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Purification and Characterization of Acetylcholine Receptor-I from *Electrophorus electricus*

(affinity chromatography/membrane-bound protein/subunit structure/sulfhydryl titration/equilibrium dialysis)

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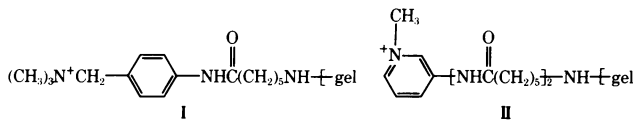
ABSTRACT **A Triton X-100 extract of electric tissue was subjected to a single step affinity chromatography using either of two affinity gels: [*N*-(6-aminocaproyl)-*p*-aminobenzyl]trimethylammonium bromide or methyl[(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide attached to Sepharose 4B. Specific elution of the acetylcholine receptor-I (AcChR-I) with low concentration of a bis-quaternary agonist, 3,3′-bis[α-(trimethylammonium)methyl]-azobenzene bromide (Bis-Q), gave a 35% yield of toxin-binding components in the crude extract. The purified AcChR-I readily underwent aggregation, which appeared to arise from the oxidation of titratable free sulfhydryl on the protein. The protein was characterized by the binding capacities for [^{125}I]α-bungarotoxin (α-Bgt), [^{3}H]acetylcholine, and [^{14}C]Bis-Q; the ratio of these capacities were approximately 2:1:2, respectively, with 5–6.5 nmole of α-Bgt sites per mg of protein. Analysis by sodium dodecyl sulfate gel electrophoresis of the disulfide-reduced and nonreduced polypeptide components indicated that a 41,500 dalton species was the major subunit component of AcChR-I. The binding of [^{14}C]Bis-Q with a Triton X-100 crude extract showed sites with both high and low dissociation constants, whereas purified AcChR-I contained only high-affinity sites. A biphasic double-reciprocal plot and a Hill coefficient of 0.7 suggested negative cooperativity in the binding of Bis-Q with the purified AcChR-I.**

During the past few years many investigators have made significant progress in the purification of the acetylcholine receptor (AcChR) and the description of its properties (1–10). In spite of many advances, the establishment of sufficient criteria for the identification of the AcChR protein(s) that are responsible for the specific change in membrane ion permeabilities has still not been achieved. Although the binding of various radio-isotope labeled snake neurotoxins (2–4, 11–13) has been widely accepted as a specific and convenient marker for the AcChR, it may be a necessary but not a sufficient criterion by itself. Evidence from this laboratory (Chang, unpublished work) and others (4, 13) indicates that there is more than one type of α-toxin-binding component present in the cholinergic membrane. Emphasis on the natural agonist, acetylcholine (AcCh), particularly when judging the purity of the isolated AcChR by the extent of AcCh binding alone (8), also can be misleading without full knowledge of the AcCh affinities of other cholinergic proteins which might easily contaminate the AcChR preparation. An approach to the identification of AcChR that is based on affinity labeling a reduced disulfide bond near the AcChR active site, a bond that has been identified physiologically in the intact monocellular electroplax (6), may provide a more useful criterion to distinguish isolated AcChR from other cholinergic components.

As purification methods for the AcChR were being developed in this laboratory, large variations in the AcCh-binding capacity but not in that for [^{125}I]α-bungarotoxin (α-Bgt) were observed among different preparations. Such observations finally led to a reasonable fractionation of two components, both of which bind α-Bgt and AcCh competitively. The major component is referred to as AcChR-I in this report. The second component was responsible for the large variations in AcCh binding among preparations and is referred to as AcChR-II. The present paper describes purification methods for AcChR-I from *Electrophorus electricus* by affinity chromatography and characterizes its subunit structure and binding capacity with [^{125}I]α-Bgt and the reversible agonists, [^{3}H]AcCh and 3,3′bis[α-([^{14}C]trimethylammonium)methyl]azobenzene bromide ([^{14}C]Bis-Q) (15). Some interesting preliminary observations on the characteristics of AcChR-II are briefly mentioned in the *Discussion*.

