PROGRESS IN THE PURIFICATION OF THE CHOLINERGIC RECEPTOR PROTEIN FROM *ELECTROPHORUS ELECTRICUS* BY AFFINITY CHROMATOGRAPHY*

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1. Introduction

An increasing number of studies have been recently undertaken on the cholinergic receptor protein since the discovery that this macromolecule can be selectively labelled by snake venom α-toxins [1–3] and solubilized by mild detergents without loss of the ability to bind cholinergic agonists and antagonists [3–8]. Several more or less successful attempts to purify the receptor protein from detergent extracts of fish electric organs have been reported during the past few months [9]. We describe in this letter an affinity column which gives, in a single step and with a particularly high yield, an approx. 150-fold purification of the nicotinic receptor protein from *Electrophorus electricus.* The purified protein still binds cholinergic ligands and a tritiated α-toxin from *Naja nigricollis* [10].

2. Materials and methods

2.1. *Synthesis of the affinity column*

A sufficient quantity of Sepharose 2B (Pharmacia) was washed several times in distilled water at 4° to yield 100 ml of beads after decantation. The total volume of the suspension was made 200 ml with distilled water and the pH adjusted to 11 with one drop of 50% NaOH. To the suspension was added 5.0 g of CNBr dissolved in 100 ml H_2O. The pH was maintained at 11.0 ± 0.05 for 10 min and the temperature at 0° with frozen distilled H_2O. The beads were then washed on a Buchner funnel with 1 l of 0.05 M $NaHCO_3$ buffer, pH 9.8, at 0°, and transferred with 1 vol of this buffer to a reaction vessel containing 11.61 g (100 mmole or 1 mmole per ml) hexanediamine (Aldrich) in a small volume of buffer which had been adjusted at pH 9.8 with concentrated HCl. The reaction was carried out with gentle agitation for 21 hr at 4°, after which the Sepharose was filtered and washed with 1.5 l of distilled H_2O. The product gave an orange color in the trinitrobenzene sulfonate test [11] indicating an amino derivative.

The amino-Sepharose was then transferred to a reaction vessel containing 5.0 g (0.03 mole or 0.3 mmole per ml beads) *N*-acetyl homocysteine thiolactone (Cyclo Chemicals) in 100 ml of 1.0 M $NaHCO_3$ buffer, pH 9.7, and agitated gently at 4° for 70 hr. The sulfhydryl Sepharose was washed with 10 l of 0.1 M NaCl at 4°. To insure that there were no disulfide bonds present, the product was incubated for 60 min at 25° in 100 ml of 0.05 M dithiothreitol (Sigma) in 0.5 M Tris-HCl, pH 8.0, followed by washings first with 2 l of 0.1 M acetate buffer, pH 5.0 at 4°, then with 1 l of H_2O. A red-brown color was obtained with the trinitrobenzene sulfonate test, indicating a sulfhydryl derivative. Estimation of the ligand concentration was made at this stage, utilizing 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB, according to Ellman [12]. Using 10^{-4} M DTNB in 0.05 M Tris-HCl, pH 8.0, and β-mercaptoethanol (Eastman)

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as standard, the beads were found to contain 0.8 ± 0.1 mM sulfhydryl or 0.8 microequivalents per ml.

Compound CT 5263 (formula given in fig. 2) was synthetized according to P. Roger [13] †. The sulfhydryl Sepharose was reacted for 90 min at 25° in 100 ml of 0.5 M $NaHCO_3$ buffer, pH 8.0, with 200 mg of CT 5263 (250 μmoles or 2.5 μmoles per ml beads) after which no free sulfhydryl could be detected by the Ellman test and a yellow color was obtained with trinitrobenzene sulfonate. The product was washed with 1 l of 1 M $MgCl_2$ and 2 l of H_2O.

2.2. *Assay of the cholinergic receptor site with* [^{3}H] *α-toxin from Naja nigricollis*

