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Tryptophan and Cysteine Residues of the Acetylcholine Receptors of *Torpedo* Species. Relationship to Binding of Cholinergic Ligands†

Mohyee E. Eldefrawi,* Amira T. Eldefrawi, and David B. Wilson

ABSTRACT: Several methods were used to analyze for tryptophan in the acetylcholine (ACh) receptors purified from the electric organs of the electric rays, *Torpedo californica* and *Torpedo marmorata*. The best value of tryptophan was 2.4 mol %. When excited at 290 nm, both receptors fluoresced with a maximum at 336, but there was no change in the fluorescence emission spectra upon binding of carbamylcholine, *d*-tubocurarine, ACh, or decamethonium. The free SH content of the *Torpedo* receptors varied in different preparations, and was highest in that purified from fresh *T. californica* using deaerated solutions and dialysis under nitrogen, and lowest in that prepared from the aged lyophilized membranes of *T. marmorata*. The maximum free SH content was 20 nmol/mg of protein or 0.22 mol %, equal to at most 18% of the total cysteic acid residues. Reaction of either 33% or of all the SH residues with *p*-chloromercuribenzoate reduced maximum ACh binding to the pure receptor prepared from fresh *T. californica* by only 23%.

Tryptophan and cysteine residues are of particular importance in studies of the relationship between the structure and function of proteins. This is both because they are present in relatively small amounts in most proteins and because reagents have been developed which react specifically with each of these amino acids. In addition, tryptophan has an intense fluorescence, which is very sensitive to its local environment, so that changes in conformation often result in changes in the tryptophan fluorescence.

Acetylcholine (ACh)[1] receptors have been purified from the electric organs of four fish species (Karlsson et al., 1972; Olsen et al., 1972; Klett et al., 1973; Schmidt and Raftery, 1973; Eldefrawi and Eldefrawi, 1973a; Biesecker, 1973; Karlin and Cowburn, 1973; Chang, 1974; Meunier et al., 1974; Lindstrom and Patrick, 1974; Michaelson et al., 1974; Ong and Brady, 1974) and their amino acid compositions determined in those of the electric ray, *Torpedo marmorata* (Eldefrawi and Eldefrawi, 1973a; Heilbronn and Mattson, 1974), *Torpedo californica* (Michaelson et al., 1974), and *Torpedo nobiliana* (Moore et al., 1974), as well as the electric eel, *Electrophorus electricus* (Klett et al., 1973; Meunier et al., 1974). Because of the difficulties involved in the analysis of tryptophan and the interference caused by detergents that are present in all these receptor preparations, only a few analyses of tryptophan were made. Its presence was detected in three of these studies (Eldefrawi and Eldefrawi, 1973a; Meunier et al., 1974; Moore et al., 1974), and not in a fourth (Klett et al., 1973).

In this paper, we use the ACh receptor purified from *T. californica* and compare it with that from *T. marmorata*. This *T. californica* receptor has been characterized immunologically. Rabbits and rats, immunized with this protein, developed skeletal neuromuscular defects, which seem to result from an impairment of their own ACh receptor function (Sanders et al., 1975). Similar observations were previously made on the ACh receptor purified from *Electrophorus electricus* (Patrick and Lindstrom, 1973; Sugiyama et al., 1973). This receptor has been reconstituted into black lipid membranes and carbamylcholine shown to induce monovalent cation selective conductance (Eldefrawi et al., 1975). The ACh receptor purified from the same *californica* species had also been reconstituted into lipid vesicles, and, in some preparations, activators were shown to accelerate ^{22}Na efflux (Michaelson et al., 1974). We report on the cysteic acid residues and free SH groups of the *californica* receptor and the effect on ACh binding of modifying them. Because of the very low content of detergent in our receptor

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[1] Abbreviations used are: ACh, acetylcholine; SH, sulfhydryl; PCMB, *p*-chloromercuribenzoate; NbS_2, 5,5′-dithiobis(2-nitrobenzoic acid).

preparation, we are able to distinguish the fluorescence emission spectra of the ACh receptor and to study the effect of the binding of cholinergic ligands on this fluorescence.

Experimental Procedure

Materials

Tissues. Electric organs of two fish were used. One was the lyophilized pellet of 12,000*g* from *T. marmorata,* prepared in 1968 (O'Brien et al., 1969) and kept since then frozen at −20° under nitrogen. The other was from fresh *T. californica,* purchased from Pacific Biomarine, Venice, Calif.

Radiolabeled Ligands. [*acetyl*-^{3}H]Acetylcholine (from New England Nuclear) with a specific activity of 49.5 Ci/mol was used in experiments with high ACh concentrations, and that (from Amersham Searle) with a specific activity of 290 Ci/mol was used in experiments with low ACh concentrations. Their purity was periodically checked by thin-layer chromatography (Lewis and Eldefrawi, 1974).

