Biochimica et Biophysica Acta 819 (1985) 18–22
Elsevier

BBA 72739

A comparison of the translational diffusion of a monomer and an oligomer of the acetylcholine receptor protein reconstituted into soybean lipid bilayers

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(Received February 19th, 1985)
(Revised manuscript received July 3rd, 1985)

Key words: Acetylcholine receptor; Oligomerization; Membrane reconstitution; Translational diffusion; Fluorescence (FRAP); (*T. marmorata*)

The translational diffusion of the acetylcholine receptor protein of *Torpedo marmorata* reconstituted into bilayer membranes of soybean lipids was examined using the fluorescence recovery after photobleaching (FRAP) technique. The protein was reconstituted either as a monomer or as a covalently cross-linked oligomer (predominantly tetramer). The covalent oligomerization of the protein was done in situ in the native membrane according to a recently described technique (Criado, M. and Barrantes, F.J. (1984) Biochim. Biophys. Acta 798, 374–381). Between 10 and 35°C no differences, within limits of experimental error, were observed in the value of the translational diffusion coefficient (D_t) for the two protein species. Typical values for D_t for the monomer and oligomer were approx. $1.5 \cdot 10^{-8}$ cm^2/s at about 10°C and approx. $3.5 \cdot 10^{-8}$ cm^2/s at about 35°C. These results are discussed in terms of a theory, based on continuum fluid hydrodynamic considerations, for the translational diffusion of porous discs in a thin viscous fluid sheet (Wiegel, F.W. (1980) Lect. Notes Phys. 121, 69–73). Using this theory, the solvent (lipid) penetration length into the tetrameric protein is estimated to be about 3/4 of the radius of the oligomeric protein aggregate in the membrane plane.

Introduction

The translational diffusion of several large integral membrane proteins reconstituted into lipid bilayer membranes has been examined in different laboratories in recent years [1–5]. It is evident from these studies that the translational diffusion coefficient (D_t) for proteins incorporated in lipid bilayers is only weakly dependent upon the radius of the protein in the plane of the bilayer. This fact has been taken as evidence in favour of the applicability of the continuum fluid hydrodynamic model [6,7] to the translational diffusion of proteins in lipid bilayer membranes [2,8,9]. However, the radii of all proteins so far examined lies between about 1.0 and 3.0 nm and this range of radii is not large enough to constitute a critical test of the model. In an attempt to extend the range of protein radii we have examined the translational diffusion, in a reconstituted soybean lipid membrane, of a covalently linked oligomer of an integral membrane protein, the acetylcholine receptor of *Torpedo marmorata*. The translational diffusion of this protein reconstituted into soybean lipid and dimyristoylphosphatidylcholine bilayers as a monomer and dimer had been previously reported by us [4]. For a recent review of the diffusion of the acetylcholine receptor in its native membrane the reader is referred to Ref. 10.

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Materials and Methods

The acetylcholine receptor was covalently cross-linked in situ in the native membrane by an oxidation reaction using 1 mM sodium periodate as described elsewhere [11,12]. The exact site of cross-linking is not known but possibly involves Schiff base formation between oxidized sugar residues and amino groups in the hydrophilic portion of the protein. However, since sodium periodate is not a group-specific reagent, various amino acid side chains could also be the target of the oxidation reaction (for a discussion, see Ref. 11). After cross-linking, the native membranes were solubilized and the protein oligomers were separated from monomers and dimers by sucrose density gradient centrifugation as previously described [4]. A typical separation is shown in Fig. 1. The three fractions with the highest radioactivity in the peak corresponding to oligomers (marked O in Fig. 1) were pooled and labeled with fluorescein isothiocyanate as described in Ref. 4. Collecting the other fractions in the oligomer peak gave a mixture of trimers, tetramers, and higher oligomers of the acetylcholine receptor. Gel filtration of the protein in the oligomer peak was performed on a Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated with 100 mM sodium chloride/10 mM sodium phosphate (pH 7.4)/1% sodium cholate/0.1 mM phenylmethanesulfonyl fluoride. The acetylcholine receptor monomer and dimer and ferritin were used as

