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Studies of the Composition of Purified *Torpedo californica* Acetylcholine Receptor and of Its Subunits†

Richard L. Vandlen,‡ Wilson C.-S. Wu, James C. Eisenach, and Michael A. Raftery*

ABSTRACT: Under conditions that limit proteolytic degradation, the detergent-solubilized purified receptor protein from *Torpedo californica* exists in monomeric and dimeric forms. The purified receptor complex is composed of four different polypeptide subunits of apparent molecular weights 40 000, 50 000, 60 000, and 65 000. The individual polypeptides have been purified and their amino acid compositions have shown them to be relatively hydrophobic. In addition, the carbohydrate composition of the intact receptor complex and of the individual polypeptides has been determined. Amino acid analysis provided evidence for the occurrence of a component with chromatographic properties similar to those of phosphoserine. Treatment of receptor with CH_3NH_2 in base, a condition which provided quantitative modification of *O*-phosphoserine residues in β-casein, completely eliminated the peak corresponding to phosphoserine following mild acid hydrolysis. We conclude that the receptor contains *O*-phosphoserine residues to the extent of approximately seven residues per molecule and these residues occur in all constituent polypeptides. Other forms of O-substituted serine and threonine were also shown to occur, most likely as glycosylated residues.

Acetylcholine receptors have been isolated from the elasmobranchs *Narcine entemedor* (Schmidt & Raftery, 1972), *Torpedo californica* (Schmidt & Raftery, 1973b; Raftery, 1973; Weill et al., 1974), *Torpedo marmorata* (Karlsson et al., 1972; Eldefrawi & Eldefrawi, 1973; Gordon et al., 1974), and *Torpedo nobiliana* (Ong & Brady, 1974) and from the fresh water electric eel, *Electrophorus electricus* (Olsen et al., 1972; Biesecker, 1973; Karlin & Cowburn, 1973; Lindstrom & Patrick, 1974; Chang, 1974).

Despite intensive effort, understanding of the structural properties of the receptor is rather limited. In many preparations gel electrophoresis in $NaDodSO_4$ revealed considerable variability in subunit pattern; the only common features reported from several studies were that the receptor protein was apparently larger than 250 000 daltons and contained at least one polypeptide of molecular weight 40 000. As described here, most of the variability in subunit pattern can be ascribed to the effects of proteolytic degradation during purification. By controlling this and other problems encountered during the preparation, a reproducible polypeptide pattern is observed for highly purified AcChR, and this allows study of its subunit structure.

Recently, in vitro phosphorylation of nicotinic acetylcholine receptors from electric organs of *T. californica* (Gordon et al., 1977) and *E. electricus* (Teichberg et al., 1977) has been reported. However, it is not known whether such phosphorylation of the receptor occurs in vivo. We have purified the acetylcholine receptor from the electric organ of *T. californica* in the presence and absence of a potent phosphatase inhibitor, and we present evidence for the presence of *O*-phosphoserine residues in the purified protein. In addition, we have detected other forms of O-substituted serine and threonine residues and determined that these are most likely joined in glycosidic linkage.

Experimental Section

Materials

T. californica was obtained locally in Pacific waters. Lyophilized venom of *Bungarus multicinctus* was obtained from Sigma Chemical Co. Radioiodine (as $Na^{125}I$) was obtained from New England Nuclear Co. Chromatographic resins were obtained from Pharmacia Co. DE-81 DEAE[1] disks and DEAE-cellulose were from Whatman, Ltd. All gel electrophoresis molecular weight standards were obtained from

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received July 11, 1978; revised manuscript received December 15, 1978.* Contribution No. 5822. Supported by U.S. Public Health Service Grants NS-10294 and GM-16424, by a grant from the Muscular Dystrophy Association of America, by a National Institutes of Health Postdoctoral Fellowship (R.L.V.), and by a National Institutes of Health Predoctoral Training Grant.

‡ Present address: Merck Sharp & Dohme Research Laboratories, Rahway, NJ.

[1] Abbreviations used: AcCh, acetylcholine; AcChE, acetylcholinesterase; AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; CBB, Coomassie Brilliant Blue; CM, carboxylmethyl; DEAE, diethylaminoethyl; DF, discriminant function; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; PAS, periodic acid–Schiff base reagent; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TBA, 2-thiobarbituric acid; AMAP, α-amino-β-methylaminopropionic acid; BSA, bovine serum albumin; BSM, bovine submaxillary gland mucin; P-Ser, *O*-phosphoserine; P-Thr, *O*-phosphothreonine.

