STUDIES OF MEMBRANE STRUCTURE BY FREEZE-ETCHING

Progress Report

Daniel Branton

PRESIDENT AND FELLOWS OF HARVARD COLLEGE
The Biological Laboratories
Harvard University
Cambridge, MA. 02138

MASTER

NOTICE
This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Energy Research and Development Administration, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

Reporting Period: 1 July 1976 to 30 June 1977

PREPARED FOR THE U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION
DIVISION OF BIOLOGY AND MEDICINE
UNDER CONTRACT AT (11-1)-2423

86
DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

LEGAL NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the U.S. Energy Research and Development Administration, nor any of their employees, nor any of their contractors, subcontractors, or their employees, make any warranty, expressed or implied, or assume any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represent that its use would not infringe on privately owned rights.

I. ABSTRACT

The structure and organization of biological membranes has been 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 have been 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. INTRODUCTION

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 Pinta da Silva and Branton, 1971, Chem. Phys. Lipids 7:9-14) 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 hydrophylic 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. PROGRESS REPORT

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 September 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. We use fluorescently labelled glycophorin. Covalent bond breakage is therefore 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 performed the above experiment and 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 develop a technique 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 can make vesicles with the donor on the inner surface and the acceptor on the outer surface of the membrane. We now plan to go on to monitor the exact amount of membrane splitting in our fracturing experiments. Before grinding under liquid nitrogen, energy transfer will occur and illumination of the vesicles at the excitation wavelength of the donor dye will result in emission at the emission wavelength of the acceptor dye. After grinding the bilayer halves will be too far distant for energy transfer, and acceptor emission will decrease while direct emission from the donor will increase in proportion to the amount of membrane cleavage. These changes can be measured at liquid nitrogen temperatures thus avoiding any rearrangements of half membranes during thawing after fracture.