LACK OF INTERACTION OF RHODOPSIN CHROMOPHORE WITH MEMBRANE LIPIDS

An Electron–Electron Double Resonance Study Using ^{14}N:^{15}N Pairs

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ABSTRACT Electron–electron double resonance (ELDOR) has been applied to the study of specific interactions of ^{15}N–spin-labeled stearic acid with the retinal chromophore of a rhodopsin analogue containing a ^{14}N spin-labeled retinal. Both the 5 and 16 spin-labeled stearic acids were incorporated into the lipid bilayer of rod outer segment membranes containing the spin-labeled pigment. No interaction between the ^{15}N and ^{14}N spin-labels was observed in rhodopsin or the metarhodopsin II state with either of these labeled stearic acids. Therefore in this system the ring portion of the chromophore must be highly sequestered from the phospholipid bilayer in both the rhodopsin and metarhodopsin II forms.

INTRODUCTION

Rhodopsin is the visual receptor protein in the mammalian rod outer segment (ROS). The chromophore responsible for the absorption of visible light is a molecule of retinal, the vitamin A aldehyde, which is covalently linked to the ϵ-amino group of lysine 297 by a protonated Schiff's base (Wald et al., 1963; Hargrave, 1982). The chromophore binding site in opsin, the apoprotein, has specific structural requirements for the formation of stable pigments but can accommodate retinal in various isomeric configurations and with certain structural alterations (Balogh-Nair and Nakanishi, 1984). Derivatives of retinal have successfully been used for studying both the steric limitations of the binding site and the role of the chromophore in the physiological function of this photoreceptor pigment (Crouch, 1986). We have recently reported the formation of a stable photosensitive rhodopsin analogue containing a spin-labeled retinal 1 (Renk et al., 1987). Electron spin resonance (ESR) studies on this pigment showed the label to be deeply sequestered within the membrane in a hydrophobic environment.

The use of ^{15}N spin-labels in double-labeling experiments has been suggested as a means to study protein–lipid interactions (Bienvenue et al., 1978; DeVaux et al., 1981) and has been applied to conventional ESR studies of lipid–lipid and lipid–protein interaction (Seigneuret et al., 1981). More recently, Feix et al. (1984) applied another ESR techniques, electron–electron double resonance (ELDOR) to ^{14}N:^{15}N spin-labeled pairs in liposomes. We describe here the application of ELDOR double-label experimentation to the study of the spin-labeled retinal/rhodopsin system. In this work, we are attempting to detect a specific interaction between the retinal chromophore, which contains a ^{14}N spin-label and a ^{15}N spin-labeled stearic acid probe incorporated into the lipid bilayer of the native ROS disk membrane. No effect was seen in either the dark-adapted state, as expected on the basis of known properties of the pigment, or under conditions that favor the stable formation of metarhodopsin (meta) II state.

MATERIALS AND METHODS

All experiments were performed under dim red light or in the dark except where noted. ^{14}N–Stearic acid spin-labels were obtained from Syva Co. (Palo Alto, CA). ^{15}N–Stearic acid labels were synthesized by the method of Venkataramu et al. (1983). Bovine ROS were isolated from frozen retinae (Hormel, Austin, MN) as previously described (Papermaster and Dreyer, 1974). The synthesis and characterization of the 9-*cis* spin-labeled retinal (SLR) analogue and the formation and characterization of corresponding SLR rhodopsin pigment (λ_{max} = 448 nm) have been reported (Renk et al., 1987). The meta I and II states of this pigment were characterized by absorption spectroscopy (model 2200; Varian Associates, Inc., Palo Alto, CA). The sample was suspended in 10 mM Tris acetate, at pH 8.5 containing 65 mm KCl, 0.2 mm $CaCl_2$, and 0.2 mm $MgCl_2$ or 10 mM sodium acetate/acetic acid with the same salts at pH 4.5. The pigments were irradiated as suspensions and kept at constant temperature in cuvettes in the spectrometer. Samples were irradiated with white light via fiber optics. To change the pH of the sample, the pigment was centrifuged in the cold at 1,000 *g* in the dark and resuspended in the new buffer.

