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Interaction of Rhodopsin with Two Unsaturated Phosphatidylcholines: A Deuterium Nuclear Magnetic Resonance Study†

Alan J. Deese, Edward A. Dratz, F. W. Dahlquist, and Michael R. Paddy*

ABSTRACT: Rhodopsin, prepared free of native lipid, was reconstituted with two unsaturated and specifically deuterated phosphatidylcholines: the minimally unsaturated 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine [(CD_3-16:0)(16:1)PC] and a highly unsaturated phosphatidylcholine typical of that found in native rod outer segment (ROS) membranes, 1-(16,16,16-trideuteriopalmitoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine [(CD_3-16:0)(22:6)PC]. Deuterium magnetic resonance (^2H NMR) spectra of these membranes and dispersions of the lipids alone were obtained at 23.0 MHz by using the quadrupolar echo technique. The apparent quadrupolar splittings are slightly less and the spectral features are somewhat broadened in the presence of the protein. Moment analyses of these spectra show that in the fluid phase the presence of rhodopsin at near physiological concentrations (1:1 w/w) does not change the average orientational order of either lipid. Rhodopsin does affect the structure of the bilayer, however, by causing an increase in the spread of the distribution of orientational order parameters about the average. Several interesting differences are observed in the phase behavior of the two lipids in the absence of rhodopsin. The orientational order of (CD_3-16:0)(16:1)PC and (CD_3-16:0)(22:6)PC is markedly different in the phase transition region, even though these two lipids have surprisingly similar phase transition temperatures. The 22:6-containing lipid exhibits a relatively large hysteresis (8–9 °C) in its phase transition, while no hysteresis is observed for (CD_3-16:0)(16:1)PC. Further, the phase transition for (CD_3-16:0)(22:6)PC occurs over a much smaller temperature range than that for (CD_3-16:0)(16:1)PC. Both the hysteresis and sharpness of the phase transition suggest a higher degree of cooperativity in the 22:6 lipid than in the 16:1 lipid. The presence of rhodopsin eliminates this hysteresis and sharp phase transition of the (CD_3-16:0)(22:6)PC and produces values of the average orientational order similar to those observed for the (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes, which are unchanged from the 16:1 lipid alone. Spin–lattice (T_1) and quadrupolar echo decay (T_{2e}) relaxation times were measured as a function of temperature for the lipids with and without rhodopsin. For all of the samples, T_{2e} is very sensitive to the gel to liquid crystal phase transition, while T_1 is relatively insensitive. In the fluid phase, rhodopsin decreases T_1 by approximately the same amount (about 2-fold) for both lipids while producing a 2-fold greater decrease in T_{2e} in the membranes reconstituted with the 22:6 lipid as opposed to the membranes prepared with the 16:1 lipid. This implies that rhodopsin differentially alters low-frequency reorientational motions of the two lipids. Overall, these results are consistent with our previous ^1H, ^{13}C, and ^{31}P NMR studies of native ROS membranes and ROS lipids: rhodopsin does not produce a long-lived, highly ordered population of lipids.

The vertebrate retinal rod outer segment (ROS)[1] disk membrane is the site of visual excitation. In these membranes, the chromophoric protein rhodopsin comprises at least 95% of the integral membrane protein (Krebs & Kuhn, 1977). Rhodopsin is deeply embedded in the (ROS) disk membrane bilayer (Dratz et al., 1979), and the polypeptide chain spans the membrane (Fung & Hubbell, 1978; Nemes et al., 1980). The fatty acids in the ROS membrane phospholipids are strikingly polyunsaturated; in cattle ROS, about 50% of the fatty acids are docosahexaenoic acid (22:6) (Miljanich et al., 1979; Stone et al., 1979). Most of the other fatty acids are the saturated palmitic (16:0) and stearic (18:0) acids. Although there is considerable heterogeneity in the head group and fatty acid composition of the phospholipids in the ROS membrane (Miljanich et al., 1979), it is reasonable to describe a typical phospholipid as having a saturated fatty acid at the *sn*-1 position of the glycerol backbone and a 22:6 fatty acid at the *sn*-2 position.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ROS, retinal rod outer segment; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; (CD_3-16:0)(16:1)PC, 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine; (CD_3-16:0)(22:6)PC, 1-(16,16,16-trideuteriopalmitoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; T_1, spin–lattice relaxation time; T_{2e}, time constant for the decay of the quadrupolar echo.

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The functional role of highly unsaturated phospholipids in biological membranes remains largely unknown. We would like to know if the highly unsaturated phospholipids of the ROS disk membrane have an influence on the way in which rhodopsin performs its function of converting light energy into electrical signals. The in vivo mechanism of action of rhodopsin has not been clearly established. Therefore, there is no direct means, at present, of determining the integrity of rhodopsin's native structure and function in vitro. However, there are indications from assays of the response of rhodopsin to light excitation that unsaturated phospholipids are needed to approach the native functional state of rhodopsin. For example, O'Brien et al. (1977) found that the first-order rate constant for the metarhodopsin I to metarhodopsin II transition in reconstituted rhodopsin systems decreased to a value slower than their measurement frame (greater than a 200-fold rate reduction) in saturated phosphatidylcholine bilayers as compared to the rate in the unsaturated egg PC system or native ROS. A functional role for unsaturated phospholipids is also suggested by physiological studies. For example, if rats have their diets restricted in unsaturated fatty acids, their retinas become less sensitive to light (Benolken et al., 1973).

Deuterium nuclear magnetic resonance (^{2}H NMR) has provided definitive information on the orientational order and dynamics of hydrocarbon chains in model lipid membranes (Seelig & Seelig, 1974, 1975, 1977; Davis, 1979). Over the past few years, ^{2}H NMR techniques have been extended to the study of protein–lipid interactions in model membranes (Seelig & Seelig, 1978; Oldfield et al., 1978; Kang et al., 1979a; Rice et al., 1979a,b; Paddy et al., 1981) and intact biological membranes systems (Stockton et al., 1977; Smith et al., 1979; Davis et al., 1979, 1980; Nichol et al., 1980; Kang et al., 1979b). These model membrane studies have utilized lipids with fully saturated or minimally unsaturated hydrocarbon chains.

