STUDIES ON THE CHOLINERGIC RECEPTOR PROTEIN FROM *ELECTROPHORUS ELECTRICUS*
Effect of detergents on some hydrodynamic properties of the receptor protein in solution

J.C. MEUNIER, R.W. OLSEN and J.P. CHANGEUX
Unité de Neurobiologie Moléculaire, Institut Pasteur, Paris, France

Received 5 May 1972

1. Introduction

In previous studies [1, 2] it was shown that the cholinergic receptor protein from *Electrophorus electricus* labeled with a heavily tritiated α-toxin from *Naja nigricollis* exhibited unusual hydrodynamic properties. In the presence of either a neutral detergent, Triton X-100, or of a negatively charged one, Na-deoxycholate, the sedimentation velocity estimated by sucrose gradient centrifugation appeared smaller than the one compatible with the Stokes' radius determined by gel filtration, assuming the protein to be a sphere with a density close to that of regular proteins. The same properties were found as well with the unlabeled receptor protein [2]. These data, which have been recently confirmed by Raftery and coworkers [3], were interpreted on the basis of either a nonglobular shape and/or an unusual density of the receptor protein [1, 2].

In the present paper, this problem has been reinvestigated in greater detail by ultracentrifugation in sucrose gradients where the density was varied by replacing H_2O by D_2O [4]. Several hydrodynamic parameters of the complex, the partial specific volume ($\bar{v}$) in particular, are measured in the presence of various detergents (Triton X-100, Na-cholate and deoxycholate). The results are interpreted on the basis of a differential binding of the detergents to the labeled receptor. Estimates of the molecular weight of the receptor–α-toxin complex are proposed.

2. Material and methods

2.1. *Preparation of the crude extracts*

Excitable membrane fragments from the electric organ of a freshly killed electric eel were prepared as previously described [5]. Four ml (i.e. about 25 mg proteins) of the acetylcholinesterase-enriched fraction were diluted to 8 ml with a buffer containing 5 mM Tris pH 8.0, 320 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$ and 4 mM $CaCl_2$. To the suspension, 10 μl of a [^{3}H]α-toxin solution (14.8 Ci/mmole; 0.4 mg/ml) [6] were added and the reaction allowed to proceed for 2 hr at room temp. Under these conditions virtually 100% of the receptor sites were labeled. The reaction mixture was then centrifuged for 0.5 hr at 10^5 *g* and the pellet resuspended in 2 ml of 300 mM Tris pH 8.0 containing 3% (w:v) of Triton X-100 (Calbiochem) or Na-cholate (Schuchardt). The dissolution was nearly complete after 1 hr of gentle stirring at 20°. The dissolved membranes were dialyzed for 5 hr against a large volume of 100 mM Tris pH 8.0, 100 mM NaCl and 1% (w:v) Triton X-100 or Na-cholate at 4°, and then submitted to centrifugation for 2 hr at 10^5 *g* at 4°, to remove undissolved material. The final supernatant contained 5 mg/ml protein and 13–14 nanomoles [^{3}H]α-toxin bound per gram of protein.

Alternatively, crude membranes were dissolved in a buffer containing 100 mM Tris pH 8.0, 100 mM NaCl, and 1% (w:v) Na-cholate as previously described [2]. The high speed supernatant was labeled with 5 μl of

[^{3}H] α-toxin as mentioned above and then centrifuged at 4° for 90 min at 10^5 *g*. The final cholate extract contained 10 mg/ml protein and approx. 10 nmoles of bound [^{3}H] α-toxin per gram of protein.

