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THE USE OF A PMR PROBE FOR STUDYING THE HYDROPHOBIC PROPERTIES OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANES AND MEMBRANE FRACTIONS

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SUMMARY

The hydrophobic character of the trimethyl group of sodium 2,2-dimethyl-2-silapentane-5-sulfonate, makes it an effective PMR probe for apolar sites on proteins and membranes. By comparing the spin–spin relaxation rates of the free and bound probe the extent and strength of the interaction can be qualitatively compared for bovine serum albumin, membranes from *Micrococcus lysodeikticus* and for different fractions isolated from this membrane. It is concluded that this membrane has hydrophobic sites on or near its surface and that the number of such sites is sensitive to the ionic composition and to the pH of its aqueous environment. Removal of lipid from the membrane greatly increased the binding of the probe, while vesicular preparations of the lipid fraction itself gave no evidence of an interaction with the probe. The results are discussed in terms of protein–lipid–water interactions.

INTRODUCTION

The application of physical techniques to the study of membrane structure has progressed rapidly in recent years, yet our present knowledge of specific structure–function relationships in biological membranes is still rather rudimentary. The existence of hydrophobic interactions in membranes has been demonstrated by a number of investigators[1–6], although the nature (*i.e.*, lipid–lipid, lipid–protein, protein–protein) of such interactions has not yet been clearly defined. One of the more promising methods for studying hydrophobic regions in membranes is based upon the use of hydrophobic probes. So far most of this work has involved either fluorescent[7] or spin-labelled[8,9] probes. Although important information has been obtained from these studies, there has always been some uncertainty as to the specificity of the probe for a particular hydrophobic region (*i.e.* lipids or proteins or both).

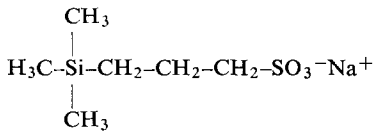
PMR spectroscopy has proven to be a valuable and interesting way of studying molecular interactions in biological membranes[1,10,11].

The experiments described here deal with the use of a hydrophobic PMR

Abbreviation: DMSP, 2,2-dimethyl-2-silapentane-5-sulfonate.

probe, which binds to hydrophobic sites on proteins. The interaction is studied by following changes of T_2 (the spin–spin or transverse relaxation time) for the probe in the presence of proteins, membranes, and membrane fractions. The relaxation time of a given molecule is a measure of its molecular motion and is (approximately) independent of the dielectric constant of the medium[12].

The probe used was sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DMSP), the structure of which is:



It is highly soluble in aqueous media and the $-Si(CH_3)_3$ end can interact with other hydrophobic moieties. The three terminal methyl groups provide nine equivalent, non-exchangeable protons, therefore the PMR signal of this group will be the sum of all nine protons and can thus be followed, even at the low concentrations needed to do binding studies (*e.g.* $5 \cdot 10^{-3}$ M).

The first part of this paper will deal with the interaction of DMSP with proteins (bovine serum albumin and lysozyme), and lipids (lecithin and cardiolipin). These results will be compared with those for membranes and membrane fractions. The effects of neutral salts, pH, organic solvents and protein denaturants have also been studied.

MATERIALS AND METHODS

Membranes were isolated and prepared from cells of the Gram-positive bacterium, *Micrococcus lysodeikticus*, by the methods described by Salton and Freer[13,14]. They were given three additional washes in 99.7% ^2H_2O and sonicated in ^2H_2O as reported previously[15]. Preparation of a lipid-free protein fraction involved dissociation of the membranes by treatment with 1% sodium deoxycholate, which yielded a soluble and a lipid-depleted, insoluble fraction[16]. Deoxycholate was removed from the soluble fraction by extensive dialysis at 4 °C. Lipids were then removed by three successive extractions with *n*-butanol in the cold (0–4 °C).

Reassociation of membrane lipids and proteins was performed in the same solvent system which was used to extract the lipids. The presence of a lipo-protein complex is known from its behavior on a sucrose density gradient[14,16].

The thiocyanate residue fraction was prepared by treating whole membranes with 0.05 M NaSCN, followed by centrifugation at 16000 rev./min for 60 min. The thiocyanate was removed from the pellet fraction by extensive dialysis in the cold.

The pH was adjusted to the desired value by addition of either HCl or KOH, and unless otherwise specified was between pH 6 and 7.

The DMSP was obtained from the Isotopic Products Division of Merck, Sharp and Dohme, ^2H_2O from New England Nuclear, bovine serum albumin from Pentex, lysozyme and the phospholipids from Calbiochem. Analytical grade, salts and all other reagents were from Fisher.