Methods

Purification of ACh Receptors. The electric organ of *T. californica* was dissected out, cut into 0.25-in. cubes, homogenized in ice-cold glass-distilled water (20% w/v), passed through cheesecloth, and then centrifuged at 16,000*g* for 1 hr. The pellets were resuspended in 1% solution of Triton X-100 in Krebs original Ringer phosphate (pH 7.4, containing 0.65 m*M* Ca^{2+}) (Dawson and Elliott, 1959). The lyophilized pellet of *T. marmorata* was homogenized in a similar Triton solution, then treated in the same manner. They were shaken for 30 min at 4° then centrifuged at 100,000*g* for 1 hr, and the supernatant was exposed to an affinity gel for purification. The specific ligand on the gel was the α-neurotoxin of the cobra (*Naja naja siamensis*) venom, purchased from the Miami Serpentarium, Fla. It was purified and attached to Sepharose 4B, then used for purifying the ACh receptors as previously described (Eldefrawi and Eldefrawi, 1973a). By increasing the volume of the desorption solution (1 *M* carbamylcholine) to 50 ml for the 20 g of gel, recovery ($\simeq$5 mg) of ACh receptors was raised to over 90% of the receptor sites present in the Triton extract. Protein was analyzed by the Lowry et al. method (Lowry et al., 1951). The receptor preparations in 5 m*M* Na_2HPO_4 were stored at 0° and used within 2 weeks.

Equilibrium Dialysis. Binding of [*acetyl*-^{3}H]acetylcholine to the ACh receptor was determined by equilibrium dialysis at 4°. Since the purified ACh receptor preparation still contained a minute amount of ACh esterase (estimated to be one catalytic site for every 20,000 ACh-binding sites (Eldefrawi and Eldefrawi, 1973a)), the preparation was preincubated with Tetram, *O,O*-diethyl *S*-diethylaminoethyl phosphorothiolate, a good inhibitor of ACh esterase, which does not affect the receptor at concentrations below 2×10^{-4} *M* (Eldefrawi et al., 1971a). For the pure receptors, Tetram was used at 10^{-5} *M* for 30 min prior to dialysis and the bath contained 10^{-6} *M* throughout dialysis, but in the case of the membrane-bound or Triton-solubilized preparations, Tetram was added to them for 30 min and to the bath at 10^{-4} *M*. For equilibrium dialysis, 0.5 ml of the purified ACh receptor (0.05–0.15 mg of protein/ml) was placed in a 0.25-in. cellophane dialysis tubing (Union Carbide) that was pretreated to eliminate contaminants as described by McPhie (1971). The dialysis bag was tied at both ends and placed into 100 ml of Krebs original Ringer phosphate (pH 7.4) containing 0.65 m*M* Ca^{2+} and [*acetyl*-^{3}H]acetylcholine, then shaken for 16 hr. Three 0.1-ml samples were taken from bath and bag contents, 10 ml of toluene liquid scintillation solution with Beckman BBS-3 solubilizer (Eldefrawi et al., 1971b) was added to each, and the radioactivity was counted in a Packard 3380 liquid scintillation spectrometer. Excess radioactivity in bag represented the amount of ligand bound. Each experiment was run in triplicate. In studying the effect of *p*-chloromercuribenzoate (PCMB) on the binding of [*acetyl*-^{3}H]acetylcholine, PCMB was incubated with the ACh receptor preparation for 30 min prior to the addition of Tetram.

Amino Acid Analysis. Cysteine was measured spectrophotometrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) by a modification of the procedure of Ellman (1959). The reaction mixture contained 0.1 *M* Tris-HCl (pH 8.0), 0.01 *M* disodium ethylenediaminetetraacetate (pH 7.5), about 0.15 mg of receptor, 0.05% sodium dodecyl sulfate, and 80 μg/ml of Nbs_2 in a final volume of 0.2 ml. The A_{412} was measured in a 1-cm path-length microcuvet at room temperature against a blank without receptor until there was no further increase in optical density. Tryptophan was determined quantitatively by both the procedures of Barman and Koshland (1967) and Penke et al. (1974).

The amino acid composotion was determined on a Beckman 120C amino acid analyzer using the procedure of Moore and Stein (1954) and analysis of cysteic acid was carried out on a performic acid oxidized sample according to Hirs (1956).

Electrofocusing. The LKB Produkter column (110 ml capacity) was used as previously described (Eldefrawi and Eldefrawi, 1972). Ampholine was used in a mixture of 1% of pH range 3–5 and 1% of pH range 6–8.

Antibody Assay. The presence of antibody reacting with the purified ACh receptor was determined by the Ouchterlony double diffusion reaction in 1% agar gel and Veronal buffer.