For ESR studies, suspensions of ROS membranes in the respective buffer were centrifuged for 5 min (Eppendorf microcentrifuge), and the pelleted membranes drawn into a capillary made of TPX, a methyl pentene polymer, and placed in the microwave cavity under a stream of nitrogen gas for 45 min to deoxygenate the sample (Popp and Hyde,

1981). Buffers used were as above. Stearic acid probes were incorporated into ROS membranes containing the SLR pigment by evaporating the solvent and leaving a film on the walls of glass bottles into which the ROS were introduced and incubated for 1–2 h. Probes were present at a concentration of 2% total lipid as estimated from the concentration of rhodopsin in the membranes, using a rhodopsin to lipid ratio of 65:1.

ELDOR instrumentation using the loop-gap resonator was as described by Hyde et al. (1985). Pump and observing frequencies are introduced directly into the loop-gap resonator, and the pump signal is isolated from the detector by using a high Q trap microwave filter. The frequency difference between the pumping and observing fields was set with a frequency counter and varied with the conditions of the experiment. A power meter (Hewlett-Packard Co., Palo Alto, CA) was used to measure the incident pump power. Temperature was controlled by a Varian nitrogen gas flow unit and was monitored by a copper-constant thermocouple immediately above the sample. Where required, ROS samples were irradiated with white light in suspension before sedimentation and introduction into the TPX capillary and cavity.

All ELDOR spectra were done in triplicate and signal intensities are reported as the average of three measurements. The observed peaks were normalized to another peak expected to be invariant as described in Results.

RESULTS AND DISCUSSION

The structures of the spin-labels used in this study are shown in Fig. 1. The SLR molecule contained a conventional ^{14}N spin-label and was combined with bleached rhodopsin to form a stable photosensitive pigment with a λ_{max} of 460 nm (Renk et al., 1987). The SLR was contained within the retinal binding site of the protein, which is expected to be sequestered from both the aqueous and lipid bilayer environments. The doxylstearic acid probes contained ^{15}N spin-labels at either the 5 or 16 position on the alkyl chain and were incorporated into the lipid bilayer.

The SLR pigment forms a meta II state (λ_{max} 380 nm) at pH 4.5 when irradiated at 2°C followed by warming to 20°C for 15 min. Resuspension at pH 8.5 at 2°C brought the λ_{max} to 440 nm, corresponding to the meta I state (Fig. 2). The equilibrium could be shifted to the meta II state by returning to the acidic pH of 4.5. The meta I species could also be formed directly by irradiation of the pigment at pH 8.5 at 2°C.

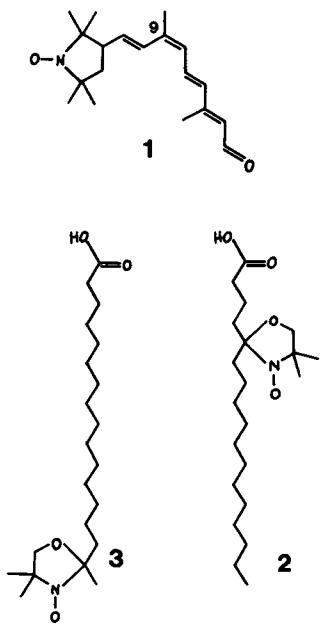

Figure 1 Structures of spin labels. (*1*) 9-*cis*^{14}N–spin-labeled retinal (SLR); (*2*) C-5 ^{15}N–doxyl stearic acid spin-label (5-SASL); (*3*) C-16 ^{15}N–doxyl stearic acid spin-label (16-SASL).