MATERIALS AND METHODS

Affinity Resins. The ligand synthesis of the monocaproyl-BTA resin (**I**), where monocaproyl-BTA is, [*N*-(6-aminocaproyl)-*p*-aminobenzyl]trimethylammonium bromide hydrobromide, was described previously (14). The synthesis of the ligand methyl[*N*-(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide hydrobromide mp 166–168° for the dicaproyl-MP resin (**II**) is similar in sequence to that of dicaproyl-PTA (14).



I II

The degree of ligand coupling to Sepharose 4B, accomplished by a reaction described previously (14), was calculated from the amount of unreacted ligand in the gel washings, measured by the absorbance at 245 nm for monocaproyl-BTA (ϵ = 16,900) and 292 nm for dicaproyl-MP (ϵ = 4,620). Both resins contained about 0.3 μmole of ligand per ml of packed gel.

Bis-Q and [^{14}C]Bis-Q Synthesis. 3,3′-Bis[α-trimethylammonium)methyl]azobenzene bromide (Bis-Q), mp 262–

Abbreviations: AcCh, acetylcholine; AcChE, acetylcholine esterase; AcChR, acetylcholine receptor; Bis-Q, 3,3′-bis[α-(trimethylammonium)methyl]azobenzene bromide; α-Bgt, α-bungarotoxin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; dicaproyl-MP, methyl[*N*-(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide hydrobromide; monocaproyl-BTA, [*N*-(6-aminocaproyl)-*p*-aminobenzyl]trimethylammonium bromide hydrobromide; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; mp, melting point.

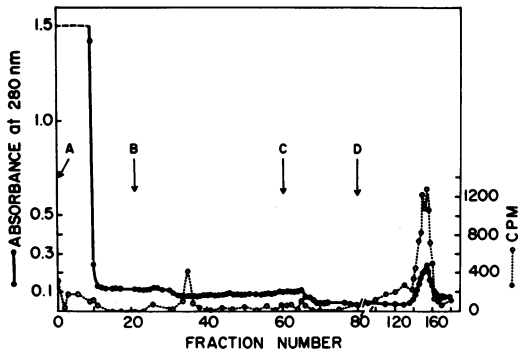


FIG. 1. Purification of toxin-binding material by affinity chromatography on dicaproyl-MP gel. After the application of 1% Triton X-100 extract, the gel was washed with three column volumes of stock buffer (20 mM sodium phosphate, pH 7.0) containing 1% Triton. The solvent was changed to stock buffer containing 1% Brij and the elution profile was followed by the absorbance at 254 nm. Detergent concentrations were lowered successively as the column was washed, and toxin-binding material was eluted with a 3 μM Bis-Q solution. *A*, 1% Brij, 17-ml fractions; *B*, 0–80 mM NaCl gradient (400 ml) in 0.5% Brij, 10-ml fractions; *C*, 0.01% Brij, 30 mM NaCl, 18-ml fractions; *D*, 3 μM Bis-Q in 0.01% Brij, 30 mM NaCl, 2-ml fractions. [^{125}I]α-Bgt binding was determined with 10 μl of each fraction. AcChE activity in the fractions was negligible.

263°, was synthesized according to the described method (15) and [^{14}C]Bis-Q was prepared by the following alternative route (personal communication of Drs. N. H. Wassermann and B. F. Erlanger, Columbia University). 3,3′-Bis(α-bromomethyl)azobenzene (15) was treated with 40% dimethylamine in water to yield 3,3′-bis(α-dimethylaminomethyl)-azobenzene. Subsequently, 3,3′-bis(α-dimethylaminomethyl)-azobenzene was quaternized by 20 mCi/mmole of $^{14}CH_3I$ (New England Nuclear Corp.), and a small amount of non-radioactive CH_3I in absolute ethanol afforded [^{14}C]Bis-Q with a specific activity 16.15 mCi/mmole; mp 247–248°. An aqueous solution of Bis-Q exhibits λ_{max} at 316 nm (ϵ = 16,000).

