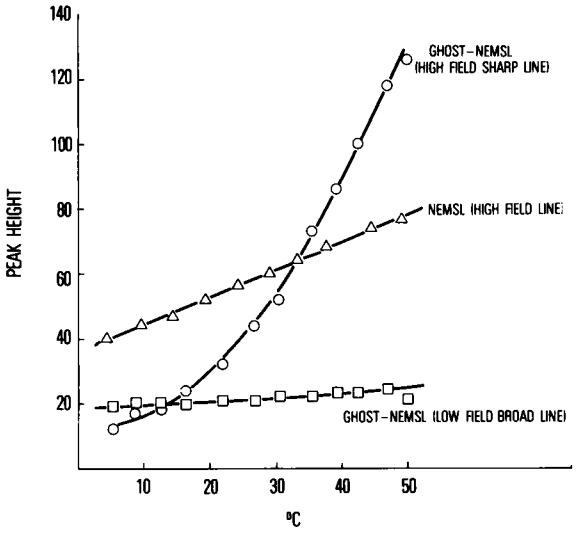

FIGURE 2 Variation of peak heights with temperature for erythrocyte ghosts labeled with NEMSL and free spin label. The instrumentation and techniques are the same as described in the legend to Fig. 1.

heights at any one set of conditions. Furthermore, the inverse r^6-dependence of the decrease (10) in signal intensity due to dipolar interactions (10) and the fact that most of these pairs of sulfhydryls are generally at the contacts between protein molecules (8, 12) provide a new, very sensitive, probe for changes in the distance and/or orientation of certain membrane proteins relative to each other.

Received for publication 1 May 1981.

REFERENCES

1. Chapman, D., M. D. Barratt, and V. B. Kamat. 1969. A spin-label study of erythrocyte membranes. *Biochim. Biophys. Acta.* 173:154–157.
2. Sandberg, H. E., R. G. Bryant, and L. H. Piette. 1969. Studies on the location of sulfhydryl groups in erythrocyte membranes with magnetic resonance spin probes. *Arch. Biochem. Biophys.* 133:144–152.
3. Butterfield, D. A., J. O. Oeswein, and W. R. Markesbery. 1977. Electron spin resonance study of membrane protein alterations in erythrocytes in Huntington's disease. *Nature (Lond.).* 267:453–455.
4. Jones, G. L., and D. M. Woodbury. 1978. Reappraisal of the electron spin resonance spectra of maleimide and iodoacetamide spin labels in erythrocyte ghosts. *Arch. Biochem. Biophys.* 190:611–616.
5. Lau, P., C. Hung, K. Minakata, E. Schwartz, and T. Asakura. 1979. Spin-label studies of membrane-associated denatured hemoglobin in normal and sickle cells. *Biochim. Biophys. Acta.* 552:499–508.
6. Fung, L. W. -M. 1981. Spin-label studies of the lipid and protein components of erythrocyte membranes. A comparison of electron paramagnetic resonance and saturation transfer electron paramagnetic resonance methods. *Biophys. J.* 33:253–262.
7. Kirkpatrick, F. H., and H. E. Sandberg. 1973. Effect of anionic surfactants, nonionic surfactants and neutral salts on the conformation of spin-labeled erythrocyte membrane proteins. *Biochim. Biophys. Acta.* 298:209–218.
8. Haest, C. W. M., D. Kamp, G. Plasa, and B. Deuticke. 1977. Intra- and intermolecular cross-linking of membrane proteins in intact erythrocytes and ghosts by SH-oxidizing agents. *Biochim. Biophys. Acta.* 469:226–230.
9. Mohanty, J. G., J. T. Wang, and J. M. Rifkind. 1981. Effect of MgATP on protein spin labeled human erythrocyte ghosts. *Biophys. J.* 33:182*a*.
10. Leigh, J. S., Jr. 1970. ESR rigid-lattice line shape in a system of two interacting spins. *J. Chem. Phys.* 52:2608–2612.
11. Taylor, J. S., J. S. Leigh, Jr., and M. Cohn. 1969. Magnetic resonance studies of spin-labeled creatine kinase system and interaction of two paramagnetic probes. *Proc. Natl. Acad. Sci. U.S.A.* 64:219–226.
12. Reithmeier, R. A. F., and A. Rao. 1979. Reactive sulfhydryl groups of the band 3 polypeptide from human erythrocyte membranes. Identification of the sulfhydryl groups involved in Cu^{2+}-*o*-phenanthroline cross-linking. *J. Biol. Chem.* 254:6151–6155.

