Pure & Appl. Chem., Vol. 62, No. 2, pp. 265–270, 1990.
Printed in Great Britain.

Studies of membrane dynamics using nitroxide spin labels

Derek Marsh

Max-Planck-Institut für biophysikalische Chemie,
Abteilung Spektroskopie, D-3400 Göttingen, F.R.G.

Abstract - Nitroxide free radicals may be covalently attached either to lipids or to proteins in order to study the mobility of the molecular components of biological membranes using electron spin resonance (ESR) spectroscopy. Simulation of the conventional ESR spectra from spin-labelled dimyristoyl phosphatidylcholine in oriented phospholipid bilayer model membranes allows a complete description of the lipid chain conformation, ordering and dynamics on the nanosecond timescale. Experiments with spin-labelled lipids in natural or reconstituted membranes reveal ESR spectral components from the lipids interacting directly with the intramembranous surface of the integral membrane proteins. This allows quantitation of the lipid stoichiometry and specificity of lipid/protein interaction, and determination of the exchange rates of the lipids at the lipid-protein interface on the submicrosecond timescale. Saturation transfer ESR spectroscopy can be used to study the motions of spin-labelled membrane-bound proteins on the submillisecond timescale. These motions include large-scale segmental mobility and rotational diffusion of integral proteins within the membrane.

INTRODUCTION

Free radicals bearing a nitroxide group flanked by quaternary carbon atoms are stable against disproportionation reactions and may be used as ESR reporter groups in biological systems. The versatile chemistry of nitroxyl radicals allows both the synthesis of derivatives of the commonly occurring biological lipids which may be used as probes in membranes, and the labelling of membrane proteins by covalent modification of the amino acid side chains.

The value of the nitroxide radical as a reporter group lies in the angular anisotropy of its ESR spectrum, combined with the sensitivity of magnetic resonance spectra to dynamic processes on the timescale of the spectral splittings and of the spin-lattice relaxation time. The spin Hamiltonian for the nitroxide free radical electron spin, $\underline{S}$, is given by

$$\mathcal{H} = \beta \underline{H} \cdot \underline{g} \cdot \underline{S} + \underline{I} \cdot \underline{A} \cdot \underline{S} \qquad (1)$$

where ß is the Bohr magneton, $\underline{I}$ is the ^{14}N nuclear spin, and $\underline{H}$ is the laboratory magnetic field vector. The angular anisotropy is expressed by the g-tensor, $(g_{xx},g_{yy},g_{zz}) = (2.0088,2.0058,2.0021)$ and the hyperfine tensor, $(A_{xx},A_{yy},A_{zz}) = (5.9,5.4,32.9)$G, where the $\underline{x}$ and $\underline{z}$ principal axes lie along the N-O bond and along the radical $2p\text{-}\pi$ orbital, respectively.

Molecular motion which is rapid compared with the spectral splittings gives rise to averaging of the anisotropy to a degree which is determined by the angular amplitude of the molecular rotation. The transverse relaxation time; T_2, which is related to the Lorentzian broadening, is determined by the rate of modulation of the spectral anisotropy and hence can be used to determine the rotational correlation times in the range 10^{-11} to 10^{-8} s. The effects of rotational modulation on the spin-lattice relaxation rate is used in

saturation transfer ESR spectroscopy to determine rotational correlation times in the range 10^{-7} to 10^{-3} s.

Biological membranes are highly dynamic structures by virtue of the molecular rotations of the lipid chains. This molecular mobility or fluidity has decisive effects on the membrane properties and is essential for many of the critical membrane functions. The lipid chain motions lie in the nanosecond time range and thus are ideally suited for study by conventional nitroxide ESR spectroscopy. Lipid exchange rates at the interface with integral membrane proteins lie at the lower limit of sensitivity of conventional nitroxide ESR and this allows spectral resolution of this specific population of lipids. The rotational motions of membrane proteins are much slower, having correlation times of tens of microseconds, and thus can be readily studied by saturation transfer ESR of nitroxide labels.

