Review Letter

PROTEIN MOBILITY IN MEMBRANES

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1. Introduction

The investigation of the mobility of components of biological membranes is currently attracting considerable attention and has recently been the subject of two detailed reviews [1,2]. The possibility of rapid diffusion arises from the concept that many membranes contain regions of lipid bilayer which are predominantly in a liquid-crystalline, that is, fluid state [3–8]. Proteins embedded in the lipid bilayer might be expected to exhibit relatively rapid rotational and translational diffusion. Of course, diffusion might also be restricted by protein–protein interactions in the membrane or by attachment to microfilaments or other assemblies within the cell [9]. The purpose of this review letter is to summarise measurements and observations of protein mobility in membranes. In addition, progress towards developing new techniques for measuring protein diffusion will be briefly described.

2. Lateral diffusion

Various methods have been devised for measuring the lateral diffusion of proteins in membranes. These methods have the common feature that proteins are labelled in one way or another so that their distribution in the membrane may be visualised. Lateral movement may then be inferred, and in some cases quantified, by changes in distribution. This has been achieved in essentially two different ways. Firstly, be various means, a non-random distribution of proteins is set up in the membrane, the time for the distribution to relax to its equilibrium state is then observed. Secondly, proteins which are initially randomly distributed may, in some cases, be caused to aggregate by a suitable stimulus, such as interaction with antibodies or lectins.

2.1. *Intermixing experiments*

The first evidence that lateral diffusion of membrane proteins may be rapid was obtained by Frye and Edidin [10]. Surface antigens of two different cell lines were visualised using immunofluorescence techniques; after fusion by inactivated Sendai virus the antigens rapidly became intermixed. Total mixing was observed in 90% of the heterokaryons after 40 min incubation at 37°C. A lateral diffusion constant in the order of $10^{-10}\,cm^2\,s^{-1}$ may be estimated from the rate of intermixing. The intermixing process is unaffected by metabolic inhibitors but is strongly temperature dependent. Petit and Edidin [11] found that the rate of intermixing decreases with temperature in the range 37 to 21°C and again below 15°C. However, in the range 21 to 15°C, the rate actually increases as the temperature falls. This interesting behaviour may be a consequence of phase separation of the membrane lipids [12].

In a further experiment, Edidin and Fambrough [13] applied a small patch of fluorescent-labelled anti-membrane antibody onto the surface of a cultured muscle fibre. Partial characterisation of the antigenic sites indicated that they are most probably proteins of at least 200 000 mol. wt The patches were observed to spread with time, from the rate of spreading a diffusion constant at room temperature of $1 - 3 \times 10^{-9}\,cm^2\,s^{-1}$ was calculated. The spreading rate is decreased by lowering the temperature and becomes zero after fixation with glutaraldehyde.

Two groups have recently measured the lateral diffusion of rhodopsin in the disc membrane of rod outer segments. Poo and Cone [14] bleached rhodopsin in one half of a single rod outer segment with a flash of light, thus creating an asymmetric distribution of rhodopsin molecules. This distribution was monitored spectrophotometrically and was found to return to a symmetric distibution in less than 60s as rhodopsin diffused laterally across the disc membrane. The data enabled diffusion constants of $(3.5 \pm 1.5) \times 10^{-9} cm^2 s^{-1}$ for frog and $(3.9 \pm 1.2) \times 10^{-9} cm^2 s^{-1}$ for mud puppy rhodopsin to be calculated. Essentially similar experiments were also carried out by Liebmann and Entine [15]. Although their diffusion constants are slightly higher [$(5.5 \pm 0.6) \times 10^{-9} cm^2 s^{-1}$ for frog and $(4.7 \pm 0.9) \times 10^{-9} cm^2 s^{-1}$ for mud puppy rhodopsin], the differences between the two sets of data are within the experimental uncertainty.

A probe method for measuring lateral diffusion which in principle is essentially similar to the rhodopsin experiments has recently been described [16]. Proteins of erythrocyte membranes were covalently labelled with fluorescein. A single ghost was observed in a fluorescence microscope and fluorescein on one half of the ghost bleached with an intense light beam. No significant diffusion of fluorescein into the bleached half of the ghost could be detected over a period of 20 min. The data apparently set an upper limit on the diffusion coefficient of the labelled components of $3 \times 10^{-12} cm^2 s^{-1}$ at room temperature. It is interesting to note that Verma and Wallach [17], on the basis of spin label studies, have also proposed that the diffusion of components of the erythrocyte membrane is restricted.

