Strategies in Applying Spin Labeling to the Structure and Dynamics of Biological Membranes*

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The spin labeling technique can provide information on molecular motion, orientation, polarity of the environment, proximity of labels, and chemical accessibility to reducing agents.

There are several general strategies for using nitroxides in the study and function of biological membranes. The first and in many ways the simplest strategy uses a small relatively hydrophobic nitroxide that can partition into the membrane lipids. This approach has been used to study micelles and lipid phase behavior. The second main strategy is to synthesize a lipid containing the nitroxide moiety. The object here is to prepare a reporter molecule that resembles as nearly as possible naturally occuring lipids.[1] Representative lipid spin labels that have been synthesized and used in membrane studies are shown in Fig. 1. The third strategy is to label covalently one of the proteins in the membrane. Still a fourth strategy is to label a natural substrate or inhibitor in order to probe the active site. The first two strategies were developed specifically for membrane studies and the last two are logical extensions of work with water-soluble proteins.

Phospholipid bilayers. The electron dipole-nuclear dipole interaction can be used directly to determine the orientation and distribution of orientations of lipids. The kinds of spin labels used are the doxyl fatty acid, phospholipid and steroid spin labels III-VIII shown in Fig. 1. By incorporating any of these spin labels into oriented hydrated phospholipid multilayers, the anisotropy demonstrates that the lipids do align as predicted by the classical bilayer model. Furthermore, the distribution of orientations and the angle of tilt present in the samples, can be determined from the splittings and lineshapes. This approach can be used to evaluate the effects of chemical treatment on the molecular organization of bilayers.

Early on, it was recognized that by synthesizing a series of fatty acid and phospholipid spin labels with the nitroxide moiety bonded at various positions along the chain one could estimate the orientation and segmental flexibility with high molecular resolution. Typical of the spin labels used in such experiments are the three doxyl fatty acids V, VI and VII shown in Fig. 1. The essential result obtained using these labels in a number of bilayer-containing systems, is that the amplitude and frequency of motion increases toward the center of the bilayer.

The electron-electron dipole and exchange interactions that occur whenever nitroxides are in close proximity have been used to determine rates of lateral diffusion of lipids, which on the order of 10^{-8} cm^2 sec^{-1} at room temperature, corresponding to a hopping frequency for lateral diffusion of 10^7 sec^{-1}.[2,3] In contrast, the half-times for passive transbilayer migration is of the order of many hours or days in pure lipid systems. The earliest determination was made by spin labeling and capitalized on the destruction of the ESR signal by a reducing agent, such as ascorbate. It was shown that the process was very slow compared to lateral diffusion and this is the essential point of the studies.

The earliest observation of regions of low viscosity in the membrane employed 2,2,6,6-tetramethylpiperidine-oxyl (TEMPO).[4] TEMPO is employed in demonstrating lateral phase separations in lipids. In binary mixtures of lipids, the TEMPO parameter can be used to construct phase diagrams.[5] The principle involved in these experiments is the solvent shift in the A and g-values. The spectral lines of a rapidly tumbling spin label in the aqueous phase and in the fluid hydrophobic regions of the bilayer gives a clear resolution of the high field line from each environment. Lateral phase separations have also been detected by exchange broadening using spin labeled lipids. Lateral segregation is induced by added divalent cations[6,7] or in one model system, charged watersoluble proteins.[8]

Several other properties of bilayers have been or are being examined by spin labeling. One of these is the polarity profile across the bilayer responsible for the permeability barrier.[9] An interesting field has developed in the use of charged labels in determinations of membrane potentials.[10] Another active area is the study of fusion and phospholipid transfer mediated by fusigens.[11]

Information in membrane immunology is being obtained by the spin labeling technique, using the nitroxide itself as the hapten. The topographical distribution of the haptenated lipid and the mobility of the hapten has been examined in order to analyze events occuring during recognition and phagocytosis.[12,13]

Biological membranes Two general strategies for asking specific questions about the structure, dynamics and function of biological membranes are to study the intact membrane or to study a homogenous preparation in which a single purified hydrophobic membrane protein is reconstituted into its native lipids or defined phospholipids as diagrammed in Fig. 2.

