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# Spatial arrangement of rhodopsin in retinal rod outer segment membranes studied by spin-labeling and pulsed electron double resonance

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ABSTRACT

We have determined the spatial arrangement of rhodopsin in the retinal rod outer segment (ROS) membrane by measuring the distances between rhodopsin molecules in which native cysteines were spin-labeled at ~1.0 mol/mol rhodopsin. The echo modulation decay of pulsed electron double resonance (PELDOR) from spin-labeled ROS curved slightly with strong background decay. This indicated that the rhodopsin was densely packed in the retina and that the rhodopsin molecules were not aligned well. The curve was simulated by a model in which rhodopsin is distributed randomly as monomers in a planar membrane.



## 1. Introduction

Rhodopsin is a member of the G-protein-coupled receptor (GPCR) family that mediates a variety of transmembrane signal transduction activities. Rhodopsin is activated by light to form G-protein transducin, which leads to vision by a series of enzyme reactions. It has been suggested that several of the GPCRs form and function as dimers or oligomers [1–6]. Although previous biochemical and biophysical studies suggested that rhodopsin was a monomer [7–13], atomic force microscopy (AFM) showed that rhodopsin aligned as an oligomer in ordered rows in the native disk membrane [14,15]. Recent fluorescence or luminescence energy transfer (FRET or LRET) and chemical cross-linking experiments also showed that rhodopsin or opsin was presented as dimers in the liposome and cultured cell membranes [16–20]. However, the image obtained from AFM could be explained as a dehydration artifact [21]. Furthermore, a recent electron cryotomography study showed that rhodopsin is not aligned well and heterogeneously distributed in the membrane [22]. FRET and cross-linking experiments do not exclude the possibility of diffusible contact between rhodopsin monomers in the crowded membrane. Therefore, it remains necessary to reexamine whether rhodopsin is a monomer or a dimer in native retinal membranes.

Recently, pulsed electron–electron double resonance (PELDOR or DEER) spectroscopy have been used to investigate the distance between intra- and inter-subunit sites of spin-labeled protein with a high accuracy of up to 6–7 nm [23–26]. Jeschke et al. succeeded in assessing the oligomerization of membrane proteins by detecting a well-defined distance between singly spin-labeled residues in neighboring molecules by a modulation in PELDOR data [27]. Here, by using their method, we have tried to measure the distance between singly spin-labeled rhodopsin molecules in the rod outer segment (ROS) from swine retina by PELDOR. The echo modulation decay curved slightly with strong background decay, indicating that the distance distribution was very wide or not determined and that the rhodopsin was not aligned well.

## 2. Materials and methods

### 2.1. Preparation of rod outer segments (ROSs)

ROSs were isolated from fresh swine retina by a conventional sucrose flotation method [28] {34% (w/v) in ROS buffer [10 mM HEPES, 65 mM NaCl, and 2 mM $MgCl_2$ (pH 7.5)]}, followed by a stepwise sucrose gradient method (0.84, 1.00, and 1.14 M). Rhodopsin in the ROS preparation was regenerated in the presence of synthesized 11-cis-retinal. The ROSs were washed twice with ROS buffer supplemented with 7 M urea and then twice with deionized water. ROS membranes were collected by centrifugation at 30,000*g* for 40 min and dissolved in ROS buffer. The rhodopsin concentration was measured by an absorbance decrease at 500 nm (molar extinction coefficient of 40,600), after exposure to light in the presence of 50 mM hydroxylamine. All samples were prepared under dim red light.

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### 2.2. Spin-labeling of rhodopsin in ROS membranes

Typically, ROSs containing 5 μM of rhodopsin were reacted with 10 μM (sample 1) or 20–80 μM (sample 2) of (1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl)methyl methanethiosulfonate (MTSL) and incubated on ice for 14 or 2 h, respectively. The labeled membrane was washed twice with ROS buffer (30,000*g* for 30 min), and suspended in a small volume to prepare the samples for CW-EPR and PELDOR measurements at high concentration.

The concentration of MTSL was measured by double integration of the continuous-wave (CW)-EPR spectrum. The molar ratio of MTSL to rhodopsin for samples 1 and 2 (see above) was estimated to be 0.4–0.6 and 1.0–1.2 mol/mol, respectively. The concentration of rhodopsin was 40–80 μM for CW-EPR and PELDOR measurements.

