EPR STUDIES ON MODEL MEMBRANES OF PHOSPHOLIPID AND OUTER MEMBRANE PROTEINS OF *PROTEUS MIRABILIS*

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1. Introduction

In [1] we described the reconstitution of model membranes from phospholipid and outer membrane proteins of *Proteus mirabilis*. Functional reconstitution was demonstrated by the ability of outer membrane proteins incorporated into phospholipid vesicles to mediate the penetration of small hydrophilic molecules through the vesicle membrane.

This report provides further evidence for the incorporation of outer membrane protein M_r 39 000 of *P. mirabilis* into the phospholipid bilayer by showing the effect of the protein on the molecular organization of the hydrocarbon chains of phospholipid vesicles. Using electron paramagnetic resonance (EPR) spectroscopy of spin-labeled fatty acids incorporated into the vesicles, it was shown that increasing amounts of protein M_r 39 000 (up to 0.25 mg protein/mg phospholipid) resulted in a marked restriction in motion of both 5-doxylstearate and 12-doxylstearate in the vesicle membranes. At higher protein concentrations, however, the freedom of motion of 5-doxylstearate and to a lesser degree of 12-doxylstearate was increased. The possibility that the tendency to a less ordered bilayer structure upon addition of large amounts of protein is due to protein aggregation or to complexing of divalent cations at the vesicle surface is discussed.

2. Materials and methods

2.1. *Bacterial strains*

Proteus mirabilis strain 19 of this laboratory was cultivated in a fermenter on complex medium to the early stationary phase of growth by E. Merck (Darmstadt). The cells were stored at −25°C.

2.2. *Extraction of phospholipids*

Phospholipids were extracted from whole *P. mirabilis* cells according to the method in [2].

2.3. *Isolation of outer membrane proteins*

The M_r 39 000 protein and the M_r 36 000 peptidoglycan-associated protein were isolated from purified cell walls of *P. mirabilis* and characterized as in [1].

2.4. *Preparation of vesicles*

Phospholipid, 12.5 μmol (~10 mg), was mixed in 1.0 ml buffer containing 0.1 M NaCl, 0.02 M Tris/HCl (pH 7.3), 1 mM $MgCl_2$ and 0.02% (w/v) NaN_3. Phospholipid vesicles containing outer membrane proteins were obtained by adding 1.0–5.0 mg protein to 12.5 μmol phospholipids. The mixtures were sonicated in the cold for 4 min at power level 3 of a model 350 Heat Systems sonicater (Heat Systems, NY) equipped with a microtip.

2.5. *EPR spectroscopy*

Vesicles were spin-labeled with *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid or 12-ketostearic acid (Syva, Palo Alto, CA). For spin-labeling, 2 μl 2.5 mM solution of the spin-label in ethanol were added to a test tube containing 0.1–0.2 ml diethylether. The solvents were then evaporated, leaving a thin film of the spin label on the bottom of the tube. Vesicle suspension,

200 μl, was subsequently added and the tube was gently shaken for 15 min at room temperature. EPR spectra of the spin-labeled membranes were obtained by use of a Varian E-4 spectrometer (Varian Associates, Palo Alto, CA). The molecular motion is reported as the order parameter S [3], which is a measure of the amplitude of motion of the molecular long axis about the average orientation of the fatty acid chains in the lipid bilayer [4], and as τ_0, an empirical motion parameter calculated from the expression in [5]. The maximum value for S is 1.0 for perfect order while complete disorder yields a value of $S = 0$. Greater freedom of motion is associated with smaller values of S and τ_0.

3. Results and discussion

The molecular motion of 5-doxylstearate and 12-doxylstearate spin labels incorporated into phospholipid vesicles containing increasing amounts of *P. mirabilis* outer membrane protein M_r 39 000 are presented in fig.1.

With 5-doxylstearate the order parameter S could be calculated from the outer and inner spectral extrema, $T_{\parallel}$ and $T_{\perp}$ [3,4]. The values obtained at 25°C and 35°C are shown in fig.1A. Since in spectra of 12-doxylstearate only the inner extrema were well resolved, order parameters could not be calculated from spectra of this probe. However, 12-doxylstearate moves in a nearly isotropic fashion [6], so that for comparative studies an empirical motion parameter (τ_0) related to the rotational correlation time can be used [5]. The τ_0 values obtained at 25°C and 35°C are shown in fig.1B.

The addition of up to 2.5 mg M_r 39 000 outer membrane protein to phospholipid vesicles (~25% protein) led to a consistent increase in the restriction of motion of both the 5-doxylstearate and the 12-doxylstearate spin labels. These results provide evidence for the actual incorporation of outer membrane protein from *P. mirabilis* into the phospholipid bilayer [4,7,8]. The amount of restriction observed is comparable to values obtained by other investigators for the incorporation of a purified myelin hydrophobic protein into phosphatidylcholine vesicles with 8-doxylpalmitate and 16-doxylstearate as spin labels [4].

