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# DISTINCT STATES OF LIPID MOBILITY IN BOVINE ROD OUTER SEGMENT MEMBRANES

## RESOLUTION OF SPIN LABEL RESULTS

A. WATTS [a], J. DAVOUST [b], D. MARSH [a] and P.F. DEVAUX [b]

[a] *Max-Planck-Institut für biophysikalische Chemie, Abt. Spektroskopie, D-3400 Göttingen (F.R.G.) and* [b] *Institut de Biologie Physico-Chimique, 13 Rue Pierre et Marie Curie, F-75005 Paris (France)*

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## Summary

Freely diffusable lipid spin labels in bovine rod outer segment disc membranes display an apparent two-component ESR spectrum. One component is markedly more immobilized than that found in fluid lipid bilayers, and is attributed to lipid interacting directly with rhodopsin. For the 14-doxyl stearic acid spin label this more immobilized component has an outer splitting of 59 G at 0°C, with a considerable temperature dependence, the effective outer splitting decreasing to 54 G at 24°C. Spin label lipid chains covalently attached to rhodopsin can also display a two-component spectrum in rod outer segment membranes. In unbleached, non-delipidated membranes the 16-doxyl stearoyl maleimide label shows an immobilized component which has an outer splitting of 59 G at 0°C and a considerable temperature dependence. This component which is not resolved at high temperatures (24—35°C), is attributed to the lipid chains interacting directly with the monomeric protein, as with the diffusable labels. In contrast, in rod outer segment membranes which have been either delipidated or extensively bleached, a strongly immobilized component is observed with the 16-doxyl maleimide label at all temperatures. This immobilized component has an outer splitting of 62—64 G at 0°C, with very little temperature dependence (61—62 G at 35°C), and is attributed to protein aggregation.

There is some disagreement in the literature regarding the interpretation of spin label experiments on lipid-protein interactions in rod outer segment disc membranes [1—4]. On the one hand an 'immobilized' lipid component, in addi-

tion to the fluid lipid bilayer component, has been observed with freely diffusable lipid spin labels [1]. This lipid component with significantly reduced mobility compared with the fluid bilayer lipids, was attributed to the first shell of lipids interacting with the membrane protein. On the other hand, experiments with spin-labelled chains covalently linked to the membrane protein revealed a mobility which was not greatly different from that of the fluid lipid bilayer lipids and only gave rise to an additional immobilized component under conditions which were said to give rise to protein aggregation: prolonged bleaching, delipidation and low temperatures [2—4].

In an attempt to resolve some aspects of this controversy we have performed a joint experiment, similar to those described in Ref. 1, and essentially confirmed the results of this latter work. A second spectral component of restricted mobility was observed with a freely diffusable lipid spin label (14-doxyl stearic acid) in a rod outer segment membrane sample which was prepared according to Ref. 2 ($A_{280}/A_{500}$ 2.8—3.5), and which had not been delipidated or extensively bleached. The more motionally restricted component was clearly visible over the temperature range from 0—30°C. Spectral subtractions and quantitation performed essentially according to the methods of Ref. 3, indicated that this second component accounted for effectively 30—40% of the total spin label intensity over the whole of this temperature range. An important feature, was that the shape of this second spectral component did not remain constant with temperature, but showed a gradually decreasing effective outer splitting, indicating that it corresponds to lipid with considerable mobility (cf. Ref. 5). The values of the outer splitting for the more immobilized component of the 14-doxyl stearic acid label are given in Table I. It is seen that not only do they decrease with increasing temperature, but also their absolute magnitudes are considerably smaller than would be expected for a rigidly immobilized spin label (approx. 63—64 G, cf. Ref. 5).

