SOME STRUCTURAL PROPERTIES OF EXCITABLE MEMBRANES LABELLED BY FLUORESCENT PROBES

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1. Introduction

Important information on the conformation and on the motion of proteins has been obtained by the use of fluorescent probes covalently bound to certain characteristic amino acid side chains [1]. In the present report we describe experiments performed *in vitro* with 5-dimethyl-amino-1-naphthalene sulphonylchloride (DNS) conjugated to fragments of excitable membranes (EME) purified from the electric organ of *Electrophorus electricus* [2]. Under our experimental conditions, DNS reacts almost exclusively with membrane proteins at sites which are different from those labelled by a noncovalent fluorophore: 1-anilino-8-naphthalene sulphonate (ANS). However, in agreement with previous findings with ANS [3], the polarization of fluorescence (p) of DNS bound at these sites is not sensitive to changes in solvent viscosity (η) upon addition of sucrose. Experiments involving the separation of lipids and proteins, and their subsequent reassociation indicate that this insensitivity requires the association of lipids and proteins into an organized membrane structure. In the presence of the non-ionic detergent Triton X-100, p decreases and becomes sensitive to η; simultaneously, an important fraction of membrane proteins becomes soluble. On the other hand, in the presence of tyrocidine (Tyr), a polypeptide antibiotic, a slight increase of p is observed. *In vivo*, on the electroplax, these two compounds show opposite effects: Triton X-100 stabilizes the membrane in a polarized state while Tyr favours a depolarized state of the membrane.

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2. Material and methods

EME were prepared from homogenates of fresh electric tissue from *Electrophorus electricus* by differential centrifugation in sucrose gradients according to the procedure of Changeux et al. [2]. The specific activity of acetylcholinesterase (AChE) in the purified EME was approx. 4.0 moles acetylthiocholine/hr/g of protein.

DNS was conjugated to EME as follows: 0.1 ml of 5×10^{-3} M DNS chloride (K and K laboratories) in acetone was added to 1 ml of a suspension of EME in 10^{-2} M $NaHCO_3$ and 1 M sucrose containing 5 mg of protein per ml. The mixture was left overnight at 4°C. The next day the conjugated membranes (DNS-EME) were separated from unreacted DNS by two successive filtrations on a G-25 Sephadex column equilibrated with 0.2 M sucrose and 5×10^{-3} M glycylglycine pH 7.0. The total amount of DNS covalently bound to EME was measured by absorbance at 340 nm (A 4.3×10^3).

Lipids were separated from the proteins according to the method of Zahler, Wallach and Lüscher [4] by filtration on an L H 20 Sephadex column equilibrated with 90% 2-chloroethanol in 0.01 N HCl, of DNS-EME dissolved in the same medium. Reconstitution of membrane structure was achieved by the extensive dialysis at 4°C of a mixture of lipids and proteins in

90% 2-chloroethanol pH 2.0 against 0.2 M sucrose, 5×10^{-3} M glycylglycine pH 7.0.

DNS-EME was digested by pronase at 37°C for 24 hr in a medium containing: 0.3 mg in proteins of DNS-EME, 0.1 mg of pronase (from Calbiochem.), 2×10^{-2} M sodium phosphate pH 7.0, in a total volume of 0.5 ml.

Separation of soluble proteins from membrane fragments after treatment of DNS-EME by 1% Triton X-100 was achieved by ultracentrifugation for 3 hr at 50,000 rpm in a SW65 rotor, of a discontinuous sucrose gradient consisting of (from the bottom to the top) 1.0 ml of 1.5 M sucrose in distilled water, 2.5 ml of 0.4 M sucrose and 0.5 ml of DNS-EME containing 1 mg of proteins per ml in 0.2 M sucrose and 0.1% Triton X-100. Membrane fragments were collected in the bottom fractions and soluble proteins in the top fractions.

Fluorescence intensity and polarization of DNS were measured according to the method of Monnerie and Neel [5] with a fluorescence polarization apparatus. The wavelength of excitation was 365 nm and the fluorescence was observed at 546 nm. Polarization, p, is defined as

$$p = \frac{2\,p_n}{p_n + 1} \text{ where } p_n = \frac{I'_v - I'_h}{I'_v + I'_h}, I'_v \text{ and } I'_h$$

being the intensity components measured along the vertical and horizontal directions with unpolarized exciting light. Except when otherwise indicated measurements were carried out with suspensions of EME in 0.2 M sucrose and 5×10^{-3} M glycylglycine pH 7.0.