Although the actual values for the molecular motions of the 5-doxylstearate and 12-doxylstearate spin labels in our system fluctuated upon addition of the M_r 39 000 protein in amounts >2.5 mg, the overall tendency was to an increase in the fluidity of the model membranes, which was somewhat more pronounced in the 5-doxylstearate than in the 12-

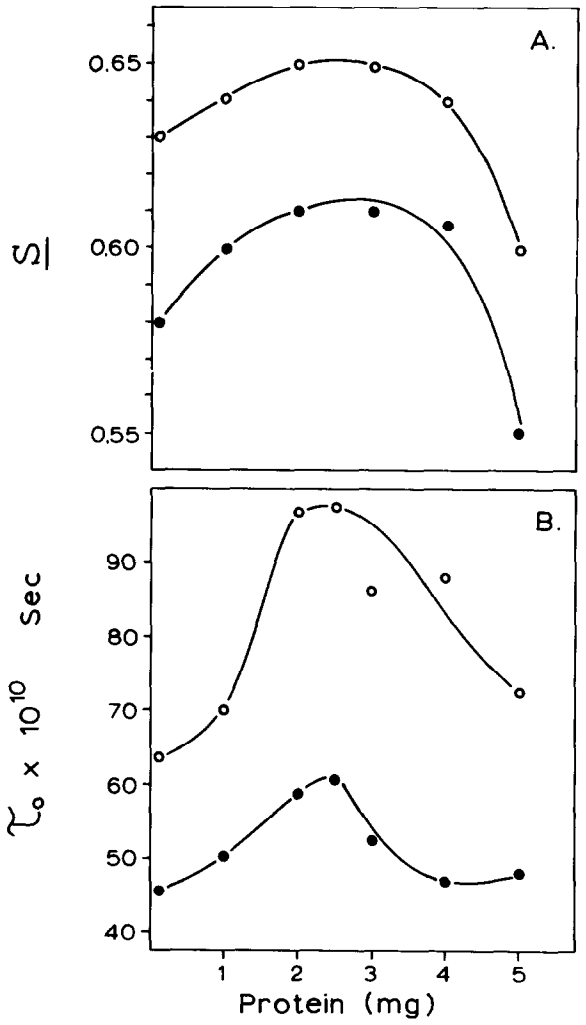

Fig.1. Dependence of the order parameter S of 5-doxylstearate (A) and the motion parameter τ_0 of 12-doxylstearate (B) on the amount of M_r 39 000 outer membrane protein of *P. mirabilis* in phospholipid vesicles. The amount of phospholipid (12.5 μmol or ~10 mg) was held constant in each case. (o–o) Measurements made at 25°C; (•–•) measurements made at 35°C. Each point on the curves represents the mean value obtained from 2–4 separate experiments.

doxylstearate region (e.g., with 5.0 mg protein, fig.1A,B). This could be an indication that the fluidity effect was to a large extent a surface phenomenon, analogous to the type 2 interaction of phospholipid and protein in bilayers as proposed [9]. The type 2 interaction results in an increase in fluidity of membranes through binding of protein to the surface of phospholipid bilayers, with partial penetration of the protein and subsequent deformation of the bilayer. This type of phenomenon might be more likely to occur in our system when large amounts of protein are aggregated on the surface of vesicles.

An alternate explanation for the fluidizing effect of large amounts of protein on our model membranes may be found in the complexing of divalent cations by the protein, which would be more prominent in the hydrophilic (5-doxylstearate) portions of the bilayer structure. Even very small concentrations of divalent cations can influence fluid → ordered transitions in phospholipid bilayers [10]. Large amounts of outer membrane protein may complex the Mg^{2+} in our system, making it unavailable for interaction with phospholipids. The resulting increase in fluidity of the model membranes might eventually override and cancel out the restriction in motion of the spin labels afforded by small amounts of protein incorporated into the bilayer in the proper manner. However, the significance of this explanation for our results is questionable as attempts to raise the concentration of $MgCl_2$ in the vesicle system to overcome this effect resulted in precipitation of phospholipids.

The results of spin-label studies reported above were obtained with the M_r 39 000 outer membrane protein of *P. mirabilis*. Parallel studies with the M_r 36 000 peptidoglycan-associated outer membrane protein gave essentially similar results, i.e., restriction in motion of spin labels at low protein concentrations and fluidity of the membranes upon incorporation of larger amounts of protein (data not shown).

The EPR studies reported here are consistent with our findings on permeability properties of reconstituted model membranes of phospholipid and outer membrane proteins of *P. mirabilis* [1]. Incorporation of 2.0–3.0 mg of either the M_r 39 000 or the M_r 36 000 proteins into phospholipid vesicles led to release of small molecular weight hydrophilic molecules (sucrose) entrapped in the vesicles, while large hydrophilic molecules (inulin) were retained. These results indicated that both outer membrane proteins were acting as hydrophilic pores mediating the penetration of small hydrophilic molecules through the model membrane in a manner similar to that observed with the native outer membrane of Gram-negative bacteria [11]. Incorporation of large amounts of protein, however, caused leakage of inulin out of the vesicles. Since our EPR experiments indicated a general tendency to less ordered bilayer structures upon addition of large amounts of outer membrane protein to phospholipid vesicles, it may be that the integrity of the bilayer can be maintained only at low protein/phospholipid ratios in the model system.

Acknowledgements

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