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Sigma Co. except rabbit muscle aldolase (provided by Dr. Y. Chao). Reagents for gel electrophoresis were obtained from Bio-Rad Laboratories, and sodium dodecyl sulfate was Sequanal grade from Pierce Chemical Co.

Bovine serum albumin (Cohn fraction V), bovine submaxillary gland mucin (type I), and prolidase were purchased from Sigma Chemical Co., St. Louis, MO; phosphatidylserine was from Supelco Inc., Bellafonte, PA; potato acid phosphatase (A grade; lot 701120; 65 IU/mL at 25 °C) was from Calbiochem, La Jolla, CA; subtilisin (subtilopeptidase A) from *Bacillus subtilis* and amino acid arylamidase (aminopeptide M) and catalase were from Boehringer Mannheim Biochemicals, Indianapolis, IN. α-Amino-β-methylaminopropionic acid was synthesized from α-acetamidoacrylic acid according to Vega et al. (1968). The compound was recrystallized from aqueous ethanol to give a white powder of melting point 165–167 °C, and the structure was confirmed by ^{1}H NMR. All other chemicals were of the highest purity commercially available.

β-Casein was purified according to the method of Manson et al. (1971). This yielded 1.67 g of purified material from 1 L of raw milk and it was stored as a freeze-dried powder at 0 °C.

Methods

Preparation of ^{125}I-Labeled α-Bungarotoxin. α-Bungarotoxin was purified from lyophilized venom as described by Clark et al. (1972) and was labeled with ^{125}I by a modified procedure of Vogel et al. (1972). The monoiodo derivative was purified from other labeled species by CM-Sephadex chromatography and usually had specific activities of 1–3 Ci/nmol with biological activity indistinguishable from unlabeled toxin (Blanchard et al., 1979). The DEAE paper disk method of Schmidt & Raftery (1973a) was used for all AcChR assays.

Purification of AcCh Receptor. Electric organs of 800–900 g from fresh *T. californica* (or from organs frozen and kept at −90 °C) were cut into small pieces and homogenized with 500 mL of buffer containing 0.4 M NaCl, 10 mM sodium phosphate, and 3 mM EDTA, pH 7.4, for 1–2 min in a Waring Blendor at high speed. All purification steps were performed at 4 °C and all buffers additionally contained 0.02% sodium azide (w/v) and 10^{-4} M PMSF. After centrifugation of the crude homogenate at 5000 rpm for 10 min in a Sorvall GSA rotor, the low-speed supernatant was filtered through cheesecloth and centrifuged at 100000*g* for 45 min in a Beckman 35 rotor. The membrane pellet was resuspended in buffer A (10 mM sodium phosphate, pH 7.4, and 1 mM EDTA) and centrifuged as before. The membrane pellet was again resuspended in buffer A, and one-tenth volume of 20% Triton X-100 was added with stirring. After 15–30 min this solution was centrifuged at 100000*g* for 1 h. The clear supernatant was then applied to an affinity column (Schmidt & Raftery, 1972, 1973b; Vandlen et al., 1976). Elution of the AcChR was achieved by a linear gradient of NaCl from 0 to 100 mM in buffer B (10 mM sodium phosphate, pH 7.4, 0.5 mM EDTA, and 0.1% Triton X-100). AcChE was eluted with 1.0 M NaCl and the resin reequilibrated with buffer B.

Sodium Dodecyl Sulfate–Polyacrylamide Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed essentially as described by Fairbanks et al. (1971) in buffers containing 0.1% $NaDodSO_4$. The samples were denatured in 2–5% $NaDodSO_4$ with 5% β-mercaptoethanol for 3 min at 100 °C. After the gels were stained with 0.5% CBB and destained, they were scanned with a Gilford Linear Transport unit at 550 nm. The standard proteins used and their subunit molecular weights were the following: myosin (210000), phosphorylase A (93000), bovine serum albumin (68000), catalase (60000), rabbit muscle aldolase (40000), and myoglobin (17000).