3. Results and discussion

3.1. *DNS binding to membrane proteins*

In the experimental conditions described in **Methods**, DNS attaches covalently to EME in suspension. In order to identify the component(s) to which DNS binds, lipids and proteins of DNS-EME were separated on an L H 20 column in the presence of 2-chloroethanol (see **Methods**). Fig. 1 shows the result of such an experiment. DNS fluorescence appears in the first fractions which contain high molecular weight material absorbing at 280 nm. Little or no DNS is associated with the material retarded on the column which, according to Zahler at al. [4], corresponds to membrane

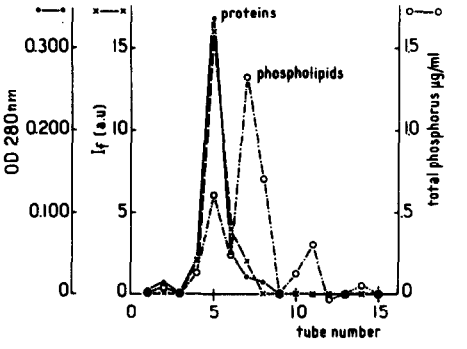

Fig. 1. Separation of lipids and proteins from DNS-EME. The optical density was followed at 280 nm as well as the intensity of fluorescence If of DNS. Total phosphorus content was estimated by the method of Taussky and Shorr [9] after evaporation of 2-chloroethanol and mineralisation by a 2.1.2 mixture of concentrated H_2SO_4, 60% $HClO_4$ and water at 165°C for 2 hr.

lipids. DNS thus seems to be largely associated with membrane proteins. This was confirmed by digestion of DNS-EME by pronase (see **Methods**). After extensive exposure to pronase, 90% of DNS becomes associated with small molecular weight compounds retarded on a G-25 Sephadex column in the presence of an aqueous solvent (in these conditions the lipids make large aggregates which are excluded by Sephadex). DNS might thus be used as a probe of membrane proteins.

The absorption spectrum of DNS bound to EME (45 µmoles/g protein) does not significantly differ from that of DNS bound to BSA (40 µmoles/g). On the other hand, its emission spectrum shows a 6 nm shift to the blue as compared to that of DNS-BSA (fig. 2). The polarization of fluorescence p of DNS bound to EME is 0.240 ± 0.003 at 20°C, a value close to that found, under the same conditions, with DNS-BSA (p = 0.255 at 20°C). For the absolute amount of DNS bound to EME in the range tested (up to 96 µmoles/g of protein) p does not change by more than 10% which indicates that, under these conditions, little or no energy transfer occurs between DNS molecules. p varies with temperature but, in contrast to our observation on ANS, this variation consistently follows the Perrin law up to 85°C, in a reversible manner (fig. 3). The apparent rotational relaxation time calculated from such values of p is ρ = 154 nsec* at 20°C, taking 14 nsec as the life

Table 1
Consequences of the reaction of DNS with EME on the reversible binding of ANS.

DNS bound (μmoles/g protein)	ANS binding	
	$K_d \times 10^{-4}$M	n (μmoles/g)
0	3.7	33
0.6	3.4	39
11.4	4.35	36
59.0	4.25	18
96.0	4.5	12

Conditions see **Methods**. EME were incubated overnight with 0, 0.4, 2, 10, 70 $\times 10^{-3}$ M DNS (from the first line to the last).

time of the excited state (1). DNS is thus strongly immobilized by coupling to EME. Its apparent slow motion is, however, too fast to be assigned to the motion of EME vesicles with DNS rigidly bound to them. Indeed, the diameters of the spherical vesicles, measured on electron micrographs (2), range from 0.1 to 1 μm and are consequently expected to show rotational relaxation times in the order of the millisecond. DNS conjugated to its receptor protein(s) thus presents an intrinsic rotational or vibrational motion within the membrane which is superimposed on that of the whole vesicle.