Fluorescence Measurements. A Perkin-Elmer MPF-3 fluorescence spectrophotometer was used for measuring the emitted fluorescence of the receptor when excited at 290 nm at equilibrium conditions. The temperature was 16° and pH 7.4. Stopped-flow experiments on the fluorescence of the receptor and its interaction with ligands were carried out at pH 7.4 with use of the spectrophotometric stopped-flow apparatus described by Sturtevant (1964). The wavelength was set at 290 nm and the solutions were measured in cells of 2-mm light path. The solutions were brought to the desired temperature in a thermostatically regulated water bath. The solutions were introduced into the apparatus by being pushed from thermostated syringes by means of a common bar through tubes to the mixing chamber. The effective dead time of the apparatus was 3 msec.

Results

Purity of the ACh Receptor Preparations. The purified *T. californica* ACh receptor preparation appeared as a single molecular species in electrofocusing with a p*I* of 4.6 (Figure 1). In the Ouchterlony double diffusion test, a single intense, sharp immunoprecipitin line was formed between antisera from a rabbit inoculated with a 1% Triton extract of *T. californica* electroplax (crude receptor preparation) and the pure receptor compared to the diffused precipitin band formed with the crude antigen used for immunization (Figure 2). When binding of [*acetyl*-^{3}H]acetyl-

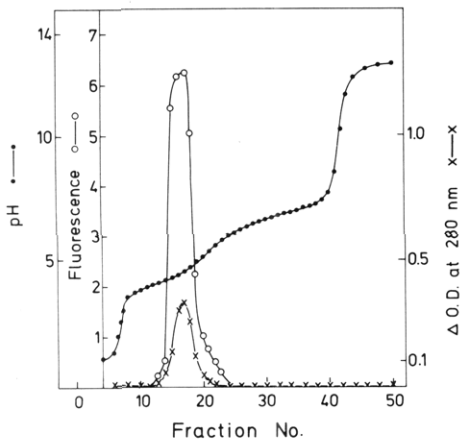

FIGURE 1: Electrofocusing of purified *T. californica* ACh receptor protein in ampholine (pH range 3–8).

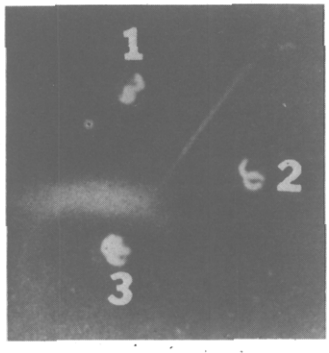

FIGURE 2: Immunodiffusion assay of pure (well 2) and crude (well 3) *californica* ACh receptor preparations against antiserum from rabbit immunized with crude ACh receptor (well 1).

choline was studied, over a wide range of concentrations (10^{-9}–10^{-6} M), to the membrane-bound and Triton-solubilized *californica* receptor, both bound it with high affinity ($K_d = 2 \times 10^{-8}$ M) with possible positive cooperativity as indicated by the upward curvature in the Scatchard plot at low ligand concentrations (Figure 3a). The Hill coefficients were calculated to be 1.6 for both membrane bound and solubilized receptor. Maximum numbers of ACh-binding sites were 1.1 and 0.5 nmol/g of electroplax, respectively. However, the pure *californica* receptor exhibited high and low affinity binding ($K_d = 2 \times 10^{-8}$ and 2×10^{-6} M) in addition to the possible positive cooperativity. The maximum number of binding sites was 10 nmol/mg of protein for either ACh (Figure 3b) or α-bungarotoxin.[2]

Fluorescence Emission of the ACh Receptor. The pure receptor of both *T. marmorata* and *T. californica* gave a peak of uv absorption with a maximum at 290 nm and emitted fluorescence with a maximum at 336 nm (Figure 4). By comparison, L-tryptophan, activated at 290 nm, emitted with a maximum at 348 nm. Thus, emission maximum for tryptophan fluorescence in the ACh receptor was shifted to shorter wavelengths by 12 nm. The peak fluorescence of a 0.0007% solution of Triton X-100 (the concentration found to be present in the pure receptor (Edelstein et al., 1975),

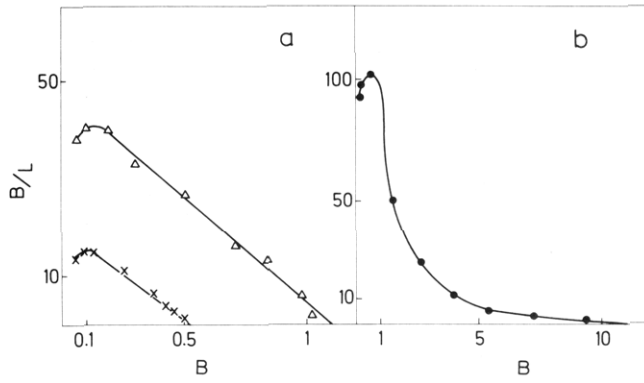

FIGURE 3: Scatchard plots of the binding of [*acetyl*-^{3}H]acetylcholine to the ACh receptor of *T. californica*. (a) Binding to the crude receptor preparations; membrane bound (△); 1% Triton extract of the membranes (x). (b) Binding to the purified receptor. B, nanomoles bound per gram of tissue in a and per milligram of protein in b. L; concentration of [*acetyl*-^{3}H]acetylcholine in μM.

