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STRUCTURAL COMPARISONS OF NATIVE AND REAGGREGATED MEMBRANES FROM *MYCOPLASMA LAIDLAWII* AND ERYTHROCYTES USING A FLUORESCENCE PROBE

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SUMMARY

1. Native and reaggregated membranes from Mycoplasma and erythrocytes have been compared using the fluorescence probe 1-anilinonaphthalene-8-sulphonate (ANS). Perturbation of both of the intact membrane structures by benzyl alcohol produces biphasic changes in ANS fluorescence intensity. Reaggregated membranes formed from membranes dissolved in sodium dodecyl sulphate show greatly reduced fluorescence intensities and lack the biphasic response to perturbation by benzyl alcohol characteristic of the intact membrane.

2. Membranes pretreated with lytic concentrations of benzyl alcohol produce increased ANS fluorescence intensities compared with the intact membranes, which decrease monotonically with increasing alcohol concentration. This fluorescence curve for pretreated erythrocyte membranes is close to the weighted mean fluorescence from the separated components, so that the pretreated membranes interact with ANS as the sum of the separated membrane components as judged by this technique. Similar quantitative relationships for Mycoplasma membrane preparations are complicated by carotenoid quenching of ANS fluorescence.

3. The ANS fluorescence curves for both types of membranes and their derived preparations are very similar to the corresponding nuclear magnetic resonance curves in their response to perturbation by benzyl alcohol. The main reason for this is the similarity in the relative binding profiles of benzyl alcohol and ANS to the membrane preparations.

4. The data from both the fluorescence and magnetic resonance experiments show that it is the membrane proteins which are incorrectly reassembled in the reaggregated structures, whereas techniques which provide information about the lipid bilayer in the reaggregated structure show that this is relatively insensitive to the conformational state of the proteins.

Abbreviation: ANS, 1-anilino-naphthalene-8-sulphonate.

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INTRODUCTION

In the previous paper we showed that the nuclear magnetic relaxation of benzyl alcohol provided a clear indication of the differences between intact membranes and reaggregated structures of both Mycoplasma and erythrocyte membranes[1]. These differences are not readily detected by other techniques and it seemed important to confirm the magnetic resonance results by an independent method. We find that the fluorescence of appropriate probe molecules also provides a sensitive measure of the state of the membrane, and particularly of the critical interactions between membrane components which are essential for the intact structure. The membranes, reaggregates, and separated components were labelled with the fluorescence probe 1-anilinonaphthalene-8-sulphonate (ANS) and perturbed with benzyl alcohol. The ANS probe has well-defined polarity-sensitive fluorescence properties and is a sensitive empirical probe for membrane perturbations[2]. Benzyl alcohol was used as the perturbing agent to allow direct comparison with the magnetic resonance experiments. The data from the two techniques have some striking similarities which can be related to the general hydrophobic binding properties of the membranes and their derived preparations.

MATERIALS AND METHODS

All membrane samples were prepared as for the magnetic relaxation experiments[1]. Technical grade ANS was precipitated from methanol with benzene and recrystallised 3 times by dissolving in acetone, adding ether to 1:1 (v/v), and cooling. The magnesium salt of ANS (Eastman-Kodak) was also used and gave quantitatively similar results at the ANS concentration used ($2.0 \cdot 10^{-5}$ M).

The fluorescence experiments were performed at 25° in the same ^2H_2O buffer used for the NMR experiments, and at fixed concentrations of membrane preparation (200 μg/ml) and of ANS ($2.0 \cdot 10^{-5}$ M). Fluorescence was excited at 388 nm and emission was measured at 480 nm from 1.0-ml samples in a Zeiss PM QII spectrophotometer with a modified detection system to increase sensitivity. Measurements were made on duplicate 1.0-ml samples containing the membrane preparation together with ANS and benzyl alcohol, and were compared with a similar standard sample without benzyl alcohol. Various blanks, containing buffer alone; buffer *plus* ANS; and membrane suspension alone, were measured with and without benzyl alcohol over the experimental concentration range. The slit widths were adjusted so that the appropriate blank in each experiment was less than 5 % of the measured fluorescence of the standard membrane preparation. Under these conditions benzyl alcohol had no significant effect on the blank correction which remained constant in all experiments. Fresh ANS solutions were made for each experiment and samples were allowed to equilibrate at 25° for 2–4 h before measurement. Manipulations of solutions containing ANS were limited to a final transfer from the mixing vial to the fluorescence cuvette. With these precautions errors due to the degradation of ANS solutions and adsorption losses in the transfer of the samples were minimised. Fluorescence yields from different experiments were compared by referring the fluorescence of the membrane preparation to a freshly prepared solution of ANS in ethanol. Different preparations of the same membrane system give fluorescence yields reproducible to within 10 %.

