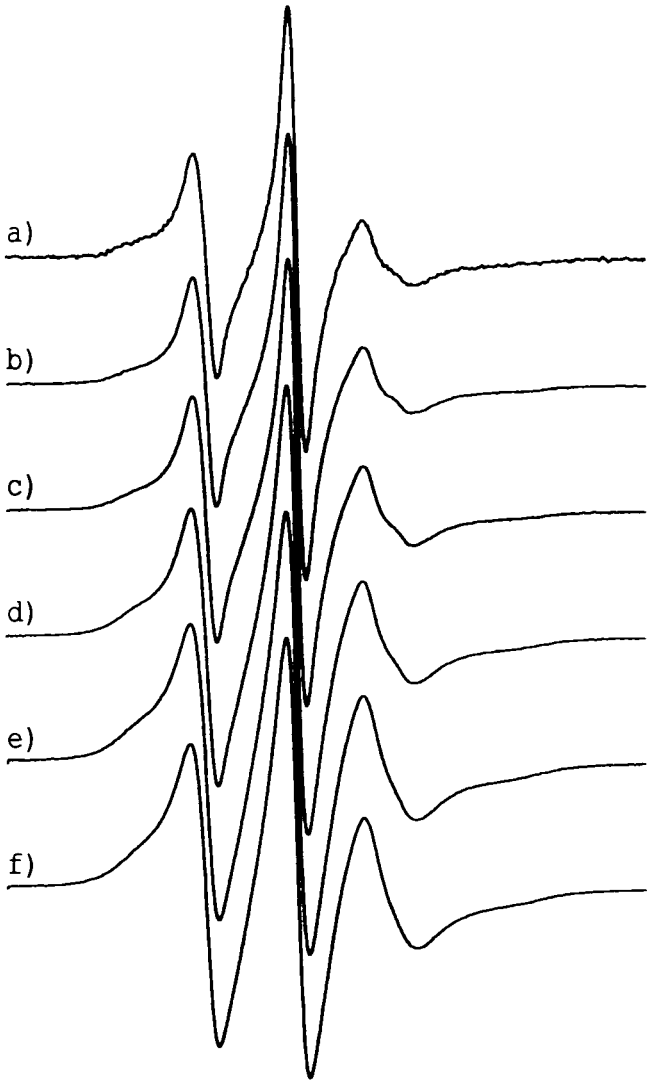

FIGURE 3 ESR spectra of spin-labeled apocytochrome *c* bound to deoxygenated DMPG bilayers that contain 1 mol % 14-PGSL spin label recorded with different root mean square microwave magnetic fields, $\langle H_1^2 \rangle^{1/2}$, at the sample. The microwave power at which the spectra are recorded, corresponds to values of $\langle H_1^2 \rangle^{1/2}$ of: (*a*) 19, (*b*) 58, (*c*) 176, (*d*) 306, (*e*) 427, and (*f*) 532 mGauss. Temperature of the sample is 30°C. Total scan width is 100 Gauss.

small relative to the line widths of the isolated components. Therefore, the CW saturation behavior of the spectra with increasing microwave power was studied, given that the effects of weak spin exchange on the spin-lattice relaxation time (T_1) are much greater than those on the spin-spin relaxation time (T_2) (cf. Marsh and Horváth, 1992).

The dependence on microwave power of the ESR spectrum of the doubly labeled deoxygenated sample consisting of DMPG bilayer membranes with the 14-PGSL spin label incorporated and spin-labeled apocytochrome *c* bound is given in Fig. 3. The spectra are normalized with respect to the central line height, and the effects of saturation on the line shape are seen as a progressive broadening with increasing microwave power. However, this saturation broadening is difficult to analyze in detail because of both the inhomogeneous broadening of the individual components (cf. Castner, 1959) and the spectral overlap. The saturation behavior of two-component inhomogeneously broadened systems is more readily analyzed in terms of the microwave power dependence of the total integrated spectral intensity (cf. Theory section, above, and Páli et al., 1993). The saturation curve for the double-integrated intensity of the spectra given in Fig. 3 is shown for increasing H_1 microwave field in Fig. 4 together with the corresponding saturation curves for the singly labeled samples. The solid lines represent nonlinear least-squares fits that are obtained by using a single-component model for the saturation of the double-integrated intensity of the ESR spectra, according to Eq. 1. The fits for the singly labeled samples are somewhat better than those for the doubly labeled sample, and result in values of $(T_1T_2)_P^\circ = 2.5 \times 10^{-14}$ s^2 for the spin-labeled apocytochrome *c* and $(T_1T_2)_L^\circ = 1.4 \times 10^{-14}$ s^2 for the 14-PGSL spin label in the presence of unlabeled apocytochrome *c*. According to Páli et al. (1993), the T_1T_2 products for these singly labeled samples are not

