Biochimca et Biophysica Acta, 465 (1977) 331—340

BBA 77615

WATER-SOLUBLE ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

SOLUBILIZATION, PURIFICATION AND CHARACTERIZATION

NURIT KALDERON * and ISRAEL SILMAN **

Department of Neurobiology, Weizmann Institute of Science, Rehovot (Israel)

(Received April 13th, 1976)
(Revised manuscript received September 28th, 1976)

Summary

The nicotinic acetylcholine receptor from electrogenic tissue of *Torpedo californica* was solubilized by tryptic digestion of membrane fragments obtained from autolysed tissue, without use of detergent. The water-soluble acetylcholine receptor was purified by affinity chromatography on a cobra-toxin-Sepharose resin. The purified receptor bound 4000—6000 pmol per mg protein of α-[^{125}I]bungarotoxin, and toxin-binding was specifically inhibited by cholinergic ligands. Gel filtration revealed a single molecular species of Stokes radius 125 ± 10 Å and on sucrose gradient centrifugation one major peak was observed of 20-22 S. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol revealed two major polypeptides of mol. wt. 30 000 and 48 000.

Solubilization of membrane proteins with retention of biological activity is one of the major obstacles to be overcome in isolation and purification of these macromolecules. This is especially true of the class of integral membrane proteins which require drastic treatments such as the use of reagents like detergents, bile acids, protein denaturants or organic solvents, to dissociate them from membranes [1]. Autolysis or proteolysis have also been adopted for solubilizing membrane proteins (see for example, refs. 2—6). The acetylcholine receptor from electric organ tissue, which is generally considered to be an integral membrane protein, has indeed been solubilized in active form only by use of the bile salt deoxycholate [7], or various nonionic detergents [8—10]. Such detergent-solubilized receptor fractions were subsequently utilized to obtain

* Present address: The Rockefeller University, New York, N.Y. 10021 (U.S.A.)
** To whom correspondence should be addressed.

purified acetylcholine receptor (see for example, refs. 11—15). In the following we describe, for the first time, a procedure which avoids the use of detergents, for solubilization in active form, and for subsequent purification and characterization, of acetylcholine receptor from electrogenic tissue of *Torpedo californica*. The procedure utilizes autolysis of the tissue followed by controlled tryptic digestion, to yield a water-soluble species which is then purified by affinity chromatography on a Sepharose-neurotoxin resin.

Experimental

Frozen electric organ tissue from *T. californica* was obtained from Pacific Bio-Marine (Venice, Calif.).

α-Bungarotoxin and cobra neurotoxin were purified from *Bungarus multicinctus* venom and *Naja naja siamensis* venom (Miami Serpentarium) according to Clark et al. [16] and Karlsson et al. [17], respectively. α-[^{125}I]bungarotoxin was prepared as described previously [18]. The cobra-neurotoxin-Sepharose conjugate was prepared according to Klett et al. [12]. 1 mg of cobra-neurotoxin was bound per gram of drained Sepharose 2B.

Trypsin (TRL, 197 units/mg) and soybean trypsin inhibitor (STI) were obtained from Worthington Biochemical Corp.

Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and sucrose gradient centrifugation on 5—20% sucrose gradients were performed as described previously [5]. Protein was determined according to Lowry et al. [19] and amino acid analysis was performed by the method of Spackman et al. [20] on samples which had been hydrolysed in vacuo for 24 h at 110°C in 6 M HCl.

Acetylcholine receptor was assayed by measuring the amount of α-[^{125}I]-bungarotoxin which coprecipitated with the receptor in 33% saturated $(NH_4)_2SO_4$[7]. Stock solutions of acetylcholine receptor and α-[^{125}I]bungarotoxin were diluted in 10 mM sodium phosphate buffer, pH 7.5, containing bovine serum albumin (1 mg per ml). The total assay volume was 0.3 ml and incubation was for 30 min at 25°C. Goat serum (0.1 ml) was then added to provide carrier protein, followed by 0.2 ml of saturated ammonium sulfate and then by 1 ml of 33% saturated ammonium sulfate. Centrifugation was performed at 4°C for 30 min at 1000 × *g*. Inhibition constants for cholinergic ligands were determined by measuring their effect on the initial rate of formation of the α-[^{125}I]bungarotoxin · acetylcholine receptor complex, as described by Schmidt and Raftery [21].

