Open Question

Diffusion of protein complexes in prokaryotic membranes: fast, free, random or directed?

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The random two-dimensional diffusion coefficient of many membrane proteins estimated from a variety of biophysical measurements is much greater than must be inferred from certain more biochemically based experiments in prokaryotes. One solution to this paradox suggests that such diffusion is neither fast nor free.

It is now axiomatic that biological membranes are generally organized as a 'fluid mosaic', consisting of a phospholipid bilayer – in, on and through which are dispersed a variety of proteins and protein complexes[1,2]. A crucial corollary of this fluid mosaic picture is that lipids, proteins and oligomolecular protein complexes can move laterally in the plane of the membrane, a view amply confirmed (e.g. Ref. 3) by a variety of biophysical techniques such as NMR, ESR and, in particular, fluorescence recovery after photobleaching (FRAP)[4–6] and 'lateral electrophoresis'[7,8].

However, as stressed in *TIBS* by Bretscher[9], a knowledge of the exact diffusion coefficients of the protein complexes is in many cases crucial to a proper understanding of the organization and functioning of such membranes. Equally, I would suggest that the *degree of randomness* of such diffusion may be a question of special significance. Yet, even at a very superficial and coarse-grained level, I perceive a serious discrepancy between many biophysical estimates of membrane protein diffusion coefficients in eukaryotic systems and those which one must infer for prokaryotes from considerations of more biochemically-based experiments.

Although, for technical reasons, the great majority of the work on membrane protein diffusion (especially that based on FRAP) has been carried out on the plasma membranes of eukaryotic cells. I wish to concentrate, in so far as is possible, on the organization of so-called energy coupling membranes, and in particular that of the bacterial cytoplasmic membrane.

The paradox I perceive is easily stated (Fig. 1). Imagine a model, spherical bacterial cell of radius $R = 0.5$ μm. A protein (complex) newly inserted at an arbitrary position in such a membrane, if free to diffuse randomly in the plane of the spherical shell membrane, has an (exponential) relaxation time for randomization, τ, given by

$$\tau = R^2/2D$$

where D is the two-dimensional diffusion coefficient (e.g. Refs 7, 8, 10). Actually, equation 1 assumes that the proteins take up a negligible area of the membrane and diffuse randomly and independently. Thus D will be somewhat overestimated if calculated, by means of equation 1, from measurements of τ[11], but we shall ignore this for the moment.

Table I gives the relaxation times calculated from equation 1 for (spherical) cell radii of 0.5 μm and 1 μm, based on a range of typical D values covering two orders of magnitude. I take it as a consensus view that phospholipid diffusion coefficients are approximately 10^{-8} cm^2 s^{-1} *in situ*[4–6,12], whilst those of proteins, in the absence of extra-membranal constraints such as membrane–cytoskeleton interactions[13,14] or the presence of 'matrix' proteins[15], lie in the range 10^{-10}–5×10^{-9} cm^2 s^{-1} (Refs 3–8, 12).

Now, because of the inverse square relationships between R and D in equation 1, the small size of bacteria (let alone membrane vesicle preparations therefrom) places much greater constraints on one's model building, for given values of τ, than the eukaryotic systems usually considered. Thus, it is evident (Table I) that even an average diffusion coefficient as slow as 10^{-11} cm^2 s^{-1} will allow an essentially complete randomization of membrane protein complexes during the time necessary for a bacterial cell to divide (say 20–120 min under typical laboratory conditions). I am not aware of any direct evidence indicating 'anchorage' of a significant percentage of bacterial membrane proteins by cytoplasmic structures, and the known sites of attachment of the cytoplasmic membrane to the cell envelope (see Ref. 16) are far too few to represent any kind of a globally restrictive barrier to lateral cytoplasmic membrane protein diffusion. Thus, by extrapolating the biophysical measurements made with eukaryotic systems to the bacterial cytoplasmic membrane, one must infer that protein complexes of a particular type should be randomly dispersed in the plane of that membrane. The present problem arises, however, from the finding that several lines of evidence indicate that they are not. Although this is an enormous (and fundamental) topic, I will briefly mention three types of approach.