The α_1-isotoxin purified from the venom of *Naja nigricollis* by the method of Boquet et al. [14] and Karlsson et al. [15] was tritiated according to Ménez et al. [10]. The stock solution contained 0.395 mg of toxin per ml with a specific radioactivity of 10.5 Ci per mmole [10]. Samples were added to eel Ringer's solution in a final volume of 0.2 ml containing 3.95 ng (0.57 pmole) of [^{3}H] α-toxin (final conc. 2.85×10^{-9} M). The mixture was then incubated 1 hr at room temp. When the total amount of protein added was lower than 2 μg then 5 μg of extraneous "helper" protein were added. The most commonly used helper was the proteins which did not adsorb on the affinity column (front peak in fig. 2). To the assay tube were then added 10 ml of 0.2 M NaCl (*without detergent*). After 10 min incubation the contents were filtered through Millipore filters (HAWP 02500) and the filters washed with 10 ml of 0.2 M NaCl. Under such conditions of low detergent concentrations (lower than 0.01% for Triton X-100 or 0.1% for NaCholate) the receptor protein and its toxin complex aggregate and are quantitatively retained on the filter while the free toxin passes through. The filters were then dried and counted in 10 ml of toluene POPOP solution in the Intertechnique Model SL 40 scintillation counter with an efficiency of ^{3}H-channel of 30%. Under these conditions the amount of [^{3}H] α-toxin bound varies linearly with the concentration of receptor protein until approx. 80% of the [^{3}H] α-toxin added to the tube is bound (fig. 1). Protein was determined with the Folin reagent [16] with bovine serum albumin as standard. In general to facilitate the estimate of the optical density, the sample was briefly centrifuged before measurement of the absorbance.

2.3. *Detergent extraction of the cholinergic receptor protein from Electrophorus electricus electric organ*

The electric organ (3–500 g) from freshly killed eels was homogenized in two volumes of cold (4°) distilled water with a Virtis model 45 for 60 sec at setting "High". Frothing was decreased by sonication (10 × 30 sec) with a Branson "Sonifier" at setting 6. Crude membranes were collected by centrifugation for 30 min at 8000 rpm in a Sorvall rotor GSA (pellet 1). The pellet was resuspended in two volumes of 0.5 M NaCl in 0.1 M Tris-HCl, pH 8, and recentrifuged as before. Pellet 2 was resuspended (30 sec, Virtis, speed "medium") in 2 vol of buffer which had a final concentration, including the volume of pellet, of 0.1 M NaCl and 0.1 M Tris-HCl, pH 8.0. To this was added enough 20% Triton X-100 (Calbiochem) to give 1% overall. The mixture was stirred for 60 min at 22°, after which it was centrifuged for 60 min at 30,000 rpm in a Spinco rotor 30 (ca. 100,000 *g*). The supernatant was filtered through cheesecloth and then used as the detergent extract.

2.4. *Affinity chromatography on the CT 5263 column*

The beads coupled to CT 5263 at a concentration of 1 μmole per ml of decanted suspension were packed in a column. In the experiment represented in fig. 2, 25 ml of the beads were used. After equilibration with the extraction buffer (0.1 M NaCl, 0.1 M Tris pH 8.0, 1% Triton X-100) 20 ml of centrifuged extract were applied at a flow rate of 30 ml per hr and fractions of 20 ml were collected. The column was then washed with 200 ml of buffer and the receptor protein eluted with a gradient of 0 to 10^{-3} M flaxedil (gallamine triethiodide, Specia). Finally the column was washed with 2 M NaCl to desorb acetylcholinesterase and other proteins. Aliquots of the fractions were extensively dialysed against the equilibration buffer and assayed for toxin binding. The column has been scaled up to a 100 ml bead volume and approx. 500 ml sample.

† A copy of the method is available upon request.

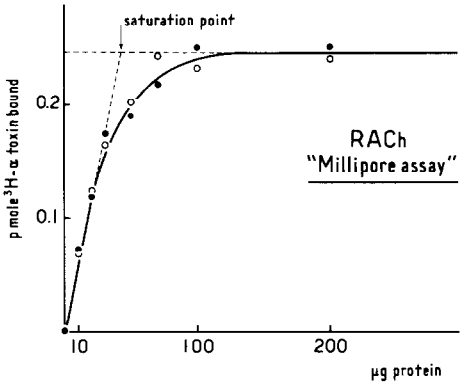

Fig. 1. Assay of the free cholinergic receptor site from a detergent extract following the method given in sect. 2.2.

2.5. *Concentration on DEAE cellulose columns*

The fractions containing the AcChR were dialysed against 0.01 M Tris pH 8.0, 0.01 M NaCl and 1% Triton X-100 for 15 hr and applied on 1–5 ml column of DEAE cellulose (Whatman DE.52) equilibrated with the dialysis buffer. The receptor protein was eluted by 0.1 M Tris pH 8.0, 1.0 M NaCl, 1% Triton X-100 and recovered in approx. 1.0 ml.