The NMR measurements were performed on a Varian A-60A. The radio frequency was set at 0.05 mG to avoid saturation effects. The sweep width was 500 cps, and the sweep time was 250 s. The samples (approximately 1 ml) were pipetted into standard glass NMR tubes. The experiments were performed at room temperature (20 °C).

The values of the relaxation rates, T_2^{-1}, of the terminal trimethyl protons of the probe, were obtained from the spectra by using the formula,

$$T_2^{-1} = \pi \Delta \nu_{\frac{1}{2}} \tag{1}$$

where $\Delta \nu_{\frac{1}{2}}$ is the spectral line width at one half maximum peak height (at 10.0τ)[17,18].

Although field-dependent and diamagnetic susceptibility-dependent broadening may occur for particulate systems, including membranes[19], it is assumed here that for a small molecule such as DMSP these effects are not significant and that the line width is a reflection of the spin–spin relaxation time, as given in Eqn 1. It is understood that without pulsed NMR data one cannot with certainty rule out other broadening mechanisms.

RESULTS

The PMR spectra of a 1% solution of DMSP is shown in Fig. 1. The peak at 10.0τ is due to the terminal trimethyl group. The multiplet peaks at 9.4τ, 8.3τ, and 7.2τ are due to the methylene protons in the α, β, and γ positions, respectively (α being the closest to the trimethyl group). The spectra of the trimethyl group at 10.0τ in the presence of bovine serum albumin (2%), cardiolipin (1%), lecithin (5%), whole *M. lysodeikticus* membranes (2%), a lipid-depleted membrane protein fraction (2%), extracted membrane lipids (2%), and lysozyme (2%) (all adjusted to w/v basis) are shown in Figs 2a, 2b, 2c, 2d, 2e, 2f, and 2g, respectively. The broadening of this peak seen in Figs. 2a, 2d, and 2e is an indication of the binding of the probe to a macromolecule. The other DMSP peaks were either not broadened at all, or only slightly during the experiments. The broadening was not dependent on the apparent viscosity of several membrane samples at various levels of pH. Furthermore, the peak for the 5% lecithin suspension was much sharper than for a 2% bovine serum albumin solution, even though the former was obviously much more viscous than the latter. Therefore it was evident that viscosity effects did not make a significant contribution to the observed variation in the line width in these experiments. Thus it appears likely that the interaction between the probe and proteins (and membranes) is an exchange process of the fast exchange limit, as defined by Fisher and Jardetzky[18]. In this case the following relationship is valid:

$$T_{2\,\text{observed}}^{-1} = \alpha T_{2\,\text{bound}}^{-1} + (1 - \alpha) T_{2\,\text{free}}^{-1} \tag{2}$$

where α is the fraction of probe molecules which is bound, and $T_{2\,\text{observed}}^{-1}$ and $T_{2\,\text{free}}^{-1}$ are obtained by applying Eqn 1 to the spectra of the probe in the presence and absence of macromolecule, respectively. By gradually increasing the ratio of BSA to DMSP it was found that $\Delta \nu_{\frac{1}{2}}$ reached a maximum value of 11.6 cycles/s when the molar ratio of BSA to DMSP was 1:12. It can be assumed that this represents saturation (*i.e.* $\alpha = 1$), and thus $T_{2\,\text{bound}}^{-1} = 36.0$.

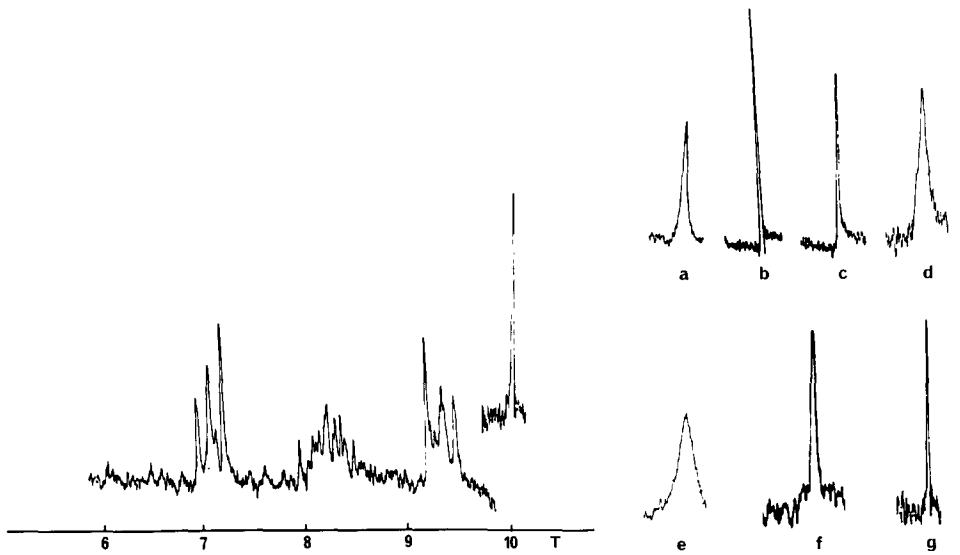


Fig. 1. PRM spectrum of a 1% solution of DMSP in ^2H_2O. The peak at 10τ is at a reduced amplitude magnification relative to the rest of the spectrum.