In this paper, we report the use of ^{2}H NMR methods to investigate the effects of rhodopsin on the reorientational motions of two phosphatidylcholines differing in the degree of acyl chain unsaturation. Rhodopsin, free of native lipid, was reconstituted with one of two specifically deuterated phosphatidylcholines: a minimally unsaturated one, 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine [(CD_3-16:0)(16:1)PC], and a highly unsaturated phosphatidylcholine, typical of that found in native ROS membranes, 1-(16,16,16-trideuteriopalmitoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine [(CD_3-16:0)-(22:6)PC]. We have analyzed the ^{2}H NMR spectra using the method of moments and have measured the spin–lattice (T_1) relaxation time and the decay of the quadrupolar echo (T_{2e}) to examine the influence of rhodopsin on the average orientational order and motional rates of the terminal methyl region of these lipids as a function of temperature. The results indicate that above the phase transition temperature the average orientational order at the methyl terminus is virtually the same whether the reconstituted membranes contain (CD_3-16:0)-(16:1)PC or (CD_3-16:0)(22:6)PC at near physiological concentrations of rhodopsin. However, as shown by the relaxation measurements, the effect of rhodopsin on the dynamical properties of these two lipids is different. These dissimilarities may reflect differential lipid–protein interactions of the two lipids, although it is possible that they may result from differences in the interactions between the lipids themselves.

Materials and Methods

Rhodopsin Isolation and Delipidation. Highly purified bovine rod outer segments were isolated by the method of Raubach et al. (1974), except that the second sucrose density gradient was centrifuged for 90 min, and all solutions contained 0.1 mM EDTA and 0.15 mM $CaCl_2$ to minimize oxidative damage (Stone et al., 1979).

Rhodopsin purification was carried out by solubilization of the ROS disk membranes in 50 mM octyl glucoside with subsequent purification and delipidation on a concanavalin A column, according to the method of Stubbs et al. (1976). Typically, rhodopsin, free of native lipids (<0.1 phospholipid/rhodopsin), was obtained with a 280/500 nm absorbance ratio of 1.6–1.8. Lipid phosphorous determinations were carried out according to a modified (Miljanich et al., 1978) method of Chen et al. (1956).

Synthesis of (CD_3-16:0)(16:1)PC and (CD_3-16:0)(22:6)PC. 1-(16,16,16-Trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine was synthesized as described by Dahlquist et al. (1977). 1-(16,16,16-Trideuteriopalmitoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine was prepared from (CD_3-16:0)-lyso-PC and docosahexaenoic acid by a modification of the method of Cubero-Robles & Van den Berg (1969) (Miljanich, 1978). The purity of each of the lipids was carefully checked by thin-layer chromatography (TLC), gas–liquid chromatography (GLC), and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Each of the lipids was observed migrating as a single spot on silica gel TLC plates (developed in chloroform/methanol/water 65:25:5) with an R_f value similar to that of a standard phosphatidylcholine sample. GLC analysis of the synthetic lipids showed that the correct fatty acids were present and in a 1:1 molar ratio with no detectable contaminants. ^{1}H NMR spectra of the lipids dissolved in chloroform–methanol (9:1) followed by integration of the spectra showed that all of the lipid protons were accountable in the proper ratios.

Rhodopsin Reconstitutions. Reconstituted membranes were prepared by combining the octyl glucoside solubilized rhodopsin (usually 40–60 mg) with octyl glucoside solubilized lipid (40–60 mg) in a 100-mL round bottom flask. This mixture was stirred for several hours at 4 °C and dialyzed against nine 4-L changes of 20 mM Hepes, 0.1 M NaCl, and 0.1 mM EDTA, pH 6.8, for 3 days at 4 °C.

The reconstituted membranes were collected by centrifugation. The pellet was resuspended in buffer, applied to a linear (15–45%) sucrose gradient, and centrifuged for 4 h at 37000*g* in a swinging-bucket Sorvall rotor. Typically, only a single sharp band of reconstituted membranes was obtained. The rhodopsin concentration of the reconstituted membranes was determined from the 498-nm absorbance of an octyl glucoside solubilized sample on a Cary 14 spectrophotometer by using a molar extinction coefficient of 40000. No detectable rhodopsin bleaching occurred during the reconstitution step, as judged by the ratio of the 280/500 nm absorbance. All of the reconstituted membrane samples used in the data reported here had a protein to lipid weight ratio of 1:1 (the ratios ranged from 0.96 to 1.04). This corresponds to about 55 lipids per rhodopsin compared to about 65 lipids per rhodopsin in the native ROS disk membrane (G. P. Miljanich, S. Schwartz, and E. A. Dratz, unpublished results).

Biochemical Characterization of Reconstituted Membranes. Regeneration and flash photolysis studies were used to assess the intactness of the native structure and functionality of rhodopsin in the reconstituted membranes. For the regeneration studies, samples were light exposed (bleached) before adding a slight molar excess of 11-*cis*-retinal. Samples were then incubated at 37 °C in the dark for 1.5 h before their visible absorbance spectrum was obtained. The kinetics of the

metarhodopsin I to metarhodopsin II transition in these reconstituted membranes was studied by observation of the absorbance transients, at several wavelengths, following flash photolysis (J. Lewis, J. Winterle, P. Powers, D. Kliger, E. A. Dratz, unpublished results).