2.2. *Sucrose density gradient centrifugation*

Stock solutions containing 5% or 20% (w:v) sucrose in 100 mM Tris pH 8.0, 100 mM KCl plus either 1% Triton X-100 (final concentration) or 1% Na-cholate or 1% Na-deoxycholate were prepared in either H_2O or D_2O as the solvent. Densities were measured with a pycnometer and refractive indices with a Zeiss Abbe's refractometer at 20°. The gradients (20 to 5% sucrose; 8.7 ml) were made with Büchler gradient-maker at 20°, stored overnight at 4° and re-equilibrated at 20° before centrifugation. A sample (0.1 ml) containing approx. 350 μg (protein) of crude extract, 400 μg of beef liver catalase (Sigma), 200 μg of yeast alcohol dehydrogenase (Sigma) and 70 μg of *E. coli* β-galactosidase (a gift for Dr. A. Ullman) was carefully layered on top of each gradient. Each tube was supplemented with 3.0 ml of paraffin oil and the centrifugation was carried out in the rotor SW 41 Ti of a Spinco model L2-65B preparative ultracentrifuge at 39,000 rpm and 20° for 6 hr. Fractions of 12 drops were collected manually with the help of a Büchler piercing unit. The drop size was nearly constant (the difference between the volume of the first and last fraction was less than 3%). Marker enzymes were assayed as reported by Racker [7] for yeast alcohol-dehydrogenase (10 μl), Chance and Maley [8] for catalase (10 μl) and Hestrin et al. [9] for β-galactosidase (10 μl). The radioactivity of the complex RACh–[^{3}H] α-toxin was measured, after dilution of 0.1 ml of each fraction in 10 ml of Bray's solution, with an Intertechnique SL 30 liquid scintillation counter. No correction was made for quenching.

2.3. *Gel filtration*

The agarose (Sepharose 6B, Pharmacia) column (2.5 × 40 cm) was equilibrated at 20° with the following buffer: 100 mM Tris pH 8.0, 100 mM NaCl, 1% Triton X-100 (w:v) or Na-cholate, and 0.02% sodium azide. The sample (1.0 ml), containing 3 mg blue dextran 2000 (Pharmacia), 0.4 mg *E. coli* β-galactosidase, 1 mg yeast alcohol dehydrogenase, 10 mg bovine serum albumin (Mann Research), 3 ng $^{22}Na^+$ (1 μCi), and 2–3 mg protein of the crude extract, was layered on top of the agarose bed. After the sample had entered the gel, elution with the equilibrium buffer was started and fractions of 1.5 ml were collected with a Gilson model "Miniescargot MTDC" automatic fraction collector, at 20° and a nearly constant flow rate of 7–8 ml/hr. β-Galactosidase and alcohol dehydrogenase were assayed as described above (sect. 2.2), bovine serum albumin according to Lowry et al. [10], and blue dextran by measuring the optical density at 650 nm. Radioactivity was counted in 0.2 ml of each fraction as already mentioned (sect. 2.2).

3. Results and discussion

We have measured the partial specific volume ($\bar{v}$) and the sedimentation coefficient ($s_{20,w}$) of the receptor–α-toxin complex by sucrose gradient centrifugation in the presence of various detergents with either D_2O or H_2O as the solvent. Sedimentation of the labeled receptor as a function of time was compared to that of marker enzymes with known $\bar{v}$ and $s_{20,w}$: yeast alcohol dehydrogenase (0.73 ml/g^{-1} and 7.4 S) [11], beef liver catalase (0.73 and 11.4) [12] and *E. coli* β-galactosidase (0.73 and 16.0) [13] (table 1).

According to Martin and Ames [12], the distance (r_i) traveled from the meniscus by any macromolecule in medium i (i = 1 for H_2O and i = 2 for D_2O) is:

$$r_i = k_i \cdot s_{20,w} \cdot (1 - \bar{v}\rho_i) \qquad 1)$$

where k_i is a constant in a medium of a given average density for any macromolecule with the same partial specific volume ($\bar{v}$) and ρ_i the density of medium i. Since the density increases linearly along the gradient ρ_i is estimated by taking its value at $r_i/2$. Under these conditions, the error in ρ_i is less than 0.5%. Thus, from

$$\frac{r_1}{r_2} = \frac{k_1(1-\bar{v}\rho_1)}{k_2(1-\bar{v}\rho_2)} \qquad 2)$$

it becomes possible to calculate $\bar{v}$ and $s_{20,w}$ for the complex receptor–α-toxin.

Fig. 1 shows the sedimentation profiles obtained in the presence of Triton X-100. The data are fitted adequately by equation 1) as shown in fig. 2 from which k_1 and k_2, the slopes of the straight relationships in H_2O and D_2O, respectively, have been calculated (fig. 2). Therefore, one can obtain estimates for

Table 1
Sedimentation of the radioactive complex receptor–α-toxin in the presence of various detergents.

