Influence of Poly(ethylene glycol) and Aqueous Viscosity on the Rotational Diffusion of Membranous Na,K-ATPase†

Mikael Esmann,‡,* Kálmán Hideg,§ and Derek Marsh‖

Institute of Biophysics, University of Aarhus, DK-8000 Aarhus, Denmark, Central Laboratory for Chemistry, University of Pécs, H-7643 Pécs, Hungary, and Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-37077 Göttingen, Federal Republic of Germany

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ABSTRACT: The Na,K-ATPase [ATP phosphohydrolase (Na^+/K^+-transporting), E.C. 3.6.1.37] in native membranes from the salt gland of *Squalus acanthias* has been spin-labeled covalently with a chloromercuri nitroxide derivative, and the rotational diffusion of the protein has been studied, as a function of the concentration of glycerol or poly(ethylene glycol) in the suspending medium, by means of saturation-transfer electron spin resonance spectroscopy. The effective rotational correlation time of the protein increases linearly with the viscosity of the aqueous glycerol medium, with a gradient whose value indicates that ca. 50–70% of the volume of the Na,K-ATPase protein is external to the membrane. The effective rotational correlation times of the protein in poly(ethylene glycol) solutions are considerably greater than those in glycerol solutions of the same viscosity and increase nonlinearly with the viscosity of the suspending medium, indicating that increasing concentrations of poly(ethylene glycol) induce aggregation of the integral proteins within the membrane. The value reached at 50% poly(ethylene glycol) corresponds to a degree of aggregation of the proteins between 2 and 5 depending on whether the ethylene glycol polymer is excluded from the membrane surface region. The results are discussed with respect to hydration forces and poly-(ethylene glycol)-induced cell fusion.

Poly(ethylene glycol) (PEG)[1] is a potent agent for induction of cell fusion (Lucy, 1984; Lane et al., 1986) that also finds wide application as a precipitant for protein purification, isolation, and crystallization (McPherson, 1985; Ingham, 1990). The mechanism of PEG-induced fusion of pure lipid vesicles is thought to arise in part from the ability of PEG to dehydrate the vesicle surfaces (MacDonald, 1985), hence overcoming the barrier to close approach created by the interbilayer hydration forces (Cevc & Marsh, 1987; Rand & Parsegian, 1989). In cellular systems, a further factor contributing to the fusogenic activity may be the induction of protein clustering within the plane of the membrane, hence promoting the close apposition of protein-free areas of lipid bilayer. A possible mechanism for such clustering could again be a reduction of the water activity at the cell surface by PEG, with a concomitant decrease of the in-plane hydration forces between the extramembranous sections of the integral membrane proteins.

In the present work, the effects of PEG on the rotational mobility and aggregation state of spin-labeled Na,K-ATPase (E.C. 3.6.1.37) in the native membrane-bound form is studied by saturation-transfer ESR spectroscopy. The steep increase found in the rotational correlation time of the spin-labeled protein indicates that aggregation of the integral membrane proteins does, in fact, take place with progressively increasing concentration of PEG in the aqueous phase. Glycerol, on the other hand, is shown not to induce protein aggregation and is used as a control for the effects of increase in viscosity of the suspending medium. From the latter, it is found that an appreciable portion of the protein is external to the membrane and hence provides a hydrated surface which can inhibit the aggregation of the protein that is demonstrated to be induced by PEG in the current STESR measurements.

MATERIALS AND METHODS

The chloromercuri spin label 5-MeClHgMSL was prepared as described in Esmann et al. (1993). Poly(ethylene glycol) with a mean molecular weight of 4000 was obtained from Sigma Chemical Co. (St. Louis, MO), and analytical grade glycerol was from Merck (Darmstadt, FRG). Membranous Na, K-ATPase was prepared from the salt gland of *Squalus acanthias* according to the method of Skou and Esmann (1979), except the treatment with saponin was omitted. The protein was spin-labeled covalently on class II -SH groups, after class I -SH groups were prelabeled with *N*-ethylmaleimide, as described earlier (Esmann et al., 1989, 1993). labeled membranes were pelleted in 30 mM histidine, 100 mM NaCl, and 1 mM CDTA, pH 7.6 (at 20 °C), and homogenized in a small volume of the same buffer. Glycerol or PEG as a concentrated solution in the same buffer was mixed thoroughly with membrane homogenate and taken up into a 1-mm-diameter glass capillary to produce a sample of length 5 mm (Fajer & Marsh, 1982).