Purification and Labeling of α-Bungarotoxin. The snake venom of *Bungarus multicinctus* (Miami Serpentarium Lab., Florida) was purified according to Clark *et al.* (16). The most potent fraction, tested on the monocellular electroplax, α-Bgt, was iodinated (17) and further purified (16) to give monoiodinated [^{125}I]α-Bgt (7.7 Ci/mmole).

Extraction of Electric Organ. The electric organ (0.5–1 kg) of *Electrophorus electricus* was homogenized in an equal volume of stock buffer (20 mM sodium phosphate, pH 7.0) with a Waring blendor for 3 × 2 sec and centrifuged at 13,700 × *g* for 1 hr. The pellet was extracted twice by homogenization (3 × 10 sec) in about an equal volume of 1 M NaCl in stock buffer, followed by centrifugations at 13,700 × *g* for 1 hr each. Two salt extractions removed most AcChE along with other soluble proteins but still 5–6% of the initial AcChE activity (6) in the original membrane homogenates remained. Then the pellet was washed twice with stock buffer and extracted by 200–350 ml of 1% Triton X-100 in stock buffer, after homogenization for 3 × 1 min and shaking for 3–4 hr at 4°. The turbid supernatant after centrifugation at 23,500 × *g* for 1 hr was used in further purification steps.

Purification by Affinity Chromatography. A solution of 1% Triton X-100 extract was applied to the affinity column (2.4 × 22 cm, 100 ml of resin) with a flow rate of 20 ml/hr. The column was washed with at least three column volumes of 1% Triton X-100 in stock buffer and subsequently with 1% Brij (absorbance above 254 nm is $\leqslant$ 0.05) in stock buffer until the eluate absorbance was constant. The column eluent was continuously monitored at 254 nm with the LKB Uvicord-I from 1% Brij wash. A linear sodium chloride gradient was applied to the caproyl-BTA column (0–0.1 M NaCl) and to the dicaproyl-MP column (0–80 mM NaCl) in 0.5% Brij in stock buffer to remove loosely bound proteins without desorbing major portions of the AcChR-I. The concentration of detergent was then lowered to 0.01% Brij in stock buffer and 30 mM NaCl, and the column was washed extensively (300–400 ml). Finally, the AcChR-I was selectively eluted from either resin by 3 μM Bis-Q in 0.01% Brij in stock buffer containing 30 mM NaCl. The major fractions eluted with Bis-Q (25–50 ml) were pooled and concentrated to one tenth the initial volume under argon by a vacuum dialysis using a collodion membrane bag. The dialysis solution (20 mM sodium phosphate buffer, 0.1 M NaCl, pH 6.9) was changed four to five times during the concentration to remove the Bis-Q from the protein solution. The concentrated solution was centrifuted to remove dust.

[^{125}I]α-Bgt-Binding was measured according to Schmidt and Raftery (19) with a minor modification. A 2- to 10-fold excess of [^{125}I]α-Bgt was incubated with a 7- to 10-μg AcChR-I sample for 1 hr in 100 μl of stock buffer. A 75-μl aliquot was pipetted onto a 2.3-cm DEAE-cellulose filter disk (DE-81, Whatman) and washed on the Büchner funnel with 12 × 2 ml of 10 mM phosphate buffer, pH 7.4, with a gentle suction. A parallel blank assay was performed with each set of measurements at the same concentrations of [^{125}I]α-Bgt and detergent in the absence of protein. The amounts of radioactivity trapped in the disks were counted in 10 ml of Bray's scintillation fluid.