Protein	Detergent	Distance travelled from meniscus (mm) $r_1(H_2O)$	$r_2(D_2O)$	$\frac{r_1}{r_2}$
ADH	Na-C	21.7 (21.4)[a]	13.4 (12.8)[a]	1.62 (1.67)[a]
	Na-DOC	21.0	12.7	1.65
	T X-100	22.0 (20.0)[b]	13.5 (12.5)[b]	1.63 (1.60)[b]
R-T	Na-C	31.3 (30.0)[a]	19.1 (19.0)[a]	1.64 (1.58)[a]
	Na-DOC	29.5	18.0	1.64
	T X-100	27.0 (24.5)[b]	14.8 (13.3)[b]	1.83 (1.84)[b]
CAT	Na-C	32.8 (30.6)[a]	19.7 (19.0)[a]	1.66 (1.56)[a]
	Na-DOC	31.0	19.0	1.63
	T X-100	33.5 (30.0)[b]	21.0 (19.7)[b]	1.60 (1.52)[b]
GAL	Na-C	45.0 (44.0)[a]	28.1 (27.8)[a]	1.60 (1.58)[a]
	Na-DOC	44.0	28.0	1.57
	T X-100	46.0 (42.5)[b]	29.5 (27.5)[b]	1.56 (1.55)[b]

Values in parentheses indicate separate experiments. [a] Sedimentation of a cholate extract from purified excitable membrane fragments. [b] Sedimentation of a Triton X-100 extract from purified membrane fragments (data from the experiment illustrated in fig. 1).

Table 2
Physical parameters of the complex receptor–α-toxin measured in various detergents.

	Detergent	$\bar{v}$ (mg/g)	$s_{20,w}$	a (Å)	Apparent molecular weight (g)	Apparent fractional ratio (f/f_0)
RACh–[^{3}H]α-toxin	1% Na-cholate	0.73(0.73)[a] ± 0.01	11.0(11.0)[a] ± 0.2	70 ± 3	323,000 ± 20,000	1.54 ± 0.02
	1% Na-deoxycholate	0.73 ± 0.01	10.7 ± 0.2	~70	315,000 ± 20,000	1.55 ± 0.02
	1% Triton X-100	0.78(0.78)[b] ± 0.01	12.5(12.5)[b] ± 0.1	73 ± 3	470,000 ± 30,000	1.39 ± 0.01

Values in parentheses have been obtained from separate experiments (see legend under table 1). Apparent M.W. and frictional ratios (f/f_0) have been calculated as recommended by Siegel and Monty [17], from

$$M = \frac{6\pi\eta_{20,w}Nas_{20,w}}{1 - \bar{v}\rho_{20,w}} \text{ and } \frac{f}{f_0} = \frac{a}{\sqrt[1/3]{\frac{3\bar{v}M}{4N}}}$$

in which M = molecular weight; $\eta_{20,w}$ = viscosity of water at 20°; N = Avogadro's number, a = Stokes' radius, $s_{20,w}$ = sedimentation coefficient, $\bar{v}$ = partial specific volume and $\rho_{20,w}$ = density of water at 20°.

$s_{20,w}$ and $\bar{v}$ (table 2) of the complex receptor–α-toxin, assuming that the hydrodynamic parameters of the markers remain unaltered in the presence of Triton X-100, and that deuterium exchange proceeds to the same extent with the receptor protein and the proteins used as markers. Interestingly, the $\bar{v}$ of the receptor–toxin complex was found significantly larger ($\bar{v} = 0.78$) than that of the proteins used as markers.