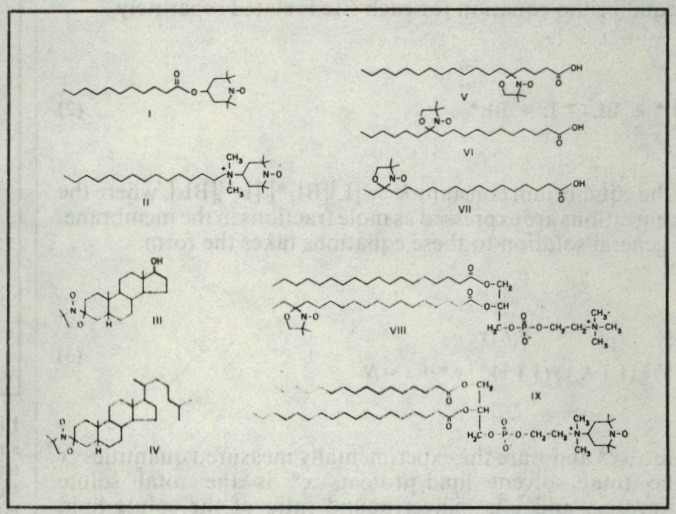

Fig 1: Examples of lipid spin labels used in membrane studies.

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Working with the intact membrane is appropriate when asking questions about the overall structural organization. For example, spin labeling is often used to determine whether or not bilayer organization is present in specialized membrane fragments or organelles. The commonly used criterion is the temperature-dependent characteristic motion of lipid spin labels. Light-induced changes in membrane potentials in photoreceptor membranes detected by spin labeling is another useful and promising approach.

Often, however, the heterogeneity of the native membrane is a real obstacle. Reconstituted systems are being used in studies of lipidprotein interactions, where the lipid to protein ratio is a variable. From these studies a general picture is emerging and can be summarized as follows. In the native membrane a significant fraction of the lipid is at the lipid-protein interface (interfacial or boundary lipid), and essentially all lipid at this interface exchanges with the bulk bilayer. Spin labeling in reconstituted systems has demonstrated that there are a large number of sites on the hydrophobic protein surface that both restrict the motion of lipid spin labels and spatially disorder the lipid chains that interact with the irregular protein surface. The influence of the protein on lipid dynamics decreases sharply with the distance of the lipid from the protein.[14,15] The next step in characterizing lipid-protein interactions is to determine the number of specific and non-specific binding sites and to obtain a measure of the relative binding constants for each class of sites. This approach is outlined below. Looking much further into the future, the determination of the exact sites that the lipid contacts the protein will require either high resolution crystallographic data or a combination of covalent lipid attachment experiments and protein chemistry. Photoreactive spin labels[16] could play a role here both to monitor the photoattachment conditions and to correlate these studies with the equilibrium binding experiments.

In membranes the problem of determining the equilibrium distribution of lipids or other molecules between the hydrophobic protein surfaces and the bilayer solvent is difficult. Solute lipids at the protein interface cannot be separated from the solute lipids in the bilayer by equilibrium dialysis or the other standard techniques of enzymology. Spin labeling provides a method of detecting both environments simultaneously, based on differences in segmental motion of the lipid.

The multiple equilibrium binding treatment for membranes[17,18] is formulated as an exchange reaction between a solute lipid L* (which is usually the spin label), and the solvent lipid L occupying a binding or contact site on the hydrophobic surface of a membrane protein P. For a protein with N binding sites there is a set of equilibrium equations of the form

$$L^* + PL_{N-i}L^*_i \rightleftarrows L + PL_{N-i-1}L^*_{i+1} \qquad (1)$$

However, since the N sites are assumed to be independent it is not necessary to consider combinations and permutations, and the equilibrium equation for each site is stated separately

$$L^* + BL \rightleftarrows L + BL^* \qquad (2)$$

and the equilibrium constant $K = [L][BL^*]/[L^*][BL]$, where the concentrations are expressed as mole fractions in the membrane. The general solution to these equations takes the form

$$\sum_{i=1}^{m} \frac{n_i K_i y}{(1 + K_i y)(1 + y)^{-1} x^* + x - N} = 1 \qquad (3)$$

where x, x^* and y are the experimentally measured quantities: x is the total solvent lipid/protein, x^* is the total solute lipid/protein and y is bilayer/bound ratio of the solute lipid determined from the spin labeling experiment. For the usual case where the concentration of spin label is small, the equation

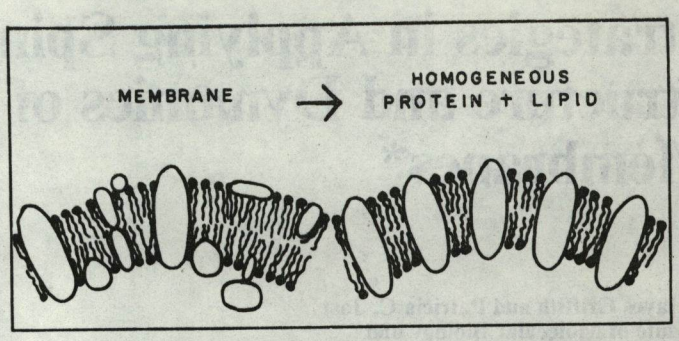

Fig. 2: Diagrammatic representation of an intact native membrane (left) and a homogeneous membrane containing a single kind of protein (right).