Equilibrium Dialysis-Binding Assays. Aliquots of either crude or purified AcChR-I (0.3 ml) were dialyzed against 75 ml of 20 mM phosphate buffer, 0.1 M NaCl, pH 7.0, containing various concentrations of [^{14}C]Bis-Q for 20–24 hr. Blank dialysis runs with the same concentration of detergent as the sample were carried out. Binding inhibition by α-Bgt was monitored in dialysis runs after preincubation of the sample with 2 μg of α-Bgt for 1 hr. Equilibrium dialyses in [^{3}H]AcCh (49 mCi/mmole), New England Nuclear Corp.) were carried out in the same solvent containing 5 μM eserine (Merck) and a given concentration of [^{3}H]AcCh for 8 hr. All samples were incubated for 1 hr in 5 μM eserine prior to the dialysis. Concentrations of eserine higher or lower than this value did not give maximum binding. After the dialysis was terminated, 0.2-ml aliquots were transferred from both the inside and the outside of the dialysis bags to 1 ml of NCS (Amersham), and 10 ml of toluene-based scintillator was added for counting.

AcChE Assay. Enzyme activity was measured by the pH-stat method using a Radiometer automatic titrator (14).

Free —SH Titration. About 50 μg of purified protein was treated with 3 mM [^{14}C]*N*-ethylmaleimide (NEM), 2.4 mCi/mmole(Amersham), for 30 min to 1 hr in an ice bath at pH 6.9. The unreacted [^{14}C]NEM was either directly dialyzed away or quenched with a calculated amount of 2-mercaptoethanol. The reaction mixture was dialyzed with two changes of the 1-liter dialysis solution. Aliquots of the bag content and the dialysis fluid were taken for radioactivity determinations. A blank experiment carried out in the absence of protein was used as a correction.

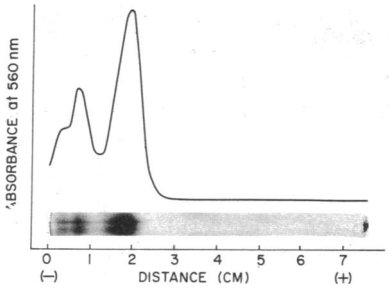


FIG. 2. Polyacrylamide gel (5% acrylamide, 0.25% bis-acrylamide) electrophoresis of purified AcChR-I (specific activity, 5.6 nmole of [^{125}I]α-Bgt binding per mg of protein) and absorbance scanning at 560 nm. The sample was 30 μl containing 20 μg of protein. A constant current of 1.8 mA per tube was applied for 2 hr at 4°. Under these conditions the tracking dye moves out of the bottom of the gel after 55 min, but a control gel shows no other protein-staining bands between the major band and the dye front.

Binding Assays after —SH Modification. Stock solutions (10–20 mM) of either NEM, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), or *p*-chloromercuribenzenesulfonate were added to 10–20 μg of purified AcChR-I to a final concentration of 5 mM and incubated for 30 min at 4° prior to the binding assays.

Protein Determination. Protein concentrations were measured (20) using bovine-serum albumin in equivalent concentrations of detergents as a standard.

Gel Electrophoresis. A procedure based on the alternate method of Davis (21) was followed, using Tris–glycine buffer at pH 8.3, 5% acrylamide–0.25% bis-acrylamide gel (pH 8.9). Gels were stained with Coomassie brilliant blue (22).

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis. Gels were run in 1% SDS according to the procedure of Fairbanks *et al.* (22). Protein samples from the purified AcChR-I in non-ionic detergent were first precipitated either by 60% $(NH_4)_2SO_4$ or by acetone (6). In the former case, the precipitate was separated, dialyzed, and lyophilized. Samples were incubated for 4 hr at 50° either with disulfide reduction in a medium containing 40 mM dithiothreitol (DTT) (22) or without disulfide reduction in the same medium minus DTT. Parallel gels were run with phospholipase A, catalase, ovalbumin, γ-globulin, and pepsin for molecular-weight markers.