NITROXIDE SPIN LABELS

Lipid spin labels

Spin-labelled phospholipid and glycolipid probes can be synthesized in which the nitroxide ring is attached in a sterically specific manner at a defined position in the fatty acyl chain. Labelled probes containing the 4,4-dimethyloxazolidine-N-oxyl moiety can be conveniently synthesized by condensation of aminoisopropanol with the appropriate n-keto fatty acid (ref. 1). A more versatile scheme of attachment, based on 2,2,5,5,-tetramethyl-N-oxyl-pyrrolidine, involves permutation of pairwise substitutions by carboxyalkyl and alkyl groups of the requisite chainlengths at the 2- to 5- positions in the pyrrolidine ring (refs. 2,3). The oxazolidine labels, and the corresponding 2,2-substituted pyrrolidine labels, place the nitroxide free radical z-axis parallel to the long axis of the fatty acid chain in its all-trans configuration. Pyrrolidine labels, with carboxyalkyl and alkyl substituents not at the same C-atom position, incorporate the ring into the aliphatic chain and have different orientations of the nitroxide axes relative to the chain axis (refs. 4-6).

The nitroxyl fatty acid may be condensed with sn-2 lyso phosphatidylcholine, via the carboxyimidazole derivative, to yield nitroxyl-labelled phosphatidylcholine (ref. 7). Other nitroxyl-labelled phospholipid species may be synthesized from the corresponding phosphatidylcholine using headgroup exchange catalyzed by phospholipase D, in the presence of an excess of the appropriate alcohol (ref. 8). Nitroxyl diphosphatidylglycerols can be synthesized from condensation of the labelled phosphatidylglycerol with phosphatidic acid (ref. 9). Collected details of the various spin-labelled phospholipid syntheses can be found in ref. 10. Glycolipids require protection of the sugar residues prior to acylation with the nitroxyl fatty acid. Details for glycosphingolipids can be found in ref. 11 and for glycoglycerolipids examples are found in ref. 12.

Protein spin labels

Membrane proteins can be spin-labelled using electrophilic reagents bearing a nitroxyl group. The coupling takes place by addition at nucleophilic centres in the amino acid side chains of the protein; these are principally cysteine residues at physiological pH, although lysine groups may be labelled to a lesser extent. A common reagent is the maleimide derivative bearing the 2,2,5,5-tetramethyl-N-oxyl-pyrrolidine or pyrroline group (ref. 13). This reacts by addition across the double bond, according to the general scheme

$$R\text{-}SH + R^{*}CH{=}CHCOR' \rightarrow R^{*}CH(RS)CH_2COR'$$

More recently, a series of novel vinyl ketone reagents has been introduced (refs. 14, 15) which show considerable promise in saturation transfer ESR studies of protein rotational diffusion (refs. 16, 17). Sulphydryl addition takes place at the ß-position across the vinyl bond and, by suitable design, the length of linkage to the nitroxide-bearing group, R^{*}, may be minimized.

LIPID CHAIN MOTION

The equation of motion for the nitroxide unpaired electron spin is given by the stochastic Liouville equation in which the time dependence is contained solely in the elements of the spin density matrix $\rho(\Omega,t)$

$$d\rho(\Omega,t)/dt = -i[\mathcal{H}(\Omega),\rho(\Omega,t)] - \Gamma_{\Omega}\rho(\Omega,t) \qquad (2)$$

where the spin Hamiltonian, $\mathcal{H}(\Omega)$, depends on the orientation and conformation of the spin-labelled chain segment, specified by the Euler angles, Ω (cf. eqn. 1). Here Γ_{Ω} is the Markov operator which appears in the extended rotational diffusion equation (refs. 18,19)

$$\Gamma_{\Omega} = \underline{L}\cdot\underline{D}_R\cdot\underline{L} + \underline{L}\cdot\underline{D}_R\cdot\underline{L}\, U(\Omega)/kT + \underline{k}_K \qquad (3)$$

where $\underline{L}$ is the operator which generates an infinitesimal elementary rotation, $\underline{D}_R$ is the rotational diffusion tensor, $U(\Omega)$ is the potential of mean torque that limits the angular fluctuations of the chain axis, and $\underline{k}_K$ is the rate constant tensor for transitions between the different chain conformations.