3.2. *Comparison between membrane sites for ANS and DNS*

Since ANS and DNS are structurally analogous, it was of interest to investigate whether they labelled the same sites in the membrane. Thus we tested the effect of ANS (5×10^{-3} M) on the reaction of DNS (5×10^{-3} M) with EME (5 mg/ml); no significant effect of ANS on the amount of DNS covalently bound to EME was found. In a second group of experiments we followed the binding of ANS as a function of the absolute amount of DNS attached to EME. As shown in table 1, the affinity and number of ANS binding sites do not change up to a ratio of 60 μmoles of DNS per g of

Table 2
Variation of the polarization of fluorescence (p) of DNS bound to EME with membrane environment.

	0.2 M sucrose		1.5 M sucrose	
Reconstitution experiment:				
Native EME	0.240 ± 0.003		0.240 ± 0.003	
EME proteins	0.250		0.277	
Reconstituted EME				
(1) After separation of lipids and proteins	0.24 ± 0.01		0.23 ± 0.01	
(2) Without separation	0.240		0.240	
Crude lipid extract of electric organ	0.137		0.160	
	EME	BSA	EME	BSA
Effect of detergents:				
Native EME	0.240	0.250	0.240	0.301
EME + 1% Triton X-100	0.170	0.252	0.182	0.302
EME + 1% SDS	0.136	0.213	0.156	0.262

Conditions of the reconstitution experiment are described in **Methods**. In the presence of 90% 2-chloroethanol pH 2.0, the quantum yield of DNS-EME decreases by a factor of ten and p = 0.077. Measurements were carried after extensive dialysis against 0.2 M sucrose and 5×10^{-3} M glycylglycine pH 7.0. In (1) lipids were separated from the proteins on a LH 20 column; In (2) EME were dissolved only in 2-chloroethanol pH 2.0. When the effect of detergents was studied, the compound was added to the suspension of EME in 0.2 M sucrose pH 7.0 at 22°C. The amounts of DNS bound were: 39 μmoles per g of EME protein, 5.5 μmoles per g of crude lipid extract (chloroform-methanol) and 20 μmoles per g of BSA. All the measurements were carried out at 22°C.

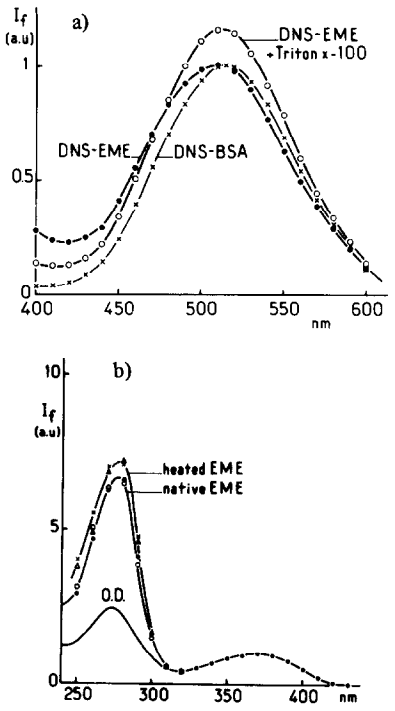

Fig. 2. Fluorescence spectra of DNS-EME and of ANS-EME. a) Emission spectra of DNS-BSA and of DNS-EME in the presence and in the absence of 0.1% Triton X-100. Spectra for BSA and DNS-EME where normalized to the same maximal intensity. 0.2 mg of protein/ml, 40 (resp. 45) μmoles of DNS were bound per g of BSA (resp. EME) protein. Wavelength of excitation: 350 nm. b) Excitation spectra of ANS-EME: ●—●, △—△ 2×10^{-5} M ANS; ○—○, x—x 8×10^{-5} M ANS. 0.2 mg of EME protein/ml. Dots: native EME; triangle and crosses: EME were heated at 80°C for 10 min. All measurements were made at 25°C. The spectra were recorded on a Jobin and Yvon Spectrofluorometer and normalized to the same number of quanta using correcting factors computed from the comparison of the observed excitation spectrum of 10^{-5} M quinine sulphate in 1 M H_2SO_4 to its absorption spectrum. Fluorescence was observed at 470 nm. Continuous line: absorption spectrum; absorption and emission spectra are normalized to the same maximal value at 380 nm. Light absorption due to scattering by EME (obtained by extrapolation of the absorption curve recorded at longer wavelengths) was substracted from the total absorption of the sample. The amounts of ANS bound per g of EME protein were 0.32 (●), 1.0 (○), 0.67 (△) and 2.05 μmoles (x). The corresponding yields of energy transfer were 3.9, 12.3, 9.3 and 28.1%.