Gel filtration was performed on Sepharose 4B columns (1.3 × 90 cm), at a flow rate of 5 ml/h, at room temperature. The buffer used was 0.5 mM sodium azide/0.05 M Tris, pH 7.5, containing either 0.1 M NaCl or 1.0 M NaCl. Samples of 2 ml containing about 10 mg total protein were applied and 2 ml fractions collected. The following markers were taken for calibration: bovine serum albumin, catalase, β-galactosidase, rabbit muscle myosin, dextran blue and potassium ferricyanide, employing the values for Stokes radii used by Bon et al. [22]. The Stokes radius (R_e) was plotted against $\{\log (1/K_{av})\}^{1/2}$ where K_{av} is the partition coefficient defined by Laurent and Killander [23].

Receptor extraction. Electric tissue from *T. californica* (150 g) was thawed

and kept at 4°C for 10 days. The tissue was homogenized in 300 ml of 0.1 M NaCl/0.05 M Tris, pH 7.5, in a Sorvall Omnimixer for 2 min at maximal speed. The homogenate (H) was filtered through gauze and centrifuged at 26 000 × *g* for 60 min at 4°C. The pellet (PI) was made up to 200 ml with 0.1 M NaCl/0.05 M Tris, pH 7.5, dispersed with a Teflon homogenizer and recentrifuged as above. The second pellet (PII) was made up to 300 ml with 1 mM sodium EDTA, pH 8.0, and dispersed as above. PII was dialyzed against 1 mM sodium EDTA, pH 8.0, at 4°C for 48 h. The dialysate (DPII) was diluted to a total volume of 1000 ml with 1 mM sodium EDTA, pH 8.0 (final protein concentration ≈1 mg/ml). To this solution was added 3 mg trypsin (i.e., 3 μg/ml). After incubation for 60 h at room temperature 3 mg of soybean trypsin inhibitor were added. The tryptic digest (TDPII) was centrifuged at 35 000 × *g* for 1 h at 4°C; the supernatant (STDPII) was collected and will also be referred to as the water-soluble acetylcholine receptor fraction. All buffers employed in the above extraction procedure and in the following purification contained 0.5 mM NaN_3.

Affinity chromatography of STDPII. 500 ml of STDPII were passed over a 2 × 4 cm column of the cobra-neurotoxin-Sepharose resin, at room temperature, at a rate of 50 ml/h. The resin was then removed from the column and subsequent operations were performed batchwise. The resin was washed by gentle stirring for 1 h at 4°C with 200 ml volumes of 1 M NaCl/0.05 M Tris, pH 7.5 (once) and 0.1 M NaCl/0.05 M Tris, pH 7.5 (five times). Elution of acetylcholine receptor was accomplished by incubating the resin with 10 ml of 0.5 M hexamethonium/0.1 mM dithiothreitol/0.05 M Tris, pH 7.5, with gentle stirring, for 20 h at 4°C. To collect acetylcholine receptor in the eluate, the mixture was filtered on a sinter-glass, and the filtrate dialysed against 5 l of 1 mM sodium EDTA, pH 8.0, with 4 changes of buffer, for 3 days at 4°C.

Results

The procedure for obtaining the water-soluble form of acetylcholine receptor is described under Experimental; yields of toxin-binding capacity and specific activity are summarized in Table I. Starting from a homogenate (H) of specific toxin-binding activity 54 pmol/mg protein, a water-soluble fraction,

TABLE I

TOXIN-BINDING CAPACITY OF ACETYLCHOLINE RECEPTOR FRACTIONS OBTAINED IN THE SOLUBILIZATION AND PURIFICATION PROCEDURE

Fraction	Protein * (mg)	Binding capacity * (pmol)	Specific activity (pmol/mg protein)	Recovery (%)
H	15.6	840	54	100
PI	9.4	1220	130	145
PII	5.3	1200	226	143
DPII	5.3	1900	358	226
TDPII	5.3	1600	302	190
STDPII	4.0	580	145	69
Purified acetylcholine receptor	0.037	200	5486	24

* Per g tissue.

