MASTER**STUDIES OF MEMBRANE STRUCTURE BY FREEZE-ETCHING****Progress Report****Daniel Branton**

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I. ABSTRACT

The structure and organization of biological membranes is being studied using a variety of physical and biochemical techniques together with electron microscopy and freeze-etching. These studies are providing knowledge relating the architecture of cellular membranes to their role in modulating cell behavior in response to environmental perturbants and in regulating the transport of molecules into and out of cells. As model systems, human erythrocyte membranes and their ghosts are being probed to determine the distribution and asymmetry of component lipids and proteins. For this purpose, a novel combination of freeze-fracture and biochemical techniques using fluorescent resonance energy transfer has been developed and tested and ultra low temperature freeze-etching has been evaluated as part of a program to develop covalent labelling techniques that will identify specific transmembrane proteins in freeze-fractured replicas.

II. BACKGROUND

My laboratory has been concerned with the relationship between the structure of membranes seen in the electron microscope and the details of their physical, chemical and functional organization. Two short reviews (Branton, 1971, Phil. Trans. Royal Soc. London B, 261:133-138 and Branton, D. & Kirchanski, S., 1977, J. Microsc. 111:117-124) as well as more complete analyses (Branton and Deamer, 1972, Protoplasmatologia, Vol. II E1; Southworth, Fisher and Branton, 1975, Techniques of Bioch. Biophys. Morphology 2:247-282) have been published. These reviews explain the rationale for our efforts and may be consulted for a more detailed introduction and background for this report. These reviews show how the approaches and methods we have developed provide clear evidence for the existence of discrete, structurally differentiated sites within the plane of most biological membranes. Recent work shows that in many membranes these sites correspond to transmembrane proteins whose distribution is dependent on chemical gradients in both the hydrophilic and the hydrophobic portions of the membrane. Although physical and averaging techniques such as X-ray diffraction, circular dichroism, and paramagnetic resonance have proven to be powerful tools for studying certain aspects of the overall membrane matrix, they have not indicated much about the distribution, structure, and associations of individual transmembrane components. In view of evidence that the in plane distribution of transmembrane proteins is responsive to external factors and can trigger a variety of cellular responses (Freedman, Raff and Gomperts, 1975, Nature 255:378; Edelman, Wang and Yahara, 1976, Cold Springs Harbor Conference on Cell Proliferation, Vol. 3), it becomes clear that averaging techniques must be supplemented by and combined with methods capable of exposing structure within individual areas of a single membrane. It is in this context that we have applied freeze-etch methods of electron microscopy. These methods have unique advantages as they provide extensive high resolution views of structure within the plane of single membranes and are readily combined with a variety of physiological, chemical and physical manipulations. This combination of ultrastructural and experimental approaches is the basis for current work in my laboratory.

III. CURRENT ACTIVITY

This report is a summary of our activities during the past year only. The application and progress report submitted for the period 1 July 1976 - 30 June 1977 should be consulted for more detailed background information.

One of the major advantages of freeze fracture as a technique for studying membranes is that it allows the visualization of integral proteins within a fractured biological membrane. In some cases, the arrangements of the particles seen on the faces of fractured membranes give clues to the identity or function of the particles. However, in most cases freeze fracture gives no clues to the nature of the proteins that are responsible for the visualized particles. If one could use freeze fracture to reveal the distribution of various receptors and transport proteins, valuable insight into the detailed structure and function of biological membranes would be available. It is possible to use ferritin-conjugated antibodies to label proteins at the surface of membranes. Deep etching following freeze fracture can then reveal the location of the ferritin-labelled proteins on the membrane surface, but fracture of the membrane to reveal intramembrane particles will remove the ferritin and direct identification of the labelled particles will not be possible. We have been studying the process of freeze fracture to develop surface labelling techniques so that the identity of intramembrane particles may be ascertained directly.

