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Variation in protein lateral diffusion coefficients is related to variation in protein concentration found in mitochondrial inner membranes

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The electrophoretic freeze-fracture electron microscopy method (Sowers, A.E. and Hackenbrock, C.R. (1984) Proc. Natl. Acad. Sci. USA 78, 6246–6250) for measuring the lateral diffusion coefficient of integral proteins was applied to a large population of spherical-shaped mitochondrial inner membranes. Membrane integral protein concentration was estimated by determining the intramembrane particle concentration. Analysis of the data reveals that: (a) the radii of the spherical inner membranes in the selected population ranged from 0.22 to 1.2 μm, (b) the intramembrane particle concentrations ranged from 2300 to 6400 per μm^2, and (c) the calculated lateral diffusion coefficients of the intramembrane particles ranged from $1.3 \cdot 10^{-10}$ to $3.35 \cdot 10^{-9}$ cm^2/s. The data clearly show a naturally occurring large range in protein concentration in the mitochondrial inner membrane and an inverse correlation of lateral diffusion coefficient with the membrane protein concentration. This study is the first to show that the lateral diffusion coefficient of integral proteins in a native membrane varies as the membrane protein concentration.

Introduction

Membrane proteins and lipids are known to diffuse laterally in the plane of biological membranes. Some catalytic events appear to depend on lateral diffusion and random collisions between specific membrane components [1–3]. Studies in our laboratory reveal that the average distance between the electron transfer components of the mitochondrial inner membrane increases when exogenous lipid is incorporated into the inner membrane, resulting in proportional decreases in the rates of electron transfer between specific components [4,5]. More recently, the lateral diffusion coefficients of the major electron transfer components have been assessed by fluorescence recovery after photobleaching, and it has been determined that electron transfer between these components is coupled to their lateral diffusion [6].

In the present study we: (a) report the lateral diffusion coefficient for integral proteins (intramembrane particles) in the mitochondrial inner membrane using the electrophoretic freeze-fracture electron microscopy technique [7], (b) estimate the range of protein concentrations (intramembrane particle concentrations) in inner membranes, and (c) conclude that the lateral diffusion coefficients for integral proteins in a given inner membrane are related inversely to membrane protein concentrations.

Methods

Preparation of spherical mitochondrial inner membranes (osmotically swollen mitoplasts), electric field-induced patching of integral proteins,

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release of the integral proteins, from the patch, analysis of post-electrophoretic gradients of intramembrane particles in freeze-fracture electron micrographs, and calculations of the lateral diffusion coefficient were carried out as previously described [7]. For this study, a total of 22 membranes were analyzed.

The protein concentration in each mitochondrial inner membrane was estimated in terms of the number of uniformly distributed intramembrane particles per unit area of mitochondrial inner membrane as follows. Huang's mathematical model [8] enables the prediction of the change in particle concentration for particles diffusing laterally from a hemispherical patch in a spherical membrane to a random distribution in the spherical membrane (Fig. 1). A feature of this model is that the particle concentration at the border between the particle-rich hemisphere and the particle-poor hemisphere is always equivalent to the average particle concentration at all locations in the membrane after equilibrium (i.e., a random distribution) is attained. Since the center sample area of the three linearly adjacent sample areas used to measure intramembrane particle concentrations along the gradient is on this border, the particle concentration in this center area is equivalent to the intramembrane particle concentration at equilibrium.

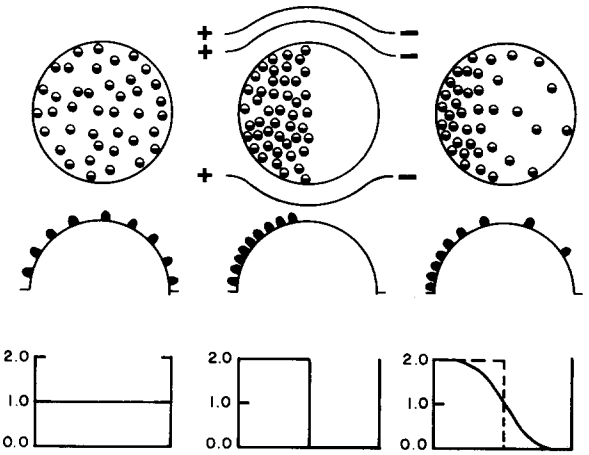

Fig. 1. Three representational views of a spherical-shaped membrane with three lateral distributionsof intramembrane particles. The representational views are: (1) Freeze-fracture face (top row), (2) cross-sectional (middle row) and (3) relative particle concentrations as a function of location along the gradient (lower row). The three lateral distributions are: (a) random (left column), (b) complete electrophoretic migration of IMP into a hemispherical patch (middle column) and (c) an IMP distribution with a gradient during post-electrophoretic re-randomization (right column).