The residual sodium dodecyl sulphate concentrations in the reaggregated membrane perparations were estimated as described by REYNOLDS AND TANFORD[3].

RESULTS

The changes in ANS fluorescence induced by benzyl alcohol in erythrocyte and Mycoplasma membranes are shown in Fig. 1. In the prelytic concentration range there is a slight decrease in ANS fluorescence from the erythrocyte membranes with

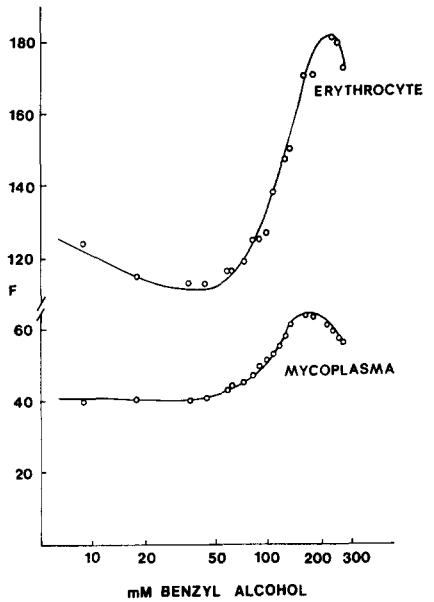

Fig. 1. The fluorescence of $2 \cdot 10^{-5}$ M ANS in the presence of 0.02 % membrane suspensions as a function of benzyl alcohol concentration at 25°. (The same concentrations of ANS and membrane preparations were used in all subsequent experiments).

increasing alcohol concentration, whereas the Mycoplasma membranes produce a constant fluorescence intensity. The membranes also differ in that the fluorescence intensity obtained from the Mycoplasma membranes is approximately 30 % of the intensity from the erythrocytes. For both membranes there is a progressive increase in fluorescence above the lytic concentration which turns over at the highest alcohol concentrations.

These fluorescence experiments were repeated on the same series of membrane preparations described for the NMR experiments (Fig. 2). Qualitatively the two sets of curves are similar; the fluorescence intensities from the two protein fractions are comparable and decrease with increasing alcohol concentration so that at 250 mM benzyl alcohol the intensities relative to zero alcohol are 67 % and 61 % for erythrocyte and Mycoplasma proteins, respectively. The lipid components from both membranes produce the lowest fluorescence intensities; there is probably a minor inflection in the erythrocyte lipid curve at 80 mM alcohol, although the effect is small. At this alcohol concentration there is a pronounced upswing in the partition coefficient of the alcohol into the lipid vesicles[4].

Membranes pretreated with 260 mM benzyl alcohol show enhanced fluorescence compared with the original membranes and the intensities are approximately doubled

for both pretreated membranes over the prelytic concentration range. In the lytic range the pretreated membrane curve coincides with the original membranes above 250 mM for the Mycoplasma membranes. The results were more variable for erythrocyte membranes above 200 mM alcohol, but are qualitatively similar to the Mycoplasma curves.