STDPII, is obtained of specific activity 145 pmol/mg protein. The yield of toxin-binding sites in STDPII is 69% of the total binding sites in H, and 31% of the number in TDPII. The intermediate fractions, DPII and TDPII, display more toxin-binding sites than the original homogenate H, apparently because "latent" binding sites are exposed during the solubilization process (see Discussion). Essentially all acetylcholinesterase activity disappeared during the prolonged dialysis at low ionic strength.

The water-soluble acetylcholine receptor in STDPII could be purified by affinity chromatography on a cobra-neurotoxin-Sepharose conjugate resin, just as had been previously reported for detergent-solubilized acetylcholine receptor preparations [11,12,24]. It was found that 8 g of the cobra-neurotoxin-Sepharose resin employed could adsorb 20 000 pmol of acetylcholine receptor toxin-binding sites. 30—40 percent of the bound acetylcholine receptor could be subsequently eluted by hexamethonium in the presence of dithiothreitol and displayed a specific activity of 4000—6000 pmol toxin-binding sites per mg protein. The receptor thus obtained was, therefore, purified about 100-fold as compared to the original homogenate H, and about 40-fold as compared to the soluble extract STDPII which was the material applied to the column. The overall yield, starting from H, was 20—30 percent.

Acrylamide gel electrophoresis of the purified acetylcholine receptor in the presence of sodium dodecyl sulfate and β-mercaptoethanol revealed two major polypeptides of mol. wt. 30 000 and 48 000 and minor components of about 67 000 and 120 000 mol. wt. (Fig. 1).

The amino acid composition of the purified acetylcholine receptor is sum-

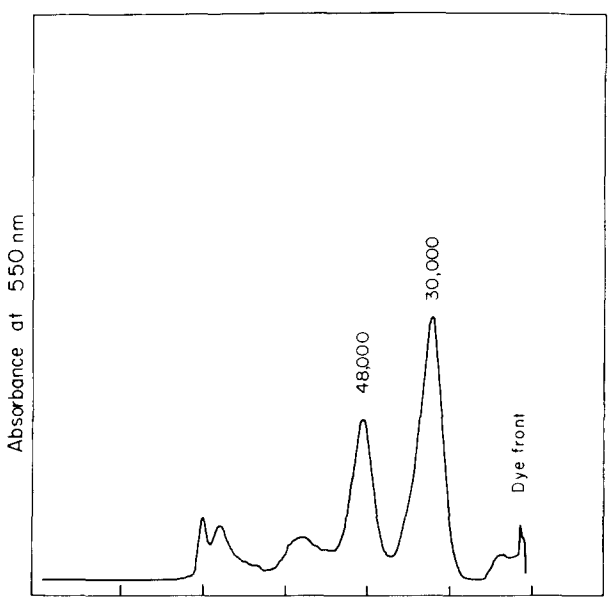

Fig. 1. Gel scan analysis of purified acetylcholine receptor, on 7.5% sodium dodecyl sulfate-acrylamide gels in the presence of β-mercaptoethanol. Samples of 100 μg protein were applied and stained with Coomassie blue. The molecular weights of the major polypeptide bands are indicated.

TABLE II

AMINO ACID COMPOSITION OF PURIFIED ACETYLCHOLINE RECEPTOR FROM *T. CALIFORNICA*

Values are expressed as mol per 100 mol of total amino acid recovered.

