USE OF AFFINITY CHROMATOGRAPHY FOR ACETYLCHOLINE RECEPTOR PURIFICATION*

Jakob Schmidt and Michael A. Raftery**

Division of Chemistry and Chemical Engineering†
California Institute of Technology
Pasadena, California 91109

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Summary

An acetylcholine receptor (AChR) affinity resin was prepared by covalently linking [N-(ε-aminohexanoyl)-3-aminopropyl]trimethyl ammonium bromide hydrobromide to agarose. A partially purified membrane protein preparation from the electroplax of Narcine was resolved on this resin into a peak of inactive protein and a peak of protein displaying α-bungarotoxin binding activity. Analysis of the purified toxin binding material by SDS polyacrylamide electrophoresis shows a major band of apparent molecular weight 28,000 and two minor bands of approximately 45,000 and 38,000.

The application of affinity chromatography for the isolation of neurotransmitter receptors is a very recent development. Raftery has described the use of agarose-linked cobrotoxin in studies on AChR from Torpedo californica (1). This procedure gives rise to purified toxin receptor complex rather than free receptor. Since isolation of free receptor appears preferable for the further characterization of structure and function, we have begun to study the use of resins which contain covalently bound quaternary ammonium functions. Such resins have been employed successfully during the past few years for the purification of acetylcholinesterase from the electric organ of Electrophorus electricus (2-5) and from bovine erythrocytes (4).

In the present communication we wish to describe the synthesis of an AChR affinity resin and its successful application for the purification

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of the AChR from the electroplax of a narcine from the Gulf of California.

Experimental

Synthesis of affinity resin: The cholinergic ligand, [N-(ε-amino-hexanoyl)-3-aminopropyl] trimethyl ammonium bromide hydrobromide, was prepared by coupling the N-benzyloxycarbonyl derivative of 6-amino-hexanoic acid (Eastman) with N,N-dimethyl-1,3-propane diamine (Eastman) and methylating the product with methyliodide, followed by deprotection. The affinity resin was then synthesized according to Cuatrecasas' procedure for the coupling of amines to agarose (6): Sepharose 2B was activated with 25 mg CNBr per ml of settled resin; the washed resin was allowed to react with the ligand (0.15 mg per ml of resin). After three days of stirring at 4°, the resin was washed and stored in 0.02% sodium azide or used immediately. Ligand concentration was determined by the amount of 6-aminohexanoic acid released upon acid hydrolysis; it was found to be 0.4 μmoles per ml.

Electroplax preparation: Narcine was caught off Puerto Peñasco, Sonora, Mexico, and shipped frozen to California. After slow thawing in the cold room the electric organs were removed, and a crude membrane fraction was obtained following the procedure of Miledi et al. (7). Membranes were extracted by stirring with 1% Triton X-100 (v/v) in 10 mM sodium phosphate (pH 7.4) at 4° overnight (approximately 3 gm original tissue per ml extract), followed by centrifugation at 140,000 x g for 2 hrs.

Assay procedures: Receptor activity was measured by a toxin-binding assay utilizing DEAE-cellulose filter disks (8). Specific radioactivity of the ^{125}I-α-bungarotoxin was about 10^6 cpm/μg, or less, depending on the age of the preparation. One unit of receptor activity is defined as that amount of receptor which binds 1 μg of bungarotoxin. Protein was determined by the method of Lowry (9) or by amino acid analysis.

Polyacrylamide gel electrophoresis: SDS gel electrophoresis of receptor preparations was performed according to Fairbanks (10).

Densitometric traces were obtained using a Gilford spectrophotometer with a Model 2410 linear transport attachment.

Results

Narcine electroplax (110 gm) was processed and extracted as described above. The extract was subjected to gel filtration as seen in Fig. 1. Fractions 25-35 of the Sepharose 6B eluate were pooled and

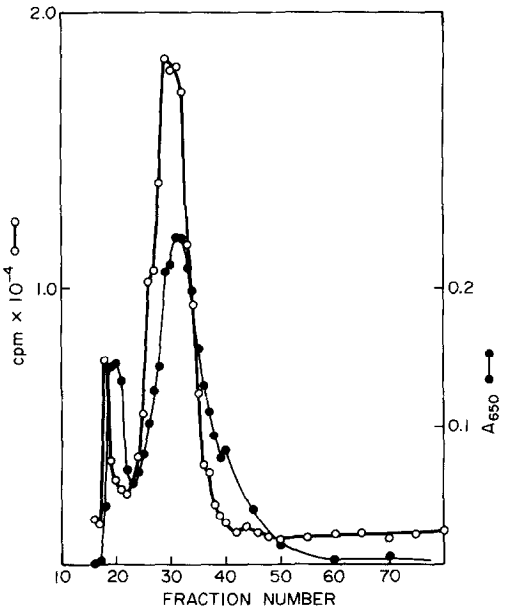

Figure 1: Gel filtration of Membrane Extract

The Triton X-100 extract (ca. 35 ml) was applied to a column of Sepharose 6B (5 x 80 cm) equilibrated in the eluent (10 mM sodium phosphate pH 7.4, 0.1% Triton X-100). Fractions of ca. 25 ml were collected. Aliquots of 0.05 ml were assayed for protein; toxin binding activity was determined by incubating 0.010 ml of eluate with ^{125}I-α-bungarotoxin in a total volume of 0.135 ml and pipetting 0.1 ml onto DEAE-cellulose disks (see Methods).

immediately applied to an affinity column (see Fig. 2). The second half (fractions 110-130) of the activity peak was pooled and designated "purified receptor". Protein and activity data are summarized in the