### 2.3. CW-EPR and PELDOR measurements

CW-EPR spectroscopy was performed using a Bruker ELEXSYS E500 spectrometer equipped with a dielectric resonator, as described previously [24–26]. Samples (~15 μL) containing 0% or 30% sucrose were loaded into capillaries (inside diameter, 1.0 mm) and inserted into the resonator, and EPR spectra were acquired using 1-G field modulation amplitude at 100 kHz and 5- or 0.1-mW incident microwave power at 10–12 °C or 170 K, respectively.

We used pulsed electron–electron double resonance (PELDOR) to measure the distance between two spin labels. PELDOR was performed with an ELEXSYS E580 FT-EPR spectrometer (Bruker Biospin) according to Nakamura et al. [27]. Spin-labeled ROS samples (100 μL) containing 30% sucrose were loaded into quartz tubes (inside diameter, 4.0 mm). The measurement temperature was set at 80 K to lengthen the spin–spin relaxation time. The time trace of the electron spin echo (ESE) intensity was measured and analyzed as described previously [27–29]. To quantify the label-to-label distance distribution, we must separate the contribution of labels within the same rhodopsin molecules from the contribution of labels in neighboring molecules. The latter contribution corresponds to a smooth decay and is of the form $\exp(-kt)$ for a homogeneous spatial distribution of spins in three dimensions and of the form $\exp(-kt^{2/3})$ for a homogeneous planar distribution, as expected for molecules distributed in a membrane [30].

For light-activated ROSs, CW-EPR or PELDOR experiments were performed after rhodopsin in the ROSs was photolyzed at 1.0 cm with a fiber illuminator (Nikon, model C-fl115) through a cut-off filter (Toshiba, Y-52) for 10 s.

## 3. Results and discussion

We have searched for evidence of rhodopsin–rhodopsin interaction in the retinal outer segment (ROS) using spin labeling and the pulsed double electron resonance (PELDOR) method. This technique made it possible to extract a specific interaction from the background and nonspecific interactions at high protein concentrations.

### 3.1. Spin-labeling of ROS

The cysteines Cys140 and Cys316 were highly reactive to fluorescence and spin-labeling reagents [31,32]. We found that the labeling efficiency increased from ~0.5 mol of MTSL/mole of rhodopsin and almost saturated at ~2 mol/mol when the concentration of MTSL in the reaction mixture containing 5 μM rhodopsin was varied from 10 to 80 μM (Fig. S1 in the Supplementary information), which was consistent with earlier spin-labeling studies [32]. Most of the PELDOR experiments were performed for the preparations at ~1.0 or ~0.5 mol/mol in which Cys140 and Cys316 were spin-labeled but the latter was dominant, as the detailed characterization was described in the Supplementary information [32].

### 3.2. CW-EPR from spin-labeled ROS

The CW-EPR spectrum from the swine ROS was very similar to that from bovine ROS when the efficiency was 0.5–2 mol MTSL/mol rhodopsin [32] and showed fast, moderate and slow motional components whose relative populations depended on the labeling efficiency (Fig. 1). A small amount of fast component was observed in the 0.5 mol/mol preparation (Fig. 1A) and might be due to spin label that was unbound or bound weakly to some sites in the membrane. The fraction of fast component was quantified as the fraction of the spectrum of free MTSL which was subtracted from the whole spectrum to remove the fast component, and resulted in only a small amount (~1%). The 2 mol/mol preparation showed light-dependent mobility changes and reverse changes (Fig. 1C and D), but the 1 mol/mol preparation did not (Fig. 1B). Because it was reported that the spin label at Cys140 but not at Cys316 showed a light-dependent mobility change [32], Cys316 was dominantly labeled in the 1 mol/mol preparation, whereas Cys140 was additionally labeled in the 2 mol/mol preparation. This is also consistent with characterization of spin labeled residues as described in