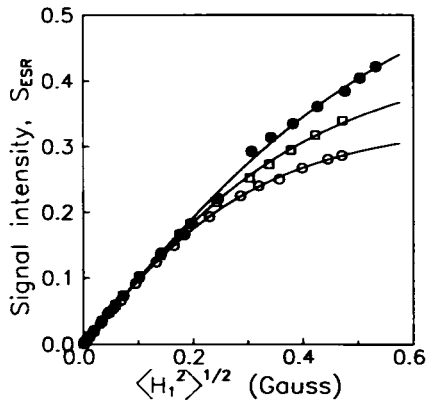

FIGURE 4 Dependence of the double-integrated intensity, S_{ESR}, of the ESR spectrum recorded at 30°C from singly and doubly spin-labeled deoxygenated complexes of apocytochrome *c* bound to DMPG bilayers on the root mean square microwave magnetic field, $\langle H_1^2 \rangle^{1/2}$, at the sample. (○) Spin-labeled apocytochrome *c* bound to DMPG bilayers. (□) Unlabeled apocytochrome *c* bound to DMPG bilayers that contain 1 mol % 14-PGSL. (●) Spin-labeled apocytochrome *c* bound to DMPG bilayers that contain 1 mol % 14-PGSL. The signal intensities are normalized to the total number of spin labels in each sample, such that $S_{ESR}^{\circ}/\langle H_1^2 \rangle^{1/2} = 1$ in Eq. 1. The solid lines show the results of the nonlinear least-squares fits of the saturation curves to Eq. 1, yielding $T_1T_2 = 2.5 \times 10^{-14}$ s^2 for singly labeled apocytochrome *c*, $T_1T_2 = 1.4 \times 10^{-14}$ s^2 for singly labeled 14-PGSL in the presence of unlabeled apocytochrome *c* and $T_1T_2 = 6.9 \times 10^{-15}$ s^2 for doubly labeled apocytochrome *c* and 14-PGSL.

TABLE 1 Effective relaxation rates ($1/T_1T_2$) for spin-labeled lipids (*L*) plus spin-labeled protein, $(1/T_1T_2)_{PL}$, spin-labeled lipid plus unlabeled protein, $(1/T_1T_2)_L^{\circ}$, and spin-labeled protein plus unlabeled lipid, $(1/T_1T_2)_P^{\circ}$, obtained from CW saturation ESR of horse heart apocytochrome *c* (apo *c*) and yeast cytochrome *c* (cyt *c*) bound to negatively charged lipid bilayers [DMPG, DOPG and DMPG/DMPC (15:85 mol/mol)]. Values of the fractional population, *f*, of the spin-labeled protein are also given. T = 30°C.

L	*f*	$(1/T_1T_2)_{PL}$ ($10^{13} \times s^{-2}$)	$(1/T_1T_2)_L^{\circ}$ ($10^{13} \times s^{-2}$)	$(1/T_1T_2)_P^{\circ}$ ($10^{13} \times s^{-2}$)
DMPG-apo *c*				
14-PGSL	0.31	14.5	7.1	4.0
5-PGSL	0.36	5.6	6.1	4.0
T-PASL	0.29	6.3	8.3	4.0
DMPG/DMPC (15:85)-apo *c*				
14-PGSL	0.11	7.8	7.1	5.1
5-PGSL	0.06	6.7	4.4	5.1
T-PASL	0.12	5.9	5.3	5.1
DOPG-apo *c*				
14-PGSL	0.41	11.3	6.65	5.0
5-PCSL	0.45	5.8	7.6	5.0
T-PASL	0.70	12.6	10.9 (9.0)*	5.0
DOPG-cyt *c*				
14-PGSL	0.72	5.7	4.8 (3.4)‡	7.2
5-PGSL	0.65	7.3	6.9 (3.1)‡	7.2
T-PASL	0.65	8.0	5.1 (3.7)‡	7.2

* Apocytochrome *c* alkylated with NEM.
‡ Horse heart cytochrome *c*.

sufficiently different to be distinguished in a fit to a two-component model for the saturation curve of the doubly labeled sample, which gives the rationale for using the one-component model that reasonably approximates the saturation curve and results in an effective value of $(T_1T_2)_{PL}^{eff} = 6.9 \times 10^{-15}$ s^2. Both singly labeled samples saturate more readily than does the doubly labeled sample as is seen clearly both from the relative values of the T_1T_2 products and from the saturation curves given in Fig. 4. This enhanced relaxation observed for the doubly labeled samples must arise from spin-spin interactions between the two labeled components, as outlined in the Theory section, and demonstrates that the mutual accessibility of the two spin-labeled components can be probed by studying their CW saturation behavior.

