A TEST OF MODELS OF MEMBRANE STRUCTURE BY MOLECULAR HYBRIDIZATION*

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SUMMARY: A considerable quantity of cytosol ('extrinsic') protein is incorporated during reformation of a particulate 'unit membrane' structure from detergent-solubilized bacterial membranes, as measured by differential labelling with radioisotopes. This finding argues against an all-or-none type of inclusion mechanism resident in the architecture of membrane-associated ('integral') proteins, if the reformed material is a reliable facsimile of the original bacterial membrane.

A current concept of membrane structure demands a class of proteins capable of preferential association with a lipid bilayer: integral proteins intrinsic to the membrane, in distinction to extrinsic cytosol proteins. This concept has evolved from the finding of an apparent majority structural protein to a complex of integral proteins (1-9). Alternate possibilities for the mechanism of incorporation of integral proteins into the membrane exist. On one hypothesis the molecular architecture of the integral protein obligates it to solution in or attachment to the lipid bilayer; possibly as a consequence of a predominance of apolar amino acid side chains being exposed on the protein surface (10, 11). Alternately, information as to cellular location is not coded in the protein but a mechanism exists for segregating and directing proteins to their final location; for instance, via a separate class of membrane-associated ribosomes (12).

If locality is encoded within individual protein species, then it

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would be predicted that solvated membrane proteins would preferentially reassociate with reforming lipid bilayers while cytosol proteins would not be captured in the non-aqueous phase. Alternately, on the opposing view, reformed lipid membranes could include proteins of the cytosol as well as original membrane-associated proteins. The finding that particulate membrane proteins can be solvated and then reassociated into at least a morphologically similar structure provides the possibility of an experimental test (13).

We describe such a test of these models. Detergent-solvated bacterial membranes (M) were reassociated, following removal of excess detergent, in the presence of differentially labelled supernatant (S) cytosol protein. A crop of particulate vesicles, the reassociated fraction (R), showing typical 'unit membrane' structure in the electron microscope (Dunlop and Nelson, unpublished) resulted. On the first hypothesis the M proteins should constitute the bulk of the R protein, little if any S protein being incorporated. On the second hypothesis both M and S protein should be found in the R fraction. The results of our test support the second hypothesis.

MATERIALS AND METHODS

A prototrophic stem line of Escherichia coli strain K 12 was grown in a tryptone (0.5%) glucose (0.2%) salts (0.2% NaCl, 0.1% KH_2PO_4, 0.4% K_2HPO_4) medium supplemented with either 15 microCi of [^3H] L-leucine or 3 microCi [^{14}C] L-leucine per 100 ml. The cultures were aerated on a rotary shaker held at 37°. Growth was followed turbidimetrically and harvested by centrifugation in mid-exponential phase. Spheroplasts were prepared in isotonic sucrose solution by treatment with lysozyme (14). All enzymes and sodium dodecyl sulfate, which was recrystallized from ethanol, were obtained from Sigma and isotopes from New England Nuclear. Protein was determined by the Lowry method using Fisher phenol

reagent and fraction V bovine albumen as a standard. Procedures for obtaining the operationally defined M, S, and R fractions are contained in the footnotes to the Table.

RESULTS

The input of radioactivity and protein in the M and S fractions, and the output of radioactivity in the reassociated R fractions, are given in Table 1. Using the input and output volumes (given in the footnotes) the ratios of input to output S to M protein in the reciprocally labelled couples can be calculated. The similarity of these ratios for each couple indicates that the proportion of S to M protein in the reassociated fractions is not markedly different from the input ratios. Hence there is no evidence from our test for preferential incorporation of previously membrane-associated protein into the reformed 'unit membrane'-like structure.

DISCUSSION

It is possible that the reassociation experiment described here is not a valid test of actual membrane formation, regardless of the morphological similarity. The known drastic action of sodium dodecyl sulfate may be such as to convert membrane non-associable cytosol proteins to associable polypeptides, coating the surface with the lipid phase soluble lauryl hydrocarbon 'tails', or inverting the presumed apolar core of the protein to the surface (15, 16). The relative volume of the R fraction pellet, when compared to the supernatant, is too small to permit explaining the bound S protein by mechanical entrapment within the globular membrane vesicles. Attachment of cytosol protein to the inside and outside surfaces of the vesicles may account for the results, rather than an incorporation of cytosol protein into a lipid bilayer; a possibility we are investigating by attempts at differential dislodgement of M and S protein from the R fraction.

Table 1

input into M and S fractions[i]

fraction	protein (mg/ml)	isotope[ii]	activity (dpm/ml)
M	3.98	^{14}C	70,069
	4.03	^{3}H	182,012
S	7.17	^{14}C	12,045
	7.27	^{3}H	26,688

output from R fractions[iii]

input fraction	[^{14}C] M + [^{3}H] S couple			[^{3}H] M + [^{14}C] S couple		
		S/M ratio			S/M ratio	
	activity (dpm/ml)	input	output	activity (dpm/ml)	input	output
M	59,208	0.954	1.02	246,296	0.166	0.140
S	60,278			34,399		

i The fractions were obtained by lysing spheroplasts from 300 ml of growth by rapid dilution into 100 ml of cold 3.3 mM Tris chloride buffer, pH 7.4, digesting with 1 microgram/ml deoxyribonuclease type I with $MgSO_4$ added to 1 mM for thirty minutes at 37°, followed by centrifugation in the cold in an angle rotor at 13,000 g for thirty minutes. The pellets, washed and resuspended in 4.0 ml buffer, constituted the M fractions. The supernatants were lyophilized and dissolved in 10.0 ml of water as the S fractions.

ii All counts have been corrected for background, and, in the R fractions, for diffusible and precipitable material occurring in the M and S fractions respectively, reassociated separately as controls. Ratios of input and output protein are calculated from the dpm/ml multiplied by the actual input and output volumes in ml.

iii Aliquots of one-third of the volume of the fractions (1.33 ml of the resuspended M fractions and 3.33 ml of the dissolved S fractions) were mixed, singly and in cross-coupled configuration viz a viz the labelling isotopes, made to similar minimal volumes with buffer, and solvated by addition of 10% sodium dodecyl sulfate to 0.30% final concentration, using Visking dialysis bags as containers. The reassociated fractions were formed by dialyzing off excess detergent against 100 volumes of cold buffer (phospholipid does not dialyze), followed by redialysis against cold buffer containing 30 mM $MgSO_4$. The resulting precipitates were collected by centrifugation, washed and made to 0.55 ml with buffer as the R fractions.

Since the R fraction is operationally defined and is characterized at present solely by its appearance in the electron microscope, and static biochemical parameters (light density in the ultracentrifuge, phospholipid content by Folch solvent extraction and thin layer chromatography, and incorporation of the oil-soluble dye Oil Red O), it is conceivable that the reassociation phenomenon has little relationship to actual membrane formation. A firm assessment of this criticism must await functional criteria applicable to the R fraction: recoverable enzyme activities or active membrane functions (17, 18).

Further questions are open to study using the technique of molecular hybridization described here: whether a special group of cytosol (and membrane) proteins is more likely to associate, whether affinity or solubility constants can be determined, at what stoichiometry phospholipid and divalent cations are required in membrane structure, and, eventually, a method for analysis of molecular architecture of the proteins and lipids involved.

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