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* Corresponding author: Institute of Biophysics, Ole Worms Alle 185, DK-8000 Aarhus, Denmark. E-mail: me@mil.AAU.DK.

‡ University of Aarhus.

§ University of Pécs.

‖ Max-Planck-Institut für biophysikalische Chemie.

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[1] Abbreviations: ESR, electron spin resonance; STESR, saturation-transfer ESR; V_1, first harmonic ESR absorption signal detected in phase with respect to the field modulation; V_2', second harmonic absorption ESR signal detected 90° out-of-phase with respect to the field modulation; Na,K-ATPase, Na^+/K^+-transporting ATP phosphohydrolase (E.C. 3.6.1.37); PEG, poly(ethylene glycol); 5-MeClHgMSL, *trans*-3-(methoxycarbonyl)-4-(4′-(chloromercuri)benzamido)methyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl.

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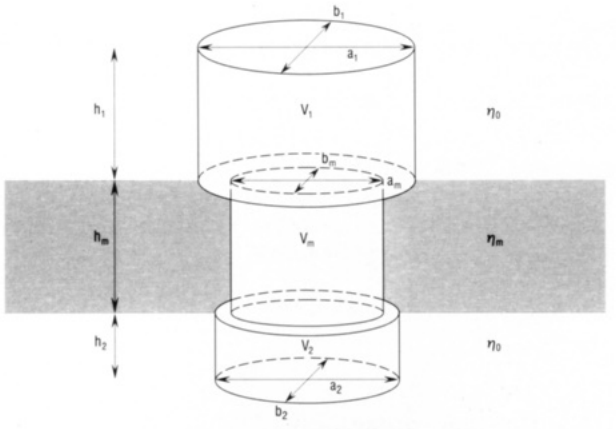

FIGURE 1: Hydrodynamic model for rotational diffusion of an integral membrane protein consisting of a central cylindrical section of volume V_m embedded in the membrane of viscosity η_m and two extramembranous cylindrical sections of total volume V_o (= V_1 + V_2) in an aqueous medium of viscosity η_o. The heights, h_i, and cross-sectional dimensions, a_i and b_i, of the three different sections of the protein are also indicated.

Conventional (V_1 display) and saturation-transfer (V_2'-display) ESR spectra were recorded as described earlier (Esmann et al., 1987, 1989) on a Varian Century-Line 9-GHz spectrometer. Standardized sample geometry and spectrometer settings and calibrations were employed (Fajer & Marsh, 1982; Hemminga et al., 1984). Calibrations of the diagnostic STESR line height ratios (L''/L and H''/H) and normalized integral intensities (I_{ST}), in terms of the rotational correlation times of spin-labeled hemoglobin, were taken from Horváth and Marsh (1988).

The kinematic viscosity of glycerol and PEG solutions of the required concentrations in the measurement buffer were determined in a thermostated Ubbelhode viscometer (Schott, Mainz, FRG) and converted to units of centipoise by using the measured densities of the solutions.

THEORETICAL BACKGROUND

The diffusion coefficient for uniaxial rotation of an integral protein about the membrane normal is defined by

$$D_{R\|} = kT/f_{R\|} \tag{1}$$

where $f_{R\|}$ is the rotational frictional coefficient, T is the absolute temperature, and k is Boltzmann's constant. To take into account the dependence of the rotational diffusion coefficient on the external viscosity, the situation is considered in which different sections of the protein, of volume V_i, are immersed in media of different viscosities, η_i (cf. Figure 1). Because the torques experienced by the separate sections of the protein are additive, so also are the individual contributions to the rotational frictional coefficient. Therefore, the latter is given by [cf. Marsh and Horváth (1989)]

$$f_{R\|} = \sum_i f_{R\|,i}^{\circ}/F_{R\|,i} \tag{2}$$

where the frictional coefficients, $f_{R\|,i}^{\circ}$, of right circular cylinders with volumes equal to those of the different sections of the protein are given by