2.2. *Protein aggregation*

Over the past few years, aggregation of antigenic sites on cell surfaces following antibody binding has been repeatedly observed [18–26]. The distribution of bound antibodies is normally detected by labelling them with fluorescent or ferritin markers. The main effects which are observed following antigen–antibody interaction may be summarised as follows. Initially the surface antigens are either randomly distributed over the cell surface [26] or aggregated in small clusters [24]. Interaction with multivalent antibodies induces aggregation of the surface antigens into patches. All the available evidence indicates that patch formation proceeds independently of metabolic processes but its rate is temperature dependent. That is, patch formation may be simply a consequence of random lateral diffusion in the plane of the membrane. As collisions take place, aggregation results from crosslinking by the multivalent antibodies. No aggregation is observed when monovalent antibody fragments are used. In some cases the patches subsequently aggregate together to form a single aggregate or 'cap'; this may then be followed by pinocytosis. These latter processes are evidently complex phenomena which require metabolic energy.

Aggregation effects are also observed when lectins bind to cell surfaces [22,27–30]. The phenomena are essentially similar to those induced by antigen–antibody interaction.

Redistribution of membrane components has also been detected using the technique of freeze-fracture electron microscopy. Pinto da Silva [31] investigated the effects of pH and ionic strength on the distribution of membrane particles in erythrocytes. The particles are randomly distributed at pH 7.5 or pH 9.5 but become strongly aggregated at pH 5.5. At lower pH (4.5) the particles are again dissociated but further aggregation occurs below pH 4.0. The aggregation is rapid, reversible and inhibited by high ionic strength or glutaraldehyde fixation. In other experiments, temperature-dependent particle aggregation has been observed in alveolar membrane of *Tetrahymena* [32,33] and in plasma membranes of *Acholeplasma laidlawii* [34,35] and *E. coli* [36]. Particle aggregation induced by proteolysis [37,38], lectin binding [39] and treatment with glycerol [40] has also been reported. On the other hand, temperature-dependent particle aggregation is not observed in plasma membranes of lymphoid cells [41]; it is proposed that this is due to restriction of free diffusion of the membrane proteins [42].

3. Rotational diffusion

3.1. *Fluorescence measurements*

The technique of fluorescence polarization has long been used to measure the rotational diffusion of proteins in aqueous solution [43]. Early studies of steady state polarizations of fluorescent probes bound to various membranes have been summarised by

Radda and Vanderkooi [44]. None of these experiments give any clear information concerning the rotation rates of membrane proteins.

Wahl et al. [45] used nanosecond pulse techniques to investigate the fluorescence polarization of analino naphthalene sulphonate (ANS) and dansyl chloride bound to electroplax membrane fragments. A very rapid partial depolarization was attributed to independent but restricted motion of the probe in its binding site. The residual polarization did not decay during the time over which fluorescence could be observed, setting a lower limit on the rotational relaxation time (ρ_0) of the membrane proteins of 700 ns.

A somewhat different approach was employed by Inbar, Shinitzky and Sachs [46,47]. They labelled lectins with fluorescein and determined rotational relaxation times in solution. The fluorescent lectins were then bound to normal and transformed lymphocytes and fibroblasts and values of ρ_0 again obtained. It is claimed that rotational relaxation times of the bound lectins are a measure of the rotational mobility of the lectin receptors in the membrane, so that differences in ρ_0 reflect differences in receptor mobility.

A puzzling feature of these experiments is that the values of ρ_0 for the bound lectins are not much different from those found in solution. Thus for concanavalin A, in solution ρ_0 = 58 ns whilst the membrane bound lectin exhibits values of ρ_0 in the range 70–160 ns. Since there is a good deal of evidence that the 'viscosity' of membranes is effectively about one hundred times that of water [2,48], it would be expected that rotational relaxation times of proteins embedded in the membrane would be in the order of microseconds or longer. The reason for this discrepancy is not clear, but it seems unlikely that the rotational relaxation times of the bound lectins relate to the rotation of proteins which form an integral part of the membrane.