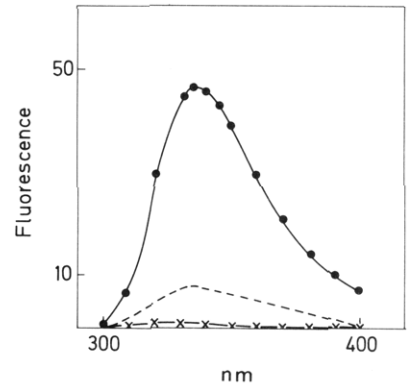

FIGURE 4: Fluorescence emission spectra of the purified ACh receptor of *T. marmorata* or *T. californica* (0.1 mg/ml), excited at 290 nm (●), Triton X-100 (0.0007%) (x), and bovine serum albumin (0.1 mg/ml) containing 0.0007% Triton X-100 (- - -).

was only 4% of the fluorescence found in the purified receptor preparation (0.1 mg/ml). A concentration of ACh receptor as low as 40 ng/ml was easily detectable by its fluorescence. For comparison, the addition of 0.0007% Triton X-100 to 0.1 mg/ml of bovine serum albumin did not appreciably change its fluorescence emission.

When pure *T. marmorata* receptor was chromatographed on Sepharose 4B, fluorescence, protein, and ACh binding were coincident and gave reasonably constant ratios across the peak (Figure 5). This provides added evidence that the tryptophan found in the receptor preparation is present in the receptor itself. In an attempt to detect a conformational change upon the binding of cholinergic ligands to the receptor, the effect of carbamylcholine, *d*-tubocararine, ACh, or decamethonium on the fluorescence of the two *Torpedo* receptors was measured. In order to obtain reproducible results, the receptor solution had to be thoroughly mixed before measurements and excitation was limited to 1–5 sec. Under these conditions, there was no detectable change in protein fluorescence upon the addition of 10^{-7}–10^{-5} M ACh, decamethonium, carbamylcholine, or *d*-tubocurarine. Neither were there changes in fluorescence in the millisecond range upon the addition of these ligands (Figure 6).

The amino acid composition of the *T. californica* receptor is presented in Table I along with the published values for the receptors of *T. marmorata, T. nobiliana,* and *E. el-*

[2] A value obtained by Dr. R. Gibson using gel filtration on Sephadex G-50 after incubation at 23° of the ACh receptor with [^{3}H]-α-bungarotoxin for various time intervals up to saturation. An identical value was also obtained by Dr. P. Wins using ion exchange chromatography on CM-50 and [^{125}I]-α-bungarotoxin.

Table I: Amino Acid Composition (mole %) of ACh Receptors from the Electric Organs of Several Fish.

	T. californica		*T. marmorata*		*T. nobiliana*	*E. electricus*	
Amino Acid	I[a]	II	III	IV	V	VI	VII
Lysine	5.4	6.1	6.1	5.0	4.5	4.6	6.3
Histidine	2.4	2.7	2.1	2.5	2.5	2.2	2.5
Arginine	3.9	4.1	3.5	3.3	3.7	4.2	4.2
Aspartic acid	11.6	11.9	11.8	12.4	12.2	11.4	9.8
Threonine	6.4	6.3	6.3	6.2	6.8	5.6	6.0
Serine	7.9	6.6	7.1	8.1	6.4	6.2	8.2
Glutamic acid	10.0	10.2	10.7	8.7	9.7	10.2	9.0
Proline	5.9	5.9	6.2	5.6	7.1	5.7	6.7
Glycine	4.6	4.9	6.4	5.0	5.0	5.9	4.8
Alanine	5.0	5.1	6.0	5.0	4.5	5.8	5.4
Cysteic acid	1.2	0.9	2.0		2.8	2.0	1.7
Valine	7.1	7.0	5.5	7.3	6.2	8.6	6.9
Methionine	2.0	1.8	1.7	2.5	1.6	2.0	3.4
Isoleucine	8.2	7.5	5.2	7.4	6.2	6.4	8.1
Leucine	9.5	9.7	9.3	10.1	10.2	10.5	10.7
Tyrosine	3.7	3.8	3.6	3.5	4.2	4.0	3.8
Phenylalanine	4.5	4.6	4.4	4.5	4.2	5.7	5.1
Tryptophan	2.4[b]		2.1[c]		1.5	0	2.4
Glucosamine		0.9			2.0		+[d]
% Polar residues[e]	47	48	48	46	46	44	46