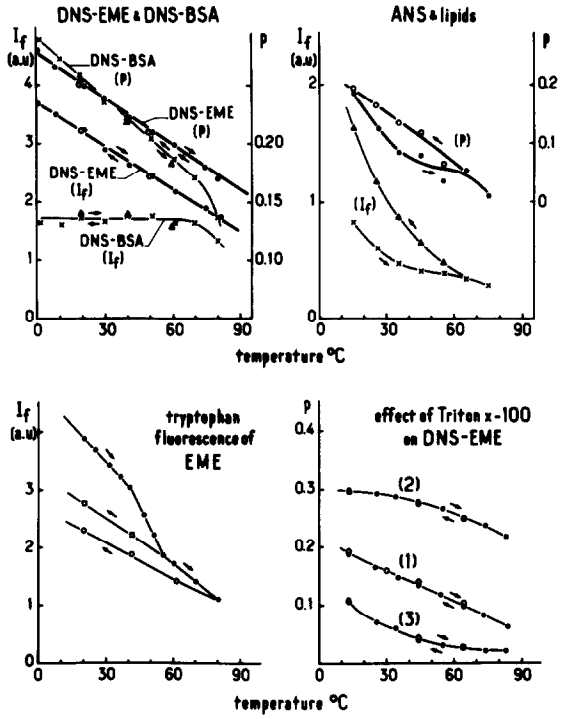

Fig. 3. Effect of temperature on the fluorescence parameters of various preparations of EME or lipids. The concentration of EME was 0.2 mg protein/ml, except when tryptophan fluorescence was measured (0.4 mg/ml). When DNS-EME were used, the amount of DNS covalently bound was 45 μmoles/g of EME protein. The concentration of DNS-BSA was 0.2 mg/ml. In the experiment with "ANS and lipids", the lipid suspension was obtained by sonication of 0.2 mg/ml of a crude lipid extract of electric organ in a Mullard apparatus for 2 min at 0°C. ANS concentration was 5×10^{-5} M. Fluorescence of tryptophan in native EME was measured at 350 nm with an excitation wavelength of 280 nm. In the experiment with DNS-EME and Triton X-100, (1) represents an unfractionated mixture of EME and 0.1% Triton X-100, (2) the membrane fragments (0.16 mg protein/ml) and (3) the soluble proteins (0.46 mg/ml) obtained by fractional centrifugation on sucrose gradient (see Methods).

EME protein. These results suggest that, under our experimental conditions, ANS and DNS label different sites on the membrane.

DNS reacts almost exclusively with membrane proteins. Could ANS, in contrast, label membrane lipids? The fact that ANS binds to a suspension of crude lipids extracted from electric tissue (fig. 3) supports this hypothesis. However the following observations dispute this interpretation: 1) p of ANS incorporated to the lipid suspension is 0.15 at 20°C and corresponds to a relaxation time of $\rho = 20$ nsec which is much

faster than that of ANS bound to EME; 2) energy transfer occurs with a high efficiency between the tryptophans of membrane proteins and ANS (fig. 2). ANS thus binds in close vicinity with membrane proteins.

We then compared the effects of temperature on the fluorescence parameters of ANS and DNS bound to EME. We first confirmed that following the fluorescence parameters of ANS, both with DNS-EME and EME, a discontinuity in the temperature curve occurs around 40°C which can be interpreted on the basis of a structural transition of the membrane. In contrast, this discontinuity, is absent when p of DNS covalently bound to EME is measured (fig. 3). This indicates that DNS is a less sensitive probe than ANS to the thermal transition of EME. The irreversible thermal transition was also detected at about 40°C with suspensions of both crude EME lipids (in the presence of ANS) or intact EME (following tryptophan fluorescence of EME proteins) (fig. 3). Moreover the energy transfer between ANS and tryptophans slightly, but significantly, increased in the course of the heat treatment (fig. 2). The thermal transition of the membrane thus seems to affect both the lipid and protein components in EME and their mutual relationships, leaving the properties of DNS binding sites unchanged. The apparent contradictory observations that ANS binds in close vicinity with EME proteins and is an excellent probe for a thermal transition present with isolated lipids in suspension, might be reconciled if it is assumed that the ANS binding sites in EME are primarily located in the areas of contact between lipids and proteins and that the structure of these hydrophobic areas is altered in the course of the thermal transition.