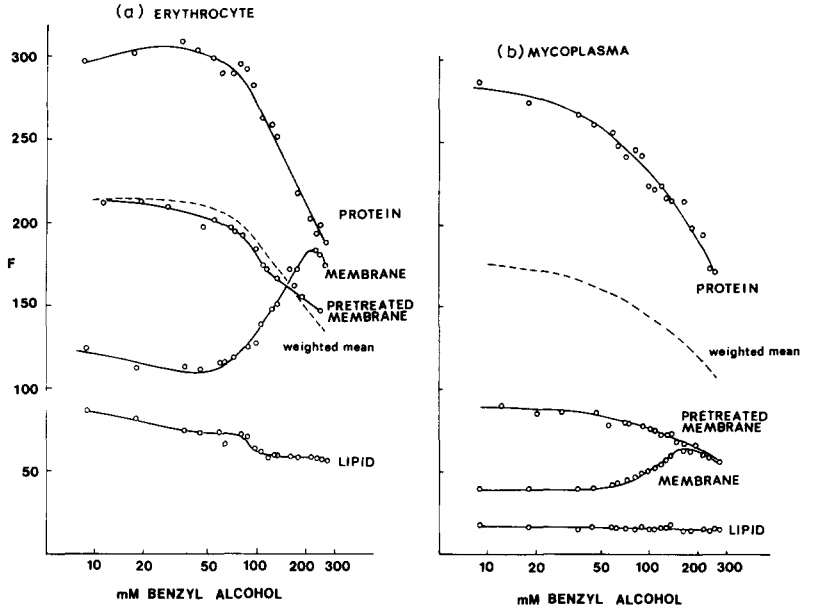

Fig. 2. ANS fluorescence from various membrane preparations at 25° (see text for details). The dashed curves are the weighted mean fluorescence intensities of the separated components corresponding to the compositions of the intact membranes. (a) Erythrocytes. (b) Mycoplasma.

For the erythrocyte membranes the weighted mean lipid and protein intensity lies close to the pretreated membrane curve (Fig. 2a), but the pretreated Mycoplasma membranes yield only 51–53 % of the weighted mean curve over the entire alcohol concentration range. The fluorescence yields of all the Mycoplasma preparations containing lipid are lower than the corresponding erythrocyte preparations, by a factor of 0.21 for the lipid and 0.33 and 0.42 for the membrane and pretreated membrane, respectively, whereas the protein ratio is 0.96. At least part of the intensity reduction in preparations containing lipid is due to the presence of intensely yellow-coloured carotenoids in the lipid, which absorb strongly in the ANS emission region and quench the fluorescence. This quenching will depress the fluorescence of the pretreated membranes in comparison with the weighted mean value for the separated components, since the protein fraction does not contain any carotenoid.

Both erythrocyte and Mycoplasma reaggregates prepared from original membranes which had been dissolved in sodium dodecyl sulphate, produce fluorescence intensities much lower than the intact membrane curves, and for the erythrocytes even lower than the lipid component (Fig. 3). Although the reaggregates of both membranes are clearly distinguishable from the original membranes, the results differ from the magnetic resonance experiments in which the reaggregate curves fall within the range

of the pretreated membranes and the intact membrane curves. The greatly reduced fluorescence from the reaggregates could be due to several causes which have been examined in preliminary experiments.

For example, the reaggregates may exclude a fraction of the ANS binding sites through interactions between the components, as suggested to account for the NMR curves for the Mycoplasma reaggregates. Some support for this was obtained by pretreating reaggregates with 280 mM benzyl alcohol. This resulted in an increase in fluorescence of about 40 % for both membrane reaggregates at prelytic concentrations, suggesting that there may be occluded ANS binding sites in the reaggregates. However, these increased fluorescence intensities are still lower than for original membranes pretreated in the same way, by a factor of about 2 for Mycoplasma membranes and 5 for erythrocytes (Fig. 3).