Preparative scale $NaDodSO_4$ gel electrophoresis was utilized for isolation of AcChR subunits. A 6% acrylamide slab gel with dimensions of 13.5 × 9.5 × 0.6 cm or 30 × 9.5 × 0.6 cm was prepared with the same buffer system that was used for analytical $NaDodSO_4$ gels. A sample of 3–5 mg of purified AcChR protein in 1 mL of solution was denatured as described above and layered onto the slab gel. The sample was applied at 50 mA and electrophoresed at 100–150 mA for 3–5 h. Following this, five narrow vertical slices were cut from the gel, stained with CBB, and destained. The remainder of the slab was tightly wrapped and stored at 4 °C during the staining and destaining. The destained slices were reinserted into place and used to locate positions of the individual subunits. Horizontal strips were cut from the gel, chopped into 1-mm pieces, covered with 15–20 mL of 10 mM sodium phosphate buffer and 0.1% $NaDodSO_4$, and incubated at 37 °C with occasional stirring to elute the protein from the gel. After 24 h, the gel pieces were removed by filtration and the solutions lyophilized. The resultant powder was dissolved in 1 mL of phosphate buffer (10 mM, pH 7.4) and dialyzed extensively against this buffer to reduce the $NaDodSO_4$ concentration.

Nondenaturing Polyacrylamide Gel Electrophoresis. Nondenaturing gel electrophoresis with 4% acrylamide and 0.14% bis(acrylamide) gels was performed with the same buffer system that was used for $NaDodSO_4$ gels except that the $NaDodSO_4$ in all buffers was replaced with 0.1% sodium cholate or 0.1% Triton X-100. Staining and destaining were performed as described above.

Carbohydrate Determination. Quantitation of neutral sugars was achieved by ion-exchange chromatography in 75% ethanol (Heller & Raftery, 1976) or by the phenol–sulfuric acid reaction for carbohydrate (Dubois et al., 1956). For the latter method, a standard curve was constructed from a sample containing neutral sugars in their relative proportions observed for native AcChR, as determined by ion-exchange chromatography. Sialic acid was determined by the resorcinol (Svennerholm, 1957) and thiobarbituric methods (Warren, 1959). Amino sugars were determined during amino acid analysis following hydrolysis in 6 N HCl for 6 to 24 h.

Amino Acid Analysis. Protein samples (0.2 mg) were dialyzed for 24 h to remove salts and excess detergent before hydrolysis in vacuo with 6 N HCl at 110 °C for 24, 48, and 72 h. Hydrolysates were dried under vacuum and analyzed according to Spackman et al. (1958) on a Beckman Model 120C amino acid analyzer with norleucine as internal standard. Cysteine and methionine were determined after performic acid oxidation (Moore, 1963). Tryptophan was determined by the method of Liu & Chang (1971) after hydrolysis in 3 N *p*-toluenesulfonic acid in the presence of 0.02% indole or after hydrolysis in 6 N HCl with 2% mercaptoacetic acid (Matsubara & Sasaki, 1969).

Chemical Modification of Proteins. Proteins were oxidized by the method of Hirs (1956). Reduction and carbamidomethylation was carried out in 0.5 M Tris-HCl, pH 7.8, containing 1% Triton X-100 in the case of AcChR samples. These solutions (2 mg/mL in protein) were brought to 0.1 M in β-mercaptoethanol and incubated at room temperature for 3 h. Solid iodoacetamide was then added to a final concentration of 0.2 M, and the solution was stirred in the dark at room temperature for 0.5 h. Samples were extensively dialyzed against several changes of water at 4 °C lyophilized.

Table I: Acetylcholine Receptor Purification

	nmol of α-BuTx bound		nmol of α-BuTx mg of protein	
	protein (mg)	act. (nmol)	sp act. (nmol/mg)	recovery (%)
homogenate	5040	960	0.19	100
membrane suspension	2695	921	0.34	96
Triton extract	1404	813	0.58	85
affinity pool	78	750	9.6	78

The procedure for methylamine reaction was adapted from that described by Kolesnikova et al. (1974). Protein samples (1–5 mg) were taken up in 1 mL of 1 N NaOH and 1 M CH_3NH_2. The solutions were stirred at 37 °C for 8 h and then neutralized (to litmus) by addition of 6 N HCl. Samples were extensively dialyzed against several changes of water at 4 °C before hydrolysis.

Reaction of proteins with Na_2SO_3 was essentially by the method of Weber & Winzler (1970). Proteins were taken up in 0.25 N NaOH and 0.5 M Na_2SO_3 (freshly prepared) to a concentration of 5 mg/mL. The solutions were incubated at room temperature for 70 h, neutralized (to litmus) by addition of 6 N HCl, and dialyzed against water at 4 °C before acid hydrolysis.