Fig. 2. The PRM spectra of the peak at 10τ due to the trimethyl protons in the presence of: (a) bovine serum albumin, (b) cardiolipin, (c) lecithin, (d) whole membrane, (e) lipid-depleted membrane protein fraction, (f) extracted membrane lipids dispersed by sonication, and (g) lysozyme.

TABLE I

OBSERVED VALUES OF $\Delta\nu_{\frac{1}{2}}$ FOR DMSP IN THE PRESENCE OF BOVINE SERUM ALBUMIN UNDER THE FOLLOWING CONDITIONS

Condition	$\Delta\nu_{\frac{1}{2}}$ (*cycles/s*)
pH 7.0	4.6
pH 10.4	4.1
pH 10.4, 0.1 M $CaCl_2$	19.9
1 M NaCl	4.9
0.1 M $CaCl_2$	5.7
0.1 M NaSCN	3.0
6 M urea	3.1
100 °C for 5 min	30.2
0.013 M sodium hexyl sulfate	2.5
0.026 M sodium hexyl sulfate	1.2
30% methanol	2.1
L-α-Lecithin (β,γ-dipalmitoyl)*	4.1
Lysolecithin*	3.4

* Concentration equal to that of bovine serum albumin by weight.

Heating a 2% solution of bovine serum albumin at 90 °C for 5 min, by which time a noticeable increase in turbidity occurs, resulted in a sharp increase in binding of DMSP. Treatment with 6 M urea produced a decrease in binding of the probe. The addition of small molecules with hydrophobic groups (*e.g.* methanol, sodium hexyl sulfate), resulted in a decreased binding of the probe. Lecithin and to a greater extent, lysolecithin, also reduced the binding of the probe by bovine serum albumin. The effects of various additives on the bovine serum albumin–DMSP interaction are reflected in the values of $\Delta\nu_{\frac{1}{2}}$ for the DMSP peak at 10τ, and these are listed in Table I.

The results of the membrane-probe experiments are presented in Figs 3 and 4, where the value of $\Delta\nu_{\frac{1}{2}}$ at 10τ is plotted against the weight ratio of membrane to DMSP. In Fig. 3 a comparison of the results of experiments done at pH 7.0 and pH 10.8, at very low ionic strength, and at pH 7.0 in 0.1 M NaCl and in 0.1 M $CaCl_2$, is shown. In Fig. 4, a similar plot compares the results for whole membrane with those for a lipid-depleted membrane protein fraction.

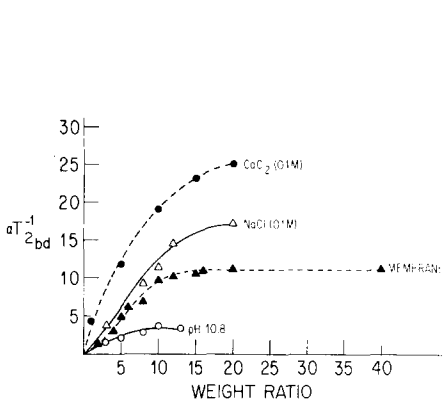


Fig. 3. Variation of $\alpha T_{2\,\mathrm{bd}}^{-1}$ with the ratios of membrane to probe at alkaline pH, in the presence of 0.1 M NaCl, and in the presence of 0.1 M $CaCl_2$, compared to that for membranes in distilled water at neutral pH.

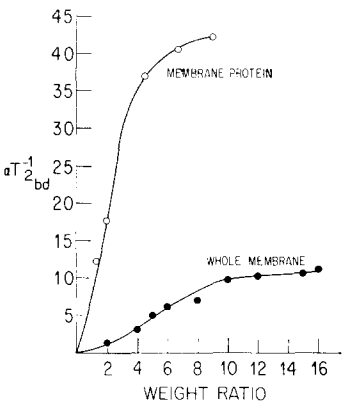


Fig. 4. Variation of $\alpha T_{2\,\mathrm{bd}}^{-1}$ with ratio of membrane to probe for whole membrane, compared to that for the lipid-depleted, membrane protein fraction.