Amino acid	Acetylcholine receptor		
	Water-soluble [a]	Triton-soluble [b]	Triton-soluble [c]
Lys	5.2	5.9	6.1
His	2.6	2.7	2.7
Arg	4.2	4.1	4.1
Asp	10.3	11.3	11.9
Thr	5.7	5.9	6.3
Ser	7.6	7.8	6.6
Glu	11.7	11.4	10.2
Pro	5.8	5.9	5.9
Gly	7.5	5.4	4.9
Ala	6.2	5.5	5.1
1/2 Cys	1.9	0.8	0.9
Val	6.3	6.3	7.0
Met	1.9	1.6	1.8
Ile	5.3	6.3	7.5
Leu	10.5	9.8	9.7
Tyr	3.5	3.6	3.8
Phe	4.4	4.5	4.6

[a] Average values for three different preparations.
[b] Aharonov et al. [42].
[c] Data of Michaelson et al. [32] for Triton-solubilized acetylcholine receptor.

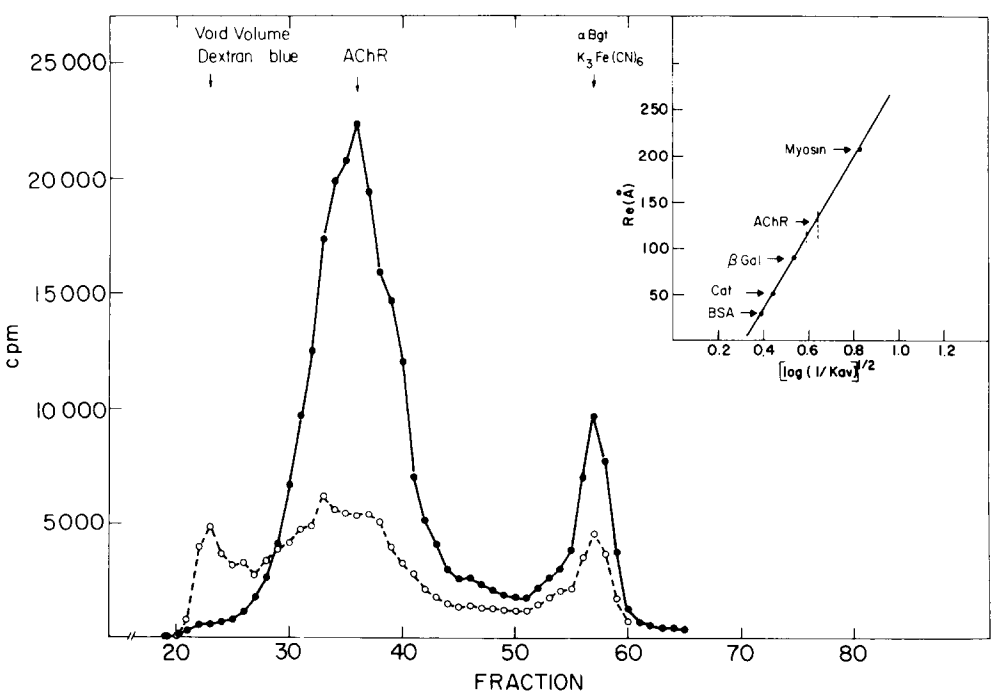

Fig. 2. Gel filtration of the α-[^{125}I]bungarotoxin · acetylcholine receptor complex on Sepharose 4B, in 0.1 M NaCl/0.05 M Tris, pH 7.5. ●———●, elution pattern of purified acetylcholine receptor; ○———○, elution pattern of STDPII.

TABLE III

I_{50} VALUES FOR PROTECTION OF THE WATER-SOLUBLE ACETYLCHOLINE RECEPTOR BY CHOLINERGIC LIGANDS AGAINST α-[^{125}I]BUNGAROTOXIN BINDING

Ligand	I_{50} (M)
D-Tubocurarine	$5.6 \cdot 10^{-7}$
Decamethonium	$6.8 \cdot 10^{-6}$
Acetylcholine	$7.7 \cdot 10^{-6}$
Hexamethonium	$3.3 \cdot 10^{-5}$
Nicotine	$7.6 \cdot 10^{-5}$
Carbamylcholine	$1.6 \cdot 10^{-4}$

marized in Table II and resembles those found for preparations of purified Triton-solubilized acetylcholine receptor from *T. californica* which are shown for comparison.