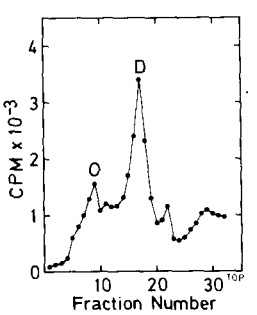

Fig. 1. Separation of the covalently cross-linked acetylcholine receptor protein on a continuous 5–20% sucrose density gradient in the presence of 1% sodium cholate. The acetylcholine receptor protein was detected by trace-labeling with α-[^{3}H]bungarotoxin as described in Ref. 4. Peaks labeled O and D correspond to the oligomer and dimer, respectively.

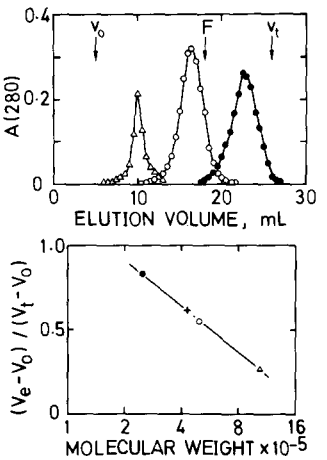

Fig. 2. Gel filtration on a column of Sepharose 4B in the presence of 1% sodium cholate. The upper panel shows the elution profiles of monomeric (●), dimeric (○), and oligomeric (△) acetylcholine receptor protein. The elution position of ferritin is indicated by the arrow labeled F. The column void and total volumes are indicated by the arrows labeled v_0 and v_t, respectively. The lower panel shows an estimation of the molecular weight of the oligomeric protein using the information from the upper panel.

calibration standards. As seen in Fig. 2, the elution of the oligomeric protein indicates that it is a tetramer (the monomer acetylcholine receptor protein has a molecular weight of approx. 250 000).

Reconstitution of the fluorescein-labeled proteins was done as described earlier [4] and fluorescence recovery after photobleaching (FRAP) experiments were performed as described in Ref. 13 using a uniform circular beam profile with a radius of 5.5 μm and a Zeiss 'Plan' 16/0.35 objective. D_t was calculated from the halftime for complete fluorescence recovery as described in Ref. 14. Fluorescence recovery was $\geqslant 95\%$ in all cases. As described elsewhere [4], the FRAP experiments were done on large (radius $\geqslant 100$ μm) proteoliposomes which do not move during the course of the experiment. The fluorescence recovery is, therefore, not due to proteoliposome movement.

Results and Discussion

In Fig. 3 the experimental FRAP curve for the acetylcholine receptor oligomer in soybean lipid multibilayers is compared with a theoretical curve for fluorescence recovery due to one diffusing

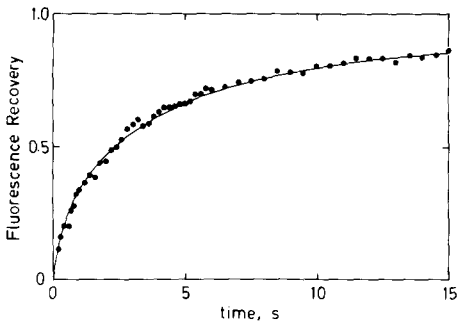

Fig. 3. FRAP curves for the fluorescein-labeled acetylcholine receptor oligomer in soybean lipid bilayers at approx. 25°C. The points are experimental and the curve is a theoretical FRAP curve calculated according to Ref. 14.

component. The agreement between experiment and theory is quite good. This was earlier also shown to be the case for the monomer and dimer [4]. In Table I we compare the temperature dependence of D_t for the monomer and tetramer. It is evident that, within limits of experimental error, there is no difference between the values of D_t for the monomer and the tetramer of the acetylcholine receptor in soybean lipid bilayers. We try below to understand this, at first sight surprising, result.