[a] Roman letters represent the various amino acid analyses: I, present data; II, Michaelson et al. (1974); III, Eldefrawi and Eldefrawi (1973a); IV, Heilbronn and Mattson (1974); V, Moore et al. (1974); VI, Klett et al. (1973); VII, Meunier et al. (1974). [b] Measured by the procedure of Penke et al. (1974). When measured by the procedure of Barman and Koshland (1967) the value obtained was 1.0%. [c] Previously measured by the procedure of Bencze and Schmid (1957) and Liu (1972). When presently determined by the method of Penke et al. (1974), it was found to be 1.9% and by the method of Barman and Koshland (1967) it was 0.8%. [d] Based on the binding of the ACh receptor to several plant lectins, it was suggested that the receptor carried a carbohydrate moiety containing at least D-mannose and *N*-acetyl-D-galactosamine. [e] The sum of Asp, Glu, Lys, Ser, Arg, Thr, and His as classified by Capaldi and Vanderkooi (1972). The percent polar residues of the ACh receptor are higher than those of most membrane proteins that require detergents for their extraction.

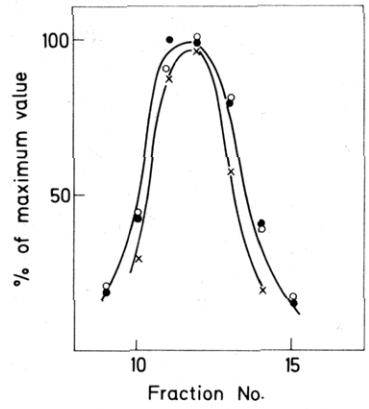

FIGURE 5: Chromatography of the purified ACh receptor from *T. marmorata* on Sepharose 4B. Each fraction is 1 ml and column void volume is 8 ml; protein (●), fluorescence at 348 nm (○), [*acetyl*-^{3}H]acetylcholine binding at 10^{-6} *M* (x).

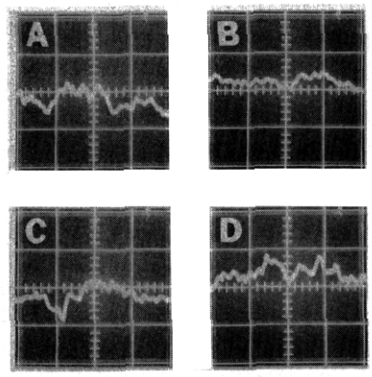

FIGURE 6: Oscilloscope scans obtained on mixing purified ACh receptor of *T. californica* (72 μg/ml) or cholingergic ligand (2×10^{-5} *M*) in 5 m*M* Na_2HPO_4 (pH 7.4) with equal volume of same buffer, or receptor with cholinergic ligand in stopped-flow apparatus. (A) Receptor–buffer; (B) ACh (2×10^{-5} *M*)–buffer; (C) receptor–ACh (2×10^{-5} *M*); (D) receptor–decamethonium (2×10^{-5} *M*). Each horizontal division represents 10 msec, and each vertical division represents 50 mV.

ectricus. The compositions of the four receptors are fairly similar, as the sums of the absolute value of the deviations for all amino acids are 7.8, 9.8, 12, 10.7, and 10.4% for the *T. californica* vs. *T. marmorata*, *T. marmorata* vs. *T. nobiliana*, *T. californica* vs. *T. nobiliana*, *T. californica* vs. *E. electricus*, and *T. marmorata* vs. *E. electricus*, respectively. Where two analyses are made on the receptor of one species, the averages of the two sets are used in these calculations. The value for the difference between the two *E. electricus* determinations is 17.2%.

The concentration of tryptophan in the *marmorata* receptor was found to be equal to that of the *californica* receptor by each of the two methods of analysis used (Table I). However, the procedure of Barman and Koshland (1967) gave half the values obtained after hydrolysis for both receptors; this may be a result of the incomplete denaturation by the acid–urea treatment.

The Free SH Groups and Their Effect on ACh Binding. The free SH content of the different ACh receptor preparations varied and so did their binding of ACh at 10^{-6} *M* (Table II). The ACh receptor prepared in the regular way from lyophilized *T. marmorata* membranes (Eldefrawi and Eldefrawi, 1973a) contained 2.4 nmol of SH/mg of protein, equivalent to 0.3 SH/ACh binding site. If the ACh receptor was extracted and purified using deaerated solutions and dialysis under nitrogen, the free SH groups increased to 6.4 nmol/mg of receptor (Table II) with a slight, but insignificant, increase in the amount of ACh binding at 10^{-6} *M*. When the receptor was prepared from a fresh *T. californica* electric organ, using deaerated solutions and dialysis under nitrogen, there were 20 nmol of SH/mg of protein, equivalent to 2 SH groups/ACh binding site. Treatment of the purified *T. californica* receptor with PCMB concentrations that blocked either 33 or 100% of the SH groups reduced ACh binding at 10^{-6} *M* by only 10%. A more accurate evaluation of the effect of PCMB on the binding of [*acetyl*-^{3}H]acetylcholine to the *T. californica* receptor was obtained by studying its effect on binding at a series of ACh concentrations. Maximum binding was 10 nmol of ACh/mg of protein. Inactivation of all the SH groups reduced binding of ACh by a maximum of 23% and did not cancel the cooperative interactions (Figure 7).