^2H NMR Methods. The ^2H NMR spectra and relaxation times were obtained at 23.0 MHz on a Nicolet NT-150 spectrometer equipped with an Oxford Instruments superconducting solenoid. All spectra were acquired by using the Fourier transform quadrupolar echo technique (Davis et al., 1976), which is capable of providing nearly distortion-free spectra (Davis, 1979; Valic et al., 1979). In obtaining the quadrupolar echo, 90° pulse lengths of 6.5–8.2 μs were used. The delay between the two pulses was 30–40 μs in the solid phase and 100 μs in the fluid phase. The pulse sequence was repeated at a rate of 4 s^{-1}. The rate of decay of the deuterium quadrupolar echo, $1/T_{2e}$, was measured from the maximum amplitude of the echo as a function of twice the separation of the two pulses in the quadrupolar echo pulse sequence. The deuterium spin–lattice relaxation time, $1/T_1$, was measured from the maximum amplitude of the echo signal as a function of the time between application of an inverting (180°) R_f pulse and the quadrupolar echo pulse sequence.

We have analyzed the ^2H NMR spectra using the method of moments. This method has recently been extensively applied in the analysis of ^2H NMR spectra of model (Davis, 1979; Paddy et al., 1981) and natural (Stockton et al., 1977; Smith et al., 1979; Davis et al., 1979, 1980; Nichol et al., 1980) membranes and thus will only be briefly discussed here.

For quadrupolar interactions in which the ^2H NMR line shape is symmetric about the Larmor frequency, the moments M_n of the half spectra are related to the moments S_n of the distribution of order parameters such that (Bloom et al., 1978)

$$M_n = A_n \times \frac{3}{4}\left(\frac{e^2qQ}{\hbar}\right)^n S_n \qquad (1)$$

where $e^2qQ/\hbar = 2\pi(1.67 \times 10^5)$ s^{-1} is the quadrupolar coupling constant for hydrocarbon chains (Burnett & Muller, 1971). In this paper, we use only $n = 1$ or 2 and thus require the values $A_1 = 2/[3(3)^{1/2}]$ and $A_2 = {}^1/_5$ (Bloom et al., 1978). The first two moments of the half-spectra, M_1 and M_2, determine the mean orientational order parameter $S_1 = \langle|S_{cd}|\rangle$ and its mean squared value $S_2 = \langle|S^2_{cd}|\rangle$. A comparison of S_1 and S_2 provides a measure of the fractional mean squared width of the distribution of orientational order parameters (Davis, 1979):

$$\Delta_2 \equiv \frac{S_2 - S_1^2}{S_1^2} = \frac{M_2}{1.35M_1^2} - 1 \qquad (2)$$

Hence, the method of moments provides a direct measure of the average orientational order of the sample and the width of the distribution of order parameters within the sample.

Results

Biochemical Characterization of Reconstituted Membranes. The absorption spectrum of (CD_3-16:0)(16:1)- and (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes, in 50 mM octyl glucoside, exhibits relatively low 280/500 nm (1.6–1.8) absorbance ratios, indicative of little or no bleached rhodopsin present. All of the reconstituted membrane samples have lower 280/500 nm absorbance ratios than rhodopsin in native membrane preparations, which presumably results from the removal of bleached rhodopsin and traces of other retinal photoreceptor proteins during rhodopsin purification. Regeneration and flash kinetic spectrophotometry studies were used to assess the intactness of the native structure and functionality of rhodopsin in the reconstituted membranes. All of the samples showed high regeneration values (>80%) of the characteristic 498-nm absorbance of rhodopsin. The kinetics of the metarhodopsin I to metarhodopsin II transition in the reconstituted membranes was quite comparable to that of native ROS disk membranes. For example, the metarhodopsin I to metarhodopsin II rate in the 22:6 lipid reconstituted membranes was 90 s^{-1} at 20 °C, compared to 70 s^{-1} for the native membranes.

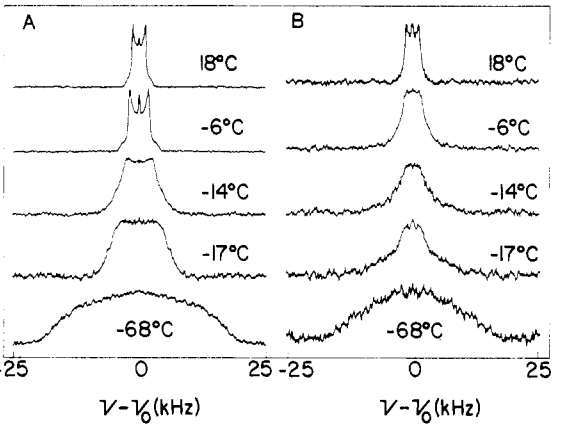

FIGURE 1: Figure 1: (A) ^2H NMR spectra of a dispersion of (CD_3-16:0)(16:1)PC as a function of temperature. Spectra resulted from 10 000–20 000 acquisitions at a rate of 4 s^{-1}. (B) ^2H NMR spectra of 1:1 (w/w) (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes as a function of temperature. Spectra resulted from 20 000–40 000 acquisitions at a rate of 4 s^{-1}.

^2H NMR Spectra of Pure (CD_3-16:0)(16:1)PC and (CD_3-16:0)(16:1)PC/Rhodopsin Reconstituted Membranes. Figure 1A shows ^2H NMR spectra of a dispersion of the pure, protein-free lipid (CD_3-16:0)(16:1)PC, as a function of temperature. The spectra exhibit the characteristic line shape of fluid phase phospholipids above about −5 °C. Below this temperature, spectral components typical of gel phase lipid begin to appear and presumably represent the onset of the fluid to gel phase transition. We have previously observed essentially identical temperature-dependent ^2H NMR spectra of this phospholipid in another buffer system (Paddy et al., 1981).

The spectra of pure (CD_3-16:0)(16:1)PC are to be compared to those of the (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes shown in Figure 1B. In this paper, the reconstituted membranes are prepared with a lipid/protein weight ratio of 1 which is near the physiological level. At temperatures well into the fluid phase of the pure lipid (5 °C or above), the two sets of spectra are very similar. The apparent quadrupolar splittings are slightly less in the presence of rhodopsin, but the main differences are that the spectral features of the reconstituted membranes are slightly broadened and less well-defined. The dissimilarities in the two sets of spectra are greatest in the region of the gel to liquid crystal phase transition. For example, at −6 °C, the spectra of (CD_3-16:0)(16:1)PC show the sharp features of the fluid phase, while the spectrum for the (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membrane is broad, rounded, and generally featureless. Discernible differences persist until well below the phase transition of the pure lipid, where both sets of spectra have features characteristic of the 16:1 lipid in the gel phase. There is no evidence at any temperature for a second, more highly ordered population of lipids in the (CD_3-16:0)(16:1)PC/rhodopsin sample.