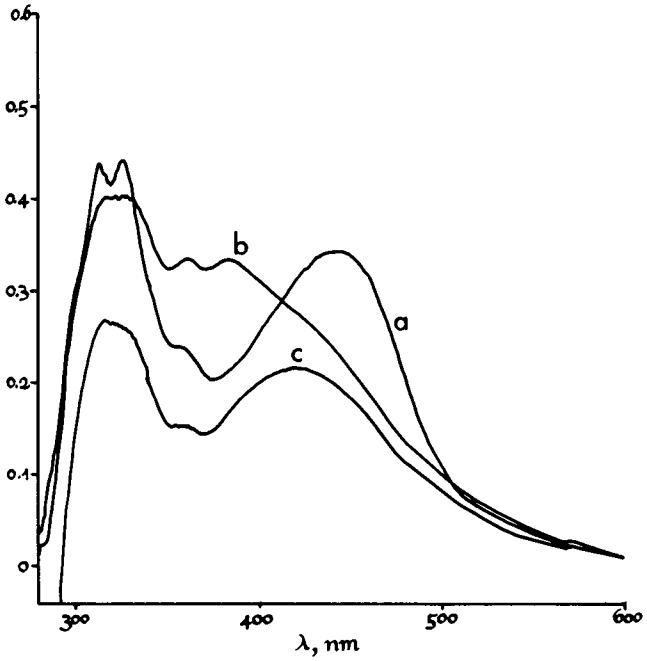

Figure 2 Absorption spectra of spin-labeled pigment and metarhodopsin states. (*a*) 9-*cis* SLR pigment at pH 7.4, 2°C, dark adapted; (*b*) meta II state formed at 2°C, pH 4.5 and maintained at 20°C for 15 min; (*c*) meta I, formed at 2°C, pH 8.5.

The conventional (V_1) ESR spectrum of the SLR pigment in the dark at 2°C (Fig. 3 *A*) is typical of a nitroxide, which is stongly immobilized with a $2A_{zz}$ value of 64.1 G.

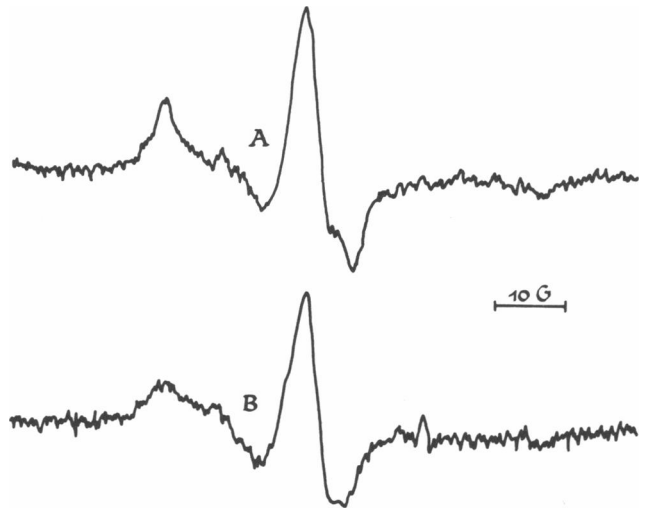

Figure 3 ESR spectra of SRL pigment. (*A*) SLR pigment ROS membrane containing 9-*cis* SLR; pigment dark adapted at 2°C in 10 mM phosphate buffer, pH 7.4. (*B*) Meta II state formed at 2°C in 10 mM NaAc/HAc, pH 4.5. Moderation amplitude 1.25 G, microwave power 7.5 mW.

When the pigment is irradiated at pH 4.5, 2°C, no significant change in the ESR spectrum is produced. When the pigment is allowed to reach 20°C for 15 min, the conditions that led to the formation of a meta II state, the ESR spectrum shows some subtle changes (Fig. 3 *B*). Specifically, there is a small decrease in the separation of the hyperfine extrema as well as some minor lineshape changes. These may result from changes in the rotational mobility of the spin-label or from differences in the relationship of the nitroxide to its surrounding protein environment.