Spin exchange frequencies and location of spin-labeled proteins with respect to spin-labeled phospholipids

The values of the reciprocal T_1T_2 products determined from fitting the saturation curves of the different singly and doubly spin-labeled systems (i.e., spin-labeled lipid, L, spin-labeled protein, P, and spin-labeled protein plus spin-labeled lipid, PL) to Eq. 1, as is illustrated in Fig. 4, are given in Table 1. In certain cases, for samples where only the lipid was labeled, there was evidence that the lipid spin label might have been partially reduced by the free sulfhydryl groups on the protein. For this reason, some values of $(1/T_1T_2)_L^{\circ}$ for samples with apocytochrome *c* alkylated by *N*-ethylmaleimide (NEM) and for horse heart cytochrome *c*, which contains no sulfhydryl groups, are given in parentheses in Table 1. It is seen from the table that the effective relaxation rates obtained from systems in which both components are labeled differ from those in which only one of the two components is labeled, as must be expected whenever the relaxation rates of the singly labeled components differ. In certain cases, e.g., with the 14-PGSL label, and spin-labeled apocytochrome *c* bound to DMPG (the data for which was presented above), the relaxation rate is faster than that of either of the two similar systems in which only one component is labeled. This gives clear evidence for relaxation enhancement by spin-spin interaction between the two components. In general, however, this is not always the case, and the presence or absence of an exchange-induced relaxation enhancement can only be obtained by quantitative analysis using the methods outlined in the Theory section. For this it is necessary to know the fraction, *f*, of one spin-labeled species (in this case the spin-labeled protein) relative to the total spin label population, which has been obtained by subtraction (see Marsh, 1982) of the spectra of the single spin-labeled samples from those of the corresponding doubly labeled samples. These values of *f* are also given in Table 1.

The values for the relaxation rates in the doubly labeled samples assuming no spin exchange between the two species, $(1/T_1T_2)_{PL}^{\circ}$, have been predicted from the values of *f* and the relaxation rates, $(1/T_1T_2)_L^{\circ}$ and $(1/T_1T_2)_P^{\circ}$, of the corresponding singly labeled species by using Eq. 3. For this purpose, the microwave power was taken to be that corresponding to half-saturation of the doubly labeled system (cf. Theory section). These calculated values in the absence of exchange are

compared with the experimental relaxation rates, $(1/T_1T_2)_{PL}$, in Table 2, for all the different doubly labeled systems. Whenever the experimentally determined value is greater than the rate predicted without exchange, there is positive evidence for an enhanced relaxation induced by Heisenberg spin exchange and therefore of accessibility of the spin label on the lipid to that on the protein. In some cases, the experimental rate is somewhat slower than that predicted with no exchange, which is physically unrealistic and must correspond to small differences in the lipid/protein ratios and overall spin label concentration between the double and single spin-labeled samples, and other experimental and theoretical (cf. above) uncertainties. It will be noted that in some of these cases a better agreement is obtained with the data given in parentheses in Table 2 than with that from the samples containing free sulfhydryl groups. Table 2 shows that for the doubly labeled samples a clear distinction exists between the relaxation enhancements corresponding to the different positions of labeling of the lipid spin labels, and also between the resulting positional profiles of the relaxation enhancements for the different lipid-protein systems, when comparison is made of the experimental rates with those predicted without spin exchange contributions. However, a quantitative comparison between the different positions of lipid labeling is best made by estimation of the resulting exchange frequencies.

The normalized exchange frequencies, $T_{1,P}{}^{\circ}\tau_{ex}{}^{-1}$, calculated according to Eqs. 7–9 for the different systems are given also in Table 2. For this purpose, the microwave power corresponding to half-saturation was taken, and the value required for $T_{1,L}{}^{\circ}/T_{1,P}{}^{\circ}$ was approximated by the ratio $(T_1T_2)_L{}^{\circ}W_L{}^{\circ}/(T_1T_2)_P{}^{\circ}W_P{}^{\circ}$ obtained from the CW saturation experiments, where $W_L{}^{\circ}$ and $W_P{}^{\circ}$ are the central line widths for the spin-labeled lipid and the spin-labeled protein, respectively, in the spectra from the singly labeled samples. The latter is not exact but serves as a reasonable approximation for estimating the exchange rates, especially when the line widths are comparable. In Table 2, the values for the normalized exchange rates that are designated by 0 correspond to situations where the calculated value was even slightly negative. These nonphysical calculated values arise because of the experimental uncertainties mentioned above. Similarly, the values that are designated by ≥5 in Table 2 correspond to situations in which no consistent solution to Eqs. 7–9 was obtained, even at very high exchange rates. Nevertheless, it is clear that in these cases the exchange frequency is much greater than the values for the other systems.