The similarities and differences in the spectra of (CD_3-16:0)(16:1)PC in the presence and absence of rhodopsin may

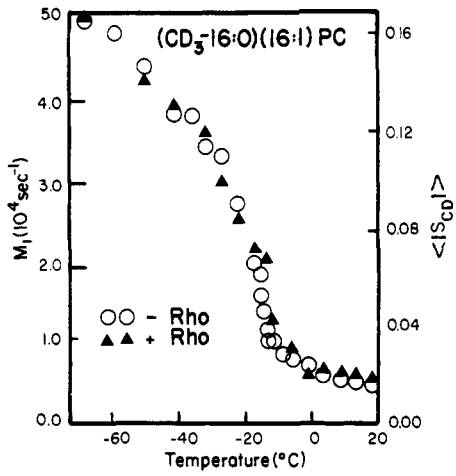

FIGURE 2: First moment (M_1) and average orientational order parameter ($\langle|S_{cd}|\rangle$) as a function of temperature for (CD_3-16:0)(16:1)PC alone (O) and 1:1 (w/w) (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes (▲).

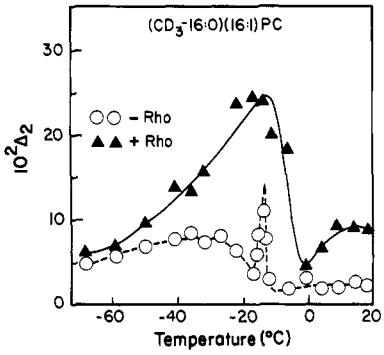

FIGURE 3: Mean squared fractional width in distribution of order parameters (Δ_2) as a function of temperature for (CD_3-16:0)(16:1)PC alone (O) and 1:1 (w/w) (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes (▲).

be characterized quantitatively by comparing the moments of the two sets of spectra. Figure 2 shows a plot of the first moment, which is proportional to the average orientational order of the system, as a function of temperature for (CD_3-16:0)(16:1)PC alone and the (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes. From Figure 2 it is clear that the average orientational order in the rhodopsin-containing sample is virtually the same as that which is observed in the sample without rhodopsin at all temperatures. This is in marked contrast to the large difference in the spread in the distribution of order parameters for the two samples, as shown in Figure 3. Here, the mean squared fractional width in the distribution of order parameters, Δ_2, is plotted as a function of temperature. At all temperatures the spread in the distribution of orientational order parameters is greater for the rhodopsin-containing samples. In the fluid phase, the rhodopsin-containing samples have Δ_2 values which are 3–4 times those of the lipid alone. Below the phase transition, Δ_2 of the reconstituted membrane does not approach the same value as that of the pure lipid until about −70 °C, and then only after a very gradual decrease from a maximum value near the phase transition of the pure lipid. Interestingly, Δ_2 has its largest value at about the same temperature in the presence and absence of rhodopsin, indicating that the phase transition temperature is changed little, if any, in the presence of rhodopsin. In summary, most of the qualitative differences in the appearance of the two sets of spectra in Figure 1 result from an increase in the width of the distribution of order parameters in the presence of rhodopsin, for it is clear from the first moment data that the presence of protein at this concentration has no effect on the average orientational order of the system.

^2H NMR Spectra of Pure (CD_3-16:0)(22:6)PC and (CD_3-16:0)(22:6)PC/Rhodopsin. ^2H NMR spectra of dispersions of (CD_3-16:0)(22:6)PC alone and a 1:1 (w/w) (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membrane are shown in Figure 4. The most striking feature in the spectra of (CD_3-16:0)(22:6)PC is the large hysteresis around the phase transition of this phospholipid, which is readily visible from about −3 to −12 °C in the spectra of Figure 4A. This hysteresis is also clearly shown in Figure 5, where the average orientational order is plotted as a function of temperature. Here one observes about an 8 °C difference in the temperature at which the average orientational order is midway between its high and low temperature limits, depending on whether spectra are acquired with decreasing or increasing temperature. Similarly, this hysteresis is observed in the plots of Δ_2 as a function of temperature, as shown in Figure 6, where there is about a 6 °C difference in the maxima of the Δ_2 values. This hysteresis is observed even though the sample temperature was changed quite slowly (~1–2 °C/h) in the hysteresis region.

Another interesting feature of the (CD_3-16:0)(22:6)PC spectra is the relatively narrow temperature range over which M_1 changes between its maximum and minimum values (Figure 5). The range of about 5 °C (with increasing temperature) is much smaller than the corresponding range for