RESULTS

Purification of AcChR-I. When a 1% Triton X-100 extract (200 ml, 1–1.5 g of protein, 41 nmoles of α-Bgt-binding sites, average of 0.083 nmole of toxin-binding sites per g of fresh tissue, 1.2 mg of AcChE) from 500 g of electric organ was chromatographed on the affinity column, 60–70% of the α-Bgt-binding components were selectively retained. An elution profile for a dicaproyl-MP column, after a 1% Triton–20 mM phosphate buffer wash is shown in Fig. 1. The AcChR-I binds with much lower affinity to the dicaproyl-MP gel than AcChE. Therefore most of the AcChE is retained tightly during the elution of AcChR-I. A solution of 3 μm of Bis-Q containing 0.01% Brij and 30 mM NaCl in stock buffer was added, and it selectively eluted AcChR-I in a relatively small volume. Bis-Q concentration at the fraction of maximum peak corresponded to about 0.2 μM. The elution profile for a monocaproyl-BTA column is essentially similar to that in Fig. 1 except that the AcChR-I is more tightly bound to monocaproyl-BTA gel than AcChE. AcChE can, therefore, be removed from this column along with other loosely bound proteins by a low sodium chloride gradient buffered Brij.

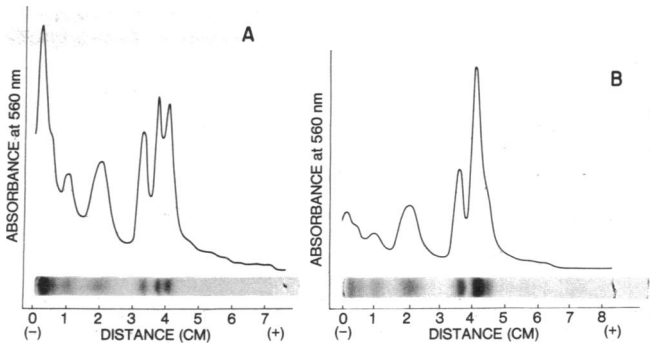


FIG. 3. SDS polyacrylamide gel (5.6% in acrylamide) electrophoresis of the purified AcChR-I and absorbance scanning at 560 nm. Electrophoresis was carried out at 8 mA per tube for 75 min. (*A*) Sample run in the absence of reducing agent. Approximately 40 μg of AcChR-I from the dicaproyl-MP column which had been stored at 4° for 2 weeks was incubated in 1% SDS, 10 mM Tris·HCl (pH 8), 1 mM ethylenediaminetetraacetate (pH 8) at 50° for 4 hr. (*B*) Parallel sample run after disulfide bond reduction at 50° for 4 hr in the same medium as *A* containing 40 mM DTT.

The AcChR-I fractions eluted by Bis-Q, after a 10-fold concentration by vacuum dialysis in a collodion bag gave 2.5–3 mg of protein in a 3-ml buffered solution containing 0.1–0.15% Brij (estimated from the original Brij concentration) and 0.1 M NaCl. From the Lowry protein determination (20) and the spectrophotometric reading at 280 nm, an estimate of the extinction coefficient was made, $E^{1\%}_{280\ nm} = 17 \pm 0.5$. An approximate 200-fold purification of AcChR with a specific activity ranging from 5 to 6.5 nmoles of α-Bgt-binding sites per mg of protein was obtained. The recovery corresponds to 55–65% of the gel-bound AcChR and 35% (average) of Triton X-100 crude extract. The amount of active AcChE present in the purified AcChR-I was only 0.007–0.05% assuming a specific activity of 10.0 mmole/(min) (mg protein) (14). Several previous reports of specific activity of AcChR determined by toxin-binding assays range from 3.3 nmole/mg to 7.15 nmole/mg of protein (1, 4, 5, 7, 9).

Homogeneity of Protein. The polyacrylamide gel electrophoresis (21) of freshly purified AcChR-I in the absence of SDS is shown in Fig. 2. There are slower-migrating components in addition to the major single band. The relative intensities of these bands change rapidly with storage; the major peak becomes smaller than the slower-moving (higher-molecular-weight) components after storage at 4° for 4 weeks.

Preincubation of purified receptor with [^{125}I]α-Bgt followed by gel electrophoresis and gel slicing showed that radioactivity corresponded to all the protein-strained bands. A similar receptor aggregation phenomenon was observed in gel exclusion chromatography (unpublished observation).