While the mathematical model predicts pre-equilibrium gradients which are symmetrical about the midpoints of the three areas, the points derived from the measured gradients fell on curves (not shown) which were usually but slightly concave upward. To derive the relaxation time-constant (needed to calculate the lateral diffusion coefficient, D), the average slope was calculated for the three midpoints. To determine whether the concave upward nature of the measured gradients was due to sample size and/or variability of data or a real effect, the slopes of the gradient including the high concentration measurements and the midpoints were statistically compared with the slopes of the gradient including the low concentration measurements and the midpoints.

Results

The sample population of the 22 convex membrane fracture faces produced a set of data witht he following ranges of values: (a) radii: 0.22–1.15 (average: 0.52) μm (Fig. 2); (b) time-constants (τ): 1.12–2.75 (average: 1.70) s (Fig. 3); (c) equivalent (random equilibrium lateral distribution con-

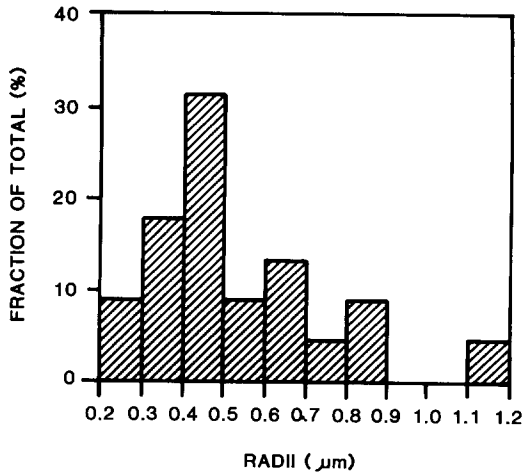

Fig. 2. Histogram of measured radii of spherical inner membranes in the sample population.

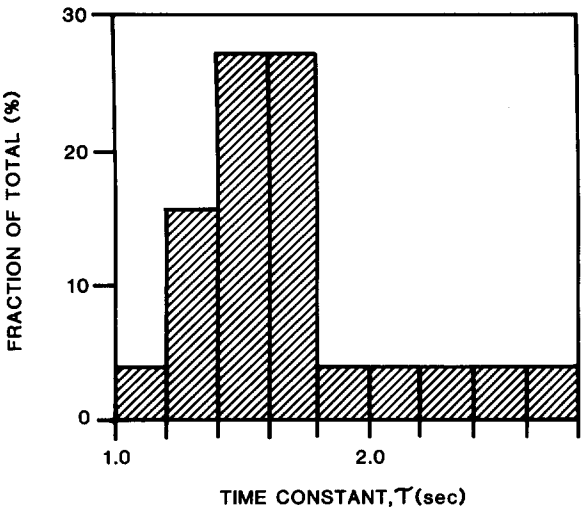

Fig. 3. Histogram of values of the relaxation time constant, τ, estimated from comparison of average gradients with gradients predicted by theory (Fig. 8 of Ref. 4) for spherical inner membranes in the sample population.

centrations of intramembrane particles on individual membranes: 2300–6400 (average: 3700) per μm^2 (Fig. 4); and (d) calculated diffusion coefficients: $1.3 \cdot 10^{-10}$–$3.5 \cdot 10^{-9}$ (average: $9.4 \cdot 10^{-10}$) cm^2/s (Fig. 5). The highest and lowest values for R and τ ranged over a factor of about 5 and 2.5, respectively, and appeared to reflect both a normal biological variability and near normal distribution. However, when the radius and time-constant for each membrane were substituted into the equation

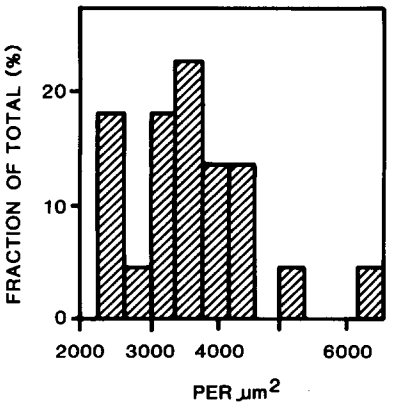

Fig. 4. Histogram of measured intramembrane particle concentration (see Methods) on spherical inner membranes in the sample population.