ADVANTAGES AND LIMITATIONS OF SPIN LABELING IN QUANTITATING PROTEIN-LIPID ASSOCIATIONS

O. HAYES GRIFFITH, J. CRAIG BAUMEISTER, AND PATRICIA C. JOST
Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403 U. S. A.

A central problem in lipid-protein interactions is how the hydrophobic regions of the irregular protein surface are interfaced with the fluid lipid bilayer of the membrane. The proteins are well-defined three-dimensional structures around which the lipids must conform in a way that leads to a minimum energy configuration of the system. Interesting aspects of this problem include segmental motion of the lipids in contact with the protein, conformation and orientation of these lipid chains solvating the protein, kinetics of the exchange between these lipids and the bulk bilayer solvent, and the relative binding constants of different kinds of phospholipids. The sensitivity and short time scale of ESR and the fact that spectral line shapes are sensitive to motion has made this one of the most useful spectroscopic probes for studying lipid-lipid and lipid-protein interactions. Although this field is relatively new and there is still some controversy, the current picture is that most, if not all, lipids in contact with the protein exhibit reduced motion compared with lipids in the bilayer (1). The lipid is somewhat spatially disordered by the irregular protein surface, and this lipid exchanges with bulk bilayer. (This exchange is evidently rapid on the

BIOPHYS. J. © Biophysical Society · 0006–3495/82/01/152/03 $1.00
Volume 37 January 1982 152–154