$$f_{R\|,i}^{\circ} = 4\eta_i V_i \tag{3}$$

and the corresponding shape factors, $F_{R\|,i}$, are given by

$$F_{R\|,i} = 2(a_i/b_i)/[1 + (a_i/b_i)^2] \tag{4}$$

where a_i and b_i are the semi-axes of the (assumed) elliptical cross section of the cylindrical section of the protein (see Figure 1). Hence from eqs 1–3, the rotational correlation time of the protein, defined by $\tau_{R\|} = 1/6D_{R\|}$, is given by

$$\tau_{R\|} = (2/3kT)\sum_i \eta_i V_i/F_{R\|,i} \tag{5}$$

The dependence of the measured rotational correlation time on external viscosity, η_o, therefore, is given by

$$\tau_{R\|} = \tau_{R,memb} + (2\eta_o/3kT)\sum_j{}' V_j/F_{R\|,j} \tag{6}$$

where $\tau_{R,memb}$ is the effective rotational correlation time for the internal membrane section of the protein (i.e., when η_o = 0), and the summation $\sum'$ is now only over the extramembranous sections of the protein (cf. Figure 1). Departures from this viscosity dependence may therefore indicate the influence of solutes added to the aqueous medium on the aggregation state of the protein in the membrane.

RESULTS AND DISCUSSION

The effects of both glycerol and poly(ethylene glycol) in the suspending medium on the rotational mobility of the spin-labeled Na,K-ATPase in native membranes were studied by using ESR spectroscopy. The conventional ESR spectra (V_1 display) of the 5-MeClHgMSL spin label attached to the Na,K-ATPase evidence little or no segmental motion of the label relative to the protein, and contain only a very small proportion of a highly mobile spin label component (data not shown). Addition of either glycerol or PEG has little effect on the conventional spectra, except that the small, highly mobile component is broadened to beyond detectability at both 4 and 20 °C. This spin label is therefore very suitable for studying the effects of glycerol and PEG on the overall rotational diffusion of the protein by means of STESR spectroscopy.

The saturation-transfer ESR spectra (V_2' display) recorded at 4 °C of the Na,K-ATPase membranes labeled with the 5-MeClHgMSL nitroxide derivative and suspended in aqueous solutions of PEG of different concentrations are given in Figure 2. It can be seen that the line shapes of the STESR spectra, and in particular the line heights in the diagnostic regions of the spectra at low and high field, change progressively in the direction expected for decreasing rotational mobility of the spin-labeled protein with increasing PEG concentration in the suspending medium [cf. Thomas et al. (1976)]. The STESR spectral changes observed at high PEG concentrations were found to be reversible on resuspending the membranes in buffer without PEG, and enzymatic activity was also fully restored for unlabeled samples after PEG was removed. Qualitatively similar changes to those with PEG, but to a smaller extent for comparable values of the aqueous viscosity, were observed in the STESR spectra of the spin-labeled protein from membranes suspended in aqueous glycerol solutions of increasing concentration (spectra not shown). The parameters of the STESR spectra—diagnostic line height ratios in the low-field (L''/L) and high-field (H''/H) regions of the spectra and the normalized integrated intensity (I_{ST})—for membranes suspended both in PEG and in glycerol solutions are summarized in Table 1. From this, it can be seen that both the diagnostic line height ratios and the integral intensities are lower in the glycerol solutions than in the PEG solutions of the same viscosity. This indicates that the rotational mobility of the spin-labeled Na,K-ATPase is reduced much more