Discussion

To determine the homogeneity of a protein in a preparation, several molecular properties may be utilized, such as molecular weight, subunit weight, and isoelectric point. In the case of the ACh receptor, this is complicated by the presence of detergent molecules, and the aggregation that occurs if they are removed (Edelstein et al., 1975). There-

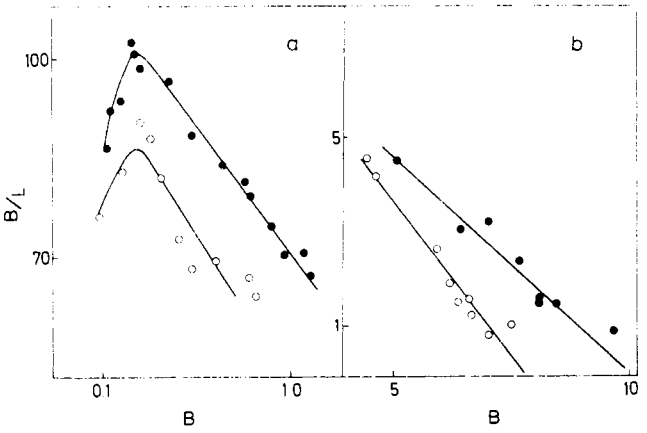

FIGURE 7: Scatchard plot of the binding of [*acetyl*-^{3}H]acetylcholine by the purified ACh receptor of *T. californica:* control (●); receptor pretreated with 4×10^{-5} *M* PCMB before dialysis (○). B, amount bound in nanomoles/milligram of protein; L, concentration of [*acetyl*-^{3}H]acetylcholine in μM. (a) Binding at low ACh concentrations (9×10^{-10}-10^{-8} *M*); (b) binding at high ACh concentrations (10^{-6}-8×10^{-6} *M*).

fore, multiplicity of forms may not necessarily be indicative of impurity, and may only reflect the variety of forms of the molecules. Molecular weight determinations, from sedimentation equilibrium experiments, with absorption optics and a scanner-computer system, indicated that the *T. californica* ACh receptor was present in molecular weights of 330,000 and 660,000 when its content of Triton X-100 was 0.0007% (w/v). When 0.1% Triton X-100 was added, the preparation showed a single component of molecular weight 330,000. Judging from the single protein peak appearing in electrofocusing (Figure 1) and column chromatography (Figure 5), the sharp immunoprecipitin line (Figure 2), the sedimentation equilibrium data (Edelstein et al., 1975), and the single protein band appearing in electrophoresis (Eldefrawi et al., 1975) one may conclude that the *T. californica* ACh receptor is a single molecular species. As for the *T. marmorata* ACh receptor preparation, this was previously reported to be ≃87% pure (Eldefrawi and Eldefrawi, 1973a).

Relative functional purity of various ACh receptor preparations is easily obtained by comparison of their specific binding. Based on α-bungarotoxin, α-cobra toxin, or maleimide binding, values of 2-4 (Karlin and Cowburn, 1973), 3-6.5 (Olsen et al., 1972; Meunier et al., 1974), 6.7 (Klett et al., 1973), and 7.7 (Lindstrom and Patrick, 1974) nmol/mg of protein (assayed by Lowry et al. method) have been reported for the *Electrophorus* ACh receptor. As for that of *Torpedo marmorata* or *californica,* values of 1.7 (Karlsson et al., 1972), 6 (Schmidt and Raftery, 1973), and 10 (Michaelson et al., 1974) have been reported. The *T. californica* and *T. marmorata* ACh receptor preparations used in the present study bind 7.8 (Eldefrawi and Eldefrawi, 1973a) and 10 (Figures 3 and 7) nmol of [*acetyl*-^{3}H]acetylcholine/mg of protein, respectively. The *californica* receptor also binds 10 nmol of [^{3}H]-α-bungarotoxin/mg of protein.[2] These values are based on protein concentrations obtained by the method of analysis of Lowry et al. (1951). The protein concentration is usually lower and consequently the specific binding is higher if amino acid analysis is used to determine the protein concentration (Eldefrawi and Eldefrawi, 1973a; Schmidt and Raftery, 1973; Meunier et al., 1973).

The presence of tryptophan in the ACh receptor of *T.*

Table II: Free SH Groups in Different ACh Receptor Preparations.