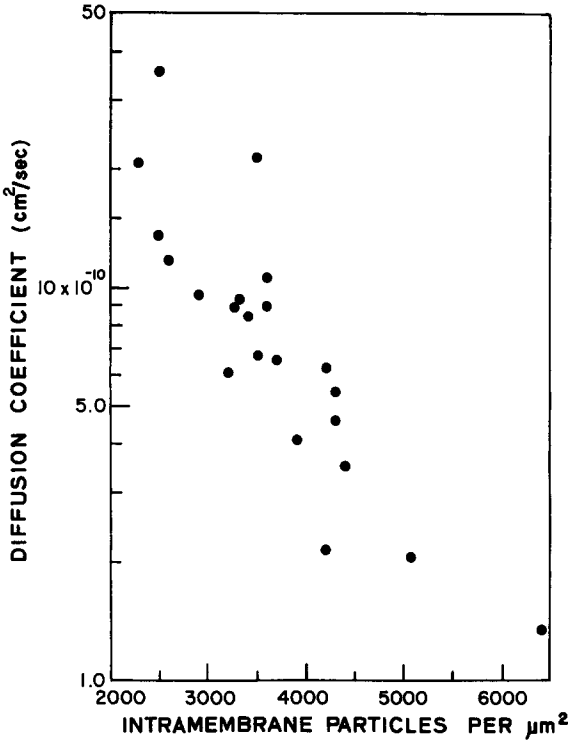

Fig. 5. Lateral diffusion coefficients calculated from the estimated relaxation time constant for each membrane in the sample population plotted as a function of membrane radius.

$D = R^2/2\tau$ [7], the largest and smallest values for D were different by a factor of more than 25. Plots of the individual diffusion coefficients against concentration of IMP in the membrane (Fig. 5) and the membrane sphere radius, R (Fig. 6) showed that the lateral diffusion coefficient was correlated with both intramembrane particle concentration and membrane radius. A plot of the radius against the intramembrane particle concentration for each membrane also showed that membranes with the samllest radii had the highest intramembrane particle concentrations (Fig. 7).

The mean intramembrane particle concentration in the sample areas with the high concentration of intramembrane particles was 15% higher than for the midpoint, while the mean intramembrane particle concentration in the low intramembrane particle concentration sample areas was only 9% below that for the midpoint. The difference in the means was significant at the 0.05 level according to Student's t-test. Thus, the shape

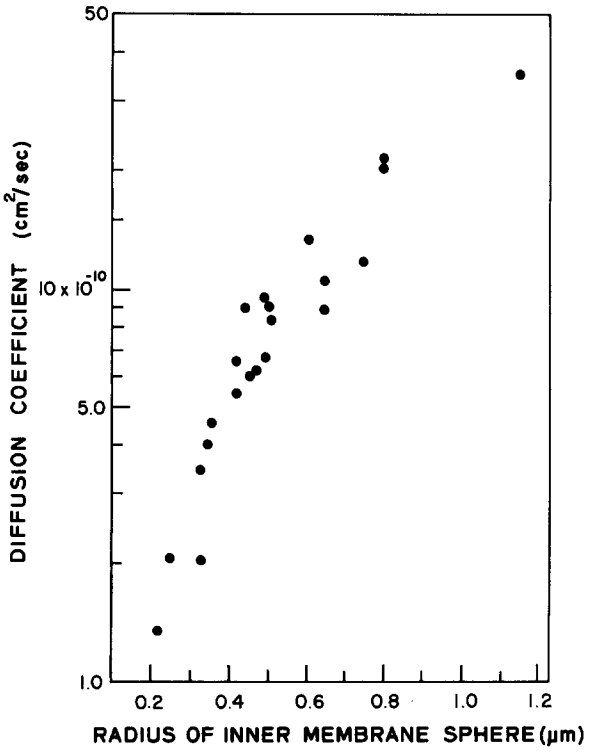

Fig. 6. Lateral diffusion coefficient calculated from the estimated relaxation time constant for each membrane in the sample population as function of intramembrane particle concentration.