Correlation times can be defined for rotational diffusion of the chain axis: $\tau_{R//} = 1/(6D_{R//})$ and $\tau_{R\perp} = 1/(6D_{R\perp})$, where $D_{R//}$ and $D_{R\perp}$ are the principal elements of the diffusion tensor, corresponding to rotation about and perpendicular to the chain axis, respectively. The elements of the conformational transition rate matrix are related to the mean lifetime, τ_J, of a given chain conformation and to its population. For example, for transitions from a *trans* conformation to a *gauche* conformation, $k_{tg} = p_t/\tau_J$, where p_t is the fractional population for the *trans* conformation at the given chain segment. In the simple case, in which only single *trans* and *gauche*$^{\pm}$ conformations are considered: $p_t + 2p_g = 1$, where p_g is the fractional population of the *gauche*$^{\pm}$ conformation.

The orientational dependence of the potential of mean torque is given, correct to second order, by:

$$U(\Phi,\Theta) = \varepsilon_1\cos^2\Theta + \varepsilon_2\sin^2\Theta\cos\Phi \qquad (4)$$

where $(\Phi,\Theta) \equiv \Omega$ are the Euler angles relating the chain axis to the membrane normal. Orientational order parameters may be defined that correspond to ensemble averages of Legendre polynomials, P_L. The principal order tensor element is given by:

$$S_{zz} = \int P_2(\cos\Theta)\exp[-U(\Omega)/kT]d\Omega / \int \exp[-U(\Omega)/kT]d\Omega \qquad (5)$$

For axial symmetry, the remaining order tensor elements are given by: $S_{xx} = S_{yy} = -\frac{1}{2}S_{zz}$.

Spectral simulations, based on the stochastic Liouville equation and using the above motional model, have been performed for chain labelled lipids in oriented bilayers of dimyristoyl phosphatidylcholine (refs. 18, 19). In the fluid lamellar phase the rotational correlation times for the chain axis motion are found to be: $\tau_{R//} \approx 0.1$ $\tau_{R\perp} \approx 1$ ns with an activation energy ≈ 12 kcal/mole, and for *trans-gauche* isomerism: $\tau_J \leq 0.1$ ns. The order parameter for the chain axis is found to be $S_{zz}^J \approx 0.5$, and population of *trans* rotational isomers is $p_t \approx 0.5$ in the upper part of the chain and $p_t \approx 0.2$ closer to the terminal methyl group (values at 40°C). Comparison with corresponding results from ^{2}H NMR reveal that only the parameters relating to the chain conformation are appreciably distorted by the presence of the spin label group.

LIPID–PROTEIN INTERACTIONS

The ESR spectra of chain-labelled lipids in membranes consist of two components, one of which represents the fluid bilayer lipid environment, and the other of which represents the lipid population in direct contact with the hydrophobic intramembranous surface of the integral membrane proteins. The chain mobility of the latter lipid component is restricted relative to that of the fluid bilayer lipids by direct

interaction with the protein. Quantitation of the relative intensities of the two components, yields information on the stoichiometry and specificity of the lipid-protein interaction. The appropriate equation for lipid-protein association is (see e.g. ref. 20)

$$(n_f^*/n_b^*) = (n_t/N_1 - 1)/K_r \quad (6)$$

where (n_f^*/n_b^*) is the ratio of fluid to motionally restricted spin-labelled lipid components in the ESR spectrum, n_t is the total lipid/protein ratio in the sample, N_1 is the number of first-shell lipid sites on the protein, and K_r is the association constant of the spin-labelled lipid relative to the unlabelled background host lipid. The stoichiometry of the interaction, specified by N_1, correlates well with the size of the intramembranous surface of the proteins involved. The lipid specificity pattern, specified by the relative values of K_r, is complex and is characteristic of the particular membrane protein. These results have been reviewed in refs. 20-23.