A similar experiment performed in the presence of Na-cholate led to somewhat different results. The radioactive complex solubilized in Na-cholate had a density equal to that of the marker enzymes ($\bar{v} = 0.73$)

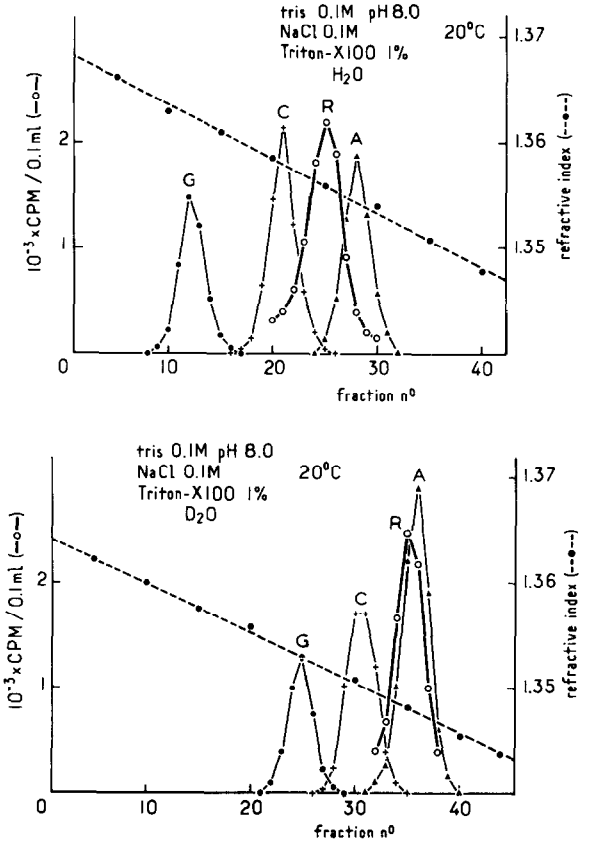

Fig. 1. Sedimentation profile of the complex receptor–α-toxin in H_2O or D_2O in the presence of Triton X-100. A = alcohol dehydrogenase, C = catalase, G = β-galactosidase, R = receptor–α-toxin.

and its sedimentation coefficient was smaller than that determined in Triton X-100: $s_{20,w}$ = 11.0 S instead of 12.5 S.

In these experiments the same detergent was used for excitation and centrifugation. We therefore considered the possibility that the differences in behavior observed were due to the extraction by Triton X-100 and cholate of different molecular forms of the receptor protein. In order to test this possibility, labeled membrane fragments were first solubilised by Na-cholate (see sect. 2.1) and subsequently centrifuged in the presence of either 1% Na-cholate, 1% Na-deoxycholate or 1% Triton X-100. Again, the $\bar{v}$ measured in the presence of Triton X-100 was larger than that measured in the presence of the other detergents (tables 1 and 2). The variability among the hydrodynamic parameters thus depends on the nature of the surfactant present in the centrifugation medium.

Finally, the Stokes radius of the complex RACh–[^{3}H]α-toxin was determined by gel filtration through Sepharose 6B in the presence of either Triton X-100 or Na-cholate (1%; w:v). K_{AV}, the partition coefficient defined by Laurent and Killander [14] was calculated from the equation:

$$K_{AV}^{1/3} = \left(\frac{v_e - v_0}{v_t - v_0}\right)^{1/3} = \alpha - \beta a \qquad 3)$$

where v_e = elution volume, v_0 = void volume (elution volume for blue dextran 2000), v_t = total volume (elution volume for $^{22}Na^+$) and a = Stokes' radius. α and β are constants.

Fig. 3 shows that Porath's correlation [15] is still followed by the enzyme markers in the presence of either Na-cholate or Triton X-100. Furthermore, no

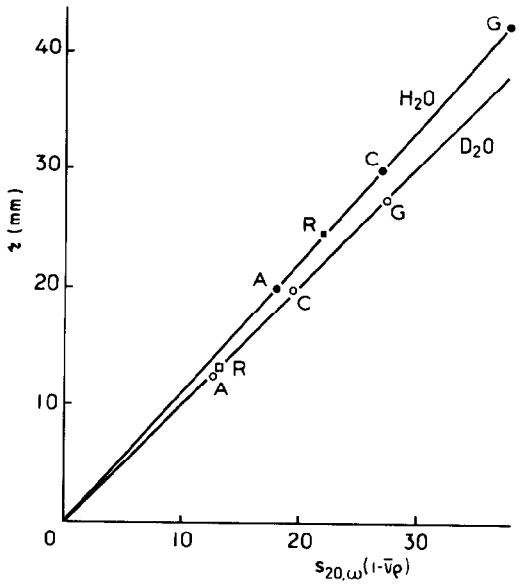

Fig. 2. Relationship between r_i and $s_{20,w} \times (1-\bar{v}\rho_i)$. Data have been taken from the experiment described in fig. 1. Symbols are the same (see text).