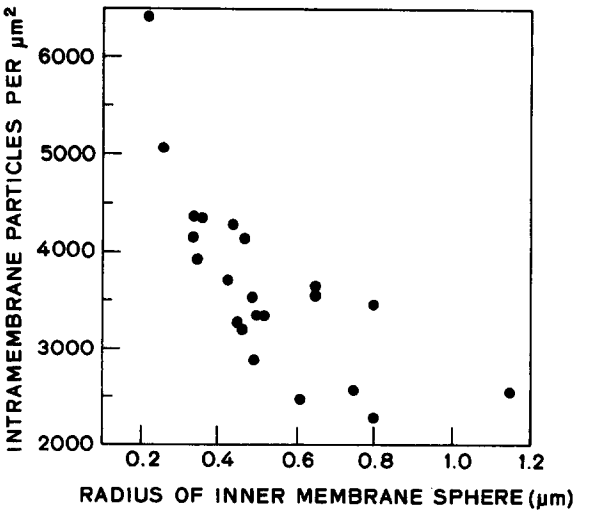

Fig. 7. Intramembrane particle concentration as a function of the radii of spherical inner membranes in the sample population.

of the concentration gradient determined from the data from all 22 membranes appeared to be slightly but significantly concave upward.

Discussion

An average lateral diffusion coefficient of $9.4 \cdot 10^{-10}$ cm^2/s was determined from the calculated values of D for all membranes in the present study. This calculation used: (a) the average slope across both segments of the measured intramembrane particle gradient on each membrane to determine the time-constant, (b) the radius of the membrane calculated from measurements on tilted replicas, and (c) the formula $D = R^2/2\tau$ [7]. This value is in order of magnitude in agreement with the mid 10^{-10} cm^2/s values found by our fluorescence recovery after the photobleaching study of Complex III (cytochrome b-c_1) and Complex IV (cytochrome oxidase), although that study was conducted on giant membranes formed by fusion of rat liver mitochondrial inner membranes or inner membranes from megamitochondria from cuprizone-fed mice [6].

The range in values for the lateral diffusion coefficient, D, was much larger than the expected experimental error in the technique (about 10%). Large ranges in the lateral diffusion coefficient of diffusible membrane components measured in natural membranes have been observed previously [9–11]. However, upon closer examination of the values for the lateral diffusion coefficient, the intramembrane particle concentration and the radius for each membrane, it was found that each of the three variables could be correlated with the other two. First, the lateral diffusion coefficient was inversely proportional to intramembrane particle concentration in the membrane (Fig. 5). As the intramembrane particle concentration increased from 2400 to 6400 per μm^2, the lateral diffusion coefficient decreased in an approximately linear relationship from $3.5 \cdot 10^{-9}$ to $1.3 \cdot 10^{-10}$ cm^2/s. Second, as the radius of the spherical membrane increased, the intramembrane particle concentration in the spherical membrane decreased (Fig. 7). This relationship clearly indicates that larger mitochondrial inner membranes have lower concentrations of integral proteins per unit surface area. Lastly, the lateral diffusion coefficient was

proportional to the radius of the given membrane sphere (Fig. 6). As the membrane sphere radius increased from 0.2 μm to 1.2 μm, the lateral diffusion coefficient increased in a slightly convex curve from $1.3 \cdot 10^{-10}$ to $35 \cdot 10^{-10}$ cm^2/s.

The finding that the lateral diffusion coefficient of a membrane component is inversely proportional to integral protein (IMP) concentration is of interest and may be physiologically important. A study with fractionated rat enterocyte membranes showed that the microviscosity of the membrane was proportional to the protein/lipid ratio [12]. In another study, the lateral diffusion coefficient of labeled lipid and lipopolysaccharide was measured in vesicular membranes reconstituted with various amounts of *Escherichia coli* membrane matrix protein reconstituted into the membranes [13]. Of particular interest is the recent study of Peters and Cherry [14] in which the lateral and rotational diffusion coefficients were determined for bacteriorhodopsin reconstituted into dimyristoylphosphatidylcholine vesicles at molar lipid/protein ratios of from 30:1 to 210:1 and over a wide range of temperatures. Above the gel state-liquid crystal phase transition temperature, the measured integral protein lateral diffusion coefficient was found to be strongly dependent and proportional to the lipid/protein ratios. While the highest lipid concentration used was a factor of 8 higher than the lowest lipid concentration used, the corresponding lateral diffusion coefficient for the integral protein at the highest lipid concentration was as much as a factor of 20 higher than at the lowest lipid concentration.