Simulation of the two-component ESR spectra, using the exchange-coupled Bloch equations, yields information on the dynamics of lipid exchange at the protein interface (refs. 24, 25). The exchange rate is determined by τ_b^{-1}, which is the first-order rate constant for exchange of the protein-interacting lipid component. These off-rates are in the region of 10^7 s^{-1}, with the exception of M-13 coat protein where the rate is approximately twice as slow. The selectivity of the lipid-protein interaction is reflected in the exchange dynamics. Combination of eqn. (6) with the condition for mass balance, yields the following relationship between the lipid exchange rate and relative association constant

$$\tau_b^{-1}/\tau_f^{-1} = (n_t/N_1 - 1)/K_r \quad (7)$$

If it is assumed that the on-rate, τ_f^{-1}, is the same for all lipids (i.e. is diffusion-controlled), the ratio of the off-rate constants for different lipids would be expected to be in the inverse ratio of the relative association constants. This is found to be the case both for the myelin proteolipid protein (refs. 24-26) and for the M-13 coat protein (ref. 27).

SATURATION TRANSFER ESR OF MEMBRANE PROTEINS

Saturation transfer ESR (STESR) exploits the fact that the spin-lattice relaxation times, T_1, for nitroxide free radicals in the very slow motional regime of conventional ESR spectroscopy lie in the microsecond time regime. Saturation can then be alleviated by rotational diffusion of the nitroxide group on the timescale of T_1.

Spectral simulations appropriate for the highly anisotropic rotational diffusion of spin-labelled membrane proteins have been performed by Robinson and Dalton (ref. 28). It was found that the effective correlation times deduced by comparison with isotropic model systems, are related to the orientation, Θ, of the nitroxide z-axis with respect to the rotational diffusion axis by:

$$\tau_R^{eff}(\pm 1) = 2\tau_{R//}/\sin^2\Theta \quad (8)$$

where $\tau_{R//}$ is the rotational correlation time for rotation of the protein about the membrane normal. In the absence of detailed knowledge of the orientation of the spin label with respect to the protein, the maximum value of the rotational correlation time, corresponding to $\Theta = 90°$, is therefore given by: $\tau_{R//} = \tau_R^{eff}/2$.

The maximal values of the rotational correlation times deduced from STESR spectra of the Na,K-ATPase, cytochrome oxidase and the ADP-ATP carrier in natural or reconstituted fluid membranes are: 25, 25 and 2 μs, respectively (refs. 16, 29-31), when deduced from isotropic calibrations, assuming $\Theta = 90°$ in eqn. (8). These values display a very clear dependence on protein size. The Na,K-ATPase and cytochrome oxidase are large integral proteins of monomer molecular weights 147 and 165 kDa, respectively, whereas the ADP-ATP carrier is a small hydrophobic protein with molecular weight 39 kDa. An important con-

sideration in such studies of overall protein rotational diffusion is that the spin label shall be rigidly attached to the protein backbone. The new vinyl ketone labels seem to be particularly favourable in this respect (ref. 16), whereas the maleimide spin label registers also the effects of segmental motion, at least for the Na,K-ATPase and the Ca^{2+}-ATPase.

The rotational diffusion coefficient can be related to the protein size using hydrodynamic theory. For a right cylindrical protein of elliptical cross-section embedded in a membrane of viscosity η, the diffusion coefficient is given by:

$$D_{R//} = kT/[2\pi\eta h(a^2+b^2)] \qquad (9)$$

where h = 45 Å is the height of the membrane-spanning region of the cylinder, and a and b are the elliptical semi-axes. Appropriate values for the membrane viscosity are η = 2-5 P (ref. 32), which were obtained by calibration with bacteriorhodopsin whose intramembranous cross-sectional dimensions are known. The data given above are found to be consistent with a diprotomer or higher oligomer for the Na,K-ATPase (refs. 16, 29), with a dimer of approximate cross-sectional dimensions 100x60 Å for cytochrome oxidase (ref. 30), and with a dimer of size approximately 46x40 Å for the ADP-ATP carrier (ref. 31).