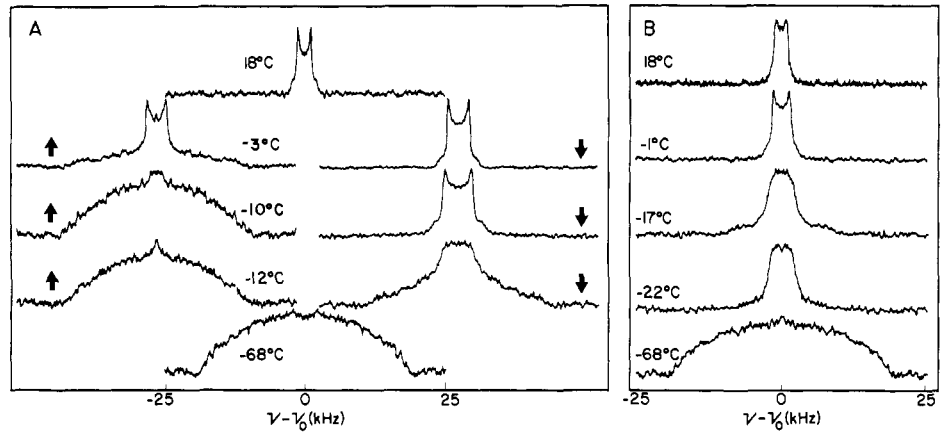

FIGURE 4: (A) ^2H NMR spectra of a dispersion of (CD_3-16:0)(22:6)PC alone as a function of temperature. ↓ indicate spectra acquired while decreasing temperature. ↑ indicate spectra acquired while increasing temperature. Spectra resulted from approximately 40 000 acquisitions at a rate of 4 s^{-1}. (B) ^2H NMR spectra of 1:1 (w/w) (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes as a function of temperature. Spectra result from approximately 40 000 acquistions at a rate of 4 s^{-1}.

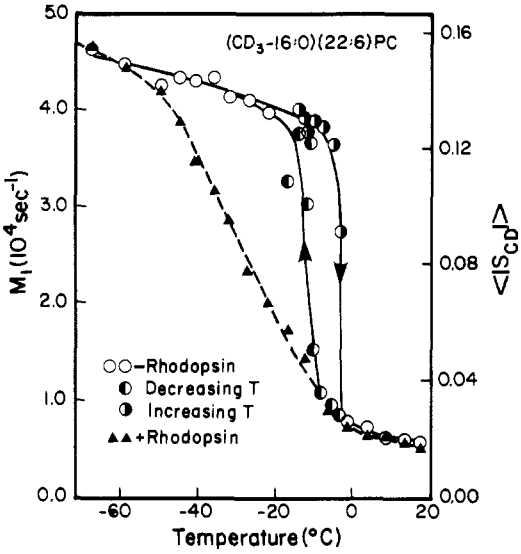

FIGURE 5: First moment and average orientational order parameter as a function of temperature for (CD_3-16:0)(22:6)PC alone (○, ◑, ◐) and (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes (▲) as a function of temperature. The symbol ◑ corresponds to spectra taken with increasing temperature, and the symbol ◐ corresponds to spectra taken with decreasing temperature.

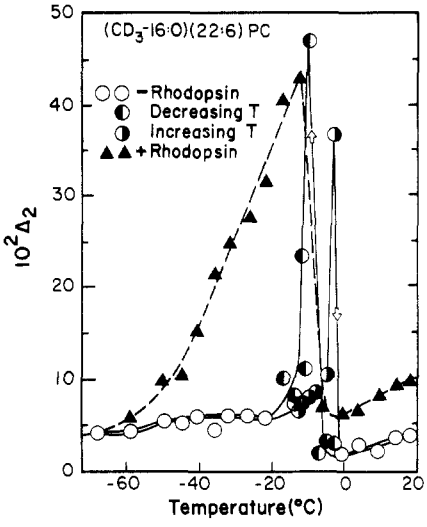

FIGURE 6: Mean squared fractional width in distribution of order parameters (Δ_2) as a function of temperature for (CD_3-16:0)(22:6)PC alone (○, ◑, ◐) and (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes (▲). The symbol ◑ corresponds to spectra taken with increasing temperature, and the symbol ◐ corresponds to spectra taken with decreasing temperature.

the (CD_3-16:0)(16:1)PC containing samples (compare to Figure 2).

The hysteresis and sharpness seen in the phase transition region for (CD_3-16:0)(22:6)PC alone is not observed when rhodopsin is incorporated in the membrane, as shown in Figures 5 and 6. However, in the fluid phase, (CD_3-16:0)-(22:6)PC and the reconstituted membrane formed from this lipid have the same average orientational order. Careful comparisons of the fluid phase spectra for (CD_3-16:0)(22:6)PC in the presence and absence of rhodopsin (see parts A and B of Figure 4) reveal the same general trends found for the (CD_3-16:0)(16:1)PC samples: very similar spectra with slightly lower apparent quadrupolar splitting in the presence of rhodopsin and significant differences in the sharpness of the spectral features. Dissimilarities become increasingly apparent as the temperature approaches the phase transition. The variation in temperature for (CD_3-16:0)(22:6)PC reconstituted with rhodopsin, shown in Figure 6, is generally similar to that observed for the (CD_3-16:0)(16:1)PC/rhodopsin sample (compare to Figure 3).

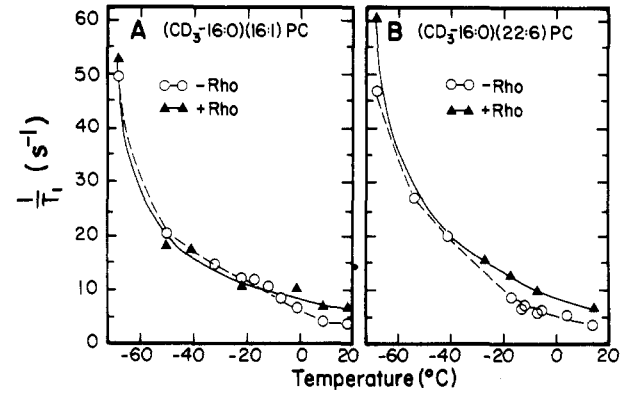

FIGURE 7: (A) Reciprocal of spin–lattice relaxation time ($1/T_1$) vs. temperature for (CD_3-16:0)(16:1)PC alone (○) and (CD_3-16:0)-(16:1)PC/rhodopsin reconstituted membranes (1:1 w/w) (▲). (B) Reciprocal of spin–lattice relaxation time ($1/T_1$) vs. temperature for (CD_3-16:0)(22:6)PC alone (○) and (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes (▲).