Finally, the lateral diffusion coefficients for DiI and proteins in mitochondrial inner membranes enriched with various amounts of exogenous lipid were found to be inversely related to the protein concentration in the membrane [15,16]. Our findings are consistent with these results.

The lateral diffusion coefficient appeared also to be dependent on inner membrane radius. Since the radius was measured very accurately and was taken into account in the calculation of the lateral diffusion coefficient, the radius dependency of the lateral diffusion coefficient most likely originated from a factor intrinsic to the membranes, protein concentration or otherwise. It is possible that, in addition to the relationship found between protein concentration and membrane radius, a relationship may exist between inner membrane lipid composition and membrane radius. This possibility is suggested by the report that lipid analysis of light and heavy sedimenting fractions of mitochondria show that light fractions of mitochondria are 30% higher in sphingomyelin, and 45% lower in cardiolipin [17]. Such differences in the membrane composition of mitochondria fractionated according to sedimentation density may be in part responsible for the range of lateral diffusion coefficients found. It remains to be determined if there is, indeed, a relationship between inner membrane area and lipid composition.

In this paper, the experimental data were compared with predictions based on a mathematical solution to diffusion of non-interacting points in a spherical-shaped model membrane which involves one mobile species diffusing according to Fick's law. Fick's law states that the relationship between the time rate of change in concentration (dc/dt), and the rate of change in the distance rate of change in concentration (d^2c/dx^2), is a term or coefficient D. Our calculated diffusion coefficients shows an inverse correlation with intramembrane particle concentration (Fig. 5). It is clear that diffusion in the mitochondrial inner membrane must be treated with the rigorous form of Fick's second law wherein D depends upon concentration, i.e.

$$dc/dt = \frac{\partial}{\partial x}(D(c))\frac{\partial c}{\partial x}$$

This is in contrast to the typical assumption of D independent of concentration, i.e., $dc/dt = D(d^2c/dx^2)$. Concentration dependence of D is usually taken to imply interaction among the diffusants (frictional or otherwise).

In addition to the concentration dependence found in the lateral diffusion coefficients calculated for each individual membrane, we also observed that when the data for all of the membranes were averaged together, the average gradient in the membrane hemisphere with high intramembrane particle concentration is steeper than the gradient in the membrane hemisphere with the low intramembrane particle concentration. The difference between the slope of the high intramembrane particle concentration segment of

the gradient and the slope of the low intramembrane particle concentration of the gradient was statistically significant at the 0.05 level. For this distribution of intramembrane particles to exist at 2.0 s after the release from the electrophoretic holding force, the intramembrane particles must have been diffusing faster in the hemisphere with the lower intramembrane particle concentration than in the hemisphere with the higher intramembrane particle concentration. Hence, from this finding, the lateral diffusion coefficient can again be interpreted to be dependent on the inverse of intramembrane concentration.

Not only does little theoretical and experimental work exist by which we can treat our data and compare our results, respectively, but the problem is beyond the scope of the present paper. However, a recent review [18] indicates that our understanding of the integral protein concentration effect on the lateral diffusion of mobile membrane components will require considerably more experimental investigation and new theoretical approaches. Moreover, modeling studies using Monte Carlo computer simulations [19], may be a promising way to study how one integral protein, otherwise free to laterally diffuse, may interfere with the diffusion path of another nearby integral protein.

In conclusion, our study is the first to indicate that the lateral diffusion coefficient of proteins in the intact native membrane varies approximately inversely with the concentration of those proteins in the membrane. Since the rate of electron transport in the mitochondrial inner membrane is coupled to the diffusion of the electron transfer components [6], the normal variations in protein concentration and consequent diffusion coefficients determined in the present study may have an important influence on the overall rate of electron transport. Our study also suggests that the mitochondrial inner membrane as a model system and the electrophoretic freeze-fracture electron microscopy method of measuring lateral diffusion and concentrations of integral proteins may provide useful data on both electron transport and lateral diffusion.

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