TABLE 2 Experimental relaxation rates $(1/T_1T_2)_{PL}$ for spin-labeled lipid (*L*) plus spin-labeled protein in complexes of horse heart apocytochrome *c* (apo *c*) and yeast cytochrome *c* (cyt *c*) bound to negatively charged lipid bilayers [DMPG, DOPG, and DMPG/DMPC (15:85 mol:mol)], together with values $(1/T_1T_2)_{PL}{}^{\circ}$, predicted according to Eq. 3 assuming no exchange between spin labels, and estimates of the normalized exchange frequency, $T_{1,P}{}^{\circ}\tau_{ex}{}^{-1}$, from Eqs. 7–9 (see text). T = 30°C.

L	$(1/T_1T_2)_{PL}$ $(10^{13} \times s^{-2})$	$(1/T_1T_2)_{PL}{}^{\circ}$ $(10^{13} \times s^{-2})$	$T_{1,P}{}^{\circ}\tau_{ex}{}^{-1}$
DMPG-apo *c*			
14-PGSL	14.5	6.0	≥5
5-PGSL	5.6	5.3	0.2
T-PASL	6.3	6.8	0
DMPG/DMPC (15:85)-apo *c*			
14-PGSL	7.8	6.8	1.6
5-PGSL	6.7	4.4	≥5
T-PASL	5.9	5.3	0.9
DOPG-apo *c*			
14-PGSL	11.3	5.9	23
5-PCSL	5.8	6.3	0
T-PASL	12.6	6.4 (7.0)*	≥5
DOPG-cyt *c*			
14-PGSL	5.7	6.4 (5.9)‡	0
5-PGSL	7.3	7.1 (5.4)‡	0.1 (1.0)
T-PASL	8.0	6.4 (5.7)‡	0.8 (1.4)

* Apocytochrome *c* alkylated with NEM.

‡ Horse heart cytochrome *c*.

A clear pattern emerges from the estimated exchange rates and the comparison of the experimental data with the predictions in the absence of exchange (see Table 2). The labeled cysteine groups in apocytochrome *c* are located closest to the label group of 14-PGSL when the protein is bound to DMPG, to that of 5-PGSL when the protein is bound to DMPG/DMPC (15:85 mol/mol), and to that of 14-PGSL and T-PASL when the protein is bound to DOPG. On binding to DOPG, the labeled cysteine residue in yeast cytochrome *c* is located closest to the label group of T-PASL, but possibly between that of T-PASL and 5-PGSL. Interestingly, the largest exchange rates with a particular protein are greater for spin-labeled apocytochrome *c* than for spin-labeled cytochrome *c* consistent with a more intimate association of the apoprotein (or at least its N-terminal segment) with the lipid bilayer.

It is also of interest to compare the estimated exchange frequencies between the spin-labeled protein and the spin-labeled lipid with those measured between spin-labeled lipids alone undergoing translational diffusion in fluid lipid bilayers. The exchange frequency between unlike spin labels is given by the following equation (see, e.g., Sachse et al., 1987):

$$\tau_{ex}{}^{-1} = \tfrac{1}{2}k_{coll} \cdot c \qquad (10)$$

where k_{coll} is the bimolecular collision rate constant and c is the total mole fraction of spin label. For spin-labeled lipids in DMPC at 30°C, $k_{coll} = 4.5 \times 10^8\ s^{-1}$ (Sachse et al., 1987), and the effective spin-label concentration (spin-labeled lipid plus spin-labeled protein) in the present experiments, with a lipid/protein ratio of 12:1 mol/mol, is $c \approx 0.02$. Hence, the corresponding exchange frequency deduced from Eq. 10 would be $\tau_{ex}{}^{-1} \approx 5 \times 10^6\ s^{-1}$; and the value of $T_{1,P}{}^{\circ}$ is likely to be in the region of 1 μs (Thomas et al., 1976). Thus the values estimated for the protein-lipid spin exchange are of a reasonable order of magnitude when compared with the translational diffusion in homogeneous systems where the accessibility factor is unity. It is possible that some values in Table 2 could be greater than the above estimate because of the nonhomogeneous nature of the system containing pro-

tein, which might give rise to higher effective local collision rates with the protein.