Initial experiments have confirmed that covalently bound ferritin is detached from a membrane during freeze fracture of that membrane. It is our hope that a surface label can be found that is large enough to be discernable on freeze fracture replicas and yet be small enough that it is not removed during the fracture process. We have decided that a membrane system containing a single transmembrane protein would be an ideal model to use in this study, and we have settled upon recombinant lipid vesicles containing the glycoprotein glycophorin (one of the two major transmembrane proteins of the erythrocyte membrane). Glycophorin is relatively simple to prepare and purify and the sugar groups of this protein are useful in affinity chromatographic analysis of covalent bond breakage.

Before observing the effects of various covalently bound surface proteins upon the fracture of glycophorin-containing lipid bilayers it is necessary to know whether fracture of unmodified glycophorin bilayers results in any breakage of covalent bonds. It is generally assumed that freeze fracturing does not break any covalent bonds in membranes. This assumption is usually tested by thoroughly freeze fracturing a membrane sample and running the resulting material on a sodium dodecyl sulfate polyacrylamide gel to look for any new peptides caused by covalent bond breakage. The problem with such an approach is that the unbroken proteins

overload the gels and make low level detection of any new peptides virtually impossible. Glycophorin membranes, however, contain a built in system to allow for enrichment of fragments due to covalent bond breakage. We have taken advantage of this by preparing vesicles with uniformly fluorescent-labelled glycophorin; after fracturing these vesicles under liquid nitrogen we have dissolved them in a detergent buffer and passed the resulting solution through a wheat germ agglutinin sepharose affinity column. Under these conditions the agglutinin binds tenaciously to the N-Acetyl-D-Glucosamine residues of the glycophorin. Thus, all intact glycophorin molecules fail to pass through the columns and the eluate contains protein only if the fracturing process breaks covalent bonds. Because we use fluorescently labelled glycophorin, covalent bond breakage is signalled by fluorescence (detected with a sensitive fluorescence spectrophotometer) in the column eluate. After initial detection the eluate can be concentrated and electrophoresed on polyacrylamide gels to visualize the glycophorin fragments.

We have found that extensive freeze fracturing of the glycophorin vesicles does not result in any detectable breakage of covalent bonds. This is important because if normal freeze fracture caused the glycophorin molecule to fragment, there could be no hope of covalently attaching a surface label that would not break off during freeze fracture. The major difficulty with our findings is the meaning of "extensive freeze fracturing". In the present experiments we are fracturing the vesicles by prolonged grinding under liquid nitrogen. We assume that such a treatment cleaves a majority of the membranes; if our assumption is correct, we are correct in stating that freeze fracture normally breaks no covalent bonds in integral proteins (assuming glycophorin to be typical integral protein). However, if our treatment fails to cleave an appreciable percentage of the membranes, we cannot determine anything about the breakage of covalent bonds in the membranes.

Therefore another thrust of our research project has been to monitor the percentage of membranes which are split during freeze-fracturing. We have taken advantage of the properties of fluorescent energy transfer between two fluorophores. If two dyes are selected such that the emission spectrum of the first (donor) dye closely overlaps the excitation spectrum of the second (acceptor) dye, illumination of a sufficiently concentrated solution of the dyes at the excitation maximum of the donor will result only in emission from the acceptor. In other words, the energy absorbed by the donor is transferred to the acceptor and re-radiated as fluorescence. This process of energy transfer is strongly distance dependent and can exceed 90% efficiency at an average donor to acceptor distance of 30 Å. Beyond this distance the transfer decreases as the sixth power of the separating distance.

In considering the magnitude of the distances involved in such energy transfer it can be calculated that appreciable energy transfer

should occur between properly matched dyes attached to the phosphate head groups on opposite sides of a lipid bilayer (40-50 Å). Since the transfer is strongly distance dependent,

splitting of the bilayer will reduce the transfer of energy to essentially zero. For such an experiment to work, carefully matched dyes must first be selected. Their spectra must overlap to give a high efficiency of energy transfer; at the same time the Stokes Shift of each dye must be maximal so that the donor can be excited by a wavelength that will not directly excite the acceptor. In addition, dyes that can be easily chemically coupled to the lipids must be chosen. Finally, a method for synthesizing vesicles with the proper donor on one surface of the bilayer and the correct acceptor on the other surface must be developed.