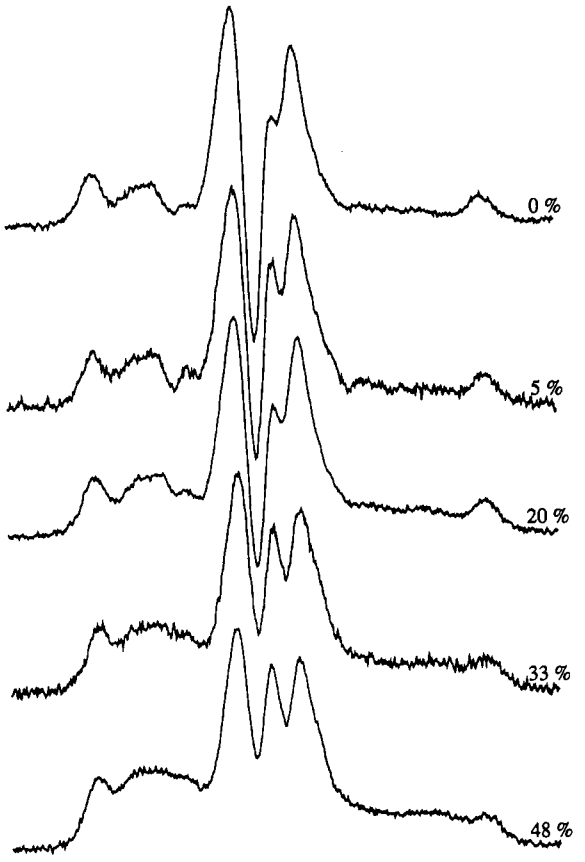

FIGURE 2: Second harmonic, 90° out-of-phase absorption STESR spectra (V_2' display) of chloromercuri-spin-labeled (5-MeClHgMSL) class II SH groups on Na,K-ATPase at 4 °C. The poly(ethylene glycol) content (%, w/v) of the suspending medium is indicated for each spectrum. Total scan width = 10 mT.

effectively by addition of PEG to the suspending medium than by addition of glycerol.

Although the different STESR parameters display the same qualitative dependence on PEG or glycerol concentration, the high-field line height ratio has been used to extract effective rotational correlation times [see Horváth and Marsh (1988)], because this region of the spectrum is free from any overlap with the mobile spectral component. The dependence on the external viscosity of the effective rotational correlation times obtained from the STESR spectra of the spin-labeled Na,K-ATPase in membranes suspended in different concentrations of glycerol and of PEG is given in Figure 3. For membranes in aqueous glycerol, the approximately linear dependence of the effective rotational correlation time on the viscosity of the suspending medium conforms to the predictions of eq 6, suggesting that the effects of glycerol result primarily from changes in the aqueous viscosity and that the aggregation state of the protein in the membrane does not change appreciably with increasing glycerol concentration. The intercept of the viscosity dependence of the effective rotational correlation time with increasing glycerol concentration yields a value of $\tau_{R,memb}^{eff}$ = 50 μs at 4 °C. The true rotational correlation time, $\tau_{R\parallel,memb}$, is related to this effective STESR correlation time by (Robinson & Dalton, 1980)

$$\tau_{R\parallel,memb} = (\tau_{R,memb}^{eff}/2)\sin^2\theta \qquad (7)$$

where θ is the angle which the principal spin-label z-axis makes with the membrane normal. The upper limit for the rotational correlation time is therefore $\tau_{R\parallel,memb}$ = 25 μs, which is comparable to that obtained previously (Esmann et al., 1987, 1989) with other spin labels. As discussed in this previous work, this result is consistent with the Na,K-ATPase being present as a diprotomer or higher oligomer in the membrane.

Table 1: Diagnostic Line Height Ratios, L''/L and H''/H, and Normalized Integrated Intensities, I_{ST}, from STESR Spectra of Membranous Na,K-ATPase Labeled with 5-MeClHgMSL, for Membranes Suspended in Aqueous Solutions of PEG or Glycerol[a]

[PEG] or [Glycerol] (%, w/v)	T (°C)	η_o (cP)	L''/L	H''/H	I_{ST} (×10²)
0	4	1.52	0.83	0.57	0.222
	20	1.00	0.78	0.46	0.144
		Glycerol			
52.1	4	15.4	0.76	0.63	0.345
65.1	4	47.1	0.71	0.68	0.383
73.6	4	101	0.83	0.75	0.428
80.8	4	201	0.82	0.81	0.408
52.1	20	8	0.66	0.51	0.233
65.1	20	20	0.58	0.55	0.318
73.6	20	39.5	0.64	0.68	0.277
		Poly(ethylene glycol)			
5	4	2.9	0.91	0.56	0.243
10	4	5.1	0.97	0.71	0.296
20	4	14.0	1.00	0.75	0.344
25	4	22.5	1.04	0.80	0.411
32.9	4	40	1.03	0.93	0.472
43.2	4	86	1.11	1.05	0.521
47.6	4	123	1.09	1.13	0.570
51	4	161	1.16	1.15	0.568
5	20	1.8	0.65	0.49	0.130
10	20	3.1	0.89	0.57	0.174
20	20	8.0	0.95	0.63	0.225
25	20	12.2	0.98	0.75	0.267
32.9	20	21.2	1.01	0.90	0.304
43.2	20	46.0	1.05	0.94	0.352
47.6	20	61.2	0.99	0.98	0.438

[a] For definition of L''/L and H''/H, see Thomas et al. (1976), and for I_{ST} see Horváth and Marsh (1983).