3. Results

The following scheme gives the results of the solubilisation of the receptor protein according to the procedure given in sect. 2.3. The results are expressed for 1 kg wet wt. electric tissue although extraction was generally carried out on electric organs of 3–500 g from one eel. The quantities of acetylcholinesterase *molecules* (AcChE) are given in moles assuming

1000 g Fresh electric organ
38 ± 4 g Protein
110 ± 20 nmoles RAcCh
310 ± 40 nmoles AcChE

homogenize | centrifuge

Pellet 1: 350 ± 35 g
26 ± 3 g Protein
108 ± 3 nmoles RAcCh
200 ± 20 nmoles AcChE

Supernatant 1
12 g Protein
2 nmoles RAcCh
110 nmoles AcChE

Salt wash | centrifuge

Pellet 2: 270 ± 25 g
24 ± 3 g Protein
107 ± 20 nmoles RAcCh
40 ± 5 nmoles AcChE

Supernatant 2
2.1 g Protein
1 nmole RAcCh
160 nmoles AcChE

Triton X-100 | solubilization

Supernatant 3: Extract
3 ± 0.5 g Protein
100 ± 20 nmoles RAcCh
40 ± 5 nmoles AcChE

Pellet 3: 200 ± 20 g
21 g Protein

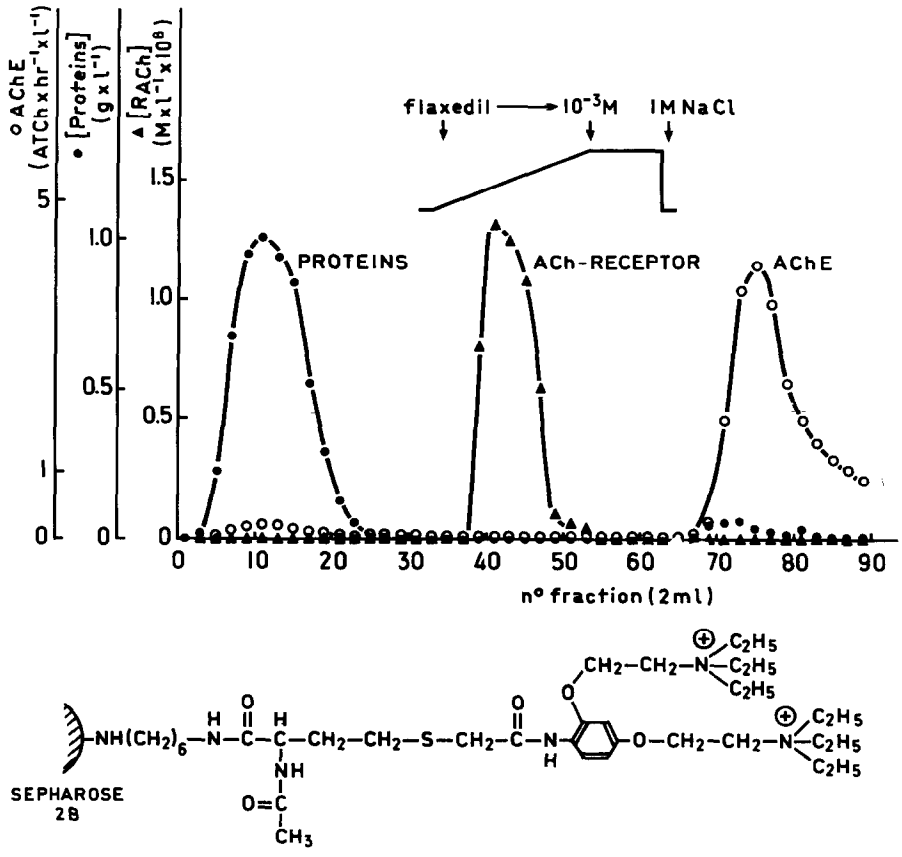

Fig. 2. Chromatography on a CT 5263 column of a Triton X-100 extract of membrane fragments from *E. electricus* electric organ.

for the time being a molecular weight of 260,000 and a specific activity of the pure protein of 750 moles of acetylthiocholine per hr per g protein at 27° in the previously given conditions of assay [3]. The quantities of cholinergic receptor *site* (RAcCh) are expressed in nmoles of [^{3}H]α-toxin molecules bound per g of protein using the millipore assay.

