

## BBA Report

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### A POSSIBLE ROLE OF RHODOPSIN IN MAINTAINING BILAYER STRUCTURE IN THE PHOTORECEPTOR MEMBRANE

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#### Summary

<sup>31</sup>P-NMR measurements demonstrate that at 37°C, independent of the photolytic state of the photopigment rhodopsin, the lipids in the photoreceptor membrane are almost exclusively organised in a bilayer. In strong contrast, the <sup>31</sup>P-NMR spectra of the extracted lipids are characteristic for the hexagonal H<sub>II</sub> phase and an isotropic phase. The isotropic phase is characterised by freeze-fracture electron microscopy as particles and pits on smooth surfaces, possibly indicating inverted micelles. These results suggest a structural role for rhodopsin in maintaining the photoreceptor membrane lipids in a bilayer configuration.

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It has recently been demonstrated that the lipid bilayer, while still the basic feature of biological membranes, is not the only conformational state in which the lipids in membranes can occur. <sup>31</sup>P-NMR studies have presented strong evidence that in the endoplasmic reticulum of rat, bovine and rabbit liver the phospholipids can undergo isotropic motion, which suggests that inverted micellar structures may be present in conjunction with the bilayer [1]. Model studies of water-phospholipid mixtures show that the preferred phase depends on the type of lipid [2–6]. Under most conditions the thermodynamically most stable phase of phosphatidylcholine is the bilayer. Unsaturated phosphatidylethanolamine also prefers the bilayer conformation at lower temperatures, but at increasing temperature it undergoes a phase

transition to the hexagonal  $H_{II}$  phase. This transition temperature is strongly dependent on the fatty-acyl chain composition. Saturated species can only form a bilayer, while egg phosphatidylethanolamine adopts the hexagonal  $H_{II}$  phase at about 25°C and dioleoylphosphatidylethanolamine at about 5°C. Other membrane lipids like cardiolipin [4, 6, 7] and monoglucosyldiacylglycerol [8,9] also adopt the hexagonal  $H_{II}$  phase at physiological temperature. Most interestingly, in mixtures of these lipids with phosphatid choline, lipidic particles of an inverted micellar nature associated with the bilayer have been observed both by freeze-fracture electron microscopy and by  $^{31}\text{P}$ -NMR [9, 10].

The presence of these different phases will considerably increase the dynamics of a biological membrane and might have important functional implications. It occurred to us, that the rod photoreceptormembrane might be a possible candidate for the occurrence of these other phases in view of its high content of exceptional highly unsaturated phosphatidylethanolamine.\* If so, this might be related to its very specialised function in light absorption and signal transduction, where in addition to the photosensitive membrane protein rhodopsin also the membrane lipids may be involved [11].

We therefore undertook a  $^{31}\text{P}$ -NMR and electron-microscopic study of both the isolated intact photoreceptor membrane and aqueous dispersions of its extracted lipids.

Rod outer segments are isolated in dim red light by means of sucrose density gradient centrifugation [12] or by means of isosmotic low ionic-strength Ficoll-sucrose gradients, which better preserve some functional aspects of the outer segments [13]. The rod outer segments isolated from the gradient are sedimented at low speed ( $3000 \times g$ , 15 min, 4°C). The sediment is cautiously resuspended in Mops-buffer (20 mM Mops, 140 mM NaCl, 5 mM KCl, 3 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 1 mM dithioerythritol, 0.1 mM EDTA, pH 7.2) to a final concentration of about 1 mM in rhodopsin, as determined by its absorption at 500 nm [12].

Retinyl-opsin membranes are obtained via reductive fixation of the chromophore (retinal) to the protein moiety of rhodopsin (opsin) by illumination in the presence of sodium borohydride [14]. In the concentrated bulk quantities required for NMR, quantitative fixation could not be achieved (range 70–80%), but this range should easily permit detection of larger changes.

Lipids are extracted from either isolated, washed rod outer segments or isolated retinas by means of a slightly modified Folch-extraction [15]. In order to exclude Schiff base formation between retinal and amino-phospholipids the lipid source is illuminated in the presence of 100 mM hydroxylamine prior to extraction so as to convert all retinal into the oxime, and washed several times to remove excess hydroxylamine. The extracted lipids are finally suspended in Mops-buffer by shaking at 4°C under nitrogen (final concentration: 40–50 mg lipid per ml).