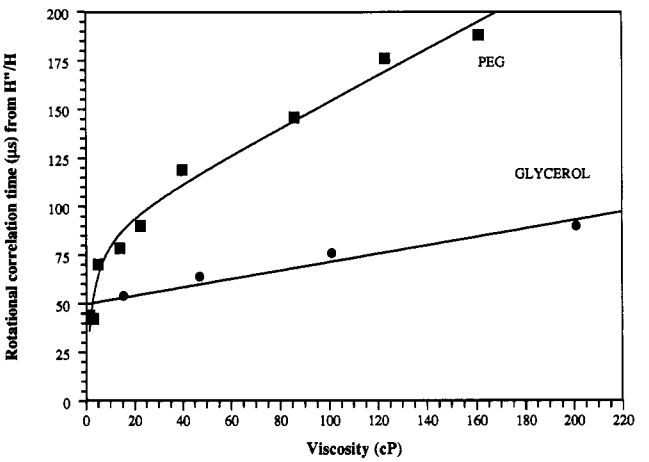

FIGURE 3: Dependence on viscosity of the suspending medium of the effective rotational correlation time, $\tau_R^{eff}(-1)$, deduced from the H''/H line height ratio in the STESR spectra of Na,K-ATPase membranes labeled with 5-MeClHgMSL: (●) membranes suspended in aqueous glycerol; (■) membranes suspended in aqueous PEG solutions. The straight line is a linear regression for the glycerol data with intercept 50 μs and gradient 22 μs P^{-1}. The line for the PEG data is simply to guide the eye.

The gradient of the dependence on viscosity of the glycerol solutions gives information on the size of the extramembranous portion of the protein (cf. eqs 5 and 6). For relatively small cross-sectional asymmetries, the shape factors may be approximated by $F_{R\parallel,i} \approx 1$ [cf. Marsh and Horváth (1989)]. Therefore, the ratio of the intercept to the gradient of the glycerol viscosity dependence in Figure 3 yields a value of $\eta_m V_m/V_o$ = 2.3 P, where V_m and V_o are the total volumes of

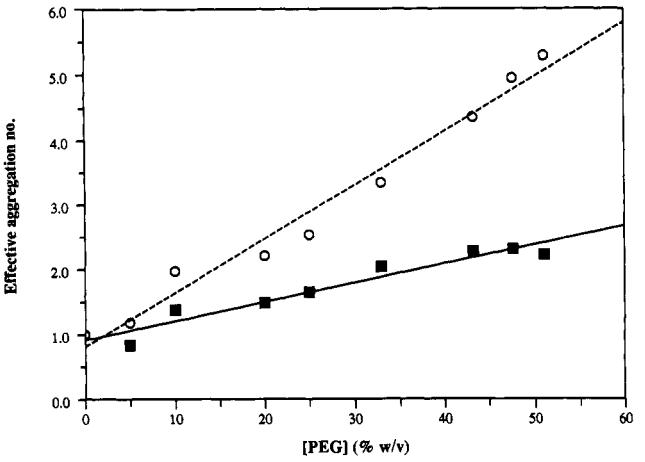

FIGURE 4: Dependence on PEG concentration (%, w/v) in the suspending medium of the ratio of the effective rotational correlation time (deduced from H''/H) of spin-labeled Na,K-ATPase to that obtained from membranes suspended in aqueous glycerol of the same viscosity (■) and to that obtained from membranes suspended in water (○). The ratio of the rotational correlation times represents the effective aggregation number of the spin-labeled protein (see text).

the intra- and extramembranous portions of the protein, respectively. It will be noted that, because this is a ratio, the orientation of the spin label does not enter. For a value of the intramembranous viscosity of η_m = 2–5 P [cf. Cherry and Godfrey (1981)], this yields a value of $V_o/(V_m + V_o)$ = 0.5–0.7; i.e., 50–70% of the Na,K-ATPase protein is external to the membrane. For comparison, from electron microscope imaging of two-dimensional crystals of the Na,K-ATPase, it was estimated that 42% of the volume of the protein is within the membrane, 40% is on the cytoplasmic side, and 18% is on the extracellular side (Maunsbach et al., 1989). The present measurements on the rotational dynamics of the protein are therefore in reasonable agreement with the structural information currently available on the protein.