Fig. 2 shows the elution profile on a CT 5263 column of a crude Triton X-100 extract of electric organ membrane fragments. Approx. 90% of the proteins present in the extract did not adsorb while almost all AcChE and AcChR remained bound to the column. A single peak of AcChR was eluted by a gradient of 0 to 10^{-3} M flaxedil. 1 M NaCl released 80% of AcChE applied and proteins which did not desorb in the presence of 10^{-3} flaxedil. The fractions containing the AcChR were pooled and concentrated to 1 ml by a 1 ml DEAE column. The results of the purification are given table 1. The yield of the column was close to 50% in cholinergic receptor sites and the purification factor more than 150-fold in a single step. The final material contained less than one catalytically active site of AcChE (assuming 65,000 g/site) per 100 cholinergic receptor site. Its specific activity was 2,000 ± 500 nmoles of cholinergic receptor site per g protein using our Millipore assay, a value close to that reported independently by Reich and co-workers [9] using a different assay. However experiments in progress indicate that our Millipore assay gives a significant underestimation of the total number of AcChR sites present: adding more helper protein to the reaction medium caused an 80% increase in the specific activity measured. Nevertheless the purified material still gives several bands by gel electrophoresis in the

Table 1
Result of the fractionation on the CT 5263 column shown on fig. 2

	[^{3}H] α-toxin binding sites (nmole)	Proteins (mg)	Specific activity (nmole/g protein)	AcChE molecules (MW 260,000) (nmole)	AcChR/ AcChE
Fresh electric tissue (15 g)	0.80	550	1.5 ± 0.4	2.0 ± 0.25	0.4
Triton X-100 extract (10 ml)	0.75	50	15 ± 2	0.5 ± 0.05	1.6
Affinity column (CT 5263)	0.37	0.185 ± 0.04	2000 ± 500	0.0007	480

presence of sodium dodecyl sulphate.

The purification step by affinity chromatography has been scaled up starting from a crude extract containing 30 nmoles [^{3}H] α-toxin binding sites and 1.5 g protein, a 70 ml affinity column yielded 3–4 mg protein with a specific activity of 3300 nmoles [^{3}H] α-toxin binding sites per g protein and a recovery of 30–50%.

The purified receptor protein still binds cholinergic agonists and antagonists as well as the [^{3}H] α-toxin. As expected from our previous work [3–5], [^{14}C]-decamethonium bound to this protein is *completely* displaced by *N. nigricollis* α-toxin.

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References

[1] C.Y. Lee and C.C. Chang, Mem. Inst. Butantan Simp. Internac. 33 (1966) 555.
[2] C.Y. Lee, Clinical Toxicology 3 (1970) 457.
[3] J.P. Changeux, M. Kasai and C.Y. Lee, Proc. Natl. Acad. Sci. U.S. 67 (1970) 1241.
[4] J.P. Changeux, M. Kasai, M. Huchet and J.C. Meunier, Compt. Rend. 270 (1970) 2864.
[5] J.P. Changeux, J.C. Meunier and M. Huchet, Mol. Pharmacol. 7 (1971) 538.
[6] R. Miledi, P. Molinoff and L. Potter, Nature 229 (1971) 554.
[7] M. Eldefrawi and A. Eldefrawi, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1776.
[8] B. Fulpius, S. Cha, R. Klett and E. Reich, FEBS Letters 24 (1972) 323.
[9] Reports by J.P. Changeux, E. Heilbronn, P. Molinoff, R. O'Brien, M. Raftery and E. Reich at the Work Session on Receptor Biochemistry and Biophysics, in Neuroscience Research Program, March (1972), Boston, Mass., at the Symposium on Drug Receptors, April (1972), London, at the 5th International Congress on Pharmacology, San Francisco, July 1972 and the 8th FEBS Meeting, Amsterdam, August (1972), etc.
[10] A. Ménez, J.L. Morgat, P. Fromageot, A.M. Ronseray, P. Boquet and J.P. Changeux, FEBS Letters 17 (1971) 333.
[11] P. Cuatrecasas, J. Biol. Chem. 245 (1970) 3059.
[12] G.L. Ellman, Arch. Biochim. Biophys. 82 (1959) 70.
[13] P. Roger "Inhibiteurs irréversibles de l'acétylcholinesterase et marqueurs sélectifs du récepteur cholinergique"; Mémoire présenté au Conservatoire National des Arts et Métiers pour le Diplôme d'Ingénieur CNAM, Paris 1970.
[14] P. Boquet, Y. Izard, M. Jouannet and J. Meaume, Compt. Rend. 262 (1966) 1134, D.
[15] E. Karlsson, D. Eaker and J. Porath, Biochim. Biophys. Acta 127 (1966) 505.
[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.