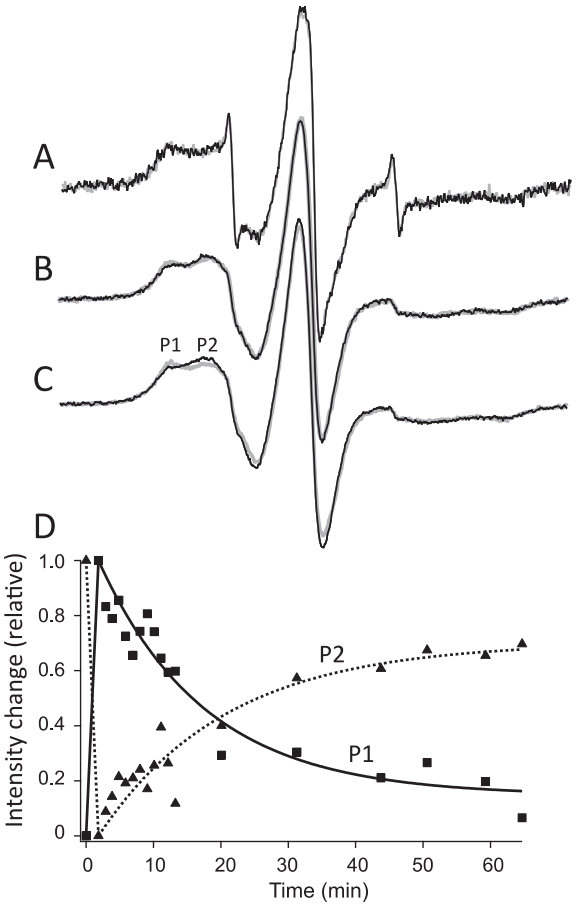


**Fig. 1.** The EPR spectra of spin-labeled rhodopsin with various labeling efficiency recorded before (dark line) and within 30 s after (gray line) illumination. The scan width is 100 G. (A) ~0.5 mole MTSL/mole rhodopsin. (B) ~1 mole MTSL/mole rhodopsin. (C) ~2 mole MTSL/mole rhodopsin. (D) Decrease and increase in amplitude of P1 and P2 in the lower magnetic field of (C) plotted against time, respectively.

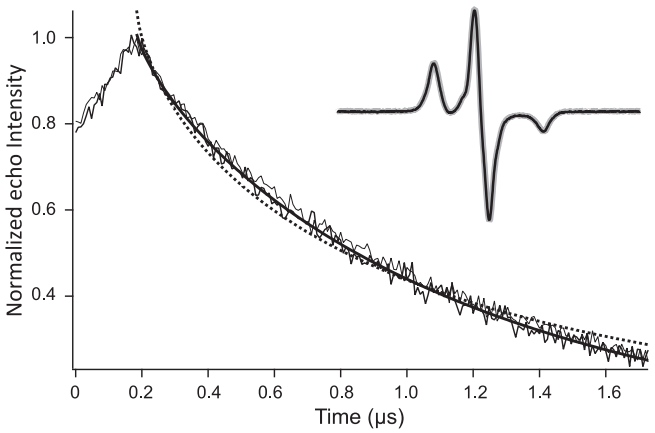


**Fig. 2.** Normalized PELDOR time-domain data for spin-labeled rhodopsin in a ROS membrane before (thick line) and after (thin line) illumination. The dotted and solid lines indicate smooth decay curves that fit the experimental curve obtained before illumination by assuming a planar distribution and a planar plus spatial distribution, respectively. The inset showed CW-EPR spectra from spin-labeled rhodopsin in the ROS membrane before (dark line) and after (gray line) illumination. The scan width is 200 G.

Section 3.1. Therefore, for measuring rhodopsin–rhodopsin interactions using PELDOR spectroscopy, we have used the preparation with ~1.0 mol/mol efficiency in which both Cys140 and Cys316 were spin-labeled but the latter was dominant.

### 3.3. PELDOR from spin-labeled ROS

To investigate how rhodopsin molecules distribute in the membrane of ROS, we measured the inter-rhodopsin distance using PELDOR at 80 K. The modulation of the electron spin echo (ESE) from the ROS containing 1 mol MTSL/mol rhodopsin decayed monotonically but slightly curved in a downward convex form (Fig. 2). On the other hand, CW-EPR spectrum from the same preparation at 170 K did not show any broadening in the line shape (Fig. 2, inset). These results suggest that a majority of spins are densely packed but have weak spin–spin interactions with broad distance distributions. These distance parameters could not be determined solely by spectral fit because the background curve is difficult to subtract from the monotonic decay curve. However, we assumed that the modulation depth was an ideal value (0.15) for the fraction of spins excited by the second frequency pulse. To obtain the best fit, we applied a single Gaussian distribution and a single background. The background curves were well fitted with a homogeneous spatial (three-dimensional) for the rhodopsin concentrations of 5–6 and 2–3 mM for labeling efficiencies of 1.0 and 0.5 (Table 1), respectively, which were consistent with the values estimated previously for ROS [22]. The background curves could not be successfully fitted with a homogeneous planar model. The resultant distances for the spatial background were similar between two different labeling efficiencies: 3.6 (full-width at half maximum of 2.7 nm) and 4.2 nm (full-width 3.1 nm), respectively (Table 1). However, this model might be unreasonable because the distance should have become longer and broader when the labeling efficiency decreased.

