INTERACTION BETWEEN SPIN-LABELED RHODOPSIN AND SPIN-LABELED PHOSPHOLIPIDS IN THE RETINAL OUTER SEGMENT DISC MEMBRANES

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1. Introduction

In order to understand the role of phospholipids in biological membranes, it is very important to be able to determine whether specific proteins and specific phospholipids can come into contact. This relates to the problem of the specificity and on the frequencies of the interactions between phospholipids and proteins. We will show here that these problems can be approached by the spin-label technique, if one takes advantage of the spin–spin interactions between spin-labeled proteins and spin-labeled phospholipids.

Rhodopsin, an intrinsic protein, is chosen to study protein–lipid interactions. However line-broadening due to spin–spin interactions between spin-labeled lipids and spin-labeled rhodopsin could only be seen in bleached rhodopsin (opsin) preparations. Therefore these spin-label experiments give some insight not only into protein–lipid interactions but also into the localization of sulfhydryl sites of rhodopsin and on the modification of the configuration of the protein under the influence of light.

2. Materials and methods

2.1. *Preparation of rod outer segment membranes*

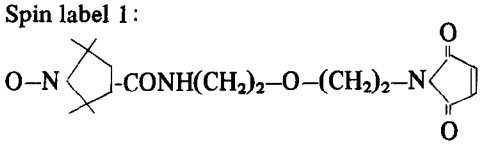
Rod outer segment (ROS) membranes were prepared from cattle retina as in [1]. Membranes were used fresh. All experiments were carried out using Na phosphate buffer, 20 mM, pH 7.4, bubbled with argon. Membrane suspensions were kept under argon during all incubations. Rhodopsin concentration was determined by $A_{500\,nm}$ after solubilization in 3% Ammonix LO. The ratio A_{280}/A_{500} was 2.1. Total protein content was determined by the Lowry method, using serum albumin as standard. Membranes were bleached by exposure to daylight, at room temperature, for 30 min.

2.2. *Spin labeling of the ROS membranes*

Two spin labels were used.

Spin label 1 was purchased from Synvar (Palo-Alto). It reacts with sulfhydryl groups of proteins.

Spin label 1:

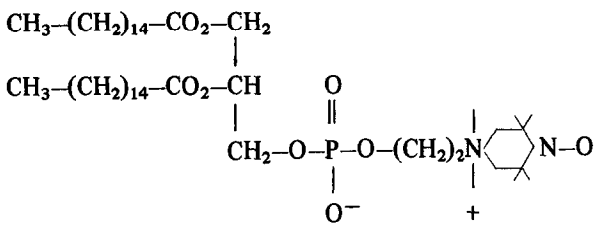


Membranes were labeled with spin label 1 either in the dark or after photobleaching. In a typical experiment, ROS membranes were incubated overnight, at 20°C, with 5 spin label molecules/rhodopsin. The unreacted spin labels were washed away by centrifugation (3 centrifugations in phosphate buffer at 100 000 × *g* for 30 min). Double integration of the ESR spectra combined with the optical absorption characteristics, permitted the determination of the amount of spin label bound/rhodopsin.

Spin label 2 is a phosphatidylcholine analog. It was synthetized as in [2].

Spin label 2 was incorporated into ROS membranes by fusion. However, it was found useful to react the SH groups of rhodopsin either with spin label 1 or with *N*-ethyl maleimide before the fusion process was performed. This preincubation was necessitated by the tendency of the pyrrolidinyloxyl group of spin

Spin label 2:



label 2 to react chemically with exposed sulfhydryl groups of rhodopsin. The fusion was performed with sonicated vesicles containing a 1:1 mixture of egg lecithin and spin-labeled phosphatidylcholine (spin label 2), because it is difficult to sonicate high concentrations of purely saturated lipids. The incubation medium (37°C) contained 10 mM spin label 2 and 1 mM membrane bound rhodopsin. Incubation was proceeded for 2 h. Unfused vesicles were washed away by centrifugation at 100 000 × *g*.

2.3. *ESR experiments*

A Varian E109 X band spectrometer was employed in the absorption mode. A 50 μl flat quartz cell was used together with a quartz dewar and a temperature control system. A capillary tube containing powdered manganese sulfide was attached to the flat part of the cell. It was used to give reference peaks, facilitating the subtraction of spectra. The spectrometer was connected to a computer (Tektronix 4051, 16 k memory), allowing accumulation, storage, subtraction and integration of the spectra.