Immediately below the phase transition of the pure lipid, one begins to observe what appears to be two component type spectra for the (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes (see Figure 4B at −17 °C). This is not observed for the membranes prepared with the 16:1 lipid and rhodopsin. Interestingly, if one compares the first moment as a function of temperature for the 22:6 lipid reconstituted membranes (Figure 5) and the (CD_3-16:0)(16:1)PC/rhodopsin membranes (Figure 2), one observes that the plots are nearly superimposable, except for small differences in the temperature region where two component type spectra are observed for the membranes containing the 22:6 lipid. In this region, the first moment values for the 22:6 lipid are slightly smaller than those of the corresponding 16:1 lipid samples. Though the first moment vs. temperature plot for (CD_3-16:0)(22:6)PC differs significantly from those of the other three samples in the phase transition region, above 0 °C the first moment values for all four samples are equal within experimental error. That is, in the fluid phase, the average orientational order at the methyl terminus of the *sn*-1 palmitoyl chain is the same for all of the samples, irrespective of the lipid used or the presence of rhodopsin.

Spin–Lattice (T_1) Relaxation Measurements. The pure lipids and reconstituted membranes showed single exponential T_1 relaxation rates at all of the temperatures studied. A plot of $1/T_1$ vs. temperature for the (CD_3-16:0)(16:1)PC-containing samples is shown in Figure 7A. Figure 7B shows the corresponding data for the (CD_3-16:0)(22:6)PC-containing samples. In all cases, T_1 is fairly insensitive to the lipid phase transition. Indeed, Arrhenius plots of the $1/T_1$ relaxation rates (data not shown) show no apparent discontinuity in the region of the phase transition for any of the samples. Rhodopsin increases the $1/T_1$ relaxation times of the terminal methyl resonance of both lipids about 2-fold relative to the pure lipid samples in the fluid phase. The two lipids alone have about the same T_1 values in the fluid phase. Thus, the rhodopsin-containing membranes have essentially the same T_1 values in the fluid phase.

Quadrupolar Echo Decay (T_{2e}) Measurements. The decay of the ^2H quadrupolar echo (T_{2e}) was measured for all the samples as a function of temperature. At all temperatures and in all samples, this decay is well fit by a single exponential process. Plots of $1/T_{2e}$ vs. temperature are shown for the

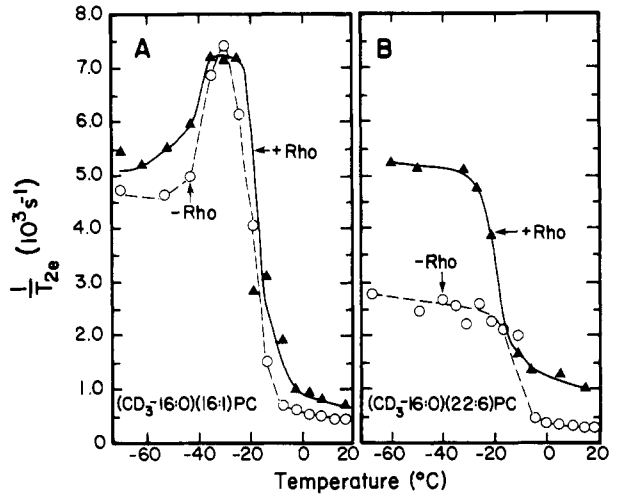

FIGURE 8: (A) Rate constant for the decay of the quadrupolar echo ($1/T_{2e}$) vs. temperature for (CD_3-16:0)(16:1)PC alone (○) and (CD_3-16:0)(16:1)PC/rhodopsin reconstituted membranes (▲). (B) Rate constant for the quadrupolar echo ($1/T_{2e}$) vs. temperature for (CD_3-16:0)(22:6)PC alone (○) and (CD_3-16:0)(22:6)PC/rhodopsin reconstituted membranes (▲).

(CD_3-16:0)(16:1)PC samples in Figure 8A and the (CD_3-16:0)(22:6)PC samples in Figure 8B. In contrast to $1/T_1$, $1/T_{2e}$ is highly sensitive to the lipid phase transition. In all of the samples, $1/T_{2e}$ increases dramatically as the temperature is lowered through the phase transition. For the samples containing (CD_3-16:0)(16:1)PC, $1/T_{2e}$ decreases again as the temperature is lowered beyond about −25 °C.

Of special interest to us is the effect of rhodopsin on $1/T_{2e}$ in the fluid phase. In the presence of rhodopsin, $1/T_{2e}$ of the (CD_3-16:0)(16:1)PC lipid increases 1.5–2-fold relative to the lipid alone. A much greater effect of rhodopsin is observed with the (CD_3-16:0)(22:6)PC lipid, where $1/T_{2e}$ is increased 3.5-fold from the value for the lipid alone.

Discussion

The results presented here are consistent with the idea that in reconstituted membranes prepared from rhodopsin and (CD_3-16:0)(16:1)PC or (CD_3-16:0)(22:6)PC, there does not exist a long-lived population of lipids that is strongly ordered by rhodopsin. Several workers have recently reached the same general conclusion in ^2H NMR studies of other integral membrane proteins reconstituted with a single type of phospholipid (cf. Oldfield et al., 1978; Seelig & Seelig, 1978; Paddy et al., 1981). The lipids used in these other experiments contained either saturated or minimally unsaturated fatty acids. We have used both minimally unsaturated and highly unsaturated lipids characteristic of the ROS membrane and studied their interactions with rhodopsin.

Our observation that the average orientational order of (CD_3-16:0)(22:6)PC or (CD_3-16:0)(16:1)PC alone is unaffected by physiological concentrations of rhodopsin does not imply that rhodopsin is not influencing the ordering or motional properties of these lipids. Rhodopsin produces a substantial increase in the fractional width of the distribution of orientational order parameters as reflected by the 2–3-fold increase in Δ_2. Some of the lipids must have slightly increased average order while others have decreased order in the presence of rhodopsin. This may mean that in the presence of rhodopsin some lipids experience smaller amplitude fluctuations while others undergo larger amplitude fluctuations, producing the same average orientational order as observed for the lipids alone. While we are unable to give a detailed molecular explanation for this phenomena, such a change might result from a general steric perturbation of the fatty acid chains upon insertion of a membrane protein into a lipid bilayer. A similar result has been previously observed with cytochrome *c* oxidase (Paddy et al., 1981).