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\*Phosphatidylethanolamine amounts to about 45% of the phospholipids and in our preparations its main fatty acyl chains are on a weight base: 16 : 0 (9%), 18 : 0 (23%), 18 : 1 (3%), 20 : 4 (3%), 22 : 5 (4%) and 22 : 6 (54%). The cholesterol content of the lipids is less than 5% on a weight base.

Freeze-fracturing was performed according to well-established procedures [10].

$^{31}\text{P}$ -NMR measurements are performed at 36.4 MHz under conditions of high power proton decoupling on a Bruker WH-90, as described before [2]. Typically, 1.5 ml samples containing 60–75 mg of lipid (in the membrane corresponding to 1.2–1.5  $\mu\text{mol}$  of rhodopsin) were used, to which for the deuterium-lock 10% of the  $^2\text{H}_2\text{O}$  analog of the above-mentioned buffer was added. About 10 000 transients are accumulated with an 0.17 s inter-pulse time, using  $45^\circ$  radio frequency pulses. The resulting free induction decay was in most cases exponentially filtered resulting in a 50 Hz line broadening, which enhances the signal to noise ratio but decreases the resolution. Therefore, in spectra containing narrow resonances sometimes no filtering was applied.

Fig. 1 presents the NMR-spectra obtained for various photoreceptor membrane preparations. The unbleached rod outer segment preparation (Fig. 1A and B), which still has the stacked-disc structure as shown by electron microscopy [12], yields a spectrum with a number of sharp resonances superimposed on a typical broad asymmetrical resonance signal, which by comparison with model spectra [2, 3, 5, 16–19] indicates an almost perfect bilayer assemblage. This corroborates results obtained by röntgen diffraction [20, 21]. The sharp resonances, which are best resolved in Fig. 1B, represent soluble phosphate compounds (phosphate, sugarphosphates, nucleotides etc.), since they disappear upon hypotonic lysis of the outer segment structure (Fig. 1D). Lysis produces mainly large single-walled vesicles [12], but no change is observed in the NMR-spectra, indicating preservation of the lipid bilayer. A small isotropic component (less than 5%) derives from small vesicles, generated during lysis, washing and resuspension of the sample.

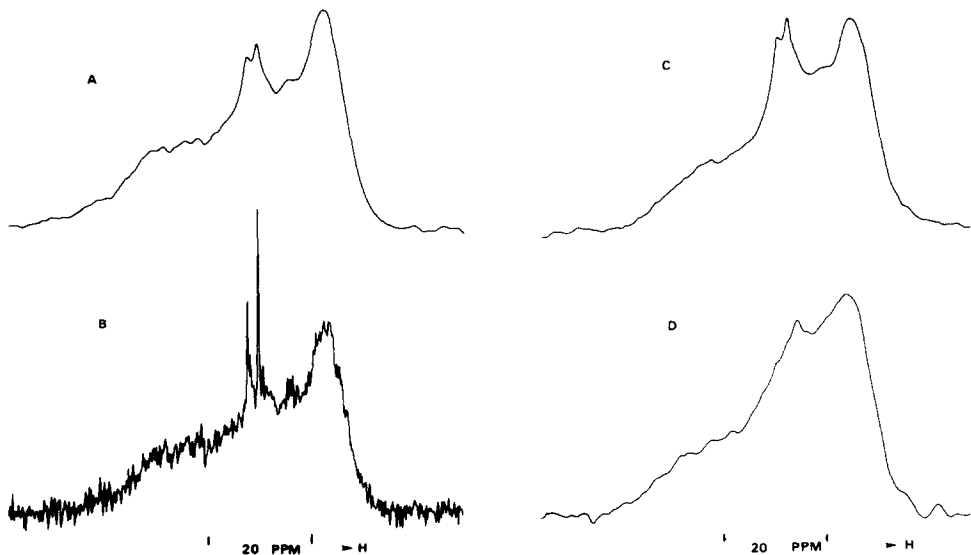


Fig. 1.  $^{31}\text{P}$ -NMR spectra obtained at  $37^\circ\text{C}$  of rod outer segments in the dark (A,B), after illumination (C) and following hypotonic lysis (D). Spectrum B is identical to spectrum A except that no filtering of the free induction decay was performed.