The conventional (V_1) ESR spectra of the C-5 and C-16 doxyl-stearic acid spin-labels (5-SASL and 16-SASL) are shown in Fig. 4. The spectrum of the SLR pigment is highly immobilized on the ESR time scale (Fig. 3 *A*), which makes the resolution of ^{14}N:^{15}N spectral features (the foremost requirements of dual-label ELDOR experiments) less straightforward than when both members of the pair are mobile and such resolution is easily achieved (Feix et al., 1984). The $M_I = +1$ (low field) peak from the SLR spectrum was selected for ELDOR since this resonance was the most clearly separated from both the 5-SASL and 16-SASL spectra (Fig. 4, *a* and *b*).

ELDOR experiments with the SLR and the 16-SASL were performed in two configurations. In the first, the $M_I = -1/2$ resonance of the 16-SASL was pumped and the $M_I = +1$ resonance of the SLR was observed. This corresponded to a frequency difference between pump and observing fields of 41 MHz. Alternatively, since it was considered that reduction in the amplitude of the broad SLR resonance might be difficult to observe for a weak ELDOR effect, the SLR resonance ($M_I = +1$) was pumped, and the 16-SASL resonance ($M_I = +1$) was observed for reduction in amplitude (Table I). The experiment was conducted both in the dark-adapted state and under illuminating conditions that lead to the formation of a stable equilibrium favoring the meta II intermediate. At saturating pump powers up to 100 mW, no interaction was detected between the 16-SASL and the SLR pigment either in the dark or meta II state (Fig. 4 *d*).

TABLE I
LINEHEIGHT REDUCTIONS FOR SLR: 16-SASL

P^{-1}	R
W^{-1}	
3.3	−0.015
2.5	0.0125
2.0	−0.015
1.67	−0.015
1.43	0.0
1.25	−0.015

A nonsaturating field of 1.0 mW was used for the observed frequency. Pump frequency − observe frequency = 41 MHz. P, pump power; R, normalized ratio of line height with pump power on and off. The accuracy in R is ~±0.02.