Next, we assumed that rhodopsin monomers distribute freely and randomly in the membrane plane (with no specific intermolecular interactions). Then, we assumed that the modulation depth was zero. First, we applied a planar model for fitting the overall decay curve. The local concentrations of the best fit were 5.9 and 3.0 mM for labeling efficiencies of 1.0 and 0.5, respectively (Table 1). However, the fit was much improved by assuming a sum of two distinct curves. We applied two distinct background curves produced from a spatial model at two distinct local concentrations. The result showed that 19% of the rhodopsin was packed at 10-fold higher density (22 mM) than the remaining 81% (3.8 mM). This model might be unreasonable because the higher density decreased slightly (18 mM) but the lower density decreased to half its value (1.8 mM) when the labeling efficiency decreased from 1.0 to 0.5 (Table 1). We also applied a sum of two distinct background curves produced from spatial and planar models. The result showed that half of the rhodopsin distributed on the membrane plane, whereas the other half distributed in the three-dimensional conditions. The fraction of rhodopsin on the membrane plane tended to increase as the ROS preparation was diluted (data not shown). The local concentrations were reasonably decreased from 4–6 to 2–3 mM when the labeling efficiency was reduced from 1.0 to 0.5 mol/mol, respectively (Table 1).

The spin-labeled ROS showed significant light-induced changes in the CW-EPR spectrum showing changes in the side-chain mobility of the Cys140 residue (Fig. 1C and D). We could not see the light-induced changes either in the modulation curve (Fig. 2) and the resultant parameters (Table 1) of PELDOR or in the CW-EPR spectrum (Fig. 2, inset) from the ROS containing 1 mol MTSL/mol rhodopsin. This suggested that the light-induced changes in the arrangement of rhodopsin did not occur in the ROS membrane. However, our PELDOR measurement could not sense a small change (<10%) in the populations in the spatial and planar models.

**Table 1**
Parameters of rhodopsin arrangement models for fitting PELDOR time-domain data.

| Label[a] | D/L[b] | Model | Background | Center (nm) | Width (nm) | Background parameter | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Frac. 1 | Conc. 1 (mM) | Conc. 2 (mM) | |
| 1.0 | D | Single Gaussian | Spatial | 3.6 | 2.7 | 1 | 5.2 | – | 0.00069 |
| | | Single density | Planar | – | – | 1 | 5.9 | – | 0.00192 |
| | | Two density | Spatial | – | – | 0.19 | 22 | 3.8 | 0.00068 |
| | | Two density | Planar + spatial | – | – | 0.51 | 6.0 | 4.5 | 0.00069 |
| 1.0 | L | Single Gaussian | Spatial | 3.6 | 2.1 | 1 | 7.8 | – | 0.00039 |
| | | Single density | Planar | – | – | 1 | 5.7 | – | 0.00190 |
| | | Two density | Spatial | – | – | 0.18 | 17.9 | 3.7 | 0.00037 |
| | | Two density | Planar + spatial | – | – | 0.46 | 4.0 | 5.7 | 0.00037 |
| 0.5 | D | Single Gaussian | Spatial | 4.2 | 3.1 | 1 | 2.4 | – | 0.00034 |
| | | Single density | Planar | – | – | 1 | 3.0 | – | 0.00040 |
| | | Two density | Spatial | – | – | 0.15 | 18.0 | 1.8 | 0.00034 |
| | | Two density | Planar + spatial | – | – | 0.71 | 3.3 | 1.9 | 0.00035 |

[a] Label per rhodopsin molecule.
[b] Dark or light.

### 3.4. Implications for the rhodopsin arrangement in ROS

Because the rhodopsin molecules were packed at the high-density condition, the PELDOR modulation due to labels in a specific dimer of rhodopsin molecules could not be separated from the background curve. By assuming that the modulation depth is zero and that rhodopsin distributes as a monomer, we found that rhodopsin distributed both in the homogeneous spatial (three-dimensional) and planar models in the membrane. This could be explained as follows. MTSL is located at Cys140 or Cys316 on the cytoplasmic side of the rhodopsin and then on the surface of ROS vesicles. When the ROS vesicles are crowded, the MTSL of a rhodopsin is close to that of the rhodopsin on the other ROS vesicles and that of the neighboring rhodopsin in the same ROS vesicle.

In summary, we have measured the distribution of the singly spin-labeled rhodopsin in ROS using PELDOR. The results indicate a model in which rhodopsin is distributed freely and packs as a monomer rather than a model in which rhodopsin forms a stereospecific dimer or is ordered in a constant lattice.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.040.

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