In the fluid phase, we detect no preferential ordering by rhodopsin of the terminal methyl region of one lipid over the other since the M_1 data show that the average orientational order is the same for both lipids. However, below the phase transition region for the (CD_3-16:0)(22:6)PC/rhodopsin sample, one begins to observe two component type spectra. This is not observed in the (CD_3-16:0)(16:1)PC/rhodopsin membranes. It is possible that at the low temperatures where two components are observed (cf. Figure 4B at −17 °C), the exchange rate between the free lipid and some of the lipid on the rhodopsin surface is sufficiently decreased that one is now able to observe two components on the ^2H NMR time scale. A more likely explanation is that there is some phase separation occurring to produce two distinct lipid environments and that exchange between these domains is slow. The exchange could be slow due to the size of the regions and may not be a result of an enhanced lifetime of the lipid–protein complex per se. A similar result has been previously observed by Kang et al. (1979a,b) with cytochrome *c* oxidase.

We observe large differences in the phase behavior of the 22:6 lipid and the 16:1 lipid in the absence of rhodopsin. The 22:6 lipid has about an 8 °C hysteresis in the phase transition region, as shown in Figures 5 and 6. No hysteresis is observed for the 16:1 lipid. Also, the width of the phase transition, as indicated by the range over which the average orientational order changes between maximum and minimum values, is much sharper for the 22:6 lipid than for the 16:1 lipid. Both the hysteresis and the sharpness of the phase transition suggest a higher degree of cooperativity in the phase transition of the 22:6 lipid as compared to the 16:1 lipid. It is interesting to note that the phase transition temperature (T_c) for the 22:6 lipid, as indicated by Δ_2, occurs at −10 or −3 °C (due to the hysteresis). One might have predicted that T_c for this highly unsaturated lipid would occur at a much lower temperature since the 16:1 lipid melts at about −12 °C. The apparently large degree of cooperativity and the relatively high T_c suggest that in the gel state the 22:6 lipid exists in a quite regular and relatively stable conformation. Indeed, the phase behavior of the 22:6 lipid is much more similar to that of the fully saturated phospholipids (Davis, 1979) than that of the unsaturated (CD_3-16:0)(16:1)PC.

As indicated by the first moment analysis, rhodopsin both broadens and lowers the temperature of the phase transition of the 22:6 lipid while having little or no effect on the phase transition of the 16:1 lipid. This might be interpreted as indicating a different type of interaction between rhodopsin and the two lipids. However, this result probably reflects differences in the strength of the lipid–lipid interactions in the gel phase between the 22:6 lipids compared to the 16:1 lipids. The second interpretation seems especially likely, considering that the (CD_3-16:0)(22:6)PC/rhodopsin M_1 vs. temperature data is nearly superimposable on the corresponding data for the (CD_3-16:0)(16:1)PC reconstituted membranes and the 16:1 lipid alone (compare Figures 2 and 5).

The T_{2e} relaxation data do suggest that rhodopsin may interact differently with the 22:6 lipid than with the 16:1 lipid. In the fluid phase, $1/T_{2e}$ values for the (CD_3-16:0)(22:6)-PC/rhodopsin reconstitutents are greater by about a factor of 3 relative to those of the 22:6 lipid alone. $1/T_{2e}$ for the (CD_3-16:0)(16:1)PC/rhodopsin sample is greater only by a factor of about 1.5 relative to that of the 16:1 lipid alone. The

fact that both of the rhodopsin-containing samples have the same value of $1/T_1$ indicates that the differences in $1/T_{2e}$ result from components of the spectral density at frequencies less than the deuterium resonant frequency (23.0 MHz) (Paddy et al., 1981). Several possibilities exist for these lower frequency motions. Since $1/T_1$ is essentially unaffected by the phase transition of either lipid (see Figure 7) while $1/T_{2e}$ is greatly affected (see Figure 8), the possibility exists that rhodopsin differentially influences the kinds of motional changes associated with going from the gel to liquid phase, such as low frequency, large amplitude chain wagging motions. Another possibility is a difference in a new kind of motion associated with the presence of the protein, such as that resulting from changing orientations while jumping on and off the protein surface (Paddy et al., 1981) or from sitting on the moving surface of a "squishy protein" (Bloom, 1980).

One simplified way of modeling the T_{2e} relaxation behavior is to assume a two-state model, with T_{2e} characteristic of states on or off the protein surface and a chemical exchange term which incorporates all other, slower frequency motions. Such a model has the general form (Paddy et al., 1981)

$$1/T_{2e} = X_f(1/T_{2e})_f + X_p(1/T_{2e})_p + (1.25 \times 10^{11}\ s^{-2})X_fX_p(S_p - S_f)^2\tau_{ex}$$

where, X_p and X_f represent the mole fraction of lipid on or off the protein surface, $(1/T_{2e})_p$ and $(1/T_{2e})_f$ represent the quadrupolar echo decay rate of lipid on or off the protein surface, and S_p and S_f are the orientational order parameters for lipid on or off the protein surface. τ_{ex} is the exchange lifetime for the interconversion of the two lipid domains. This model suggests four ways in which the differences in $1/T_{2e}$ observed for (CD_3-16:0)(22:6)PC and (CD_3-16:0)(16:1)PC may arise: (1) differences in the exchange lifetimes (τ_{ex}), (2) differing amounts of lipid interacting with the protein (X_p), (3) different inherent motional properties of the lipid on the protein surface ($(1/T_{2e})_p$), and (4) a variation in the order parameter difference, ($S_p - S_f$), for the two lipids on and off the protein surface. Two of these possibilities suggest stronger interactions between (CD_3-16:0)(22:6)PC and rhodopsin than between (CD_3-16:0)(16:1)PC and rhodopsin: a slower chemical exchange rate for (CD_3-16:0)(22:6)PC or more (CD_3-16:0)(16:1)PC molecules associated with the rhodopsin surface per unit amount of protein. The requirement for steric packing of the protein which is deeply embedded in the membrane lipid hydrocarbon suggests that the latter possibility is an unlikely one (Dratz et al., 1979).