Sucrose Gradient Centrifugation. Linear gradients from 5 to 20% sucrose (w/v) were prepared in 10 mM sodium phosphate, 50 mM NaCl, and 0.02% sodium azide, pH 7.4, with no detergent, with 0.05 or 1% Triton X-100, or with 2% sodium cholate. Gradients (12 mL) were prepared on a Beckman Gradient Maker and kept at 4 °C for 4 h. Receptor solutions containing 0.5 mg of protein were layered on the gradient and centrifuged for 16 h at 40000 rpm in a Beckman SW-41 rotor. Fractions of 0.6 mL were collected and analyzed for [^{125}I]-α-BuTx binding activity and for protein.

Other Assays. Acetylcholinesterase (EC 3.1.1.7) activity was assayed by the method of Ellman et al. (1961). Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard, or by total amino acid analysis on a Beckman Model 120C analyzer with norleucine as internal standard.

Lipid peroxidation of AcChR-enriched membrane fragments (Duguid & Raftery, 1973) and of purified AcChR was followed by the measurement of 2-thiobarbituric acid reactive substances (e.g., malonaldehyde) as described by Bidlack & Tappel (1973). Quantitation of the free malonaldehyde concentration was done by using a molar extinction coefficient of 1.58×10^5 for the TBA–malonaldehyde complex (Sawicki et al., 1963). Malonaldehyde cross-linked AcChR was prepared by incubation of purified receptor with various concentrations of malonaldehyde bis(dimethylacetal) for 3–8 h at room temperature.

Results

Purification of AcChR. Quantitative data on purification of the AcChR from *T. californica* electric organ of 630 g wet weight are shown in Table I. The pooled receptor fractions contained 78% of the toxin-binding sites of the crude homogenate and underwent a 50-fold purification factor from the homogenate or a 17-fold purification of the Triton X-100 extract, this latter factor being similar to that first reported for AcChR purification by affinity methods (Schmidt & Raftery, 1972) by using *N. entemedor* electroplax. Generally, additional purification steps (DEAE-cellulose chromatography and sucrose gradient centrifugation) produced little or no increase in specific activity. Routinely, specific activities of

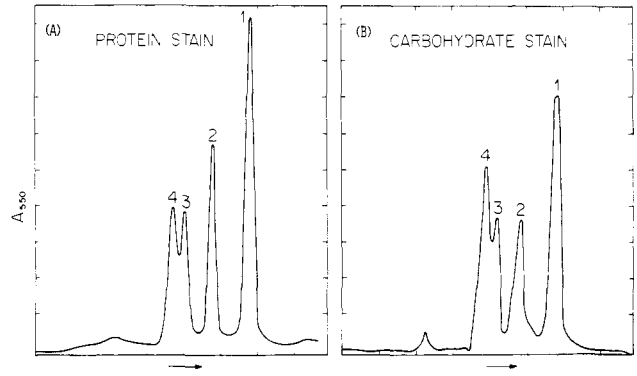

FIGURE 1: (A) Sodium dodecyl sulfate gel electrophoresis of purified AcChR on 6.5% acrylamide gels with 0.1% $NaDodSO_4$ run according to Fairbanks et al. (1971). After staining with Coomassie Blue and destaining, we scanned the gel at 550 nm with a Gilford Linear Transport unit. The direction of electrophoretic migration is from left to right. The four labeled bands, 1–4, correspond to molecular weights of 40, 50, 60, and 65×10^3, respectively. (B) Staining with the periodic acid–Schiff base reagent was performed as described by Fairbanks et al. (1971).

8–9 nmol/mg of protein were obtained, corresponding to 1 toxin molecule bound per 110000–125000 daltons of AcChR protein. These values are based on protein determinations when the Lowry method was used, which we have previously shown to yield values equivalent to 1.08-fold that obtained by amino acid analysis (Martinez-Carrion et al., 1976). This factor is roughly compensated for by virtue of the percentage of carbohydrate residues which are present (discussed later). There was a very low contamination of the pooled AcChR preparation by AcChE (0.01% by weight).

Subunit Composition of Purified AcChR. Following affinity chromatography, $NaDodSO_4$–polyacrylamide gel electrophoresis revealed the presence of four types of polypeptide. The apparent molecular weights and standard deviations (in parentheses) of the four subunits, labeled 1, 2, 3, and 4 in Figure 1A, were determined by reference to standard proteins in 5–7% acrylamide gels run according to Fairbanks et al. (1971) to be 40000 (800), 50000 (1400), 60000 (1500), and 65000 (1400). All four bands also stained positively for carbohydrate with the periodic acid–Schiff base stain (Figure 1B).