In this respect the 'immobilized' component observed with the diffusable lipid labels in normal membranes is different from that seen with covalent lipid labels in extensively bleached or delipidated membranes. It is found that this latter component, which corresponds to that observed previously [2,3], has a considerably larger outer splitting and a much smaller temperature dependence. The results for the 16-doxyl stearoyl maleimide probe in samples which had

TABLE I

VALUES FOR THE MAXIMUM OUTER HYPERFINE SPLITTING, $2A_{max}$, OF THE 'IMMOBILIZED' LIPID SPIN LABEL COMPONENTS IN BOVINE ROD OUTER SEGMENT DISC MEMBRANES

| | $2A_{max}$ (G) | | | | |
|---|---|---|---|---|---|
| | 0°C | 10°C | 18°C | 24°C | 35°C |
| 14-Doxyl stearic acid spin label | | | | | |
| normal, unbleached | 59 | 58 | 56 | 54 | — |
| 16-Doxyl stearoyl maleimide spin label | | | | | |
| normal, unbleached | 59 | — | — | — | — |
| 65% delipidated | 62 | 62 | 60.5 | — | 60 |
| totally delipidated | 64.5 | 63.5 | 62.5 | — | 61 |
| bleached 37°C, 1 h | 63 | 63 | 62 | — | 60 |

been extensively bleached or delipidated after labelling are given in Table I. The original membrane preparation was similar to that used for the diffusable labels in untreated samples. It is seen from Table I that the outer splitting at 0°C is considerably larger than observed with the diffusable label for all three cases, and for the fully delipidated sample approaches closely to the rigid limit of approx. 64 G. Moreover, the change in outer splitting with temperature is only 2 G or less between 0 and 24°C, as compared to 5 G for the diffusable label; and even at 35°C all splittings are 60 G or greater. For these reasons it seems most probable that this immobilized component arises from protein aggregation, as originally suggested [2]. Thus there are quite distinct differences in the mobility of the various lipid spin label components observed in rod outer segment membranes. In particular the component attributed to protein aggregation has a considerably greater degree of motional restriction than the protein-interacting component seen by the diffusable labels in normal membranes. The two are thus not to be confused: the former corresponds more closely to the rigidly immobilized limit of conventional spin label spectroscopy, whereas the latter has a mobility more closely approaching that of the lipid bilayer.

Most significantly it is now found that the 'immobilized' component which was observed with the covalent label in unbleached membranes at low temperatures [2] has a considerably smaller outer hyperfine splitting than found in delipidated or extensively bleached samples. A splitting of 59 G is observed for the 16-doxyl stearoyl maleimide label in unbleached membranes at 0°C, and this decreases considerably with increasing temperature such that it is no longer resolvable at 35°C. This behaviour correlates rather well with that found for the diffusable stearic acid spin label, suggesting that it arises from lipid chains interacting directly with the protein and thus is a natural consequence of lipid-protein interactions rather than a result of protein aggregation. ESR lineshape calculations show that the temperature dependence of the spectra can be explained if it is assumed that the 16-doxyl group of the maleimide-linked chain exchanges between the first and subsequent shells of lipid surrounding the protein with a frequency of approx. $10^7$ $s^{-1}$ (Davoust, J., Seigneuret, M. and Devaux, P.F., unpublished results). A somewhat similar explanation has been postulated previously [1] in an attempt to reconcile the results with the diffusable and covalently attached acyl chain labels. Support for the suggestion that the covalently attached chain exchanges between the first shell and more fluid lipids comes also from the linewidth of the fluid component which is considerably broader than that found in bilayers of the extracted lipids alone. In view of these results it seems likely that the freely diffusable lipids also exchange at a somewhat similar rate, with the first shell lipids surrounding the protein. This could be one of the reasons for the apparent decrease in outer splitting of the 'immobilized' component with temperature. If the spin labels are in a state of intermediate exchange at higher temperatures, then the quantitation in terms of a two-component spectrum must be considered only as a first approximation, and an apparent deviation from a fixed stoichiometry of the 'immobilized' component with respect to the protein, can be most probably attributed to the effects of exchange.

The most significant result is that the mobility of the first shell or boundary lipids next to the protein, is markedly less than that of the bulk, as asserted

previously [1]. But it should be emphasized that the first shell lipids are by no means rigidly immobilized, nor are they strongly bound to the protein, rather they exchange readily with the remaining lipid phase. In terms of effective rotational rates these motionally restricted lipid chains are most probably considerably closer in correlation time to the fluid bilayer lipids ($\tau_R \sim 1$ ns) than to the protein backbone ($\tau_R \sim 20$ $\mu$s).

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