SDS Gel Electrophoresis. Electrophoretic analysis of purified receptor in 1% SDS in the absence of reducing agent yielded several protein-staining bands as shown in Fig. 3*A*. The molecular weight of the lower three distinctive bands corresponded to 49,000, 42,500, and 37,000. The large amount of protein aggregates at the top of the gel did not dissociate even if the incubation was conducted in 3% SDS at 50° for 4 hr to overnight. In the presence of a disulfide reducing agent, the protein band at the top of the gel was markedly reduced

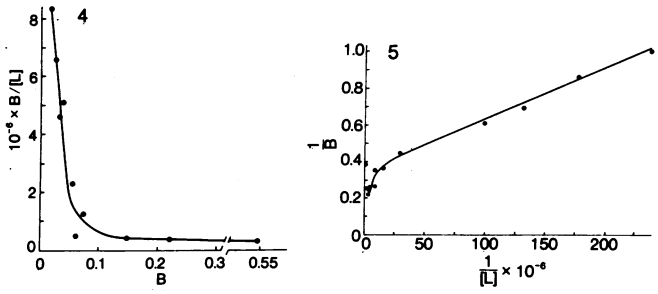


FIG. 4. Scatchard plot of equilibrium dialysis data for the binding of [^{14}C]Bis-Q to a 1% Triton X-100 extract of electric tissue. *L*, molar concentrations of free [^{14}C]Bis-Q (2.2 nM–1.8 μM); *B*, nmoles of ligand bound per g of fresh tissue extract. Solvent used was 20 mM phosphate buffer, 0.1 M NaCl (pH 7.0).

FIG. 5. Double reciprocal plot of [^{14}C]Bis-Q binding to the purified AcChR-I. *L*, molar concentrations of free [^{14}C]Bis-Q (4.2 nM–1.2 μM); *B*, nmoles of [^{14}C]Bis-Q bound per mg of purified AcChR-I. The numbers on the abscissa are the product of the indicated factor and the experimental values.

together with a sharp increase only in the 41,500 dalton band (see Fig. 3*B*). The lower three bands in Fig. 3*A* all were present in the reduced gel, but the 37,000 dalton band appeared as a descending shoulder on the 41,500 dalton band. A minor broad band in both Fig. 3*A* and 3*B* corresponded to a molecular weight of about 102,000. More recent SDS gel electrophoresis runs with freshly purified AcChR-I in the absence of DTT gave relative amounts of the lower three which appeared identical to those of the reduced gel in Fig. 3B. Fig. 3*B* has a striking resemblance to the densitometric scan reported recently by Biesecker (7). A major polypeptide species of 40,000–43,500 daltons, accompanied by other major species has been reported by several laboratories (5, 6, 23).

Equilibrium Dialysis Binding Assays. It has been observed that in the present dialysis media (20 mM phosphate buffer, 0.1 M NaCl, pH 7.0) normally 80–90% equilibration of Bis-Q is reached in 20–24 hr in the blank run and the % of equilibration in the blank is higher at the higher concentrations. Since one does not know how the receptor protein in the sample has affected the equilibration, the data presented here are uncorrected values. Fig. 4 shows a equilibrium titration of the 1% Triton X-100 crude extract with [^{14}C]Bis-Q displayed on a Scatchard plot. Two dissociation constants, one of high affinity (K_{D_1} = 6.4 nM with a maximum binding of 0.07 nmole/g of tissue) and a second of low affinity (K_{D_2} = 2.9 μM with a maximum binding of 1.4 nmole/g of tissue) were observed. Preincubation of the sample with α-Bgt blocked completely the binding of [^{14}C]Bis-Q at concentrations below 1.8 μM.