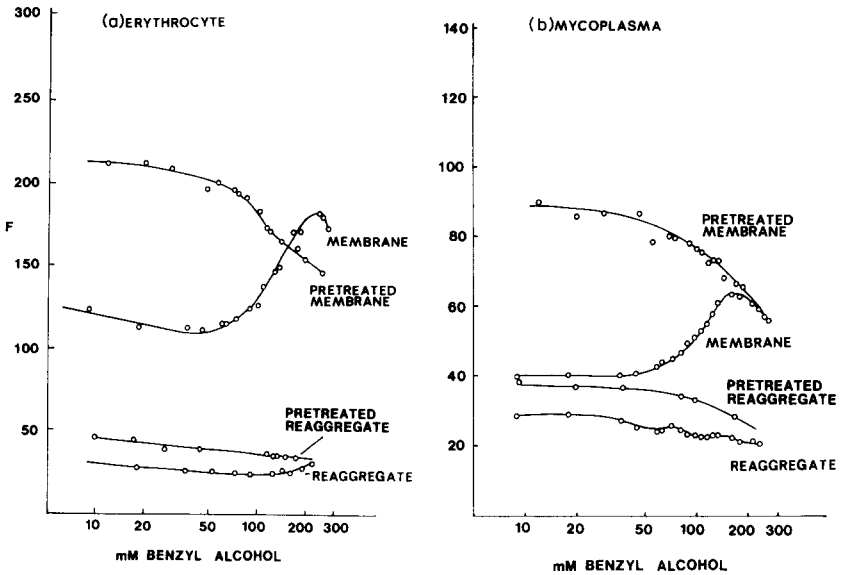

Fig. 3. ANS fluorescence from sodium dodecyl sulphate reaggregates. The curves are compared with intact membranes and membranes pretreated with 280 mM benzyl alcohol. (a) Eryhtrocytes. (b) Mycoplasma.

This suggested that the residual sodium dodecyl sulphate in the reaggregates might be responsible for the large depression in their fluorescence intensities. The residual sodium dodecyl sulphate in erythrocyte reaggregates under the experimental conditions for fluorescence measurements was $2 \cdot 10^{-5}$ M. In intact erythrocyte membranes we find that $2 \cdot 10^{-5}$ M sodium dodecyl sulphate decreases the fluorescence by about 20 % (Fig. 4). This may be due to direct competition between the anionic sodium dodecyl sulphate and ANS molecules, or to perturbation of the membrane structure by the detergent. The important point is that although this sodium dodecyl sulphate concentration depresses the fluorescence intensity from ANS in the membranes, they still retain the biphasic response to benzyl alcohol. At higher sodium dodecyl sulphate concentrations up to 10^{-4} M, the fluorescence is further reduced and starts to increase at about 40 mM benzyl alcohol, so that the characteristic response of an intact membrane is altered. The lytic concentration for sodium dodecyl sulphate

is close to 10^{-4} M[5] so that we would expect the upswing on addition of benzyl alcohol to be shifted to a lower alcohol concentration, as observed. It is clear from these experiments that the residual sodium dodecyl sulphate is grossly inadequate to account for the difference in fluorescence intensities from the original membranes and the reaggregates, and that the interaction of ANS with the two structures indicates that the reaggregates are not properly reconstituted.

To avoid the complications involved in the use of sodium dodecyl sulphate, erythrocyte reaggregates were also prepared from membranes dissolved in 2-chloroethanol as described by ZAHLER AND WEIBEL[6]. The fluorescence curve for the reaggregate was close to the pretreated membrane curve (Fig. 5) and clearly interacts with ANS as the sum of its separated components rather than as an intact membrane structure. On pretreating the reaggregated membrane with 280 mM benzyl alcohol,

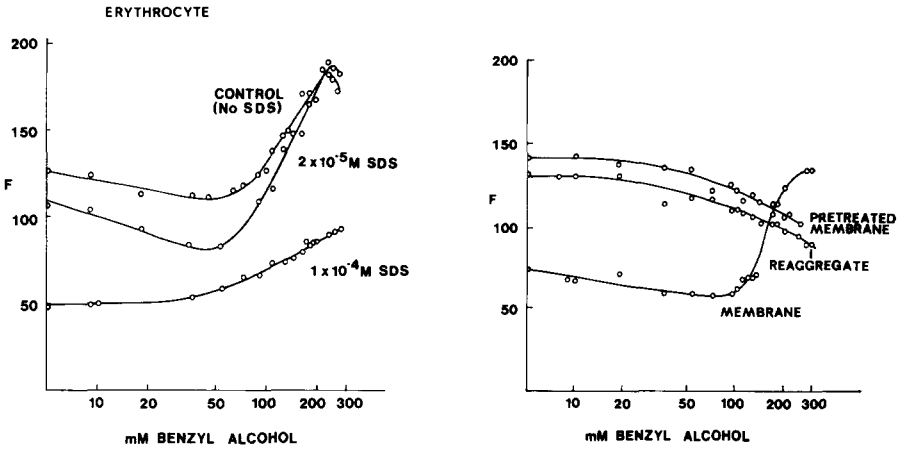

Fig. 4. The effect of the addition of sodium dodecyl sulphate (SDS) on ANS fluorescence from intact erythrocyte membranes at 25°.