Table III shows the I_{50} values for inhibition of α-[^{125}I]bungarotoxin binding by several cholinergic agonists and antagonists. It can be seen that these ligands effectively protect the acetylcholine receptor against α-[^{125}I]bungarotoxin binding.

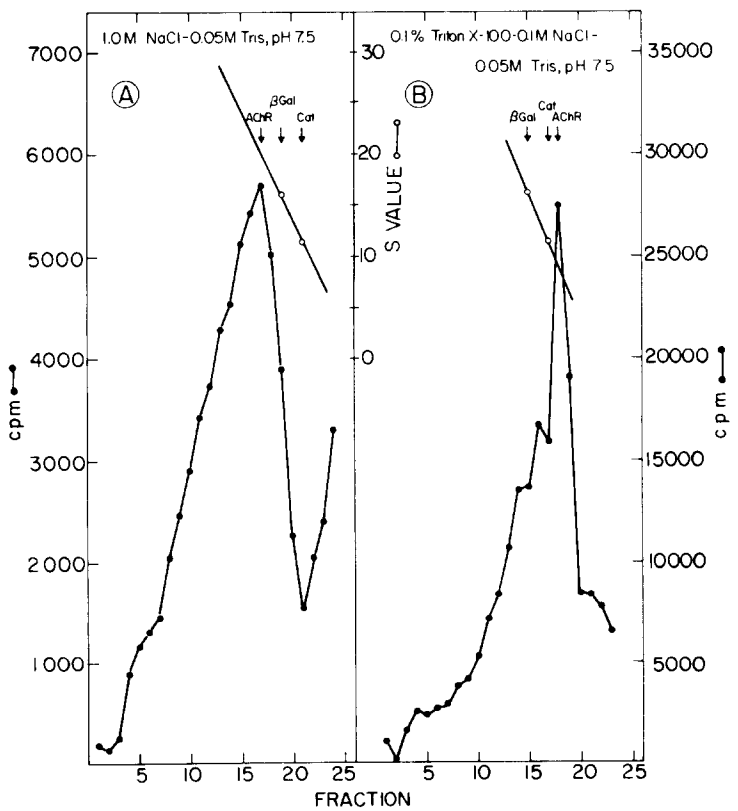

Fig. 3. Sucrose gradient centrifugation of the α-[^{125}I]bungarotoxin · acetylcholine receptor complex. The markers were catalase, taken as 11.4 S, and β-galactosidase, taken as 16.1 S. (A) Centrifugation in 1.0 M NaCl/0.05 M Tris, pH 7.5. (B) Centrifugation in 0.1% Triton X-100/0.1 M NaCl/0.05 M Tris, pH 7.5.

The molecular parameters of the water-soluble acetylcholine receptor were studied by sucrose gradient centrifugation and by gel filtration on Sepharose 4B, using preparations labelled with α-[^{125}I]bungarotoxin. Such experiments were carried out both on the crude extract, STDPII, and on the purified acetylcholine receptor eluted from the affinity column. Fig. 2 shows the gel filtration profiles obtained for STDPII and for purified acetylcholine receptor in 0.1 M NaCl/0.05 M Tris, pH 7.5. The purified acetylcholine receptor yielded a major toxin-binding peak of Stokes radius 125 ± 10 Å. Much of the acetylcholine receptor in STDPII had similar dimensions, but some larger material was totally excluded from the gel. Gel filtration in 1.0 M NaCl/0.05 M Tris, pH 7.5, yielded a similar Stokes radius for purified acetylcholine receptor. Sucrose gradient centrifugation of purified acetylcholine receptor in 1.0 M NaCl/0.05 M Tris, pH 7.5, revealed a major toxin-binding component of sedimentation coefficient 20—22 S (Fig. 3A), which partially aggregated at low ionic strength. Even at high ionic strength some aggregates were present, since the peak was skewed towards higher sedimentation values. In the presence of 0.1% Triton X-100, most of the material is converted to a 9 S species (Fig. 3B).