An increase in the ratio at which the binding curve begins to plateau is indicative of either an increase in the number of binding sites, or a decrease in the strength of the binding (*i.e.* the affinity of the site for DMSP), whereas an increase in the height of the curve at saturation (*i.e.* $\alpha=1$), indicates an increase in $T_{2\,\mathrm{bound}}^{-1}$. The value of $\Delta\nu_{\frac{1}{2}}$ at saturation gives $T_{2\,\mathrm{bound}}^{-1}$.

The effects of various organic solvents, denaturants, pH and neutral salts on the line width are seen in Fig. 5. Small molecules with hydrophobic moieties seem to be able to displace the probe from the membrane (*i.e.* $\Delta\nu_{\frac{1}{2}}$ decreases for acetone, methanol, chloroform, and sodium deoxycholate).

Urea caused a decrease in the line width at 10τ, the decrease being proportional

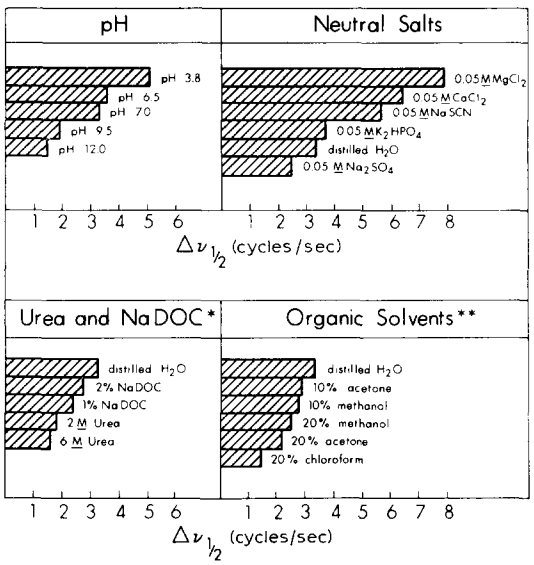


Fig. 5. The effects of pH, neutral salts, urea, sodium deoxycholate (NaDOC), and organic solvents on the line width ($\Delta\nu_{\frac{1}{2}}$) of DMSP in the presence of membrane. * Concentration by weight. ** Concentration by volume.

to the urea concentration. The addition of urea to the membrane dispersion also resulted in a marked decrease in the turbidity, indicating an increase in the membrane solvation, and in the appearance of a broad band in the 8.7–9.1 τ region.

The line width at 10 τ varies inversely with pH, from pH 3 to 12. The apparent decrease in binding which occurs at high pH (*e.g.* pH > 10), is accompanied by the appearance of the membrane lipid hydrocarbon peaks at 8.7 τ and 9.1 τ, as well as an increase in membrane solubilization (as evidenced by a decrease in turbidity). All of these pH effects are completely reversible. The spectra of sonicated aqueous disperson of *n*-butanol extracted membrane lipids did not, however, change significantly with pH.

Treatment of the membrane with 0.05 M NaSCN resulted in an increased line width, but the NaSCN residue showed less affinity for the probe (*i.e.* a decreased line width) than the untreated membrane. Since soluble lipoproteins are released from the membrane by treatment with NaSCN (Green, D. H., unpublished observations), it is possible that these proteins play a role in the binding of the probe. All neutral salts except Na_2HPO_4, K_2HPO_4 and Na_2SO_4, caused an increase in $\Delta\nu_{\frac{1}{2}}$. The HPO_4^{2-} salts had little effect, and the SO_4^{2-} caused a decrease in the $\Delta\nu_{\frac{1}{2}}$. The order of ability of cations to cause an increased binding of DMSP by the membrane is: $Mg^{2+} > Ca^{2+} > Li^+ > Na^+ = K^+$. The order of anions is: $SCN^- > Cl^- > HPO_4^{2-} > SO_4^{2-}$.

Partial extraction of membrane lipids with organic solvents, or recombination of membrane lipids with proteins, resulted in a reduced binding of the probe. By using an external reference (trimethylsilane), it was determined that the chemical shift of the methyl proton signal did not change during any of these experiments.

DISCUSSION

The above experiments demonstrate that DMSP can be used as an NMR probe for hydrophobic groups on the surface of proteins. That the interaction of the probe with proteins is mainly hydrophobic in nature is apparent from the following observations. Small molecules with hydrophobic groups can displace the probe. Increasing the ionic strength almost always results in an increased binding. At neutral pH, the negatively charged phospholipid phosphates should repel the negatively charged sulfonate end of DMSP.