3.3. *Importance of lipids on the insensitivity of fluorescence polarization to viscosity*

In agreement with our findings with ANS, p of DNS-EME is insensitive to an increase in sucrose concentration in the solvent from 0.2 to 1.5 M [3]. As a consequence of the covalent association between DNS and EME proteins, it is possible to analyse the contri-

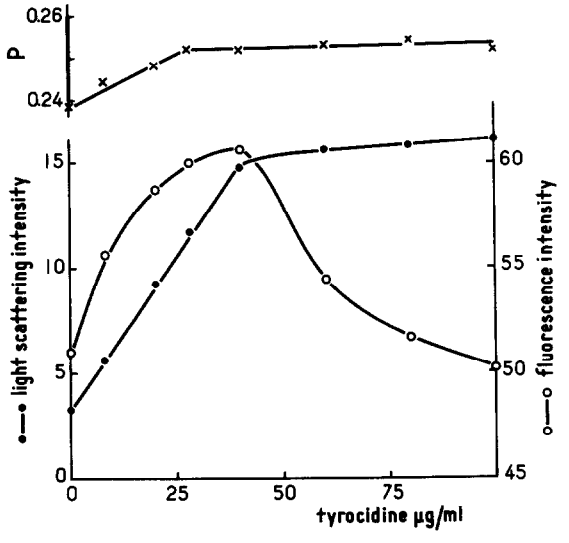

Fig. 4. Effect of tyrocidine on the fluorescence parameters of DNS-EME and on the light scattering by EME. (Concentration of EME: 60 µg of protein/ml).

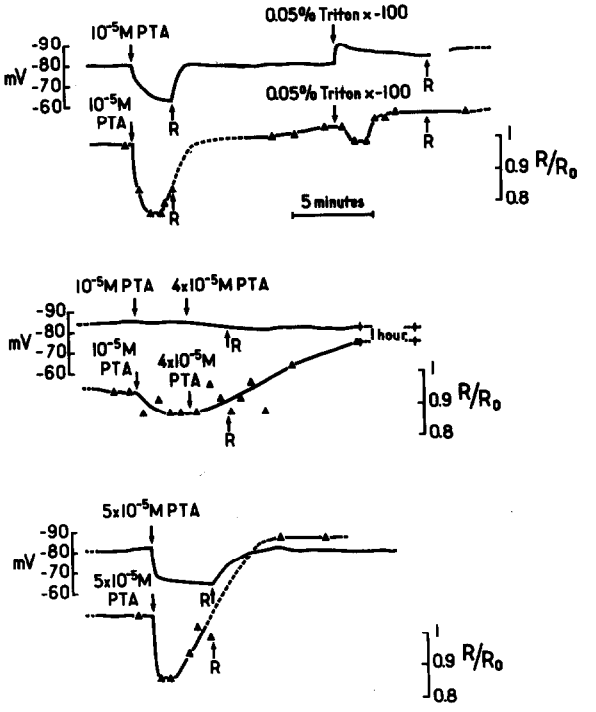

Fig. 5. Effect of Triton X-100 *in vivo* on the electrical response of the electroplax to a cholinergic agonist: phenyltrimethyl ammonium (PTA). Potential and resistance (R/Ro) of the excitable membrane of the isolated electroplax were measured according to a technique previously described [8, 10]. R represents physiological saline Ringer's solution. The blockade of the response to PTA is slowly reversed with time.

bution of membrane lipids to this effect. Removal of lipids from EME proteins is not accompanied by a siginificant change in the value of p but the latter becomes sensitive to changes in solvent viscosity (table 2).

We were unable to obtain similar information with lipids extracted from EME since, as mentioned above, DNS reacts almost exclusively with membrane proteins. However under the same conditions, DNS reacts slightly with a total lipid extract (chloroform-methanol) of the electric organ. p of DNS bound to these lipids is low ($p = 0.137$) and sensitive to changes of solvent viscosity. The insensitivity, of p to η thus seems to be a characteristic of the lipid-protein complex.