Upon illumination of the outer segments, resulting in a rather stable mixture of opsin, free retinal and metarhodopsin III [22], the NMR-spectra again remain practically identical except for a small (15 Hz) upfield shift of the largest sharp resonance, which based on its chemical shift most likely arises from free phosphate (Fig. 1C). This shift would be consistent with an acidification by 0.2 pH units. Most important however it can be concluded that the starting and end state of the photolytic reaction show a very similar organisation of the membrane lipids. Actually, this is not too surprising, since biochemical and spectroscopical evidence also indicates only minor differences between rhodopsin and opsin [23–27]. However, the excitation-transduction coupling probably is a transient process. Therefore, it cannot be excluded that during the reaction a transient change in the organization of membrane lipids does occur. Recently, it has been reported that in a blocked photolytic intermediate, retinyl-opsin, additional protein sulfhydryl groups become available and the overall sulfhydryl group reactivity is considerably enhanced [28]. This intermediate might therefore represent an active state in the excitation mechanism. However, the NMR-spectrum of this intermediate (not shown) was practically identical to the spectrum of the isolated disc membranes shown in Fig. 1D. This suggests that the protein-conformation

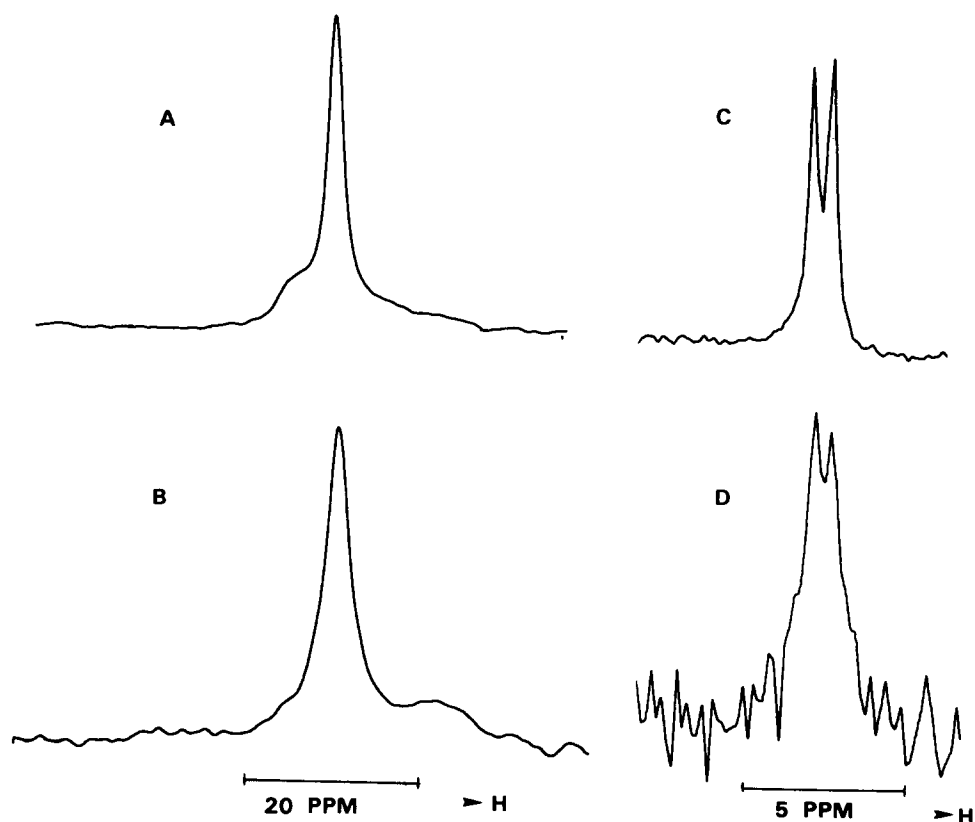


Fig. 2.  $^{31}\text{P}$ -NMR spectra of total rod outer segment lipids. (A,C), rod outer segment liposomes at  $37^\circ\text{C}$ ; (B), rod outer segment liposomes at  $4^\circ\text{C}$ ; (D), sonicated rod outer segment liposomes at  $37^\circ\text{C}$ . In spectra C and D no filtering of the free induction decay was performed.

change, manifesting itself in a different behaviour of sulphhydryl groups, does not significantly alter the organisation of the membrane lipids.