reduces to the useful linear approximation

$$y = \frac{x}{NK_{av}} - \frac{1}{K_{av}} \qquad (4)$$

where

$$K_{av} = \frac{1}{N}\sum_{i=1}^{m} n_i K_i \qquad (5)$$

K_{av} is the weighted average of the individual binding constants K_i for the various classes of binding sites i and n_i is the number of binding sites in each class. A number of specific cases including models for one class of binding sites, two classes of binding sites, excluded sites and nonexchangeable lipid as well as the more general saturation of sites treatment have been discussed elsewhere.[17] The binding site treatment has been applied experimentally to the membrane proteins cytochrome oxidase, (Na,K)-ATPase and Ca-ATPase, all reconstituted in phospholipid bilayers. An example is shown in Fig. 3, where each of three spin labels were incorporated into phospholipid bilayers containing (Na,K)-ATPase, reconstituted with phosphatidylcholine (solvent lipid). Thus, if there is no perturbation of the equilibrium by the spin label, one would expect K_{av} to approximate the value of one for the spin-labeled phosphatidylcholine solute lipid. This is observed to be the case in several examples.[15,17,18] The total number of binding sites from the PC data is roughly $N = 60$ per functional unit (314,000 daltons).

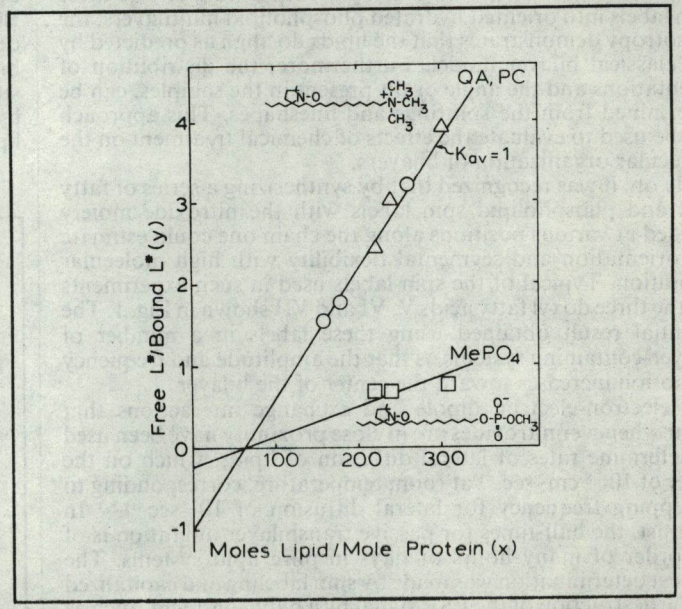

Fig. 3: The effect of solute headgroup charge on lipid binding to the shark Na, K-ATPase.[17,18] The structure of the quarternary amine (QA, triangles) and methylphosphate analogue ($MePO_4$, squares) are shown. The phopsphatidylcholine spin label (PC, circles) has the same labeled acyl chain at the 2-position on the glycerol (similar to VIII, Fig. 1). The solvent in all three cases is phosphatidylcholine. The lines drawn are for $K_{av} = 1$ and $K_{av} = 5$. →

Evidence for some selectivity is given by the $K_{av} \sim 5$ line drawn through the data points for the negatively charged methyl phosphate lipid label. The number of these selective sites must be small, since the analogous positively-charged quaternary amine spin label would be expected to be repelled by these sites. The data indicate a $K_{av} \sim 1$, consistent with the conclusion that only a few sites are selective for the negatively-charged label. In the limiting case of only one specific site for the negatively charged lipid among 59 non-specific sites, then $NK_{av} = (60)(5) = (59)(1) + (1)K_2$, giving $K_2 \sim 240$. This corresponds to a $\triangle G$ of about -12.5 kJ mole^{-1}. The data presented here are preliminary. However, experimental data on several systems exhibit the expected linearity over ranges of lipid to protein rations that bracket physiological limits. We note that relatively small binding constants may play a significant role in determining the composition of lipids containing membrane proteins due to the very high relative concentrations of the different lipid species in native membranes.

Acknowledgments

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