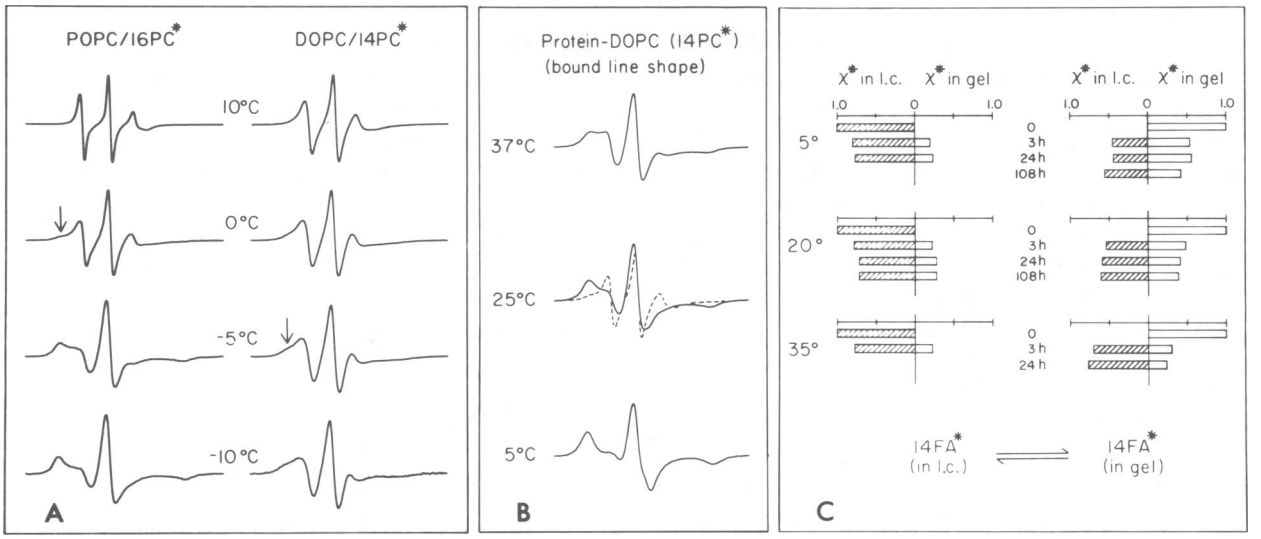

FIGURE 1 Spectral line shapes of lipid spin labels in liposomes without (*A*) and with (*B*) protein present, and the data for label distribution between two populations of liposomes, one in the gel and the other in the liquid-crystalline state (*C*). *A*, line shape as a function of temperature for 16-doxylphosphatidylcholine (16PC*) in 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine (POPC), and 14-proxylphosphatidylcholine (14PC*) in dioleoyl-*sn*-3 phosphatidylcholine (DOPC). *B*, line-shape as a function of temperature for detergent-free cytochrome oxidase in DOPC at a phospholipid:protein molar ratio of 40:1, showing the broad spectrum of the spin label in contact with protein. This spectrum is essentially pure bound component without fluid bilayer. The dashed-line spectrum (25°C) is from a similar sample but at higher lipid-to-protein ratio (90:1 molar ratio) in which the bound component is 45% of the total spectrum. The bilayer component resembles the top of spectrum of *A*, right column. *C*, distribution of the 14-proxylstearate label between liposomes of DOPC and liposomes of dipalmitoyl-*sn*-phosphatidylcholine at temperatures where DOPC is in the liquid-crystalline phase (l.c.) and DPPC is the gel phase. χ^* is the fraction of spin label in each population of vesicles as determined by separation and quantitation of the distinctive line shapes in each phase. In the left column, unlabeled DPPC vesicles (gel) were added to labeled DOPC vesicles (l.c.) and incubated at the indicated temperature. The right column summarizes the results when the label was initially in DPPC vesicles (gel) and unlabeled DOPC vesicles (l.c.) were added. The spectra are recorded at the incubation temperatures 5°, 20°, or 35°C.

NMR time-scale, but usually sufficiently slow on the ESR time-scale to preserve the separate spectral components.)

RESULTS AND DISCUSSION

Recent experiments in this laboratory have shifted from examining the kinetics of the exchange to the thermodynamics of the equilibria between the protein surface and the adjacent bilayer. There is an exchange reaction between the labeled solute lipid (L*) and solvent lipid (L) competing for the hydrophobic surface of the protein (P) that directly interacts with the lipid bilayer. This exchange yields a set of equations of the form

$$L^* + PL^*_{N-i}\,L^*_i \rightleftharpoons L + PL_{N-i-1}\,L^*_{i+1}$$

where N is the number of binding or contact sites. Using this treatment, relative equilibrium constants and the number of binding sites can be determined from spin labeling data (2). During the course of these experiments some potential pitfalls have become clear. The purpose of this note is to discuss problems that await the unwary experimenter.

The assumptions made in data analysis and spectral interpretation of the quantitative spin labeling studies of lipid-protein interactions are relatively straightforward in systems at physiological temperatures (3, 4), i.e., systems in which lipids are well above their transition temperature and the label is near the end of the acyl chain (this assumes sample homogeneity). However, substantial complexities are introduced when these labels, or indeed any spectroscopic label, are used to interpret lipid-protein interactions at lower temperatures. At lower temperatures broadened line shapes are observed in the absence of protein (Fig. 1 *A*) which substantially overlap the motionally restricted components seen in the presence of protein (Fig. 1*B*). This line overlap can lead to substantial overestimates of the amount of lipid in contact with the protein. The line shape of protein-associated lipid ("boundary lipid") is relatively temperature insensitive (Fig. 1 *B*), although in this example the outer line separations decrease from 64.5 gauss at 5°C to 60.2 G at 37°C. In pure lipid vesicles (Fig. 1 *A*), the line shape is highly sensitive to temperature and at lower temperatures becomes very broad. As the label is moved farther toward the polar headgroup, the same effect is seen at higher temperatures, especially in the gel phase, e.g., at 20°C in dimyristoylphosphatidylcholine containing 12-doxylstearic acid (5).

When the temperature used is near the transition temperature of a defined lipid, still further complexities are introduced. In the absence of protein, two spectral components can be obtained in pure lipid vesicles (note arrows in Fig. 1 *A*), reflecting more than one environment.

Gel and liquid-crystalline domains may both be present near the transition temperature of vesicles containing a defined lipid plus protein, especially at higher lipid:protein ratios. Since the line shapes from the gel phase often resemble those of the protein-associated component, an incorrect analysis of the system will lead to an apparent increase in the estimate of bound lipid near and below the transition temperature. Even if the line shapes could be separated, it is clear that the simple equilibrium described by the exchange equation given above no longer applies when the label distribution favors one phase. Measurements of the distribution of 14-proxylstearate as it equilibrates between two populations of liposomes, one gel and the other liquid-crystalline, are shown in Fig. 1 *C*. Starting with labeled liquid-crystalline vesicles (DOPC) and adding gel phase vesicles (DPPC), it is clearly seen that the fatty acid label migrating through the aqueous phase establishes an equilibrium distribution that is 75% in favor of the liquid-crystalline phase. The converse experiment, where the label is trapped in the gel phase before addition of the liquid-crystalline vesicles, shows the same trend, although the system does not reach equilibrium at lower temperatures. At 35°C the end result, however, is the same for both experiments. It is not surprising that lipid packing in the gel phase tends to exclude the lipid spin label (6), since it carries the nitroxide moiety on the acyl chain.