First, we consider the possibility that the acetylcholine receptor monomer may aggregate in the membrane to an oligomeric species so that the measured value of D_t is for an oligomer whether or not the protein was previously cross-linked by

TABLE I

TEMPERATURE-DEPENDENCE OF D_t FOR THE ACETYLCHOLINE RECEPTOR MONOMER AND OLIGOMER RECONSTITUTED INTO SOYBEAN LIPID BILAYERS

Results are reported as the mean ± S.D. of at least five FRAP experiments on different multibilayer domains of each of at least two separately prepared slides.

Temp. (°C)	D_t (10^{-8} cm^2/s)	
	Monomer	Oligomer [a]
15	1.7 ± 0.2	1.3 ± 0.3
19	1.7 ± 0.4	1.8 ± 0.5
25	2.2 ± 0.3	2.1 ± 0.2
29	2.8 ± 0.5	2.6 ± 0.6
34	3.3 ± 0.6	3.1 ± 0.4

[a] Predominantly tetramer, see text.

periodate treatment. Recent work [15] on electrical conductance in bilayer lipid membranes (black films) made from 84 mole % soybean lipid and 16 mole % cholesterol and containing the acetylcholine receptor monomer shed some light upon the conditions required for aggregation of this protein. It was found that the monomeric receptor did not aggregate at a membrane cohesive pressure $\leqslant 35$ mN/m but did aggregate to form dimers and tetramers at a cohesive pressure $\geqslant 35$–40 mN/m. In the bilayers in question, cohesive pressures $\geqslant$ 35–40 mN/m were only achieved by addition of 0.5 mM $CaCl_2$ to the aqueous phase. Earlier work from the same laboratory [16] showed that the cohesive presure of bilayers prepared from soybean lipid alone was approx. 32 mN/m. In the work reported on here, we have prepared our bilayers from soybean lipid alone. Our membranes also do not have any cholesterol and there is no Ca^{2+} in the aqueous phase. We can thus expect that the cohesive pressure in our membranes is $\leqslant 32$ mN/m and as a result the acetylcholine receptor monomer remains monomeric. We are, therefore, comparing the translational diffusion behaviour of the monomeric acetylcholine receptor to that of an oligomeric species.

Perhaps a better explanation for the lack of difference in the D_t values for the acetylcholine receptor monomer and oligomer can be obtained by examining the structure of the two protein species in the membrane. From neutron scattering studies of the structure of the acetylcholine monomer in detergent solutions Wise et al. [17] have proposed the structure shown in Fig. 4A for this protein. The same authors suggested that the center to center separation distance between the two monomers in the dimeric protein is 8.5 nm. In consideration of these neutron scattering results we propose that our acetylcholine tetramer may have a structure as shown in Fig. 4B, assuming it to have been formed by a cross-linking of dimeric protein species in the native membrane. The dimer is known to be the predominant acetylcholine receptor species in the native membrane as suggested by sucrose density gradient centrifugation and electron microscopic data [12]. That the tetramer can have the structure shown in Fig. 4B is also indicated from electron microscopic images of the receptor protein solubilized from native mem-

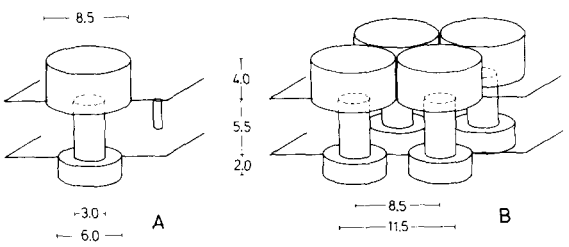

Fig. 4. Models for the acetylcholine receptor monomer (Panel A) and tetramer (Panel B). The model for the monomer is taken from Ref. 17. The size of a lipid molecule, drawn to scale as a cylinder, is also shown for comparison. The model for the oligomer is one of several possible structures and is drawn assuming that a tetramer is formed by cross-linking of two dimeric protein units, the dimer being the most prominent form of the acetylcholine receptor in its native membrane. The dimensions are taken from the proposed structure for the monomer [17] and are given in nm.

