^{1}H-NMR STUDIES OF PROTEIN-LIPID INTERACTIONS IN RETINAL ROD OUTER SEGMENT DISC MEMBRANES

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1. Introduction

Proton nuclear magnetic resonance (^{1}H-NMR) techniques have been applied extensively to model bilayer membranes. Although interpretation of the results has been controversial, some general conclusions regarding molecular motion have been drawn. Membrane phospholipid resonance linewidths, under certain conditions, are a measure of the overall transverse (T_2) relaxation rates, which in turn are related to relatively low frequency inter- and intramolecular motions. The spin-lattice (T_1) relaxation rates are determined by faster intramolecular motions. High resolution NMR spectra of several biological membranes have been obtained, but, as yet, few relaxation studies have been reported. A difference in the ^{1}H resonance linewidths and ^{13}C T_1 relaxation times of the choline methyl (N^+Me_3) resonance of sarcoplasmic reticulum vesicles and liposomes of extracted phospholipids has been interpreted to indicate protein– lipid interactions in the head-group-region of this membrane [1]. In the study reported herein we use ^{1}H relaxation behavior in a similar comparative manner to demonstrate and characterize interactions between the photoreceptor protein rhodopsin and phospholipids in the retinal rod outer segment (ROS) disc membrane.

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** Recent analyses in our laboratory (W. L. Stone and E. A. Dratz, in preparation) indicate that our preparations contain < 2% by weight cholesterol in total ROS lipids.

2. Materials and methods

Highly purified ROS membranes were isolated by the method of Raubach et al. [2]. All buffers contained 0.1 mM EDTA and 0.15 mM $CaCl_2$ and were argon saturated immediately prior to use to minimize oxidative damage to the polyunsaturated fatty acids [3]. Manipulations were performed under an argon atmosphere whenever possible. ROS membrane vesicles were prepared by centrifugation of the purified ROS membranes and vigorous resuspension of the pellet in water containing 0.1 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetra-acetic acid (EGTA), pH 7.0. This procedure was repeated three times, followed by centrifugation and resuspension of the final pellet in 0.1 M BO_3^{-3}, 0.1 M KCl, 10^{-4} M EDTA buffer in D_2O at pD 7.0 (deuterated BO_3^{-3} buffer). The membrane suspension was allowed to soak for approx. 12 h, centrifuged, and the pellet resuspended in a small volume of deuterated BO_3^{-3} buffer to yield a membrane concentration of 30–60 mg/ml.

The total ROS lipids were extracted by a modification of the procedure of Folch et al. [4]. All solvents were freshly distilled and argon saturated. All manipulations were performed under an argon atmosphere. The solvents used for lipid extraction contained 50 mg/ml of the antioxidant butylated hydroxytoluene (BHT). 20–40 mg of extracted lipids were transferred by rotary evaporation to a small volume of $CHCl_3$ and applied to a 1.5 × 20 cm column of silicic acid. Retinol, retinal, cholesterol (< 4% by weight of total ROS lipids) [5] ** and BHT were removed by elution with $CHCl_3$: MeOH, 9 : 1, and the phospholipids

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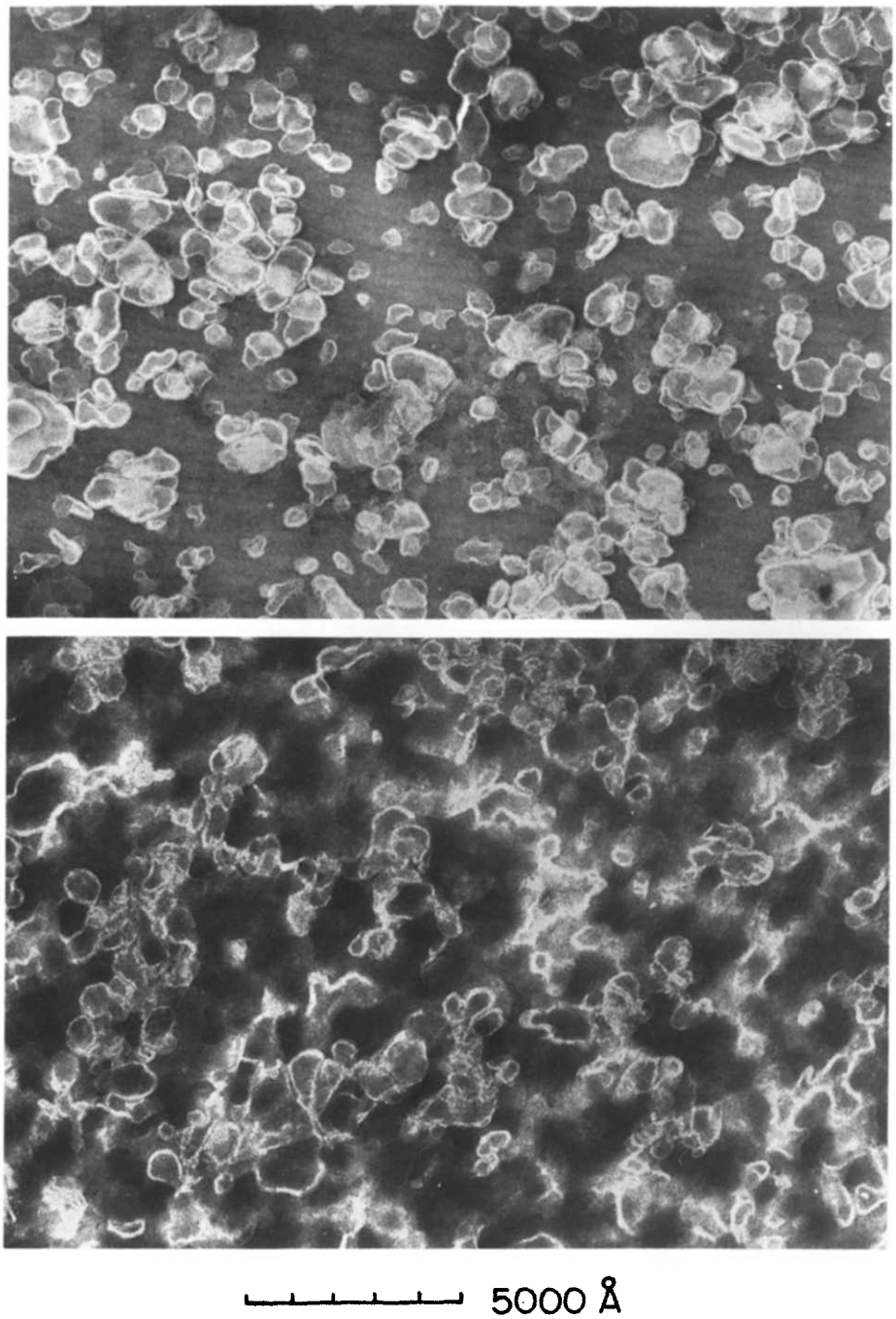