Discussion

The acetylcholine receptor is an integral membrane protein which has so far been solubilized and purified only by use of detergents. The data presented above demonstrate for the first time that it is possible to solubilize and purify acetylcholine receptor without use of detergents in a form which retains the binding characteristics of the native receptor. The possibility must be considered that the autolysis step leads to the release of lysolipids, which can act as detergents capable of solubilizing the acetylcholine receptor, as a result of the action of phospholipase A which is present in most animal cells [25]. However, neither the autolysis step itself, nor the subsequent dialysis, cause significant solubilization, so that the tryptic treatment seems to be an essential part of the process. Moreover, the acetylcholine receptor, when solubilized, does not resemble detergent-solubilized acetylcholine receptor in its physicochemical characteristics (see below).

As mentioned above, autolysis and/or proteolytic digestion have been utilized for solubilization in active form of various membrane proteins [2—6]. In the case of microsomal cytochrome b_5, proteolytic treatment actually cleaved this integral membrane protein into a water-soluble domain containing the heme group and a hydrophobic domain involved in attachment to the membrane, whereas detergent solubilized the two segments as a single polypeptide chain [2,26]. In the case of acetylcholine receptor, our present findings suggest a more subtle modification, since the amino acid composition is similar both to that of purified Triton-solubilized acetylcholine receptor from *T. californica* (see Table II) and of acetylcholine receptor purified similarly from *Torpedo marmorata* and *Electrophorus electricus* [12,24]. Moreover, it is converted, in the presence of Triton X-100, to a form with a sedimentation coefficient similar to that of detergent-solubilized acetylcholine receptor (see below). Thus proteolysis and autolysis might be destroying components of the membrane to which the acetylcholine receptor is attached. It is, however, also possible that

proteolytic cleavages occur in the acetylcholine receptor molecule itself, which modify its attachment to the membrane without concomitant disruption of its quaternary structure or release of peptide fragments. This latter possibility is supported by the observation of a major 30 000 species on sodium dodecyl sulfate-acrylamide gels (see below) and by the fact that subsequent treatment with Triton X-100 strongly diminishes toxin-binding capacity, although toxin previously bound does not seem to be released by the detergent.

The procedure for solubilization of *Torpedo* acetylcholine receptor was based on a procedure previously developed for *Electrophorus* acetylcholine receptor [18]. In order to improve the yield and activity of the solubilized acetylcholine receptor, a systematic study was carried out in which several parameters were varied (Kalderon, N. and Silman, I., unpublished results). A number of points should be stressed, autolysis was found to be a highly effective step. Although it occurred more rapidly at room temperature, considerable loss of toxin-binding activity occurred concomitantly, and prolonged autolysis at 4°C seems preferable. In the procedure for solubilizing *Electrophorus* acetylcholine receptor [18], dialysis against low ionic strength buffer was beneficial and seemed to lead to exposure of "latent" acetylcholine receptor, perhaps as a result of rupture of vesicular structures. However, since the prolonged tryptic digestion of *Torpedo* tissue is also performed at low ionic strength, where proteolysis was found to be more effective than in 0.1 M NaCl, the dialysis step may be omitted, a preparation with similar properties thus being obtained. The low concentration of trypsin employed seems important; higher concentrations of trypsin did not accelerate solubilization, but caused a deactivation not observed at the low concentrations employed. Finally, the low concentration of membrane fragments (corresponding to about 1 mg/ml protein) also seemed to be important, since at higher concentrations (e.g., 5 mg/ml) less efficient solubilization was achieved.