Segregation of membrane proteins in dividing cells

A variety of studies, though not all,

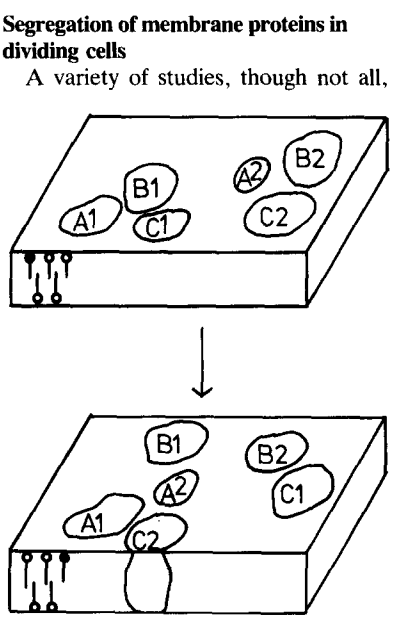

Fig. 1. Diagrammatic 'snapshots' of two possible time-dependent dispositions of protein complexes in a typical fluid mosaic membrane. The questions we wish to consider are (1) the speed at which the individual complexes can diffuse, and (2) the degree of randomness of their diffusion pathways. More explicitly, are the time-averaged, nearest-neighbour relationships for any particular complex independent of the nature of that complex, or are there preferred pathways (of whatever length) for the diffusion of such complexes?

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reviewed by Kepes and Autissier[17] and by Poole[18] indicate that the segregation of newly-incorporated bacterial membrane proteins between mother and daughter cells is significantly non-random. Nevertheless, *some* membrane proteins *do* seem (especially in experiments with non-synchronized cultures) to be more-or-less randomly arranged in the bacterial cytoplasmic membrane (see, for example, Ref. 18). It is not known with certainty whether in such cases it is their *insertion* that is random or their lateral diffusion rapid.

Freeze–fracture studies

Although many uncertainties remain concerning the interpretation of freeze–fracture experiments (e.g. Ref. 19), a number of such studies have indicated that the distribution of particles seen in the fracture faces of the inner mitochondrial[20], thylakoid[21] and bacterial cytoplasmic membranes[22], when quenched above their gel-to-liquid phase transition temperatures, are significantly non-random.

A particularly striking, and apparently unimpeachable, example of an extremely non-randomly organized prokaryotic membrane is the cytoplasmic membrane of purple non-sulphur bacteria, which I will consider in some detail.

Photosynthetic bacteria

Under aerobic conditions, many Rhodospirillaceae carry out oxidative phosphorylation, whilst under anaerobic or semi-anaerobic conditions they may be grown photoheterotrophically. Transition between these two regimes provides a convenient and interesting system for the study of prokaryotic membrane development.

The transition to phototrophic conditions is accompanied, *inter alia*, by: (1) the synthesis of massive amounts of the light-harvesting protein complexes; (2) their incorporation into special areas of the cytoplasmic membrane (CM) termed the intracytoplasmic membrane (ICM); (3) a seven-fold increase in the ratio of ICM:CM surface area; and (4) a 36% increase in the total membrane surface area[23]. These last two facts are consistent with the finding[24] that many components of the respiratory and (cyclic) photosynthetic electron transport chains are identical.

As Drews[25] has recently remarked, 'Numerous studies have shown that cytoplasmic and intracytoplasmic membranes form a continuous membrane system, but both membranes are clearly different in function and composition . . .

TABLE I. Relaxation times (τ) (Equation 1) for the lateral randomization of protein complexes newly inserted into spherical shell membranes of the radii (R) indicated, for given values of the two-dimensional diffusion coefficient D.

R (μm)	D ($cm^2\ s^{-1}$)	10^{-9}	10^{-10}	10^{-11}
0.5	τ	1.25 s	12.5 s	2 min
1		5 s	50 s	8 min

Clearly the differentiation by selective synthetic processes must be greater than equalization by diffusion and membrane flow.' In view of equation 1, we may therefore make a quantitative statement. If we take a protoplast volume of 0.4 μm^3 (Ref. 23), equivalent to a (sphere's) radius of approx. 0.5 μm, the effective *random* two-dimensional diffusion coefficients *must* be significantly less than 10^{-12} (if not 10^{-13}) $cm^2\ s^{-1}$ (Table I) to account for these observations. It should be mentioned that the lipids of these membranes are certainly in the 'fluid' state since their gel-to-liquid phase transition occurs below 0°C (Ref. 26). How do these inferred diffusion coefficients compare with those implicated in other energy-coupling membranes?

Energy-coupling membranes

The consensus view at present, based upon a variety of biophysical approaches, seems to be that the two dimensional diffusion coefficients of the protein complexes of the inner mitochondrial[3,8] and the chloroplast thylakoid membrane[27] lie in the range 10^{-10} to 10^{-9} $cm^2\ s^{-1}$. Note, however, that calculations based on the kinetics of chlorophyll fluorescence changes in thylakoids indicate that $D \approx 10^{-11}$ $cm^2\ s^{-1}$ at 25°C (Ref. 27). In this type of membrane, knowledge of the exact values of D is quite crucial in understanding the lateral transfer of reducing equivalents[27,28] and (for so-called localized coupling theories) of free energy[29]. The values cited are already significantly lower than those (approx. 3×10^{-9} $cm^2\ s^{-1}$) found for photopigment diffusion in vertebrate rods[30,31]; such latter values are much more in line with those to be expected from hydrodynamic considerations[32]. Thus, even in the case of eukaryotic energy coupling membranes, some unidentified factors seem to be operating to decrease D values below their viscous drag-constrained limits. The relatively high protein contents of biomembranes *per se* cannot alone account for this[33]. Thus, on applying the foregoing considerations to prokaryotes, where we need, most evidently in the case of photosynthetic organisms, to restrict free, random diffusion to much less than even 10^{-11} $cm^2\ s^{-1}$ to accord with the observations, we find a glaring lacuna in our knowledge.