We have already chosen a dye pair which seems ideal for preliminary experiments. The biggest difficulty has been in synthesizing asymmetrically labelled vesicles. We start by making vesicles that are uniformly labelled on both surfaces with the donor dye, Fluorescamine. After synthesis the vesicles are treated with carbodiimide and glucoseamine, non-penetrating reagents that selectively destroy the donor dye on the outer membrane surface because an amide bond forms between the free (but essential) carboxyl group on the Fluorescamine and the amino-sugar. Following this step the acceptor dye (a cyanine dye) is coupled to the outer surface using another non-penetrating reagent. With this method we have made vesicles with the donor on the inner surface and the acceptor on the outer surface of the membrane and have monitored the amount of membrane splitting in our fracturing experiments. Before grinding under liquid nitrogen, energy transfer occurs and illumination of the vesicles at the excitation wavelength of the donor dye results in emission at the emission wavelength of the acceptor dye. After grinding the bilayer halves are too far distant for energy transfer, and acceptor emission decreases while direct emission from the donor increases in proportion to the amount of membrane cleavage. Although these changes can be measured at liquid nitrogen temperatures, thus avoiding any rearrangements of half membranes during thawing after fracture, in our preliminary experiments measurements have been made at room temperature (see Kirchansky and Branton, J. Cell Biology 75: 216a, 1977).

Although the technique of producing asymmetrically labelled vesicles by chemically destroying one of the dyes (on the outer membrane surface using a non-penetrating reagent) has been quite successful, the choice of dye pairs is severely limited because, to date, fluorescamine is the only dye that we have been able to effectively inactivate chemically. Consequently, we have spent the last few months in synthesizing fluorescently labelled phospholipids in which the fluorophore is attached to the lipid polar head group by enzymatically labile bonds. The basic idea is to make vesicles with the fluorescent lipid and then digest the fluorophore off the outer surface with a suitable soluble enzyme such as an esterase. This is an attractive notion because enzymes are selective, gentle, and do not penetrate biological membranes.

Early experiments centered around attaching a suitable carboxyl containing fluorophore (Eastman Coumarin 343) to phosphatidyl glycerol via an ester linkage. In these experiments we found that the dye failed to react properly with most ester forming reagents. In addition, it appeared that direct attachment of the dye to the lipid did not provide an adequate binding site for the enzyme. As a result, we developed alternate chemical procedures for esterifying Eastman 343. Using these procedures we have attached ester containing polymers (glycol-succinyl) of various lengths to the dye. These were next tested to see if they were adequate substrates for enzymes with esterase activity. It was found that polymers containing twelve or more carbon atoms (dye-glycol-succinyl-glycol-succinyl) acted as excellent substrates and were rapidly cleared from the dye both by hog liver esterase and by chymotrypsin. Shorter polymers (dye-glycol-succinyl) were progressively poorer enzyme substrates. It would seem to be a straightforward matter to merely attach the dye with the substrate chain to a phospholipid to produce the desired fluorescent probe. However, we have experienced great difficulty at this final stage of the synthesis and this problem has significantly slowed progress. However, the hitherto unsuccessful syntheses have produced much useful information about producing fluorescently labelled membranes and have suggested other possible solutions to the problem of producing asymmetrically labelled lipid bilayers. Recent efforts using oxyl-chloride activation appear particularly promising.

Realizing that visualization of small covalent labels on trans-membrane proteins will require maximal resolution in the freeze-fracture replicas, we have modified our freeze-etch device so that fracturing and rotary replication can be done at liquid He temperatures. This device will be evaluated more completely in the coming year.

IV. PUBLICATIONS

Kirchanski, S. and Branton, D. 1977. Fluorescent energy transfer measurement of frozen membrane fracture. J. Cell Biology 75: 216a.

Branton, D. and Kirchanski, S., 1977. Interpreting the results of freeze-etching. J. Microsc. 111:117-124.