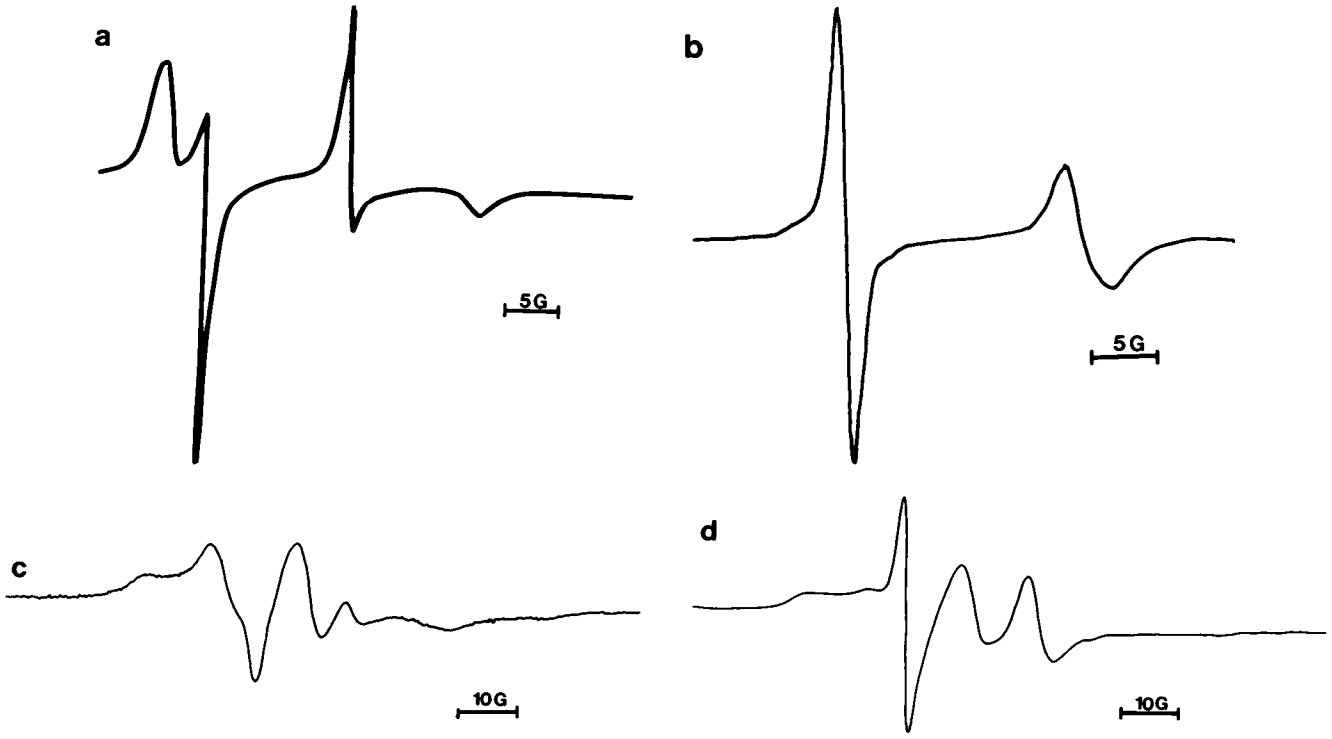

Figure 4 ESR and ELDOR spectra. (*a*) ESR spectrum of 5-SASL incorporated in ROS; (*b*) ESR spectrum of 16-SASL incorporated into ROS; (*c*) ELDOR spectrum of ^{15}N-5-SASL incorporated in spin-labeled rhodopsin pigment; (*d*) ELDOR spectrum of ^{15}N-16-SASL incorporated in spin-labeled rhodopsin pigment. All experiments on pigments performed in the dark.

Similarly, the SLR/5-SASL experiment was performed either by pumping the $M_I = -1/2$ resonance of the 5-SASL and observing the $M_I = +1$ resonance of the SLR or by reversing the pump and observed peaks. Both cases correspond to a pump-observed frequency difference of 26.6 MHz. As was the case for the 16-SASL experiment, no reduction in signal amplitude was seen at the observed frequency, indicating no interaction between the SLR and 5-SASL (Fig. 4 *c*).

The meta equilibrium of the SLR pigment requires a pH of 4.5 to favor the meta II intermediate in the SLR pigment. Under these conditions, the SASL carboxylates are substantially protonated and can undergo vertical fluctuations more deeply into the lipid bilayer (Feix et al., 1984). Thus the ESR spectrum of the 5-SASL shows a strong pH dependency between pH 4.5 and 9.5 (see Feix et al., 1984, Fig. 5) such that there appear to be two distinct environments occupied by this label, presumably corresponding to the protonated and unprotonated forms. While such low pH conditions are not ideal for examining the interactions among the SASLs (creating uncertainty as to their precise location along the bilayer normal), this should in fact provide an even greater opportunity for the stearic acid probes to encounter a given site exposed at the protein surface.

The lack of interaction between the ^{14}N-SLR and the ^{15}N-SASL molecules indicates that the chromophore of rhodopsin remains within a sequestered binding site in the opsin protein at least through the meta II intermediate in the photoactivation sequence. This result agrees with previous work with the SLR in which we demonstrated that the chromophore is also sequestered from the aqueous environment in this intermediate (Renk et al., 1987) and extends the assertion to include sequestration from the hydrophobic region of the surrounding lipid bilayer.