The binding of purified AcChR-I with [^{14}C]Bis-Q is expressed in a double reciprocal plot in Fig. 5 and indicates a complex pattern at higher concentration. The binding behavior at low [^{14}C]Bis-Q concentrations (4.2 nM–0.1 μM) gave a dissociation constant of K_D = 7.9 ± 0.8 nM and a maximum binding of 3.1 nmole/mg of protein. Between 0.15 μM and 0.56 μM additional binding was observed which corresponded to about 1.7 nmole/mg of protein and extrapolated to $K_{D_2} = 4.4 \times 10^{-8}$ on a Scatchard plot. Reduced [^{14}C]Bis-Q binding was observed at higher concentrations of 0.7 μM–1.6 μM; at a concentration of 1.2 μM, binding was 55% of the maximum binding (also see ref. 24). Preincubation of AcChR-I with [^{124}I]α-Bgt blocked [^{14}C]Bis-Q binding at both 1 μM and 0.1 μM [^{14}C]Bis-Q concentrations.

The binding capacity of AcCh to purified AcChR-I was 2.2–2.6 nmole/mg of protein and reached its saturation at 0.5 μM AcCh. The binding to AcCh was completely blocked by preincubation of the receptor with α-Bgt.

Free —SH Group Modification. Purified AcChR-I contains free —SH groups which may be titrated with [^{14}C]NEM. The number of —SH groups averages 6 ± 1 nmole/mg of protein in fresh preparations but varies from preparation to preparation and appears to be a function of the number of days of storage and the conditions under which the purified AcChR-I has been kept. When [^{125}I]α-Bgt-binding studies were conducted after treating the receptor protein with the sulfhydryl reagents, NEM, iodoacetamide, *p*-chloromercuribenzenesulfonate, or DTNB, no significant change in the binding of [^{125}I]α-Bgt was observed. Similar previous studies (25) on the effect of DTNB and *p*-chloromercuribenzoate on the binding of α-Bgt to *Electrophorus* membrane extracts reported no effect by DTNB but a small negative effect by *p*-chloromercuribenzoate. [^{3}H]AcCh binding at a 0.4 μM concentration is affected only slightly at most (10% reduction in some cases after treatment with *p*-chloromercuribenzenesulfonate) after —SH modification of the AcChR-I.

DISCUSSION

Acetylcholine receptor and AcChE display significantly different affinities toward two kinds of quaternary ammonium ligands, monocaproyl-BTA and dicaproyl-MP. A high degree of purification is achieved by the combination of using such affinity ligands for the affinity gel (0.3 μmole/ml of gel) and controlling the concentration of specific agonist, Bis-Q (3 μM), for the selective desorption of AcChR-I from the affinity column. Bis-Q is a reversible receptor agonist with the highest known physiological affinity for AcChR (15).

Polyacrylamide gel electrophoresis (Fig. 2) of freshly purified AcChR-I and gel exclusion chromatography of the [^{125}I]-α-Bgt-AcChR-I complex both show a small amount of higher-molecular-weight components in addition to the single major species. The relative amount of these minor proteins appears to increase during storage of the purified protein. While it may be possible that these components are other species of membrane-bound cholinergic protein, their relative increase on storage suggests that they arise from aggregation. Such aggregation phenomena also have been detected in other AcChR studies (3, 8, 27, 30). Incubation of purified AcChR at 1–3% SDS at 50° for 4 hr did not dissociate the aggregated material unless a disulfide reducing agent was added (Fig. 3). If aggregation were due exclusively to hydrophobic interactions or ionic interactions between charged proteins, a dissociation into smaller polypeptide chains would be expected during incubation in SDS, as is observed with a similar protein, AcChE (18). Furthermore, the quantity of free sulfhydryl groups in purified AcChR-I (6 ± 1 nmole/mg of protein by [^{14}C]NEM titration) rapidly decreased with storage. Such observations suggest that the aggregation phenomenon may be due to intermolecular disulfide bond formation caused by air oxidation. Addition of a reducing agent, such as mercaptoethanol or dilute DTT, to prevent disulfide formation is precluded because of a readily reducible disulfide bond near the active site of AcChR which is essential for its functional activity (26). Thus it is an intrinsically difficult task to obtain a completely homogeneous AcChR-I protein, and this observation may explain discrepancies existing in the literature regarding the molecular weight of AcChR (7, 18, 27, 28). The air oxidation may possibly be diminished by the protection

of detergent micelles; it has been reported that 0.5–1% detergent (Triton X-100 or deoxycholate) considerably prevented aggregation (27, 30).