Comparison with other studies and conclusions

For cytochrome *c*, the spin label is attached to the cysteine residue at position 102 at the very C-terminal of the protein. Upon binding to the DOPG bilayers, the spin-labeled cytochrome *c* is located in the phospholipid headgroup region, possibly slightly deeper. Also, the ESR spectra of spin-labeled cytochrome *c*/DOPG complexes show a largely immobilized component (data not shown), which indicates an intimate association of the spin label site with the membrane surface. FTIR studies (Muga et al., 1991b; Heimburg and Marsh, 1993) have indicated that, whereas the secondary structure is largely unchanged, the tertiary structure of cytochrome *c* is loosened or destabilized on binding to negatively charged lipid membranes. This loosening of the protein folding is consistent with the C-terminal part of cytochrome *c*, to which the spin label is attached, binding with its spin label site directed toward the DOPG membrane into which it apparently inserts slightly. In addition, NMR studies on cytochrome *c*/cardiolipin complexes (Spooner and Watts, 1991a,b; 1992) have provided evidence that the haem moiety interacts directly with the phospholipid headgroups on binding, in agreement with the location of the spin-labeled holoprotein found here.

The results with the spin-labeled apoprotein demonstrate a distinctly different membrane location from that for the holoprotein with a much greater penetration into the hydrophobic core of the membrane. The spin label on apocytochrome *c* is attached to the cysteine residues at position 14 or 17 in the N-terminus. In phospholipid membranes that are composed of DMPG or DOPG, the spin-labeled apocytochrome *c* is located at the position of the 14-PGSL spin label, i.e., deeply inserted in the membrane. This is in accord with DSC studies (Rietveld et al., 1985) in which it was observed that binding of apocytochrome *c* to DMPG abolished the gel-to-fluid phase transition of the phospholipid, and also with several other studies cited in the Introduction. In spin-labeled apocytochrome *c*/DOPG complexes, spin-spin interactions are observed also with T-PASL, which might suggest a second mode of binding as in DMPG/DMPC complexes (see below), or perhaps even a penetration right through the membrane. However, in the NEM-labeled apocytochrome *c*/DOPG complexes, still some reduction of the lipid headgroup spin label (T-PASL) was observed, which complicates the interpretation. Interestingly, the location of the spin label attached to the C-terminal of the heat-denatured holoprotein that has been proposed as a model for the interaction of the apoprotein with lipids (Demel et al., 1989) is found to be very similar to that found for the holoprotein (data not shown), i.e., quite different from that of the N-terminal of the apoprotein.

In the case where the proportion of the negatively charged lipid component in the lipid mixtures resembles that of the outer mitochondrial membrane, i.e., DMPG/DMPC (15:85 mol/mol), the spin-labeled apoprotein shows spin-spin interactions with the phospholipids spin labeled at each of the three positions in the molecule. This suggests both penetration of the protein and a location in the phospholipid headgroup region, which is in agreement with FTIR results obtained by Muga et al. (1991a). Such a coexistence of two populations has been observed for apocytochrome *c* that is bound to mitochondria (Nicholson et al., 1988). External addition of proteases digested part of the precursor protein, but apocytochrome *c* was able also to penetrate the outer mitochondrial membrane to interact with the heme lyase, which attaches the heme group to the apoprotein and is located in the space between the outer and inner mitochondrial membranes. Thus, the spontaneous insertion of the N-terminal part of apocytochrome *c*, as is observed *directly* in this study, adds strong evidence to the model for import of apocytochrome *c* into mitochondria, in which negatively charged lipids play an important role. In this respect, the studies with bilayers containing solely negatively charged lipids are crucial. For these systems, only one of the two binding modes suggested for the lipid mixtures is found. This is the productive mode of binding involving deep penetration of the N-terminal section of the protein into the membrane, which thus further emphasizes the direct involvement of negatively charged lipids in the translocation process.

Finally, the present measurements illustrate the viability of using ESR saturation studies to determine exchange interactions between spin labels attached to different biomolecules. A straightforward method of analysis that does not require good resolution of the spectral components is available by using the total integrated spectral intensity. This type of experiment therefore should be generally applicable to the determination of mutual accessibilities and locations of different spin-labeled species in a wide variety of systems, of which membranes are likely to be a particularly fruitful example.

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