It is not surprising that bovine serum albumin should exhibit an affinity for DMSP, since it is known that there are several hydrophobic residues on the surface of this protein[22]. The increase in binding which occurs upon heating bovine serum albumin, and the decrease which occurs upon denaturation by urea suggest that the binding sites are probably composed of several hydrophobic residues, which in the native conformation are in close proximity. Conversion to a random coil by urea[20] separates these groups, thus breaking up the site. Heating bovine serum albumin results in cross-linking molecules by a disulfide interchange, while retaining considerable tertiary structure in each individual bovine serum albumin molecule[21]. Thus hydrophobic residues on different molecules may be brought closer together, thereby creating new binding sites.

The experiments with whole membranes, lipids, and lipid-depleted membranes are in accord with the presence of hydrophobic sites on or near the surface(s) of whole membranes. The saturation values of $\Delta\nu_{\frac{1}{2}}$ for the membrane and for bovine serum albumin are within the same order of magnitude for similar weight ratios of protein to DMSP. Since $\Delta\nu_{\frac{1}{2}}$ is a measure of the mobility of the probe, which in turns is a function of the mobility of the protein to which it is bound, it is conceivable that at least some of the proteins within the membrane may have some mobility, (*e.g.* rotational) and are not held tightly in a static crystalline-like lattice.

Removal of the membrane lipids results in a very marked increase $\Delta\nu_{\frac{1}{2}}$ at 10τ, indicating a considerable reduction of the mobility of the DMSP–protein complex, which may result from an aggregation of the lipid-depleted proteins. This suggests that hydrophobic lipid–protein interactions in the native membrane are important in maintaining membrane plasticity. The lipid hydrocarbons provide a hydrophobic environment which can accommodate the membrane proteins, and thus prevent irreversible protein aggregation. This interpretation is reinforced by the observation that those conditions which result in an increase in the molecular motion of the lipid hydrocarbon chains, result in a decrease in the binding of the probe. The decrease in binding subsequent to partial lipid extraction or recombination of membrane lipids with proteins, probably results from non-specific lipid–protein associations (occurring either during the extraction or recombination) which block the binding sites. Sodium deoxycholate causes a decrease in the binding of DMSP, possibly by interacting with the hydrophobic sites on the membrane.

Treatment with 6 M urea results in a decrease in the binding of the probe and an increase in membrane solvation (decreased turbidity) and an increase in the freedom of movement of the lipid hydrocarbon chains as evidenced by the appearance of high resolution NMR peaks. All three effects indicate that urea causes a decrease in the hydrophobicity of the membrane proteins.

The spectra of the membranes at alkaline pH resemble those obtained by Kamat *et al.*[23] from solubilized erythrocyte membrane lipoproteins. The appearance of the hydrocarbon peaks at 8.7τ and 9.1τ can only mean that the particles are reorienting themselves much more rapidly. The optical clarity of the solution contrasts markedly with the distinct turbidity of sonicated membrane preparations. Since neither lipid-depleted membrane proteins, nor purified sonicated membrane lipids exhibited these reversible pH effects, they must depend on the interaction of the lipids and the proteins.

The results with neutral salts can best be understood as a combination of ion-binding and water structure effects. Divalent cations which are particularly effective in increasing $\Delta\nu_{\frac{1}{2}}$, are capable of cross-linking lipid phosphate groups[24–26] as well as binding to negative and neutral (*i.e.*, amide C=O) groups on proteins[27]. The NMR data (Fig. 3) indicate that $T_{2\,\text{bound}}^{-1}$ is increased by 0.1 M $CaCl_2$. The increase in $T_{2\,\text{bound}}^{-1}$ could result from the restriction of motion of the membrane proteins in the presence of Ca^{2+}.

Fig. 3 also demonstrates that the addition of 0.1 M $CaCl_2$, and to a lesser extent 0.1 M NaCl, results in a higher ratio of membrane to probe needed for saturation. As was pointed out, this could be due to an increase in the number of sites or a decrease in the affinity of the sites or both. From studies on the effects of these and other salts on the conformation and solubility of proteins, such as ribonuclease and collagen, it is known that Ca^{2+} can lead to an unfolding of the native conformation of proteins, thereby exposing hydrophobic sites[28].

The order of effectiveness of cations in increasing the binding of the probe by the membrane is approximately the order of decreasing charge density, and close to the Hofmeister series. The order for anions is also consistent with the idea that these ions may alter membrane organization through an effect on water structure[28].

The importance of charge effects in maintaining membrane integrity are obvious from the observation that changes in the pH are accompanied by changes in the binding of DMSP, the solvation of the membrane, and the mobility of the lipid hydrocarbon chains, all of which demonstrate the complex nature of the lipid–protein–water interactions in the membrane.

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