These results illustrate the fact that at low temperatures, especially near or below the lipid transition temperature, spectroscopic line overlap and general thermodynamic and kinetic considerations dictate the need for caution in analyzing lipid-protein interactions. One possible problem that has not turned out to be an obstacle in estimating the equilibrium binding of lipids to protein is the potential perturbation introduced by the spin label. This has been tested (7) and the effects on the equilibrium distribution of lipid binding to several transmembranous proteins are negligible when the temperatures used are well above the transition temperature of the lipid and the nitroxide moiety is near the end of the acyl chain of the lipid spin label (7).

This work was supported by grant GM25698 from the National Institutes of Health.

Received for publication 2 May 1981.

REFERENCES

1. Jost, P. C., and O. H. Griffith. 1980. The lipid-protein interface in biological membranes. *Ann. N. Y. Acad. Sci.* 348:391–407.
2. Brotherus, J. R., O. H. Griffith, M. O. Brotherus, P. C. Jost, J. R. Silvius, and L. E. Hokin. 1981. Lipid-protein multiple binding equilibria in membranes. *Biochemistry.* 20:5261–5267.
3. Jost, P. C., and O. H. Griffith. 1978. The spin labeling technique. *In* Methods of Enzymology. C. G. W. Hirs and S.N. Timaseff, editors. Academic Press, Inc., New York. 49:369–371.
4. Brotherus, J. R., P. C. Jost, O. H. Griffith, J. F. W. Keana, and L. E. Hokin. 1980. Charge selectivity at the lipid-protein interface of membranous Na,K-ATPase. *Proc. Natl. Acad. Sci. U.S.A.* 77:272–276.
5. Kang. S. Y., H. S. Gutowsky, J. C. Hsung, R. Jacobs, T. E. King, D. Rice, and E. Oldfield. 1979. Nuclear magnetic resonance investigation of the cytochrome oxidase-phospholipid interaction: a new model for boundary lipid. *Biochemistry.* 15:3257–3265.
6. Oldfield, E., K. M. Keough, and D. M. Chapman. 1971. The study of hydrocarbon chain mobility in membrane systems using spin-label probes. *Fed. Eur. Biochem. Lett.* 20:344–346.
7. Griffith, O. H., J. R. Brotherus, and P. C. Jost. 1981. Equilibrium constants and number of binding sites for lipid-protein interaction in membranes. *In* Protein-Lipid Interactions. P. C. Jost and O. H. Griffith, editors. John Wiley Interscience, Inc., New York.

MOLECULAR MECHANISMS OF ALAMETHICIN CHANNEL GATING

STEVEN C. QUAY
Department of Pathology, Stanford University School of Medicine and the Palo Alto Veterans Administration Hospital, Stanford, California 94305, U.S.A.

RAMON LATORRE
Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115 U.S.A.

Alamethicin is a largely hydrophobic, linear eicosapeptide, with an acylated N-terminus and a C-terminal alcohol (1). Because alamethicin is able to induce a voltage-dependent conductance in lipid bilayers similar to that found in nerve and muscle, it has often been used as a model system for studying channel gating and channel formation. Most of the models suggested for alamethicin channel gating propose that the applied field leads to the insertion of alamethicin molecules initially located at the membrane surface into the hydrocarbon region of the bilayer (2). We show here that alamethicin can be photolytically cross-linked to a phosphatidylcholine analogue containing a carbene precursor at the end of the C-2 fatty acyl chain. This result indicates that a portion of the alamethicin moelcule is present in the interior of the bilayer in the absence of an applied voltage. The alamethicin-phospholipid photoproduct is able to induce a voltage-gated conductance similar to that of natural alamethicin.

BIOPHYS. J. © Biophysical Society · 0006–3495/82/01/154/03 $1.00
Volume 37 January 1982 154–156