Very interestingly, the behaviour of the extracted rod outer segment phospholipids is quite different and more according to the expectations (Fig. 2). Under low resolution conditions at 37°C a large sharp resonance with a chemical shift of phospholipids undergoing isotropic motion is found next to a signal typical for the hexagonal  $H_{II}$  phase (Fig. 2A). Below 25°C the isotropic peak remains but is now associated with lipids in a bilayer configuration (Fig. 2B). Under high resolution conditions (no filtering of the free induction decay) the isotropic signal at 37°C consists of two narrow peaks with chemical shifts of phosphatidylcholine (upfield peak) and phosphatidylethanolamine (Fig. 2C). Sonication of the rod outer segment lipids leads to small structures of which the high resolution spectrum is shown in Fig. 2D. Interestingly, the relative intensities of the phosphatidylcholine and phosphatidylethanolamine peak are similar, but the linewidth of these peaks is much broader than in the isotropic phase of the rod outer segment lipids (Fig. 2C). Freeze-fracturing of lipid samples reveals numerous particles and pits, which at 37°C are associated with the hexagonal  $H_{II}$  phase (Fig. 3A, B), but at 4°C with a lamellar phase (Fig. 3C). In analogy with previous observations [8, 10] these results strongly suggest the existence of lipidic particles of an inverted micellar nature in the total rod outer segment lipid 'liposomes' at 37°C. Lipids extracted from entire retinas, which have a similar phospholipid distribution but are less unsaturated [15], show a similar behaviour, albeit that the isotropic component is less explicit. So far, we have not found conditions where the major part of the extracted rod outer segment lipids only adopts the bilayer conformation.

The above observations raise two important points:

1. The extracted lipids cannot function as a model system for the lipids in the photoreceptor membrane in view of their completely different configuration and behaviour. Pertinent studies comparing e.g. ion-permeability [15] or lipid-dynamics [29, 30] in rod outer segment membranes versus rod outer segment lipid 'liposomes' cannot therefore derive valid conclusions from such a comparison. Their exceptional organisation might explain why under certain conditions mere addition of 'liposomes' of rod outer segment lipids to lipid-depleted rhodopsin results in partial reconstitution without requiring the use of detergents [22].

2. Rhodopsin, being the predominant membrane protein (over 85%, w/w), must play a decisive role in organising the lipids into a bilayer. As the pure lipids avoid the bilayer configuration at physiological temperature, the following questions become intriguing: (a) how is this structuring effect of rhodopsin achieved, (b) how far does it reach laterally and (c) why has nature chosen this remarkable system for the organisation of the photoreceptor membrane. We can only guess how rhodopsin manipulates the lipid structure. One could conceive of a mushroom-like shape, the stem constructed of  $\alpha$ -helices permeating the membrane [25, 26], and the extending head interacting electrostatically with the lipids thereby forcing them into a bilayer. It is questionable, however, whether the small percentage of random structure in rhodopsin (10–20%) [24, 25] is sufficient for such a task. Alternatively, a