3.2. *Rhodopsin*

The spectroscopic events which follow excitation of rhodopsin provide a basis for measuring the rotational diffusion of this protein in the disc membrane of retinal rods. The first studies were carried out by Hagins and Jennings [49] at low time resolution. They found that no dichroism could be induced in the plane of the disc membrane following partial bleaching with a flash of plane polarized light. They suggested that this could be due to rotation of the rhodopsin molecule which is rapid compared with the time of measurement (100 ms).

Tao [50] investigated the polarization of fluorescence of the derivative of rhodopsin, *N*-retinyl opsin and established a lower limit of 0.26 μs for ρ_0. Brown [51] confirmed the earlier data of Hagins and Jennings and demonstrated that photodichroism can be detected after fixation with glutaraldehyde.

The rotational relaxation time of rhodopsin was successfully measured in an elegant experiment by Cone [52], who used a flash photolysis apparatus capable of resolving events in the microsecond time range. Following excitation by a brief pulse (5 ns) of polarized light, transient dichroism arising principally from the lumirhodopsin intermediate was detected. From the rate of decay of dichroism, a value of ρ_0 of about 20 μs at 20°C was calculated. It should be noted that this value refers to rotation about an axis normal to the plane of the disc membrane. It is particularly satisfying that the rotational and lateral diffusion measurements made with rhodopsin are mutually consistent, that is both are compatible with a particle of diameter about 50 Å freely diffusing in a medium of viscosity about 2 P.

3.3. *Bacteriorhodopsin*

Illumination of bacteriorhodopsin in purple membrane fragments of *Halobacterium halobium* produces a transient spectroscopic species centered at 410 nm of lifetime about 10 ms. This photoproduct was used to investigate rotational diffusion of bacteriorhodopsin [53]. The transient absorption was found to be strongly dichroic following flash illumination with plane polarized light; however, unlike rhodopsin, the dichroism did not decay during the period of observation. From this it was concluded that the rotational relaxation time is slower than 20 ms, that is more than 1000 times slower than rhodopsin. This difference is consistent with X-ray diffraction data which indicate a crystal-like hexagonal packing of bacteriorhodopsin in purple membranes [54] as opposed to a planar liquid-like array of rhodopsin in disc membranes [55].

3.4. *Other membrane proteins*

Junge [56] utilised the photodissociation of the cytochrome a_3–*CO* complex to investigate its rotation in the mitochondrial inner membrane. In these

experiments, no dichroism of the absorbance change resulting from photolysis by a plane polarized light pulse was detected. However, in subsequent experiments a dichroism was found. Moreover, only a slow decay of dichroism consistent with the rotation of the whole mitochondrion was observed [57].

Junge and Eckhof [58] observed photoinduced dichroism of chlorophyll a_1 in chloroplasts which persisted for at least 100 ms. This could mean that chlorophyll a_1 (which may be a chlorophyll–protein complex) is immobilised in the membrane, or alternatively that it rotates only about an axis which is normal to both the membrane and the plane of the porphyrin ring.

4. 'Slow' Probes for measuring protein mobility

4.1. *Rotational diffusion*

With one possible exception, the measurements discussed in the previous section support the expectation that rotational relaxation times of proteins in membranes should be in the order of microseconds or longer. These experiments were possible because in each case illumination of the protein produces a long-lived photoproduct. However, it is clearly desirable to develop a general technique which may be applied to any membrane protein. This in principle may be achieved by using a probe molecule in which excitation produces a spectroscopically distinguishable species of suitably long lifetime [53]. Such probes might be termed 'slow' probes to distinguish them from fluorescent or 'fast' probes which report on motions in the nanosecond time scale. Since triplet states have lifetimes typically in the order of milliseconds or longer at room temperature, measurement of the transient dichroism of the triplet-triplet absorption of suitable probes could in principle provide a general method of quantitatively investigating the rotation of membrane proteins.

4.1.1. Model systems

Some preliminary experiments to test the feasibility of the above suggestion have been reported [53]. These experiments were carried out using eosin as a probe. Eosin was bound non-covalently to bovine serum albumin and signals arising from the triplet–triplet absorption of the bound eosin were obtained. A transient dichroism of the triplet–triplet absorption is observed when the complex is dissolved in a viscous glycerol–water mixture. In this way, it was demonstrated that measurements of rotational relaxation times in the microsecond time range are feasible using this method.