Potential solutions

Two possible solutions to our problem are: (1) widespread anchorage of membrane proteins to cytoplasmic and/or cell envelope components, or (2) 'domain' formation by localized or global lipid crystallization (see Ref. 34). As indicated above, neither of these solutions seem plausible in prokaryotes on the basis of our present knowledge. One therefore seems forced to the view that the organization of (and dynamic interaction between) protein complexes in the bacterial cytoplasmic membrane is both more extensive and more subtle than that implied by a view in which they are free to diffuse rapidly and randomly in the plane of such membranes. Such an organization might require the input of electron transport- or ATP-derived free energy[29].

In concluding his recent review of lateral motions of membrane proteins, Axelrod[3] stated, 'Further advances will demonstrate . . . whether the functional mobility [of such protein complexes] is random diffusion or directed flow.' The considerations raised here would seem strongly to indicate the latter.

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Emerging Techniques

Fluorescent analog cytochemistry

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Functional molecules or organelles, covalently labeled with fluorescent probes, can be re-incorporated into living cells where they reveal native molecular activity in a wide variety of cellular processes.

Fluorescent analog cytochemistry is a new approach to elucidating the behavior, interaction and spatial organization of specific cellular components in living cells[1,2]. A fluorescent analog consists of the native cellular component plus one or more covalently attached fluorescent probes, chosen for their spectral properties and/or environmental sensitivities. The technique, previously called molecular cytochemistry[1], takes advantage of the sensitivity of fluorescence detection methods and the specificity of fluorescently-labeled molecules. Cells containing fluorescent analogs can be analysed by qualitative and/or quantitative fluorescence microscopy and by flow cytometry.

Basic principles

Five sequential steps are involved: (1) purification of the molecule or organelle, and fluorescent labeling to produce the fluorescent analog which is selected for by purifying a defined ratio of dye to molecule and an absence of unbound fluorescent dye; (2) comparison of the biochemical, biophysical and physiological properties of the fluorescent analog with those of its unlabeled counterparts *in vitro*; (3) characterization of the spectroscopic properties of the analog *in vitro*, both alone and in combination with other molecular species with which it may interact *in vivo*; (4) incorporation of the analog into living cells followed by testing the functional capability of the analog *in vivo*; and (5) analysis of the cells containing both the fluorescent analog and a soluble control molecule labeled with a distinct fluorophore. Some of the technical and biological aspects of this method as applied to contractile proteins have been reviewed recently[2–4].

A fluorescent analog of the cytoskeletal protein actin has been characterized in detail[5]. Actin labeled at Cys 374 with 5-iodoacetamido-fluorescein (AF-actin) polymerizes into F-actin filaments forms Mg^{2+} paracrystals and activates myosin magnesium ATPase, all to a degree comparable with unlabeled actin controls. The labeled filaments appear normal under the electron microscope and behave as expected in cell extracts capable of gelation, solation and contraction[1,5]. The fluorescein moiety is at the surface of labeled actin filaments, as shown by its ability to bind anti-fluorescein antibody[6,7] and by measuring distances between subunits by resonance energy transfer. Fluorescent analogs have now been prepared for some lipids as well as many cytoskeletal proteins including actin[1–3], tubulin[2,3], α-actinin[2,3], vinculin[3], fibronectin[12], calmodulin[13,14] and tropomyosin (see Ref. 2 for a review).

The spectroscopic properties of the analog (such as fluorescence quantum yield, corrected excitation and emission spectra, and lifetime of fluorescence) are determined by standard solution methods in physiological buffers. Subsequently, the effects of microenvironmental changes on these spectroscopic parameters are analysed. The most physiologically relevant effects are emphasized (e.g. ionic strength, pH, Ca^{2+} concentration, solvent polarity and binding to specific substrates or ligands)[5]. While many of the spectroscopic properties of the actin labeled with 5-iodoacetamido-fluorescein are now available[5], other analogs are less well characterized.

There are now several methods for injecting molecules into cells, including direct microinjection with electrodes, fusion with liposomes or red blood cell ghosts[2,4,15] and newer methods, including hypo-osmotic shock treatment[16] and short circuited pinocytosis[17]. As a rule, the final concentration of the analog in the cell is designed to be less than ~10% of the concentration of the endogenous cellular component[2].

Once the cells are loaded with fluor-

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