Species	Treatment[a]	Nanomoles of free SH Groups per mg of Receptor Protein[b]	Nanomoles of free SH Groups per nmole of ACh Binding Site[c]	[*acetyl*-^{3}H] Acetylcholine Binding at 1 μM (nmol/mg of Protein)
T. marmorata	A	2.4	0.3	3.1 ± 0.4
T. marmorata	B	6.4	0.8	3.7 ± 0.3
T. californica	B	20.0	2.0	5.1 ± 0.4
T. californica	C	14.0	1.4	4.6 ± 0.3
T. californica	D	0.0	0.0	4.6 ± 0.4

[a] The receptor was exposed to one of the following conditions: A, purified as usual (Eldefrawi and Eldefrawi, 1973a); B, deaerated solutions were used during purification and dialysis was under nitrogen; C, purified as in B, then 10^{-5} *M* PCMB was added to the pure receptor; D, purified as in B, then 4×10^{-5} *M* PCMB was added to the pure receptor. [b] Protein assayed by the Lowry et al. method (1951). [c] Maximum number of ACh-binding sites per milligram of receptor protein are 7.8 nmol for *T. marmorata* (A or B) and 10 nmol for *T. californica* (treatment B) (Figure 3).

marmorata (Eldefrawi and Eldefrawi, 1973a) is confirmed, and this amino acid is also found in the receptor of *T. californica* (Figures 4 and 5 and Table I). An observation of practical significance is the strong fluorescence emitted by the ACh receptor, which makes this method 1000-fold more sensitive than uv absorption for the detection of ACh receptor in solution. Similar to most proteins (Brand and Witholt, 1967), emission maximum for tryptophan fluorescence in the ACh receptor is shifted to shorter wavelengths by about 12 nm. The absence of changes in emission maximum or quantum yield, resulting from the binding of cholinergic ligands to the ACh receptor (Figure 6), does not exclude the possibility that changes do occur in the conformation of the receptor, which do not alter the environment of the tryptophan residues.

It is apparent that the number of free SH groups in the ACh receptor varies in the different preparations. This confirms a recent finding in an ACh receptor purified from the electric eel (Chang, 1974). However, using titration with [^{14}C]-*N*-ethylmaleimide, only 6 nmol of SH groups was found per mg of protein, a value similar to the one presently reported for the *Torpedo marmorata* ACh receptor purified from aged lyophilized membranes using deaerated solutions and dialysis under nitrogen (Table II). Under the same conditions the ACh receptor purified from fresh *T. californica* has 2.5 times more free SH groups (Table II). The highest value obtained of 20 nmol of free SH per mg of protein or 2 per ACh binding site (= 0.22 mol %) may still be a minimum value. Since the concentration of cysteic acid is 1.2 mol % (Table II), the maximum number of disulfide bonds in the *T. californica* ACh receptor, which is 82% of the cysteic acid, is calculated to be 4.5 disulfide bonds/ACh binding site, or 45 nmol/mg of protein.

The ACh receptors of the two *Torpedo* sp. are similar in the maximum number of active sites found in membranes (Figure 3; O'Brien et al., 1969; Eldefrawi et al., 1971a), in their amino acids and content of tryptophan (Figure 4) and their molecular weights (Edelstein et al., 1975). They also exhibit positive cooperativity in their binding of ACh (Figure 3; Eldefrawi and Eldefrawi, 1973b; Weber and Changeux, 1974), and so does the membrane-bound ACh receptor from *Electrophorus electricus* (Meunier et al., 1973). The membrane-bound ACh receptors bind ACh with a high affinity (8×10^{-9}-2×10^{-8} *M*), which is detected by equi-

librium dialysis or centrifugation in *T. californica* (Figure 3a), *T. ocellata* (Eldefrawi and Eldefrawi, 1975), and *T. marmorata* (Eldefrawi et al., 1971a; Weber and Changeux, 1974; Sugiyama et al., 1975) and by displacement of bound fluorescent ligand in the latter (Cohen and Changeux, 1973). Also, only high affinity binding of ACh is observed in membrane preparations from *E. electricus* (Eldefrawi et al., 1971c; Suszkiw, 1973; Meunier et al., 1973). Thus, one may conclude that native ACh receptors of fresh electric organs of *Torpedo marmorata, T. californica, T. ocellata,* and *Electrophorus* bind ACh with only a high affinity. Also, it may be that handling of the *T. marmorata* membranes, their lyophilization and storage at −20° until use over the past 6 years (O'Brien et al., 1969) has resulted in the conversion of some of the high affinity sites to low affinity ones. Fresh *T. marmorata* membranes bind ACh with a single high affinity (M. E. Eldefrawi, unpublished), while under the same conditions, the aged lyophilized ones bind it with high and low affinities (O'Brien and Gibson, 1974).