REFERENCES

1. J.F.W. Keana, J.B. Keana and D. Beetham, J. Am. Chem. Soc. 89, 3055-3056 (1967).
2. T.D. Lee, G.B. Birrell and J.F.W. Keana, J. Am. Chem. Soc. 100, 1618-1619 (1978).
3. K. Hideg and L. Lex, J. Chem. Soc., Chem. Commun., 1263-1265 (1984).
4. T.D. Lee, G.B. Birrell, P.J. Bjorkman and J.F.W. Keana, Biochim. Biophys. Acta 550, 369-383 (1979).
5. M. Esmann, K. Hideg and D. Marsh, Biochemistry 27, 3913-3917 (1988).
6. T. Heimburg, K. Hideg and D. Marsh, to be published (1989).
7. W.F. Boss, C.J. Kelley and F.R. Landsberger, Anal. Biochem. 64, 289-292 (1975).
8. P. Comfurius and R.F.A. Zwaal, Biochim. Biophys. Acta 488, 36-42 (1977).
9. J.F.W. Keana, M. Shimizu and K.K. Jernstedt, J. Org. Chem. 51, 2297-2299 (1986).
10. D. Marsh and A. Watts, in Lipid-Protein Interactions (P.C. Jost and O.H. Griffith, eds.) Vol. 2, pp. 53-126, Wiley, New York (1982).
11. G. Schwarzmann and K. Sandhoff, Methods Enzymol. 138, 319-341 (1987).
12. I. Nishida and M. Yamada, Biochim. Biophys. Acta 813, 298-306 (1985).
13. O.H. Griffith and H.M. McConnell, Proc. Natl. Acad. Sci. USA 55, 8-11 (1966).
14. H.O. Hankovsky, K. Hideg, L. Lex, G. Kulcsár and H.A. Halász, Can. J. Chem. 60, 1432-1438 (1982).
15. H.O. Hankovsky, K. Hideg and G. Jerkovich, Synthesis 526-528 (1989).
16. M. Esmann, H.O. Hankovsky, K. Hideg and D. Marsh, Biochim. Biophys. Acta 978, 209-215 (1989).
17. L.I. Horváth, L. Dux, H.O. Hankovsky, K. Hideg and D. Marsh, to be published (1989).
18. A. Lange, D. Marsh, K.-H. Wassmer, P. Meier and G. Kothe, Biochemistry 24, 4383-4392 (1985).
19. M. Moser, D. Marsh, P. Meier, K.-H. Wassmer and G. Kothe, Biophys. J. 55, 111-123 (1989).
20. D. Marsh, in Progress in Protein-Lipid Interactions. (A. Watts and J.J.H.H.M. de Pont, eds.) Vol. 1, pp. 143-172, Elsevier, Amsterdam (1985).
21. D. Marsh, Trends Biochem. Sci. 8, 330-333 (1983).
22. D. Marsh, J. Bioenerg. Biomemb. 19, 677-689 (1987).
23. D. Marsh, in Supramolecular Structure and Function. Dubrovnik, Yugoslavia Sept. 16.-29, 1987 (G. Pifat-Mrzljak, ed.) pp. 82-108, World Scientific Publishing, Singapore (1988).
24. L.I. Horváth, P.J. Brophy and D. Marsh, Biochemistry 27, 46-52 (1988).

25. D. Marsh and L.I. Horváth, in Advanced EPR in Biology and Biochemistry (A.J. Hoff, ed.) Elsevier, Amsterdam (1989).
26. L.I. Horváth, P.J. Brophy and D. Marsh, Biochemistry 27, 5296-5304 (1988).
27. C.J.A.M. Wolfs, L.I. Horváth, D. Marsh, A. Watts and M.A. Hemminga, Biochemistry, in press (1989).
28. B.H. Robinson and L.R. Dalton, J. Chem. Phys. 72, 1312-1324 (1980).
29. M. Esmann, L.I. Horváth and D. Marsh, Biochemistry 26, 8675-8683 (1987).
30. P. Fajer, P.F. Knowles and D. Marsh, Biochemistry 28, 5634-5643 (1989).
31. L.I. Horváth, A. Munding, K. Beyer, M. Klingenberg and D. Marsh, Biochemistry 28, 407-414 (1989).
32. R.J. Cherry and R.E. Godfrey, Biophys. J. 36, 257-276 (1981).