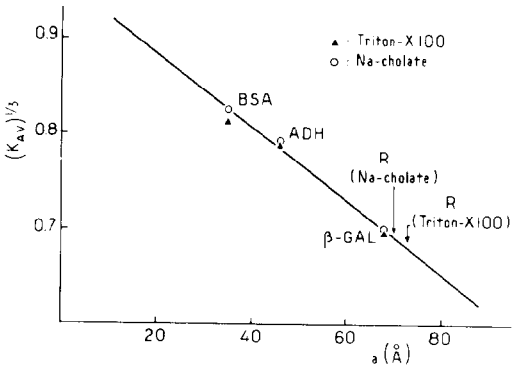

Fig. 3. Filtration through Sepharose 6B. Data are plotted according to Porath (see text). Values for the Stokes' radii of serum albumin (BSA) and alcohol dehydrogenase (ADH) have been taken from Siegel and Monty [17]. The Stokes' radius of β-galactosidase (β-GAL) has been calculated from the molecular weight (540,000) and partial specific volume (0.73).

significant difference was found in the presence and in the absence of detergent, indicating that the detergents used do not significantly bind to the markers, which are all soluble globular proteins. This finding, which confirms Helenius and Simons' results and interpretation [16], was further supported by the observation that the sedimentation velocity of one marker, catalase, did not change in the presence of either Na-cholate or Triton X-100. The Stokes' radius of the receptor–α-toxin complex in the presence of detergent was estimated by extrapolation, taking for the Stokes' radius of the markers the values given in the literature. The results are given in table 2. The Stokes radius is slightly higher in the presence of 1% Triton X-100 than in the presence of either Na-cholate or deoxycholate, an effect which parallels the effect of Triton X-100 on $\bar{v}$. In table 2, all the presently available data have been summarized and the relevant *apparent* molecular weights and frictional ratios (f/f_0) calculated. A slightly asymmetric shape is indicated by these values.

Since the detergents are required to solubilise the receptor protein from the membrane fragments and to maintain it in solution, it is expected that these detergents interact with the protein. The data obtained in the presence of Triton X-100 can be readily interpreted on this basis. Taking for Triton X-100 $\bar{v} = 0.99$ (Rohm and Haas product information) and assuming for the protein component $\bar{v} = 0.73$, then a contribution of up to 21% of the total mass of the receptor–toxin complex by Triton X-100 (from 160 to 170 molecules of detergent) must be postulated to account for a $\bar{v}$ of 0.78. The apparent molecular weight of the receptor without Triton X-100 would then be corrected from 470,000 to 360,000. Interestingly, a similar conclusion was reached, but with sodium dodecylsulfate as a detergent, by Rosenberg and Guidotti [18] with the so-called hydrophobic proteins of erythrocyte ghost: they found that the $\bar{v}$ actually measured in the presence of detergent was significantly higher ($\bar{v} = 0.78$) than the one calculated from the amino acid composition ($\bar{v} = 0.74$–0.75).

In contrast with the result obtained in Triton X-100, $\bar{v}$ of the receptor–toxin complex measured in Na-cholate or deoxycholate is 0.73, the same as that of the standard proteins. Since $\bar{v}$ of Na-cholate or deoxycholate micells are, respectively, 0.75 and 0.76 [22], i.e. close to those of globular proteins, eventual binding of these surfactants to the receptor would not manifest itself by a large change of buoyant density. It is likely, in agreement with Helenius and Simons' results, that cholate and deoxycholate do bind to the receptor but the resolution of our method is not sufficient to measure it with some accuracy. The

slightly lower *apparent* molecular weight value in Na-cholate or deoxycholate (~320,000) as compared to that in Triton X-100 (~360,000) might be due to a charge effect of the few molecules of cholate or deoxycholate bound to the protein. This would lead to an underestimate of $s_{20,w}$ and thus of molecular weight. Such an effect might account as well for the higher frictional ratio obtained in the presence of the anionic detergents. In any case, since it has been found independently by Karlin [19] and by ourselves [1, 2] and recently by Raftery [20], that the receptor protein splits in the presence of sodium dodecyl sulfate into units of 4 to 5 $\times 10^4$ M.W., the receptor protein in solution presumably possesses an oligomeric structure. If the subunits are identical, 6 to 8 would be present. It is not yet known if the receptor protein shows such a structure when integrated into the membrane framework.