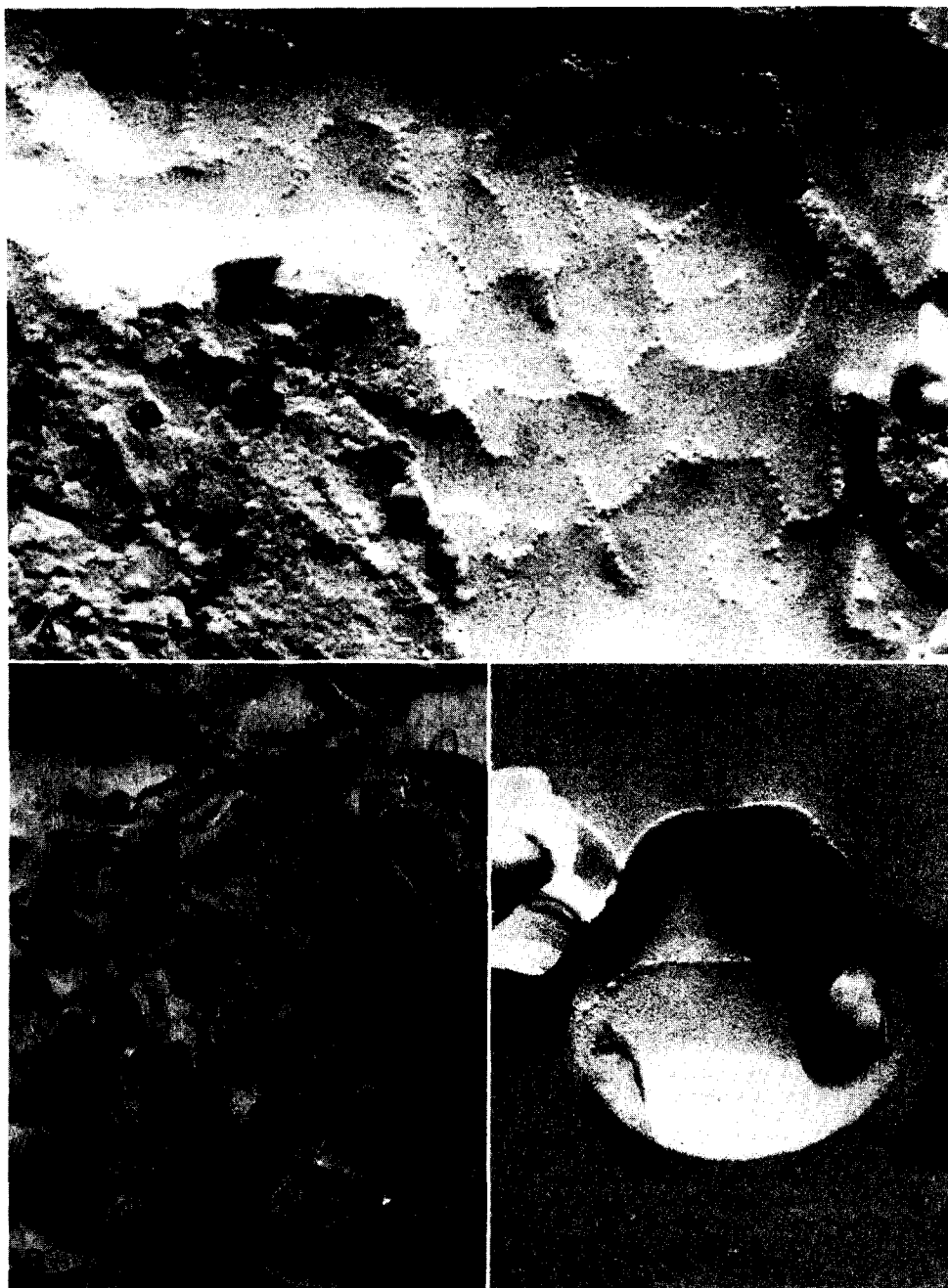


Fig. 3. Freeze fracture micrographs of total rod outer segment lipids (magnification  $\times 100\,000$ ). (A, B), rod outer segment liposomes at  $37^\circ\text{C}$ ; (C) rod outer segment liposomes at  $4^\circ\text{C}$ . The small particles and pits organised in strands probably represent inverted micelles [10], which can be associated with lamellar phases (A, C) or hexagonal  $H_{II}$  phases (like in B).

mere polarity gradient on the surface of rhodopsin might suffice to stabilise a lipid bilayer over a short range. This range must include at least two shells, as the average number of lipid shells between individual rhodopsin molecules will vary between three and five\*. Attempts to determine an approximate range by reconstitution experiments are in progress. It is tempting to suggest that the highly unsaturated lipids are required for a proper functioning of the photoreceptor membrane just because of their capability to change phases easily. However, so far we have found no evidence for major lipid changes either in the stabilised photointermediate retinyl-opsin or in the end-state of photolysis: opsin. This does of course not preclude transient local changes in lipid organisation during the excitation process, e.g. at the protein-lipid interface, which may escape detection by NMR.

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\* Taking an average lipid surface area of 1 nm<sup>2</sup>, cylindrical rhodopsin dimensions of 3 nm diameter and 7 nm length and a rhodopsin concentration in the outer segment of 3 mM. Per rhodopsin 60-62 phospholipids are present in the rod outer segments.

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