The 41,500-dalton subunit polypeptide (Fig. 3*B*) may correspond to the previously reported subunit polypeptide of 40,000 daltons which is affinity labeled in intact cells or purified AcChR (6). No new bands appear after disulfide reduction, but the relative amount of 41,500 dalton polypeptide increases as the large components at the top of the gel decrease. This suggests that the 41,500-dalton species is the major subunit of AcChR-I and is the component which undergoes disulfide polymerization. Whether the presence of additional minor bands after disulfide reduction is due to the heterogeneity of the subunit structure of AcChR-I itself or to the incomplete resolution of AcChR-I from contaminants in the purification procedure cannot be determined at this time.

The binding of [^{14}C]Bis-Q to Triton X-100 crude extracts of electroplax shows two distinct dissociation constants, one of low affinity (K_D = 2.9 μM) and one of high affinity (K_D = 6.4 nM), on a Scatchard plot (Fig. 4). The high-affinity sites are blocked by preincubation with α-Bgt. After purification only the high-affinity sites are associated with AcChR-I, but the Scatchard plot is not linear. The possibility of negative cooperativity (29) among the sites is indicated by a biphasic double-reciprocal plot (Fig. 5) and a Hill coefficient of 0.7. The appearance of negative cooperativity in the binding of acetylcholine to purified AcChR has been reported (8). While calculations of the binding capacity of AcChR-I for Bis-Q are complicated both by the failure of Bis-Q to equilibrate completely during equilibrium dialysis and by an apparent absolute decrease in the total bound Bis-Q above 0.7 μM, the observed maximum value of about 5 nmole of Bis-Q per mg of protein is in good agreement with that of 5.7 nmole of [^{125}I]α-Bgt bound per mg of protein. A report of near equivalent-binding capacities for α-toxin and the bis-quaternary agonist decamethonium for purified AcChR has appeared (30).

The specific activity of the purified AcChR-I determined by [^{125}I]α-Bgt binding, 5–6.5 nmole/mg of protein, is extremely stable and is not affected appreciably by the aggregation or by —SH modification. Purified AcChR-I exhibits high-affinity sites to [^{3}H]AcCh which are saturated at 0.5 μM ligand with 2.2–2.6 nmole of AcCh-binding sites per mg of protein. This value is close to one half of the [^{125}I]α-Bgt sites and is in good agreement with the capacity of the higher affinity-binding sites for AcCh of a previous report [2.7 μmole/mg, (8)]. In contrast to that report, however, this preparation does not appear to have additional lower affinity-binding sites for AcCh. The specific activity determined by AcCh binding in this report is subject to error and could be a low estimate because 5 μM eserine was present during equilibrium dialysis to inhibit the minute amount of AcChE contaminant in the purified AcChR-I. This concentration of eserine also may have acted as an inhibitor of the binding of AcCh to AcChR-I. A successful use of an organophosphate, tetram, instead of eserine in such a system has appeared in several reports by Eldefrawi *et al.* (ref. 8, references therein).

Many purification experiments in our laboratory have shown that another component which meets the present criteria for AcChR and which has been designated AcChR-II (see *Introduction;* Chang, unpublished work) can easily contaminate the preparation of AcChR-I. Even if the contamination by AcChR-II is small, its presence is important because of its high-binding capacity for AcCh (several times that of AcChR-I). Consequently small amounts of AcChR-II can alter considerably the apparent AcCh-binding capacity of AcChR-I. These high-capacity sites on AcChR-II have a lower affinity for AcCh and are blocked by preincubation with α-Bgt. Preliminary experiments indicate that the AcCh sites on AcChR-II, unlike those on AcChR-I, are completely blocked by sulfhydryl reagents, NEM, iodoacetamide and *p*-chloromercuribenzenesulfonate.

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