To observe bimolecular collisions between spin-labels in the ELDOR experiment, interaction must occur on a time scale comparable to the electron spin-lattice relaxation time (T_{1e}) of the observed species. Saturation recovery measurement of T_{1e} utilizing a pulse EPR spectrometer (Huisjen and Hyde, 1974; Hyde, 1979), gives a relaxation time from 4.0 + 0.5 μs (for SLR) to 2.0 + 0.1 μs (for ^{15}N-16-SASL). Consequently the ELDOR equipment would detect interaction occurring at a frequency of 0.05–0.025 MHz (i.e., 0.1 $T_{1e} - 1$), or one collision per 2–4 $\times$ 10^{-5} s, or greater. Even with a conservative estimate of 10^{-8} cm^2/s as a lateral diffusion constant for the lipid-analogue ^{15}N-SASLs, these probes would undergo a random walk over a radius of ~100Å in the above given time, allowing numerous opportunities for interaction with rhodopsin molecules. Thus the spin-label chromophore analogue is apparently not accessible to the bulk lipid of the ROS disk membrane in either the meta I or meta II state.

We considered three possible reasons for the lack of interaction between the SASLs and SLR. (*a*) The SLR could be exposed to the lipid environment, but at a site on the protein surface that is not explored by the SASLs. It is known that 16-SASL undergoes large amplitude vertical fluctuations in membrane bilayers, allowing the nitroxide moiety to approach the membrane surface even into the region of the bilayer occupied by 5-SASL (Feix et al., 1984, 1987), and we have observed interaction between ^{14}N–16-SASL and ^{15}N–5-SASL in the ROS membrane. This would appear to provide opportunity for SLR–SASL interaction regardless of the depth in the bilayer at which the pigment was exposed. Transfer of saturation between the ^{14}N and ^{15}N spin populations occurs via Heisenberg exchange (Feix et al., 1984), with an interaction distance of ~4.5 (Windrem and Plachy, 1980). Localization of the SLR chromophore in a "crevice" on the protein surface cannot be readily excluded, but this is effectively the same as sequestration within a protein core. (*b*) The SLR could be exposed at the protein surface, but interact only with a layer of boundary lipid. There is an extensive literature on boundary lipid in native and reconstituted ROS (Davoust et al., 1983; Pates and Marsh, 1987 and references cited therein). Pates and Marsh (1987) conclude that a motionally restricted population of lipids exists in ROS membranes due to association with the surface of monomeric (i.e., dispersed) rhodopsin molecules. However, it is noted that the bulk–boundary lipid exchange rate is on the order of 10^7 s^{-1} (Pates and Marsh, 1987; Davoust et al., 1983). This is similar to the exchange rate among phospholipids in the bulk phase, and more than two orders of magnitude faster than the time-scale limitations of the ELDOR experiment (discussed above). Interactions between a spin-label covalently bound to the hydrophobic surface of rhodopsin and both fatty acid (Seigneur et al., 1981) and phospholipid (Davoust et al., 1983) spin-lables have been observed. Thus interference by a long-lived boundary layer does not appear to explain our observed lack of interaction between SLR and the SASLs. In light of the above (as well as considerations of the ELDOR time scale in the preceding paragraph), the conclusion that (*c*) the SLR is sequestered within the protein structure away from the lipid environment, appears to be the most plausible explanation for our results at the present time.

It has been reported that the meta I–meta II transition is affected by the composition of the lipid bilayer (deGrip et al., 1983; Baldwin and Hubbell, 1985*a, b*). Our data indicate that the phospholipid effects do not directly involve the retinal chromophore, supporting the earlier conclusions that the influence of membrane lipids on aggregation state (deGrip et al., 1983) or protein conformation (Baldwin and Hubbell, 1985 *a, b*) are key factors in the meta I–meta II conversion.

In conclusion, the present data provide solid evidence for sequestration of the retinal chromophore from the phospholipid bilayer of the bovine ROS disk membrane through the meta II intermediate and complement previous results showing sequestration of the chromophore from the aqueous environment. The retinal chromophore seems

to interact primarily with the protein environment through the physiologically important stages of photobleaching, with only the Schiff's base able to interact with the aqueous environment. Any discussion of the photophysics of the late photoproducts must then focus on the retinal chromophore–protein interaction.

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