Fig. 5. ANS fluorescence from reaggregates prepared from erythrocyte membranes dissolved in 2-chloroethanol at 25°. The curves are compared with the intact membranes and membranes pretreated with 280 mM benzyl alcohol. (The fluorescence intensities are lower than in previous experiments because H_2O was used instead of ^2H_2O as the solvent.)

the fluorescence intensity increased by less than 10 % over the whole alcohol concentration range, so that the reaggregated structure does not differ significantly from the pretreated membrane in its interaction with ANS. In these experiments the fluorescence data for the reaggregate is very similar to the corresponding NMR data for an erythrocyte reaggregate prepared from membranes dissolved in methyl cellosolve[1]. It is also apparent from these data that the very low fluorescence intensities from the sodium dodecyl sulphate reaggregates is in some way associated with the use of sodium dodecyl sulphate as the dissociating agent.

DISCUSSION

An unexpected feature of these results is the marked similarity in the form of the fluorescence curves and the nuclear magnetic resonance line width measurements for the same membrane preparations (*cf.* Fig. 2 with Fig. 2 of the preceeding paper).

Both types of experiment give biphasic curves for the intact membrane, high relative values for the membrane protein and low values for the lipid component. It is also significant that for both experiments, the pretreated erythrocyte membrane curve lies close to the weighted mean curve for the separated components, so that we conclude that the pretreated membranes interact with both benzyl alcohol and ANS essentially as the sum of the separated components.

The magnetic resonance and fluorescence curves depend in the first instance on how much of the probe molecule binds to the various membrane preparations. Detailed binding studies by COLLEY *et al.*[4,7] have established that the relative binding capacities of the erythrocyte membrane preparations used in these experiments for benzyl alcohol and ANS are similar, although the partition coefficients for ANS are much higher than for benzyl alcohol. The conclusion from these studies was that the binding profiles of small molecules to intact membrane structures are determined primarily by the organisation of the membrane components. Many of the binding sites available to benzyl alcohol and ANS on the separated membrane components are inaccessible in the intact membrane because they are used in the assembly of the structure. Thus underlying the similarity in the data from the two techniques used in the present studies is the similarity in the binding profiles of benzyl alcohol and ANS.

The data from the two techniques also depend on independent physical parameters which are the rotational correlation times of the bound alcohol molecules[8] in the magnetic resonance experiments and the polarity of the binding sites in the sensitised fluorescence experiments[2]. It is these spectroscopic properties which account for the sensitivity to structural perturbation beyond a simple measure of probe binding. The consistent responses in the fluorescence and magnetic resonance experiments to perturbation by benzyl alcohol suggest that there may also be a qualitative relationship between rotational stabilisation and the polarity of the binding sites. A quantitative description of the intrinsic fluorescence intensities of ANS bound to the various membrane preparations and the effects of perturbation by benzyl alcohol will be presented elsewhere. It is important to note that in the fluorescence experiments perturbation by benzyl alcohol can affect the amount of ANS bound as well as the intrinsic fluorescence from the binding sites.

The quantitative fluorescence relationships in the Mycoplasma membranes are complicated by the effects of carotenoid quenching already described, and this probably accounts for the depressed fluorescence of the pretreated Mycoplasma membranes compared with the weighted mean curve for the separated components. Thus there is good agreement in the nuclear magnetic resonance data for pretreated Mycoplasma membranes and the weighted mean curve, suggesting that carotenoid quenching accounts for the loss of approximately half the ANS fluorescence from the pretreated membranes.