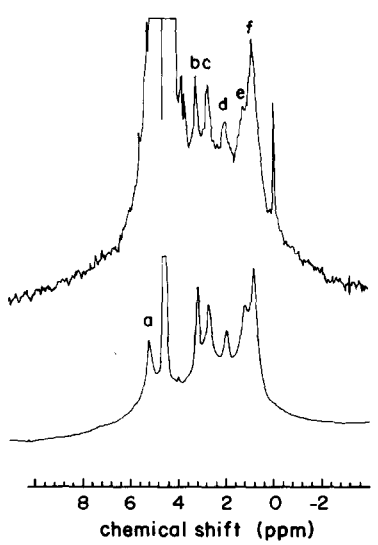
Fig.1. Electron micrographs of negatively stained ROS liposome (upper photograph) and ROS membrane vesicle (lower photograph) NMR samples. Both preparations were sonicated for 20 min as described in the materials and methods section. The ROS membranes were stained with 1% phosphotungstic acid and the ROS liposomes with 1% ammonium molybdate.

were then eluted with MeOH. The purified ROS phospholipids were dried to a film by rotary evaporation in a round bottom flask, hydrated with deuterated BO_3^{-3} buffer for approx. 1 h at room temperature, and swirled with several glass beads to disperse the phospholipids.

The water-washed ROS membranes and ROS phospholipid dispersions were sonicated for 20 min in a 10 ml conical glass centrifuge tube, in an ice bath under a stream of argon, with the microtip of a Branson W140 20 kHz sonifier at a power setting of 4. Titanium particles were removed by a short, low-speed centrifugation.

3. Results

Figure 1 shows electron micrographs of negatively stained, sonicated ROS membrane vesicles and liposomes of extracted ROS phospholipids. The average vesicle diameter of both the ROS membrane and ROS liposome preparations is approx. 1000 Å. Figure 2 shows 100 MHz ^{1}H-NMR spectra of sonicated ROS membrane vesicles and ROS liposomes. In both cases the spectra contain sharp, well resolved resonances superimposed upon a broad component. The assignments of the sharp resonances [6] are indicated in fig.2. The fraction of the ROS phospholipid protons which appear as sharp resonances was estimated by