In summary, the unusual hydrodynamic behavior of the cholinergic receptor protein, observed mainly in the presence of Triton X-100, is better explained by a decrease of density caused by detergent binding rather than by a highly asymmetric shape. Such a conclusion might be extended to other membrane proteins which are solubilized by Triton X-100 [21] and have similar physical properties. It is presumably related to the presence of areas on the surface of these proteins which establish hydrophobic contacts with membrane lipids. In solution these hydrophobic loci would be occupied by detergent molecules.

Acknowledgements

We thank Dr. R. Baldwin for essential suggestions and critical comments, Drs. J. Cohen, G. Gachelin, A. Fritsch and S. Bram for helpful discussions and Drs. Menez, Fromageot and Boquet for the gift of tritiated α_1-isotoxin from *Naja nigricollis*.

This work was supported by funds from the Centre National de la Recherche et technique, the Collége de France, the Commissariat à l'Energie Atomique and the National Institutes of Health.

References

[1] J.C. Meunier, R. Olsen, A. Menez, J.L. Morgat, P. Fromageot, A.M. Ronseray, P. Boquet and J.P. Changeux, C.R. Acad. Sci. 273 D (1971) 595.
[2] J.C. Meunier, R.W. Olsen, A. Menez, P. Fromageot, P. Boquet and J.P. Changeux, Biochemistry 11 (1972) 1200.
[3] M.A. Raftery, J. Schmidt, D.G. Clark and R.G. Wolcott, Biochem. Biophys. Res. Commun. 45 (1971) 1622.
[4] S.J. Edelstein and H.K. Schachman, J. Biol. Chem. 242 (1967) 306.
[5] J.P. Changeux, J. Gautron, M. Israel and T.R. Podleski, C.R. Acad. Sci. 269 (1969) 1788.
[6] A. Menez, J.L. Morgat, P. Fromageot, A.M. Ronseray, P. Boquet and J.P. Changeux, FEBS Letters 17 (1971) 333.
[7] E. Racker, in: Methods in Enzymology, Vol. I (Academic Press, New York, 1955) p. 500.
[8] B. Chance and A.C. Maley, ibid., Vol. II (1955) p. 764.
[9] S. Hestrin, D.S. Feingold and M. Schramm, ibid. Vol. I (1955) p. 241.
[10] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[11] E.L. Kuff, G.H. Hogeboom and M.J. Stuebich, J. Biol. Chem. 212 (1955) 439.
[12] R.G. Martin and B.N. Ames, J. Biol. Chem. 236 (1961) 1372.
[13] G.R. Craven, E. Steers Jr. and C.B. Anfinsen, J. Biol. Chem. 240 (1965) 2468.
[14] T.C. Laurent and J. Killander, J. Chromatog. 14 (1964) 137.
[15] J. Porath, Pure Appl. Chem. 6 (1963) 233.
[16] A. Helenius and K. Simons, Biochemistry, in press.
[17] L.M. Siegel and K.J. Monty, Biochim. Biophys. Acta 112 (1966) 346.
[18] S.M. Rosenberg and G. Guidotti, J. Biol. Chem. 243 (1968) 1985.
[19] A. Karlin, J. Prives, W. Deal and M. Winnik, J. Mol. Biol. 61 (1971) 175.
[20] M. Raftery, Neuroscience Research Program, Work Session on Receptor Biochemistry and Biophysics, March 5–1, 1972.
[21] P. Cuatrecasas, J. Biol. Chem. 247 (1972) 1981.
[22] D.M. Small, in: Molecular Association in Biological and Related Systems, American Chemical Society Symposium, Florida, 1967 (Advances in Chemistry Series, Washington, 1968) p. 31.