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INTERACTION OF A HYDROPHOBIC PROTEIN WITH LIPOSOMES EVIDENCE FOR PARTICLES SEEN IN FREEZE FRACTURE AS BEING PROTEINS

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Summary

Freeze fractures of liposome membranes showed a smooth fracture surface while liposomes into which a hydrophobic protein from human myelin had been incorporated showed a particulate fracture surface. The evidence suggests that at least some of the analogous particles present on fracture surfaces of biological membranes could be proteins, embedded in a phospholipid bilayer.

The freeze fracture technique has become an increasingly important tool to investigate the structure of biological membranes. Branton [1] has proposed that the fracture passes through the centre of membranes while labelling studies by da Silva and Branton [2], and Tillack and Marchesi [3] have shown smooth etch surfaces and particulate fracture surfaces in erythrocyte ghost membranes.

The liposome is regarded as a model membrane system [10] and liposomes prepared from a number of highly purified lipids show many of the structural and functional properties attributed to membranes. They possess a lipid bilayer and may be used to study the transport of ions.

Biological membranes rich in lipid seem to have smooth fracture surfaces [4, 5]. In model membrane systems of phospholipid [6—9] the fracture surface is likewise essentially smooth.

Since biological membranes are known to contain protein as well as lipid, it was of interest to see if protein could be incorporated into vesicles. For this

purpose we chose a hydrophobic protein prepared from normal human myelin. Evidence is presented to show that this protein appears to be incorporated into the lipid bilayer.

Myelin was isolated from normal human white matter by the method of Lowden et al. [11] and the hydrophobic protein (N-2) prepared according to Gagnon et al. [12]. The water-soluble form of N-2 was obtained after extensive dialysis according to the procedure of Anthony and Moscarello [13].

Phosphatidylserine was purified from bovine brain as described previously [14]. The phosphatidylserine was dissolved in chloroform at a concentration of 10 μmoles/ml and stored under nitrogen in sealed vials at −50 °C.

For the preparation of liposome vesicles, 10 μmoles of phosphatidylserine were dispersed in 2 ml of buffer (10 mM in NaCl, 2 mM in histidine, 2 mM in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 6.5) by shaking under nitrogen for 10 min after evaporation of the solvent under vacuum. The tube with the dispersion was then placed in a bath-type sonicator and sonicated for 60 min under a nitrogen atmosphere and the temperature maintained at 36 °C [15].

An aliquot of the above dispersion (3 μmoles of phosphatidylserine in 0.6 ml) was mixed at room temperature with 0.7 ml of N-2 solution containing 2 mg protein, which had been previously dialyzed against the same buffer. Cloudiness developed within a few minutes after mixing, and some white precipitate slowly settled within 1 h.

Liposomes and liposomes with protein were made 30% (v/v) with glycerol and small droplets were placed in gold cups. Specimens were frozen in Freon 22, stored no longer than 5 min in liquid nitrogen, and placed on a 4-place specimen table of a Balzer BA360 Freeze Etch Apparatus pre-cooled to −150 °C. Specimens were fractured at −115 °C at $2 \cdot 10^{-6}$ Torr and within 2 s of the last fracture were shadowed with carbon and platinum and then replicated with carbon. The cold knife holder was placed directly over the replicas until the bell jar was filled with dry nitrogen gas. Replicas were floated on water and washed for 60 min in commercial sodium hypochlorite solution (Javex). Replicas were washed twice in water, mounted on bare 75 × 300 mesh copper grids, and examined in a Philips EM 300 operated at 60 kV. An arrow in the lower left hand corner of each micrograph shows the direction of the black platinum shadow.

Fig. 1 shows a liposome without the addition of protein. The fracture surface is smooth and without particles. Most of the liposomes appear as vesicles composed of a single membrane. Both concave and convex fracture surfaces are usually seen as well as fractures which appear to cut across the membrane. After the addition of the hydrophobic protein, the fracture surface of the liposome is particulate (Fig. 2). The particles appear to be randomly distributed although in some fractures there are smooth patches devoid of particles. The latter are usually convex fracture surfaces. Some aggregation of the protein before incorporation into the vesicles cannot be excluded. Concave and convex fracture surfaces are shown in both figures. When protein was not