Recently, we have developed techniques of labelling proteins covalently using eosin isothiocyanate [59]. In studies with simple model systems we have obtained encouraging evidence that meaningful measurements of slow rotations may be made using this probe [60]. Two results are shown in figs.1 and 2. Fig.1 shows the result of an experiment in which concanavalin A was labelled with eosin isothiocyanate and then conjugated to sepharose 4B. The sample was excited with a 2 μs pulse of light of wavelength 540 nm from a dye laser. The triplet–triplet absorption transient measured at 650 nm exhibits a dichroism which does not decay for the duration of the signal. This is the result to be expected for an immobilised protein; its significance is that it demonstrates that the dye does not exhibit unrestricted independent rotation about its point of attachment to the protein. Essentially similar results have been obtained with several different proteins.

Fig.2 shows the result of an experiment designed to test whether the technique gives quantitatively

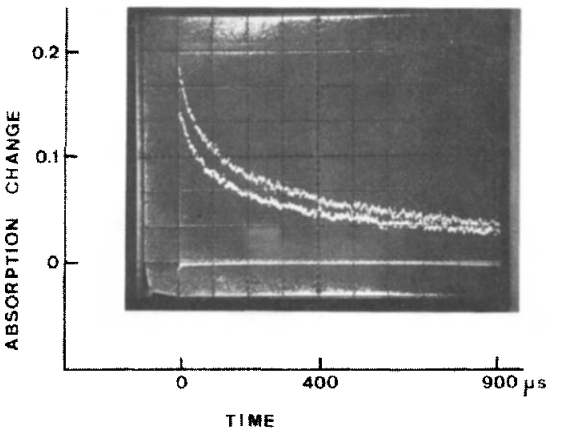

Fig.1. Triplet–triplet absorption at 650 nm of eosin–concanavalin A complex conjugated to Sepharose 4B and suspended in 80% glycerol. 7 μg eosin per mg protein, eosin concentration 1.6×10^{-4} M. Upper trace parallel, lower trace perpendicular polarization of measuring beam relative to exciting flash. Temperature 21.5°C.

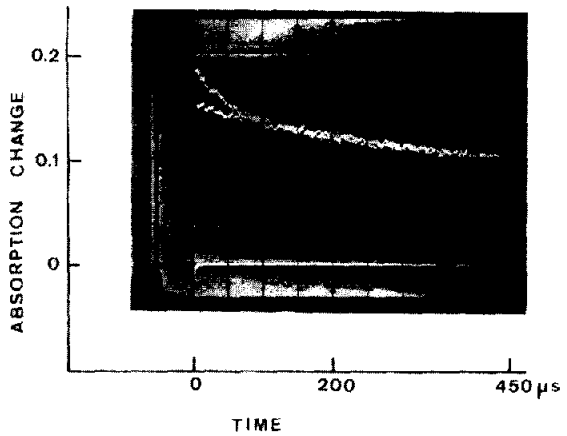

Fig.2. Transient dichroism of triplet–triplet absorption of eosin-ovalbumin complex in 97% glycerol. 11 μg eosin per mg protein, eosin concentration 8×10^{-6} M. Other details as fig.1.

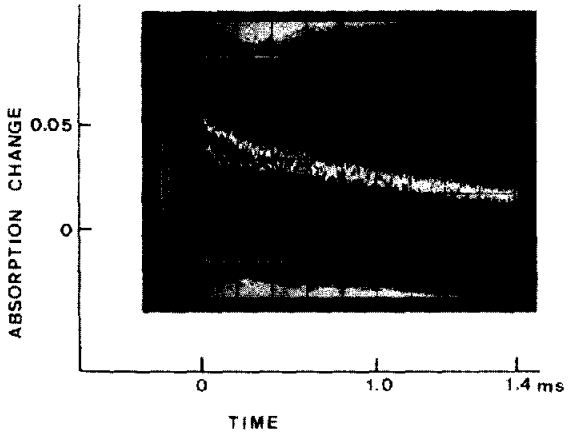

Fig.3. Dichroism of triplet–triplet absorption of eosin conjugated to bovine erythrocyte ghosts. 0.65 μg eosin per mg protein, eosin concentration $\sim 0.6 \times 10^{-5}$ M. Membranes suspended in 20 milliosmolar phosphate buffer pH 7.4. Other details as fig.1 and in text.