3. Results and discussion

ROS membranes can be labeled readily with spin label 1. A maximum of 2 spin labels/rhodopsin are bound [3,4], in agreement with the number of sulfhydryl sites labeled with radioactive *N*-ethyl maleimide [5]. The ESR spectra is compased of relatively narrow lines (see fig.2a). These lines, as will be shown, can be broadened by spin–spin interactions with a second paramagnetic center.

3.1. *Line broadening by Ni^{2+}*

If 50 mM, Ni^{2+} is added to the membrane suspension, the spectrum 2a, of fig.2, is modified into 2b.

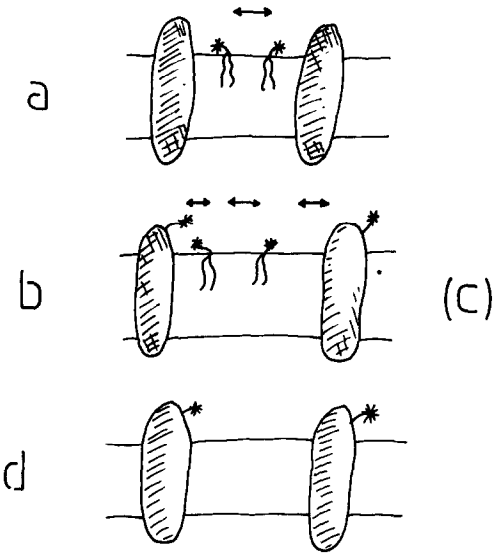


Fig.1. Schematic representation of the various types of spin-labeling used to observe lipid–protein interactions in ROS membranes. (a) 10% of the phospholipids are labeled on the polar head group (spin label 2). (b) The same ratio of phospholipids than in sample (a) are labelled, but this time the proteins also are labeled (spin label 1). This is the double-labeled sample. (c) Corresponds to the contribution of the proteins only in sample (b). (d) Only the proteins are labeled.

The broadening of the lines reveals that the paramagnetic centers in water interact directly with a large fraction of the membrane bound nitroxides. This type of experiment was shown [6] to be useful to demonstrate the accessibility of spin labels to water. Spectrum b of fig.2 therefore suggests that the 2 sulfhydryl groups of rhodopsin labeled by spin label 1 or by *N*-ethyl maleimide are located on the polar portion of rhodopsin and exposed on the outer face of the membrane.

If ROS membranes are bleached (before or after the labeling), the ESR spectra are unchanged, addition of Ni^{2+} broadens the lines to the same extent as before.

This simple experiment with Ni^{2+} illustrates how the proximity of a second paramagnetic center modifies the shape of the ESR spectra of nitroxides.

3.2. *Line broadening by spin labeled phosphatidylcholine analogs*

The ESR spectra of 2 samples containing 10% spin-labeled phospholipids, are shown in fig.3a,b. In

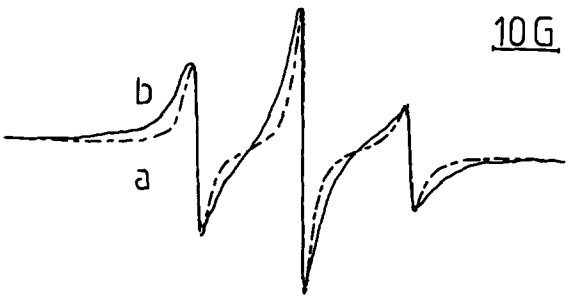


Fig.2. Effect of Ni^{2+} on the ESR spectrum of spin label 1 in ROS membranes. The spectrum (a) (dotted lines) is the spectrum before addition of Ni^{2+}; spectrum (b) was recorded after the addition of 50 mM $NiCl_2$. Spectra are normalized in such a way that the maximum has the same amplitude. Temperature is 24°C.

sample b, the proteins also are labeled. All samples used in this experiment were bleached beforehand for reasons which will be given later. Spectrum c (dotted lines) of fig.3 is the computer subtraction of spectrum a from spectrum b. The subtraction in principle should exactly cancel the contribution of the spin-labeled phospholipids in spectrum a. Yet spectrum c cannot be superimposed on the spectrum obtained directly with rhodopsin labeled with spin label 1 (spectrum d). No linear combination of spectrum d (narrow lines) and spectrum b, for arbitrary coefficients, can reproduce spectrum c. It must be concluded therefore that the double labeled sample does not give rise to a signal corresponding to the mere addition of the 2 spin contributions taken separately: the signal of spin label 1 is broadened by the presence of spin label 2 in the membranes. The broadening of spectrum c is analogous to the broadening described in section 3.1. and due to the interaction of spin label 1 with other paramagnetic groups close by. Hence a substantial fraction of the spin-labeled phospholipid interacts with the spin-labeled proteins in the double-labeled membranes.