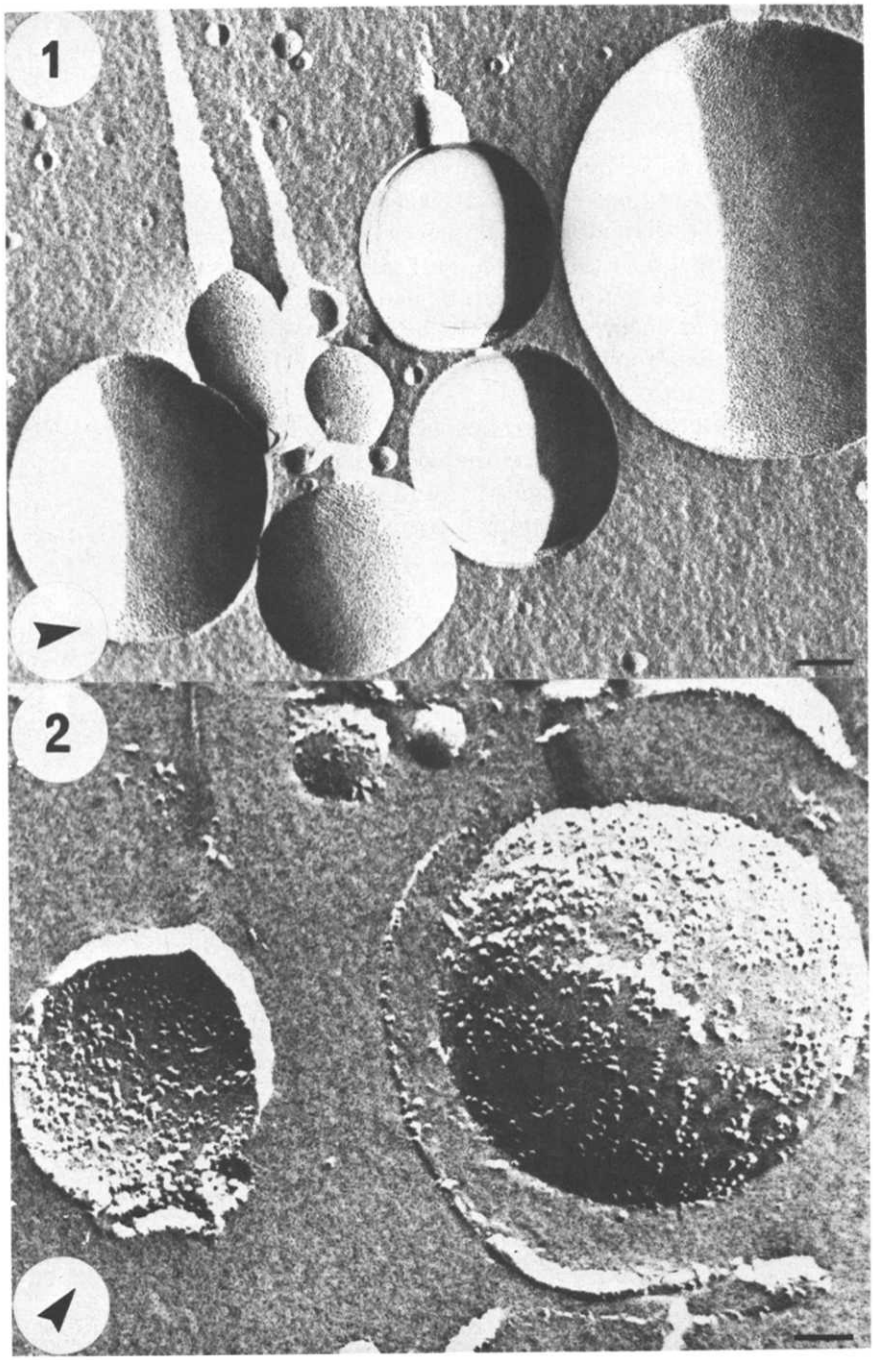

Fig. 1. Freeze fracture of a liposome. Note that the fracture surfaces are smooth without particles. Total magnification 75 000 X. Bar, 0.1 μm.

Fig. 2. Freeze fracture of liposomes after the incorporation of a hydrophobic protein extracted from human myelin. Note the particles are present on the fracture surfaces. Total magnification 75 000 X. Bar, 0.1 μm.

added, we never observed particles on the fracture surface of the liposome.

We have observed the N-2 protein using the negative stain technique as globular particles 85—90 Å in diameter. The particles on the fracture surface (shown in Fig. 2) were 115—120 Å in diameter. The difference in size could be due to the thickness of a platinum deposit of approx. 30 Å. The particle density was calculated to be 2000 ± 100 particles per μm^2 and the protein incorporated in the liposomes was 50% by weight. The surface area occupied by the particles of 90 Å diameter and a density of 2000 per μm^2 is 12% of the total area. Considering a 50% protein to lipid ratio by weight and respective values of 1.3 and 1.0 g/ml density, it can be calculated that the protein of cylindrical shape would occupy 38% of the areas if they were completely embedded in the lipid bilayer and only 12% of the area if they were embedded by 1/3 of their total volume.

We have also observed the liposomes with the hydrophobic protein using the negative stain technique. We were unable to visualize any protein on the membrane surface or in the void area surrounding the liposome. This observation suggests that most of the protein is incorporated in the liposome and embedded far enough in the lipid bilayer so as not to be visualized by the negative stain technique.

It is thus possible that the fracture removes one monolayer, exposing the particles as shown previously [2, 3] in erythrocyte ghost membranes. It seems to us likely that the N-2 protein is embedded at least partly into the bilayer by reason of its high content of apolar amino acids [11], its solubility in organic solvents, and its ability to penetrate and expand lipid monolayers [16].

MacLennan et al. [17] have shown particulate fracture surfaces of reformed membranes of purified ATPase with particles distributed on both concave and convex fracture surfaces. The reformed membranes appeared identical with freeze fracture surfaces of the sarcoplasmic reticulum. Hong and Hubbell [18] have recombined membranes of rhodopsin and phospholipid and shown that the etch surfaces of the membranes were smooth while the fracture surfaces were particulate. Control egg phosphatidylcholine surfaces were completely smooth. They concluded that the rhodopsin was located within rather than on the phospholipid bilayers.

In accordance with the above studies, we concluded that the particles present on the fracture surfaces in our model system represent proteins embedded into the lipid bilayers. By analogy, some of the particles commonly observed on fracture surfaces of biological membranes may likewise represent "intrinsic" membrane proteins. The comparatively low density of such particles in freeze fractures of myelin could be due to the fact that N-2 protein constitutes only 6% of the total dry weight [12], while in our system, the protein is approximately 50% by weight.

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