reliable information. In this experiment a transient dichroism is observed from a complex of eosin and ovalbumin dissolved in 97% glycerol; the rate of decay of dichroism gives a rotational relaxation time of 75 μs. This may be compared with the theoretical value of 28 μs which may be calculated for a spherical protein of mol. wt 43 500 in 97% glycerol at 21.5°C. The ratio of observed to theoretical values is in reasonable agreement with that of 2.2 previously obtained for ovalbumin in water by measurement of fluorescence polarization [43].

4.1.2. Red blood cell membranes

In view of the encouraging results obtained with model systems, we have recently begun experiments with membranes. A preliminary result obtained with red blood cell membranes is shown in fig.3. In this experiment, intact bovine erythrocytes were labelled with eosin isothiocyanate, then, after removing excess, label, ghosts were prepared. As may be seen from fig.3, a long-lived dichroism of the eosin triplet–triplet absorption is observed. The results place a lower limit on ρ_0 of 1.5 ms and indicate that ρ_0 most probably lies between 1.5 and 6.0 ms. Thus the labelled component(s) clearly has considerably less rotational mobility than that found for rhodopsin in the disc membrane [52]. Experiments to clarify the significance of this finding are currently in progress.

4.2. *Lateral diffusion*

It has been proposed [61] that the properties of triplet states may also be exploited to measure lateral diffusion. Triplet–triplet annihilation is normally a diffusion controlled reaction which can only occur when two triplets approach within a short (10–15Å) distance of each other. Thus measurement of the rate constant for triplet self-quenching provides a method of investigating diffusion rates. Because of the long lifetime of the triplet state, measurements can be made at quite low probe concentrations. So far this technique has only been applied to the investigation of lipid diffusion [61] but its extension to proteins may also be envisaged.

Razi Naqvi [62] has discussed some difficulties encountered in interpreting the results of lateral diffusion measurements in two dimensions. It has been known for many years that in a three-dimensional diffusion-controlled reaction, the instantaneous reactivity of a given molecule declines initially with time but thereafter attains a time-independent limiting value [63]. In two dimensions Razi Naqvi shows that a steady state is never reached, since the reactivity is a continuously decreasing function of time. This poses a serious problem in the evaluation of a diffusion constant from an experimentally measured bimolecular reaction rate.

5. Other techniques

It has been proposed that slow tumbling of spin labels may be investigated by means of non-linear electron spin resonance techniques [64,65]. Two classes of techniques, namely adiabatic rapid passage and stationary electron–electron double resonance (ELDOR) may in principle be used to characterise rotational processes with correlation times from 10^{-3} to 10^{-7}s. Stationary ELDOR responses obtained with spin labels in *sec*-butylbenzene at low temperature have been shown to be in agreement with theoretical predictions [65]. Some rotational measurements with muscle proteins in solution have also been reported [66].

Measurement of lateral diffusion of detergent micelles and liposomes has been reported using autocorrelation spectroscopy of Rayleigh scattered light [67]. This technique may also find application in the measurement of protein diffusion in membranes.

6. Conclusions

Most, but not all, of the observations summarised in this letter are consistent with rather free diffusion of proteins in membranes. At the same time, only in the case of rhodopsin has the molecular diffusion of a specified membrane protein been satisfactorily determined. In all other cases, various qualifications must be made with respect to the interpretation of the experimental data. Thus many observations are qualitative rather than quantitative and often the component of the membrane which is exhibiting diffusion is not well characterised. Some experiments may report on the diffusion of proteins which are superficially attached to the membrane rather than forming an integral part of it. Where protein redistribution is detected using an optical microscope, it is possible that movements of macroscopic areas of membrane are involved, rather than true diffusion of individual proteins.

Some of these difficulties may be overcome by the new techniques which are currently being developed to investigate protein mobility. While these methods will undoubtedly encounter problems of their own, they offer the possibility of obtaining more precise information than is currently generally available. This in turn may assist in the important task of relating the mobility (or immobility) of proteins to the functional properties of membranes.

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