Since electron spin–electron spin interaction for molecules tumbling rather rapidly can only take place at short distances, we can concluded that this experiment demonstrates the direct contact of the spin-labeled phospholipids with rhodopsin. This result also confirms the localization of the sulfhydryl groups of rhodopsin on the polar moiety of the protein.

We have mentioned that bleached samples were used for the experiments described above. The reason is that if all the procedures are conducted in the dark no interaction can be detected. This negative result gives more confidence in the former results with bleached membranes. Furthermore it indicates that bleached rhodopsin must have a somewhat different configuration from dark-adapted rhodopsin. This is consistent with the change of reactivity of the SH groups under the influence of light detected [5], and also with the modifications in the ESR spectra of spin-labeled rhodopsin following photobleaching detected

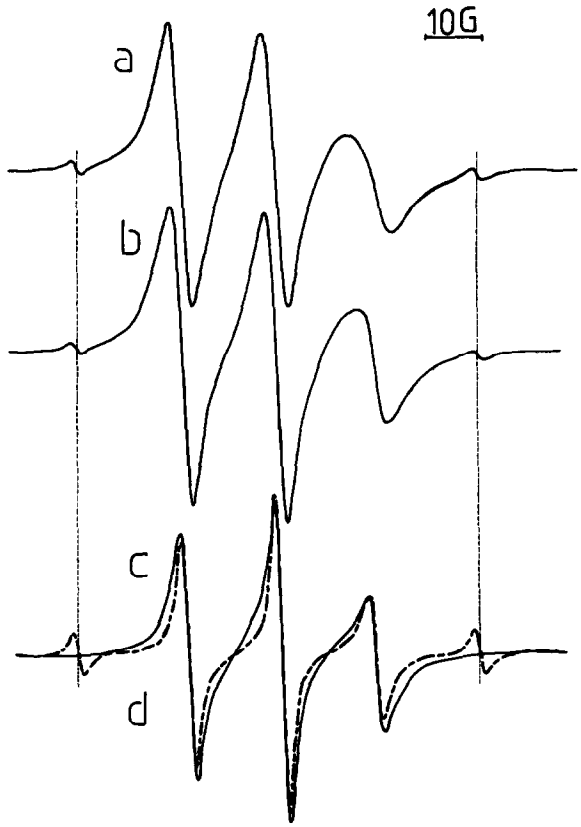


Fig.3. ESR spectra of the samples schematized in fig.1. Temperature 37°C. (a) Phospholipids are labelled; (b) phospholipids and proteins are labelled; (c) spectrum obtained by computer subtraction of (b) minus (a); (d) (dotted lines) only the proteins are labeled. Clearly (c) and (d) are not identical. The broadening of spectrum (c) was systematically observed if sample were bleached beforehand. If samples were not bleached spectrum (c) and (d) were always identical. The small peaks at the low and high field region are reference peaks (powder of manganese sulfide), these references are very important when performing the subtraction.

[7] using organomercuric spin labels. However if membranes are labeled in the dark and afterwards illuminated just before ESR recording, no spin–spin interaction is observed. This could be due to a modification in the molecular events accompanying the bleaching, attributable to the binding of spin label 1. In fact if ROS membranes are labeled with spin label 1, it takes much more time to see the usual change in color of the membranes once illuminated.

In summary, our results suggest that a possible modification of the position of 1 (or 2 ?) SH groups on rhodopsin accompanies the bleaching. However the main emphasis here is on the technical possibility of detecting direct contact between specific proteins and specifically-labeled phospholipids in a membrane. The broadening observed in our experiments (detectable even at 4°C), indicates that a phospholipid with 2 saturated chains, if diluted in the normal unsaturated phospholipids of ROS membranes, is not excluded from the direct vicinity of rhodopsin. This is an interesting observation.

A qualitative observation of the type described before does not allow a differentiation between long term interactions (dipole–dipole) or fast but frequent collisions (spin exchange). However a quantitative analysis, of the type in [8], should enable us to decide if the frequency of exchange is comparable to that of two phospholipids in the bulk of the membrane, as suggested [9]. It requires an extensive study of the dependence of the line shapes on concentration and temperature. If the exchange rates are much slower, actual numerical values would not be obtainable by conventional ESR; but a qualitative determination of whether or not the diffusion of phospholipids is slowed down in the vicinity of (or by interaction with) intrinsic proteins is itself very important.

Acknowledgements

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