Purification of water-soluble acetylcholine receptor from STDPII was performed essentially by the affinity chromatography procedure of Karlsson et al. [11] for purification of Triton-solubilized *T. marmorata* acetylcholine receptor. For good elution of acetylcholine receptor from the cobra-neurotoxin-Sepharose resin, it was found advantageous to add 0.1 mM dithiothreitol to the solution of the hexamethonium which served as the eluting ligand. This may be because dithiothreitol inactivates the neurotoxin by reducing some of its disulfide bonds [27]. Dithiothreitol may also act to prevent aggregation or deactivation of acetylcholine receptor via free sulfhydryl groups [28,29] during the elution procedure. Acetylcholine receptor itself contains a disulfide bond sensitive to low concentrations of dithiothreitol [30,31]. Reduction of this bond does not appear to decrease toxin-binding capacity [14], but does markedly modify the binding characteristics with respect to cholinergic agonists and antagonists [32]. This may explain why the I_{50} values obtained for acetylcholine and carbamylcholine (Table III) are somewhat higher than might be expected.

The water-soluble acetylcholine receptor differs markedly in physicochemical parameters from the detergent-soluble acetylcholine receptor obtained from *T. californica*. The detergent-soluble acetylcholine receptor has a sedimentation coefficient of 9.5 S [33] and an apparent mol. wt. of $\approx$300 000 [34,35], while

the principal component of the water-soluble acetylcholine receptor has a sedimentation coefficient of 20—22 S and a Stokes radius of 125 ± 10 Å, corresponding to a mol. wt. of $\approx 10^6$ if a $\bar{v}$ of 0.75 is assumed [36]. The water-soluble acetylcholine receptor retains previously bound α-[^{125}I]bungarotoxin in the presence of 0.1% Triton X-100. However, under such conditions it is converted to a 9 S species, thus suggesting that it may be composed of several molecules of the 9 S form held together by hydrophobic forces. It is of interest, in this connection, that is has been shown that, upon removal of detergent from purified Triton-solubilized acetylcholine receptor, reversible aggregation can occur, to dimers in *T. californica* and to tetramers in *T. marmorata* [34,37]. The principal molecular species observed on removal of detergent from purified *T. marmorata* acetylcholine receptor had a sedimentation coefficient, 22—26 S, and a mol. wt., ≈1300 000, similar to those observed for the water-solubilized *T. californica* acetylcholine receptor. As mentioned under Results, even at high ionic strength the water-soluble acetylcholine receptor contained components heavier than 22 S as judged from the skewed shape of the sedimentation profile. These heavier forms may result from partial aggregation under the experimental conditions employed, or alternatively they may indicate that the solubilization process was incomplete.

The sodium dodecyl sulfate-acrylamide gel electrophoretograms of the purified acetylcholine receptor reveal only two major polypeptides, at 30 000 and 48 000 mol. wt., as well as some minor heavier components which may correspond to similar species reported in purified detergent-acetylcholine receptor preparations [31,33]. However, the major 30 000 component is not found in most purified detergent-acetylcholine receptor preparations which usually contain a 40 000 component absent in our preparation. This may be a result of the autolytic or tryptic steps; indeed, various authors have reported preparations containing lower molecular weight components which may result from degradation during isolation and purification [14,38,39]. Moreover, Shamoo and Eldefrawi [40] have shown that after tryptic digestion of detergent-solubilized acetylcholine receptor which leads to proteolytic cleavage of the 40 000 molecular weight subunit with concomitant formation of a 32 000 mol. wt. polypeptide, full binding capacity for acetylcholine is maintained.

Although the acetylcholine receptor is considered to be an integral membrane protein, the results presented above show that it can be solubilized without use of detergents, with retention of its receptor characteristics. It has been proposed that the acetylcholine receptor is composed of both an anionic binding site and an ionophoric component involved in cation translocation [41]. It is possible that the ionophoric component is selectively degraded or detached either by our procedure or by the detergent method. Detailed comparison of the structure and properties of the two types of preparations, as well as their parallel utilization in reconstitution studies, should yield valuable information concerning this problem.

Acknowledgments

We wish to thank Mrs. Esther Saksel-Roth for her skillful technical assistance. This work was supported by a grant from the United States-Israel Binational Science Foundation, Jerusalem.

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