The only major discrepancy between the nuclear magnetic resonance and fluorescence experiments is for the Mycoplasma reaggregates prepared from membranes solubilised in sodium dodecyl sulphate. In the magnetic resonance experiments the reaggregate curves were intermediate between the native membrane and the separated components, which suggested that some but not all of the extra binding sites on the separated membrane protein were inaccessible in the reaggregates. In the fluorescence experiments, the reaggregate curves fall below the intact membrane curve for both erythrocyte and Mycoplasma membranes, and the residual detergent is clearly in-

sufficient to cause this degree of change in the biphasic response of an intact membrane to benzyl alcohol. The absence of a significant biphasic response in the reaggregates and the very low fluorescence intensity clearly indicate that the dissolution of the native membrane in sodium dodecyl sulphate has caused a major change in the interaction of the reaggregated structure with ANS. The differences between the magnetic resonance and fluorescence curves for the reaggregates indicate that in some structures the binding of probes as judged by the two techniques can be differentiated and the independent nature of the spectroscopic parameters involved is apparent. However, the increase in fluorescence on pretreating the reaggregated structures with benzyl alcohol is quite consistent with the magnetic resonance data which indicated substantial interaction between the components in the reaggregated structures and hence a decrease in binding sites available to probe molecules.

In contrast to the reaggregates prepared by dissolution in sodium dodecyl sulphate, there is excellent agreement between the fluorescence data and NMR data for erythrocyte reaggregates prepared from membranes dissolved in methyl cellosolve or 2-chloroethanol. These reaggregates interact with probes essentially as the sum of their separated components as judged by both techniques. However we would emphasise that our main objective here is to establish objective criteria for the state of membrane structures and reaggregates, rather than to demonstrate that particular techniques for forming recombinants are unsuccessful. In our hands, reaggregates formed from membranes dissolved in organic solvents do not show any significant reconstitution of the membrane proteins to their original state, but we have not made any systematic attempt to study the effect of reaggregation conditions on the final structure. It is clear however, that reaggregate structures which are in fact grossly disrupted can appear indistinguishable in electron micrographs from the original membranes, and that electron microscopy is quite inadequate as a criterion for structural integrity.

Finally we compare the data for the membrane reaggregates in this work with electron spin resonance (ESR) studies of spin-labelled Mycoplasma membranes. TOURTELLOTTE *et al.*[9] found that the ESR spectra from a nitroxide ($>N\dot{-}O$) labelled derivative of stearic acid incorporated biosynthetically into the polar lipids of the membrane were very similar in the intact membrane and in vesicles of the extracted lipid. The small spectral differences which were observed indicated greater motional freedom in the lipid vesicles compared with the membrane and were taken as evidence for weak lipid–protein interactions in the intact membrane. A well defined thermal transition in the membrane from lipids was observed in the spin-labelled lipid spectra at the same temperature in the intact membranes and the extracted lipids. There were no changes in the spectra due to thermal protein denaturation or glutaraldehyde fixation, so that the physical state of the lipids is not very sensitive to some conformational changes in the membrane proteins. The data suggest that the mobility of the lipid spin labels is primarily determined by lipid–lipid interactions. Similar studies by ROTTEM *et al.*[10] using several fatty acid spin labels incorporated by exchange *in vitro* into Mycoplasma membranes, reaggregated structures and extracted lipids, are in excellent agreement with these biosynthetic spin-label results. They confirm the similarity of the spectra in the membranes and extracted lipids, with a slightly greater freedom of motion of the labels in the lipid vesicles. This is reduced in reaggregates with the correct lipid to protein ratio to the same freedom of motion as the original

membranes, again indicating a small effect of the proteins on the physical state of the lipids.

These results support the conclusions we draw from the X-ray studies in the previous paper, that a lipid bilayer is substantially reformed in the reaggregates and that its properties are only slightly affected by the membrane proteins. The usefulness of the magnetic resonance and fluorescence data is that they indicate clearly that it is the membrane proteins which are incorrectly recombined with the lipids. They are therefore sensitive to structural features not readily detected by other techniques. The retention of a considerable number of abnormal binding sites for probes in the reaggregated membranes indicates that either the conformation of the proteins is not restored to the native state, or that they are not interacting in some specified way with the lipids to exclude the abnormal binding sites. It is presumably these specified conformations of the proteins and their interactions with other components which define the native structure of the membrane. The spectroscopic techniques described here allow the possibility of identifying an essential element of the intact membrane structure.

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