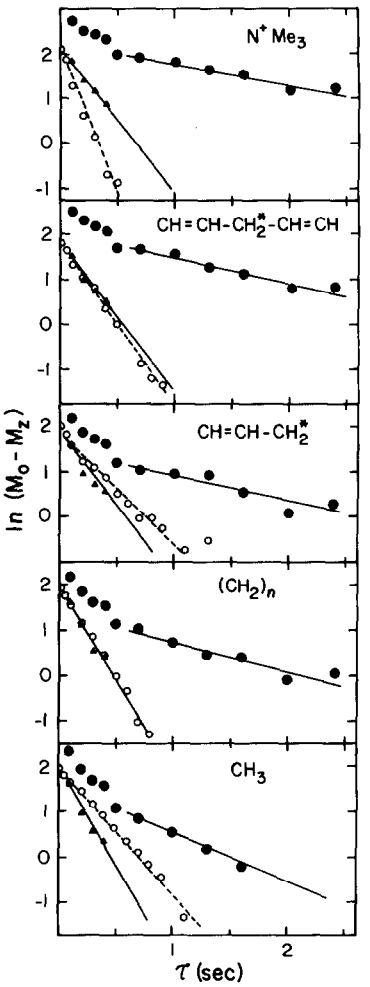


Fig.3. Comparison of 100 MHz inversion recovery vs τ plots for the sharply resolved resonances of the ROS membrane vesicles (•) and the ROS liposomes (○) at 25°C. The upper solid line in each panel is a least squares fit to the membrane recovery at long τ values and represents the slowly relaxing component. The slow component was subtracted from the membrane recovery to yield a fast component (▲), which was fit with the lower solid line using least squares. The dashed lines are least squares fits to the liposome recovery curves.

Fig.2. 100 MHz ^{1}H-NMR spectra of sonicated ROS membrane vesicles (upper spectrum) and ROS liposomes (lower spectrum). The assignments of the sharp resonances are: (a) CH=CH, (b) $N^+(CH_3)_3$, (c) $CH{=}CHCH_2^*CH{=}CH$, (d) $CH{=}CHCH_2^*CH_2$, (e) $(CH_2)_n$, and (f) CH_3. The spectra were obtained with a JEOLCO PFT-100 spectrometer in the pulse Fourier transform mode, employing a 2.5 s repetition rate between successive 90° pulses, approx. 8–10 Hz line broadening, and a 300 μs trigger delay.

choosing a curved base-line to approximate the broad, unresolved spectral component and comparing the integrated area to a known intensity standard. We estimate that 90–100% of the N^+Me_3 protons and 25–40% of the hydrocarbon side chain protons of the ROS phospholipids yield sharp resonance components in either preparation. The linewidths of the corresponding resonances of the ROS membrane vesicles and ROS liposomes are very similar.

The spin-lattice (T_1) relaxation rates of the ROS membrane vesicle and ROS liposome preparations were determined by the inversion recovery (180° – τ–90°) pulse method. Plots of $\ln(M_0\text{-}M_z)$ versus τ, where M_0 is the equilibrium magnetization and M_z is that sampled at $t = \tau$, are shown in fig.3. The spin-lattice relaxation rates of the sharp ROS liposome resonances are exponential, while those of the sharp ROS membrane resonances are biphasic, within experimental error. The rate of the more rapidly relaxing component of each membrane resonance appears roughly equivalent to that of the corresponding liposome resonance. The slow ROS membrane relaxation components have no parallel in the ROS liposome relaxation behavior. The inversion recovery behavior of the ROS disc membranes and ROS phospholipid liposomes is similar for sonicated and unsonicated samples and does not appear to depend strongly on whether a flat baseline or a curved baseline (to approximate the broad, unresolved spectral component) is used for evaluation of M_0 and M_z.

4. Discussion

We have obtained well resolved ^{1}H-NMR spectra of sonicated ROS membrane vesicles and protein-free liposomes of extracted ROS phospholipids. Differences in the relaxation behavior of these two preparations can be attributed to the presence of rhodopsin in the disc membrane since (a) rhodopsin comprises > 85% by weight of the disc membrane protein [7] and (b) the size distributions of the two preparations are similar, so that any effects of vesicle size are expected to be approximately the same.

The ^{1}H-NMR spectra of both the ROS membrane vesicles and ROS liposomes contain a comparable fraction of sharp and broad phospholipid spectral components. The linewidths of the corresponding sharp resonances of both preparations are similar, suggesting that rhodopsin does not greatly affect relatively low frequency motions of the ROS phospholipids such as large amplitude segmental excursions and lateral diffusion [8,9]. However, there are substantial differences in the ^{1}H spin-lattice relaxation behavior of the ROS membrane vesicles and ROS liposomes. The non-exponential T_1 relaxation rates of the resolved membrane resonances can be decomposed into two components, one of which seems to correspond to phospholipids interacting with rhodopsin and one to phospholipids in an environment similar to that in the protein-free ROS liposomes. Since the T_1 relaxation rates of phospholipid bilayer membranes are determined by relatively high frequency *trans-gauche* chain isomerizations [9], it appears that higher frequency segmental motions of the ROS phospholipids are predominantly affected by the interaction with rhodopsin in the disc membrane. It is very unlikely that the nonexponential T_1 relaxation behavior of the ROS membrane vesicles is due to the interaction of phospholipids with cholesterol, since cholesterol constitutes only 2–4% by weight of the ROS lipids and large changes are observed in the T_1 behavior of the N^+Me_3 resonance, which is least affected by cholesterol in model phosphatidylcholine bilayer membranes [10]. T_1 effects are observed for the terminal CH_3 and other hydrocarbon chain resonances, in addition to the N^+Me_3 headgroup resonance, so that the interaction of phospholipids with rhodopsin is propagated through at least one monolayer of the disc membrane bilayer.

Note added in proof

Recent experiments [11] indicate that the exponential spin lattice relaxation of liposomes of total ROS phospholipids is unchanged upon addition of 4% by weight cholesterol.

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