If one assumes that all of the increase in $1/T_{2e}$ upon protein insertion comes from the exchange process, one can set a lower limit for the exchange rate (Paddy et al., 1981). The exchange lifetime obtained for the 16:1/rhodopsin sample is $\sim 10^{-6}$–10^{-7} s at 4 °C. This lifetime is within 2 orders of magnitude of the time required for the lipid alone to diffuse through sites in a bilayer lattice and is roughly the same lower limit that we have previously calculated for the exchange lifetime of 16:1 on the surface of cytochrome *c* oxidase (Paddy et al., 1981). With this model, the 2-fold increase in $1/T_{2e}$ observed with the 22:6 lipid thus would reflect a 2-fold longer exchange lifetime on and off the surface of rhodopsin, but this exchange would still be quite rapid.

The data presented here are for two-model membranes with highly simplified fatty acid compositions and phosphatidylcholine head groups. As such, one must be cautious in extrapolating these results to the native ROS disk membrane. Some of the complexity (molecular heterogeneity) in ROS lipids may be important for the lipid–protein interactions that occur in the native system. However, we have also studied intact, native, ROS disk membranes by using ^1H, ^{13}C, and ^{31}P NMR, and these results, to date, are consistent with the findings reported here. Rhodopsin does alter the motional properties of some of the lipids, but we do not find evidence for a highly ordered lipid component in the native or model ROS membrane system.

Acknowledgments

We thank Jim Lewis for flash photolysis measurements of metarhodopsin I to metarhodopsin II kinetics in reconstituted membranes.

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Structural and Functional Similarities of δ-Crystallin Messenger Ribonucleic Acids from Duck and Chicken Lenses†

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ABSTRACT: δ-Crystallin of the embryonic duck lens was compared with that of the embryonic chicken lens with respect to polypeptide composition, synthesis, and messenger ribonucleic acid (mRNA) sequences. Labeling experiments with [^{35}S]-methionine revealed that the duck δ-crystallin is composed of minor amounts of polypeptides with molecular weights near 50 000 (50K) and 49 000 (49K) and much greater amounts of polypeptides with molecular weights near 48 000 (48K) and 47 000 (47K), as judged by sodium dodecyl sulfate–urea–polyacrylamide gel electrophoresis. All four sizes of polypeptides were synthesized in similar relative proportions as found in vivo in a rabbit reticulocyte lysate supplemented with δ-crystallin mRNA isolated from the embryonic duck lens. Synthesis of the 48K and 47K δ-crystallin polypeptides was differentially reduced in duck lenses cultured in the presence of ouabain. This is similar to the differential reduction of synthesis of the lower molecular weight δ-crystallin peptides in embryonic chicken lenses demonstrated previously. R loops formed between duck or chicken δ-crystallin mRNA and a cloned chicken δ-crystallin cDNA and heteroduplexes formed between duck or chicken δ-crystallin mRNA and cloned chicken genomic DNAs containing δ-crystallin sequences showed that, except for the putative 5′ leader sequence, the duck and chicken δ-crystallin mRNAs have extremely similar nucleotide sequences. These data indicate considerable conservation of δ-crystallin throughout the approximately 100 million years of divergence between ducks and chickens. The findings also suggest a possible relationship between the structure of δ-crystallin mRNA and the differential reduction in synthesis of the lower molecular weight δ-crystallin polypeptides in ouabain-treated lenses of ducks and chickens.

Crystallins are highly conserved structural proteins which comprise at least 90% of the soluble protein of the vertebrate lens [see Harding & Dilley (1976) and Bloemendal (1977)]. There are four families of crystallins (α-, β-, γ-, and δ-crystallin) which differ in their biochemical and immunological properties. Each crystallin family is composed of multiple polypeptides.

δ-Crystallin differs from the other crystallins in that it is found only in the lenses of birds and reptiles [see Clayton (1974) and Williams & Piatigorsky (1979a)]. This crystallin has been most intensively studied in the chicken. Chicken δ-crystallin is a tetrameric protein with a native molecular weight near 200 000 (200K) (Piatigorsky et al., 1974). The δ-crystallin polypeptides may be fractioned into a minor band of 50K polypeptides and a major band of 48K polypeptides by sodium dodecyl sulfate ($NaDodSO_4$)–urea–polyacrylamide gel electrophoresis (Reszelbach et al., 1977). Both δ-crystallin polypeptides are synthesized in a heterologous cell-free system, suggesting that each is encoded by a separate mRNA (Reszelbach et al., 1977). The δ-crystallin polypeptides are extremely similar (Piatigorsky, 1976) and, to date, have been found to differ from each other by only two tryptic peptides (Shinohara et al., 1980). Despite their similarity, the ratio of synthesis of the δ-crystallin polypeptides is markedly affected by the relative concentrations of Na^+, Cl^-, K^+, and acetate in the cultured embryonic chicken lens (Shinohara & Piatigorsky, 1977; Shinohara et al., 1980) and in a reticulocyte lysate containing δ-crystallin mRNA (Shinohara & Piatigorsky, 1980). The basis for this unexpected finding is not known but may reflect some interesting structural feature(s) of the δ-crystallin mRNA(s).

In the present investigation, I have examined δ-crystallin synthesis in the duck in order to determine whether the ionically controlled alteration in the ratio of synthesis of the δ-crystallin polypeptides is confined to chickens or whether it is an evolutionarily conserved characteristic found in other species. Like chicken δ-crystallin, duck δ-crystallin is a tetramer with a molecular weight of approximately 200K and is fractionated by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis into at least two bands with molecular weights near 47K and 48K (Wiliiams & Piatigorsky, 1979b). These duck

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