Reconstitution of membranes structures from their lipid and protein moieties provided further information on this point (see **Methods**). On reassociation no significant change of p was observed but solvent insensitivity reappeared. Solvent insensitivity thus seems to require the association of lipids and proteins into some organized membrane structure. One possible interpretation of these results is that DNS bound to its protein receptor(s) is buried inside the membrane in such a manner that it does not interact with the solvent during its motion. The lipids would contribute to a barrier which separates DNS binding sites on membrane proteins from the solvent.

3.4. *Effects of detergents and tyrocidine on the properties of membrane proteins labelled by DNS*

As shown in table 2 exposure of DNS-EME to detergents such as sodium dodecylsulfate (SDS) or Triton X-100 is followed by a dramatic decrease of p accompanied by a slight change in the emission spectrum (fig. 2). p still varies with temperature according to the Perrin law but becomes sensitive to changes of solvent viscosity upon addition of sucrose. However, these changes are not as large as those expected for a soluble protein, the sensitivity to solvent being only "partial". In order to investigate whether or not this partial sensitization was due to the solubilization of a fraction of membrane proteins, DNS-EME exposed to Triton X-100 were layered on top of a sucrose gradient and centrifuged as indicated in **Methods**. Under these conditions 60% of the protein remains in the supernatant. p of DNS bound to these proteins is low ($p = 0.091$ at 20°C in 0.2 M sucrose) and strongly sensitive to η ($p = 0.110$ in 1.5 M sucrose); in contrast, p of the bottom fractions is high ($p = 0.249$) and does not change with η (fig. 3). From these data, the initial value of p and its partial sensitivity to solvent in the mixture of DNS-EME + Triton can be accurately computed. The decrease of p and its partial sensitization to η observed in the presence of Triton X-100 is thus accounted for by the solubilization of an important fraction of membrane proteins. These results suggest that the proteins labelled by DNS have a much slower motion when they are integrated into a membrane structure than when they are dispersed in solution.

Tyrocidine (Tyr) has a completely different effect on EME; the intensity (If) of fluorescence, the polarization p, and the intensity of light scattering increase linearly and in parallel with the concentration of Tyr up to an end point (fig. 4) which is the same for the three parameters followed but depends on the concentration of membranes. At the end point the ratio of Tyr to membrane proteins is of about 1.5 and is the same for EME which has not been coupled to DNS. A somewhat similar effect is observed with BSA coupled with DNS. In both cases, an increase of p is noticed and the effect is reversed by Triton X-100. These results indicate that 1) as previously suggested [6] Tyr interacts with membrane proteins in EME and 2) Tyr tends to decrease the motion of the kinetic element constituted by DNS bound to its receptor proteins. In contrast with observations with Triton X-100 or sodium dodecylsulphate (SDS), Tyr tends to make the environment of the membrane proteins labelled by DNS more "rigid". These results are in complete agreement with those obtained by different groups of workers on biological or artificial membranes using different physical techniques [7]. The observation that, *in vivo*, Tyr and Triton X-100 have opposite effects on the electrical parameters of the electroplax membrane is of interest. As previously mentioned [8], Tyr promotes an irreversible *depolarization* of the electroplax membrane accompanied by a decrease of resistance. On the other hand, exposure of the innervated membrane to Triton X-100 in the concentration range used in these experiments (half maximum effect: $1/10^5$ v/v both *in vitro* and *in vivo*) does not lead to a depolarization of the membrane but to a reversible block of the response to agonists such as carbamylchloline, phenyltrimethyl ammonium or decamethonium and even to antibiotics like Tyr (fig. 5). Exposure to Triton X-100 thus tends to stabilize the mem-

brane in a "*polarized*" state. In an attempt to correlate these observations *in vivo* with those *in vitro* it can be suggested that the polarized state is stabilized by agents which tends to increase the motion of membrane proteins while the depolarized, excited, state is favoured by compounds which tend to immobilize the membrane proteins. Thus excitation promoted by physiological agonists such as acetylcholine might correspond to a decrease in motion, or immobilization of the cholinergic receptor(s).

* Note added in proof: Recent studies by Philippe Wahl of the fluorescence decay of DNS bound to EME indicate that the intrinsic motion of the *proteins* labelled by DNS is even much slower. This observation further strengthens our interpretation that proteins are strongly immobilized in the membrane phase.

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