branes in which cross-linking has been done [12]. The effective radius of the tetramer in the lipid membrane will then be expected to be approx. 5.7 nm. In the plane of the membrane, however, the hydrophobic portions of the acetylcholine receptor protein only occupy part of the cylindrical volume defined by this radius, the rest being filled up by lipid as seen in Fig. 4. Radiation inactivation studies on the acetylcholine receptor in native membranes [18] indicate that each monomer in a dimeric molecule constitutes a separate target for an inactivating electron. This could only be the case if the two monomers in a dimer have very little contact other than the disulfide bond that holds them together, the rest of the space being filled up with lipid molecules. The tetrameric protein can thus be considered to be a 'porous disc' and the hydrodynamic model for diffusion of porous discs in thin viscous fluid sheets presented by Wiegel [19] can be used to determine whether this porous disc is penetrated by the solvent (in our case, the lipid molecules). D_t, in this model, is given by Eqn. 1

$$D_t = \frac{kT}{4\pi\eta h}\left[\ln\frac{\eta h}{\eta' a} - \gamma + \frac{2}{\sigma^2} + \frac{I_0(\sigma)}{\sigma I_1(\sigma)}\right] \quad (1)$$

where k is Boltzmann's constant, T is the temperaturein degrees Kelvin, η is the viscosity of the membrane, η' is the aqueous viscosity, h is the thickness of the membrane, a is the radius of the protein (considered here to be cylindrical in the membrane), γ is Euler's constant (0.5772), I_0 and I_1 are modified Bessel functions, and $\sigma = a/(k_0)^{1/2}$ where $(k_0)^{1/2}$ is the penetration length for the surrounding fluid (in our case the lipid molecules) into the permeable cylinder. If the cylinder is impermeable, $\sigma \to \infty$ and Eqn. 1 reduces to the well known equation of Saffman and Delbrück [6] which may, therefore, be considered to be a limiting case of Wiegel's model [19]. The difference between D_t for an impermeable cylinder and D_t for a permeable cylinder of the same radius gives the value of $((2/\sigma^2) + (I_0(\sigma)/\sigma I_1(\sigma)))$. By reference to Table VI of Ref. 19 the value of σ can be deduced and the value of $(k_0)^{1/2}$ can be calculated. We assume that Eqn. 1 can be applied to the diffusion of the acetylcholine receptor monomer and tetramer in soybean lipid bilayers and that their radii are 1.5 and 5.7 nm, respectively. Using the experimental value fo D_t ($3.3 \cdot 10^{-8}$ cm^2/s) for the monomer at approx. 35°C we calculate η for the membrane to be 1.72 poise. If the acetylcholine tetramer were an impermeable cylinder of radius 5.7 nm, and the membrane viscosity were 1.72 poise, the expected D_t for the tetramer would be $1.9 \cdot 10^{-8}$ cm^2/s at approx. 35°C. The experimentally obtained value is, however, $3.1 \cdot 10^{-8}$ cm^2/s. From the difference between the experimental value for D_t and the calculated value of D_t for an impermeable cylinder of the same radius, and with reference to Table VI of Wiegel [19], we estimate $\sigma = 1.35$ from which $(k_0)^{1/2} = 4.2$ nm. This value for the penetration length is about 3/4 of the radius of the tetrameric protein, which suggests that the lipid almost freely drains through the oligomeric protein aggregate. Any further interpretation of the result in terms of a molecular model may, however, be premature. The results reported here may be of general significance for the understanding of the translational diffusion of oligomeric protein aggregates in biological membranes.

Acknowledgements

We would like to thank Dr. Thomas M. Jovin for his support and Drs. Robert M. Clegg and Jürgen Stümpel for useful discussions.

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