Our solubilization of the ACh receptor from fresh membranes with 1% Triton does not change its affinity for ACh, since those solubilized from *T. californica* or *T. ocellata* still bind ACh with a single high affinity ($K_d = 2 \times 10^{-8}$ *M*) (Figure 3a; Eldefrawi and Eldefrawi, 1975). This contrasts with another report on the *T. marmorata* ACh receptor, where in the membrane only high affinity ACh binding is observed, but, after solubilization, low affinity binding appears (Sugiyama et al., 1975). After purification of the ACh receptor in our laboratory, the receptor exhibits both high and low affinity binding of ACh, whether it is from *T. marmorata* (Eldefrawi and Eldefrawi, 1973a), *T. californica* (Figure 3b), *T. ocellata* (Eldefrawi and Eldefrawi, 1975), or *E. electricus* (unpublished). The low affinity binding that is present after purification may be related to that appearing in the aged lyophilized *T. marmorata* membranes, or it may be due to a change of constraints on the molecule in its new environment. Another factor that may contribute to the varying affinities for ACh in the different purified ACh receptors is the kind and amount of detergent that is present in all the preparations (Edelstein et al., 1975).

It had been shown that exposure of the monocellular preparation of *Electrophorus* electroplax to PCMB inhibited its depolarization by activators (Karlin and Bartels, 1966). This suggested that PCMB reacted with SH group(s) in one or more components of the ACh-activated permeability system at the junctional region of the innervated membrane. Subsequently, it was shown that PCMB reacted with an impure ACh receptor preparation and inhibited its binding of ACh (Eldefrawi and Eldefrawi, 1972).

The data in Table II and Figure 7 suggest that some free SH groups contribute to ACh binding, either directly or indirectly and their inactivation reduces, but does not eliminate, binding. The small contribution of free SH groups to ACh binding was evident in another study on purified *Electrophorus* ACh receptor, when PCMB treatment reduced ACh binding by only 10% in some cases and not [^{125}I]-α-bungarotoxin binding (Chang, 1974). However, the free SH groups in that preparation were only 6 nmol/mg of protein, compared to 20 nmol of free SH/mg of protein in the ACh receptor from *T. californica* presently studied (Table II). Since inactivation of 33% of the free SH groups has the same effect on ACh binding as inactivation of more SH groups (Table II), one may conclude that the SH groups that affect binding are more reactive with, or more accessible to, PCMB than the rest. It is interesting to note that the maximum binding of the ACh receptor purified from aged lyophilized *T. marmorata* membranes (7.8 nmol/mg of protein) with much of its free SH possibly already oxidized (Table II) is similar to that of the *T. californica* receptor purified from fresh electric organs after all its free SH groups are reacted with PCMB (7.7 nmol/mg of protein, Figure 7). One difference between the two preparations is that the inactivated SH groups of the aged *T. marmorata* receptor may be oxidized and probably form S–S bonds, whereas those of the PCMB treated *T. californica* are covalently attached to PCMB. Since several laboratories are studying the interactions of ACh with its pure receptors, it is important, for correlation of the data, to know the conditions to which each preparation is exposed and its content of detergent and of free SH groups.

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Differential Effects of 8-Anilino-1-naphthalenesulfonate upon Binding of Oxidized and Reduced Flavines by Bacterial Luciferase†

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ABSTRACT: Upon binding to bacterial luciferase, both the absorption and the fluorescence excitation maxima of 8-anilino-1-naphthalenesulfonate (ANS) shift from 353 to 370 nm while the fluorescence emission optimum shifts from 540 to 480 nm, and the fluorescence quantum yield increases from 0.003 to 0.39, indicating that the environment of the ANS binding site is hydrophobic. ANS binds to luciferase with dissociation constants of 1.9×10^{-5} and 2.3×10^{-5} *M* at 5 and 23°, respectively. As with both oxidized flavine mononucleotide (FMN) and reduced flavine mononucleotide ($FMNH_2$), ANS also binds to luciferase with a stoichiometry of 1 site per dimeric luciferase molecule. ANS acts as a luciferase inhibitor, competitive with $FMNH_2$, with an inhibitor dissociation constant of 2.3×10^{-5} *M* at 23°. However, the binding of ANS does not significantly displace FMN from binding to luciferase. Interactions of FMN and $FMNH_2$ with luciferase are thus differentially regulated by the ANS binding.

Bacterial luciferase catalyzes the bioluminescent mixed function oxidation of $FMNH_2$[1] and a long chain aldehyde to yield light (λ_{max} 490 nm), FMN, H_2O, and carboxylic acid (Hastings and Gibson, 1963; Eberhard and Hastings, 1972; Nealson and Hastings, 1972; Shimomura et al., 1972; McCapra and Hysert, 1973; Dunn et al., 1973; Hastings et al., 1973; Hastings and Balny, 1975). Luciferase has been shown to be highly specific for $FMNH_2$; a negative charge on the flavine side chain is necessary for both tight binding and good substrate activity while structural variations of the flavine ring system generally result in poor activity with luciferase (Meighen and MacKenzie, 1973; Mitchell and Hastings, 1969). The binding of $FMNH_2$ to luciferase was demonstrated by kinetic analysis to have a stoichiometry of

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[1] Abbreviations used are: $FMNH_2$ and FMN, reduced and oxidized flavine mononucleotide; ANS, 8-anilino-1-naphthalenesulfonate.