The dependence of the effective rotational correlation time of the spin-labeled protein on the viscosity of the aqueous phase for membrane suspensions in PEG solutions (see Figure 3) does not conform to the simple linear dependence predicted by eq 6 that is found for the glycerol solutions. The effective correlation times for suspensions in PEG are all considerably larger than for those in glycerol at the same viscosity, suggesting that aggregation of the protein within the membrane is taking place in the presence of PEG. If differences in the shape factors can be neglected, it follows from eq 5 that the ratio of the effective correlation times in the presence of PEG to those at the same external viscosity in glycerol will give a measure of the degree of protein aggregation. Again this ratio is independent of the spin label orientation. These ratios are plotted as a function of concentration of the PEG solutions in Figure 4. From this figure it is seen that the (viscosity normalized) apparent degree of aggregation of the protein increases with increasing PEG concentration (■, solid line), reaching an effective value of ca. 2.1 at 50% (w/v) PEG.

Differences in the response of the protein mobility to the presence of glycerol compared with that to the presence of PEG can arise for at least two reasons. These relate, on the one hand, to the effective local viscosity at the membrane surface and, on the other hand, to the quantitatively different effects of PEG and glycerol on the water activity and hence on the hydration state of the membrane surface. Discussions of these two aspects are given separately below.

First, glycerol is a small molecule that is freely accessible to the extramembranous portions of the protein at the membrane surface, and these portions of the protein therefore will experience the full effect of glycerol on the aqueous viscosity. For PEG, on the other hand, this large polymer molecule may be excluded from the regions of the membrane surface where the extramembranous sections of the protein protrude [see also Arakawa and Timasheff (1985) and Arnold et al. (1990)]. If (as is likely) this is the case, the extramembranous portion of the protein will experience a viscosity closer to that of water alone than that measured for the PEG solutions; i.e., the local and bulk-phase viscosities would be similar in the case of glycerol, but with PEG the local viscosity could be much smaller than the bulk-phase viscosity. In this case, the apparent degree of protein aggregation in the presence of PEG will be considerably higher than the value of 2.1 quoted above. The effective correlation times in PEG normalized to those in water (which is the appropriate procedure in this latter situation) are given in Figure 4 (○, dashed line). From this it can be seen that the apparent degree of aggregation deduced on this assumption increases progressively with increasing PEG concentration, reaching a value of ca. 5 in 50% (w/v) PEG.

Second, the effect of PEG on the water activity is considerably greater than that of glycerol [cf. Miner and Dalton (1953) and Arnold et al. (1988)]. Since hydration forces, which are dependent on the water activity, are found to occur between hydrated lipid bilayers (Cevc & Marsh, 1987; Rand & Parsegian, 1989), it is reasonable to assume that similar repulsive interactions may take place between the hydrated extramembranous portions of integral proteins at the membrane surface. A reduction in water activity (effectively a dehydration) at the membrane surface would then favor aggregation of the proteins within the plane of the membrane, as is observed here for the membranous Na,K-ATPase in the presence of PEG. Glycerol would be expected to be less effective in this respect, not only because it gives rise to a smaller reduction in water activity but also because such small polar molecules that are accessible to the protein surface can themselves give rise to solvation forces [cf. McIntosh et al. (1989)].

The observation that aggregation of an integral membrane protein is induced by PEG could be relevant to the ability of PEG to induce cell fusion. Not only would this tend to create areas of bare lipid surface unobstructed by proteins, but, in addition, the reduction of the water activity at the lipid surface would also tend to promote fusion of the lipid bilayer, as is observed in model systems (MacDonald, 1985; Parente & Lentz, 1986). Aggregation of intramembranous particles prior to PEG-induced fusion has been demonstrated in erythrocyte ghosts by freeze-fracture electron microscopy (Hui et al., 1985). The present dynamic measurements demonstrate that the aggregated membrane proteins still possess the necessary mobility to undergo further in-plane redistribution on subsequent osmotic swelling that is obligatory to induce PEG-mediated cell fusion, for instance, in monoclonal antibody hybridoma technology (Lane et al., 1986).

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