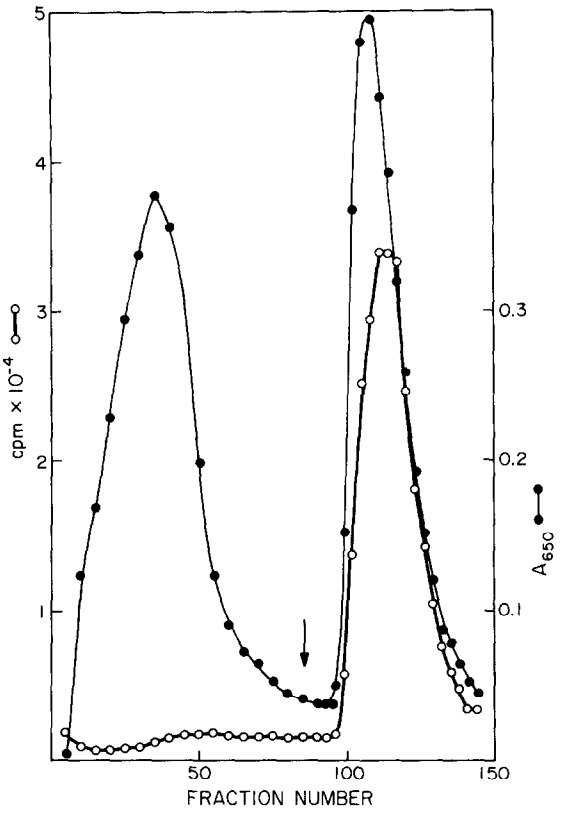

Figure 2: Affinity Chromatography of Partially Purified AChR

The active fractions in the Sepharose 6B eluate (ca. 280 ml) were pooled, and applied to a column of the affinity resin (2.5 x 17 cm), equilibrated in starting buffer (10 mM sodium phosphate; 0.1% Emulphogene). The column was washed with about 9 volumes of the same buffer at which time a linear sodium chloride gradient was commenced (250 ml of starting buffer and 250 ml of same containing 0.1 M NaCl; start of gradient is marked by an arrow). Aliquots of 0.25 ml were assayed for protein; aliquots of 0.025 ml were assayed for activity as described in legend to Figure 2.

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purification table (Table I). Results of a gel-electrophoretic analysis of "purified receptor" are shown in Fig. 3. A major component is seen with an apparent molecular weight of 28,000. In addition minor bands are discernible, peaking at 45,000 and 38,000, respectively.

Table I: Purification of Acetylcholine Receptor

Fraction	Protein (mg)	Activity (units)	Spec. Activity (units/mg)	Purification -fold	Recovery
I Membrane Suspension	512	800	1.55	1.0	100%
II Detergent Extract	190	735	3.87	2.5	92%
III Sepharose 6B Pool	95	475	5.00	3.2	59%
IV "Purified Receptor"	13*	280	21.5	13.9	35%

* determined by amino acid analysis.

Discussion

The affinity resin described above resembles resins that have been used successfully for the isolation of acetylcholinesterase (2, 3, 5). It is plausible that all macromolecules possessing a choline recognition site should be retained by such resins. The long delay in their utilization for receptor work must be attributed to certain technical difficulties which have been eliminated with the development of suitable AChR solubilization and assay procedures.

Elution of the bound receptor at fairly low salt concentrations (20-50 mM) may be due to a reduction of ligand affinity with increasing ionic strength. Such effects of ionic strength on inhibitor affinity are well documented for acetylcholinesterase (11) and explain the chromatographic behavior of the enzyme (2, 3). This behavior is undesirable as it results in contamination

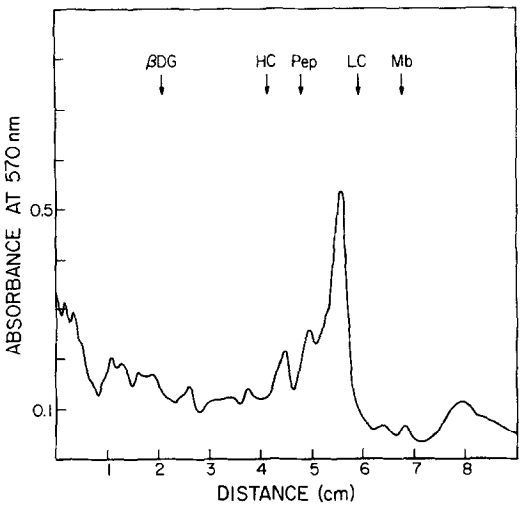

Figure 3: Gel Scan Analysis of Purified AChR

Purified receptor, representing ca. 0.2 gm of original electric tissue was electrophoresed and scanned as described in Methods. Arrows indicate positions of protein markers used for calibration. (Abbreviations: βDG, β-D-galactosidase from E coli; HC, LC, Rabbit igG heavy and light chains; Pep, pepsin; Mb, myoglobin).

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of the purified material with non-specific proteins that, during chromatography, also respond to increases in salt concentration. It is hoped that elution with specific ligands will solve this problem.

There is no doubt that the "purified receptor" still contains non-receptor proteins. Its heterogeneity, as evidenced by SDS gel electrophoresis, may however be caused not only by contamination with inert protein, but also by a multiplicity of toxin-binding sites in Narcine electric tissue, or a complex subunit structure of a single AChR in Narcine. Clarification of this point may have to await further purification, i.e., the obtainment of a product of higher specific activity and/or resolution of the toxin-binding material into several active components.

However the question of the subunit molecular weight of the narcine AChR will be settled, it appears unlikely that the major component found by SDS gel electrophoresis is a chance contaminant. Taking into account recent

studies on the AChR from Torpedo californica (1,12) and assuming a certain homology among torpedinid fish, this indicates that the subunit molecular weight proposed for Torpedo marmorata AChR by Miledi et al. (7) may be off by a factor of two.

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