The four bands on $NaDodSO_4$–polyacrylamide gel electrophoresis were characteristic of receptor preparations of high specific activity made under carefully controlled conditions. Under less than optimal conditions, the number and apparent molecular weights of the subunits varied substantially, such variability in pattern being due to at least two major factors: proteolytic degradation and subunit cross-linking. Subunits 2, 3, and 4 were especially sensitive to proteolytic degradation by endogenous enzymes which were released or activated upon membrane solubilization. This enzyme activity was apparently dependent upon the presence of divalent cations and the inclusion of EDTA throughout the preparation generally inhibited degradation. DFP or PMSF were useful but less effective than EDTA. The rate of proteolysis was temperature and concentration dependent, and receptor solutions were therefore kept at 4 °C at all times and in dilute solution (less than 0.5 mg/mL of protein). The first effect of degradation observed by $NaDodSO_4$–polyacrylamide gel electrophoresis was the loss of subunits 3 and 4. Further degradation produced a number of peptides with molecular weights in the range of 28000–40000.

CBB staining bands corresponding to molecular weights greater than 90000 were sometimes observed. These higher

Table II: Determination of Malonaldehyde in AcCh Receptor Solutions[a]

sample	malonaldehyde (μM)
membrane suspension	
1 h	<0.01
8 h	<0.01
24 h	0.26
1 week	0.9
1 month	4.0
1% Triton extract of membrane suspension after 24 h[b]	0.26
purified AcCh receptor 1 week	<0.01

[a] Measured by the method of Bidlack & Tappal (1973). Samples were stored at 4 °C without antioxidant or use of argon for the indicated times before assay. Limits of detection were 0.01 μM. [b] Extract was prepared immediately after homogenization before any malonaldehyde was detectable.

molecular weight bands were most frequently seen in preparations from older animals, from organs stored at −20 °C or warmer for any length of time, or after long-term storage of purified AcChR solutions. To test the possibility that such species were due to malonaldehyde or other reactive dialdehyde products of lipid peroxidation (Bidlack & Tappel, 1973), the free malonaldehyde concentration of several preparations was measured. The results of one such study are given in Table II. In general, those preparations in which malonaldehyde was detectable yielded receptor samples that contained a considerable amount of higher molecular weight bands on $NaDodSO_4$–polyacrylamide gel electrophoresis. In addition, the yield of AcChR extracted from the electroplax membranes by neutral detergents was reduced, consistent with the occurrence of protein–protein cross-linking (Minssen & Munkres, 1973). Similar high molecular weight bands were produced when the receptor was incubated with malonaldehyde bis(dimethylacetal) before $NaDodSO_4$–polyacrylamide gel electrophoresis. In general, lipid autoxidation was controlled by working at low temperatures, by the inclusion of sterically hindered phenols, such as 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol, and by use of argon-saturated buffers.

Amino Acid and Carbohydrate Analyses of Purified AcChR and Its Subunits. The results of amino acid analysis are given in Table III. The mole percent values for each amino acid were the average of seven determinations and were corrected for the degradation of serine and threonine by extrapolation to zero time of the values obtained after 24-, 48-, and 72-h hydrolysis. Values for valine, isoleucine, and leucine were obtained from 48- and 72-h hydrolysis results. The standard deviation for each value was generally less than ±1.0%. The partial specific volume of the detergent-free receptor, calculated from the amino acid and carbohydrate compositions (Gibbons, 1966), was 0.74 mL/g.

The presence of carbohydrate moieties on the purified receptor has been demonstrated by several methods: (1) on $NaDodSO_4$ gels (Figure 1B) the periodic acid–Schiff base method revealed that each subunit carried carbohydrate; (2) amino acid analyses of the purified receptor and of its individual subunits indicated the presence of glucosamine (Table III); (3) hydrolysis of purified receptor in 0.1 N HCl for 2 h, followed by quantitative analysis by chromatography on Aminex A5 resin, revealed the presence of the neutral sugars mannose, glucose, and galactose in the approximate mole ratio of 10:1:2, respectively (Figure 2). Neutral sugars were present at the level of 2.7 ± 0.3% by weight. Additional assay of neutral sugar content by the phenol–sulfuric acid method gave a value of 194 nmol of carbohydrate/mg or 3.5% by weight.

Table III: Amino Acid and Carbohydrate Compositions of the Acetylcholine Receptor

components	purified receptor (mol %)[a]	subunits (mol %)[b]				subunits (residues/subunit)[c]			
		1	2	3	4	1	2	3	4
amino acid									
Asp	11.4	10.4	11.1	11.8	12.1	37	49	61	68
Thr	6.2	7.0	6.2	5.3	5.3	25	27	28	30
Ser	6.3	7.5	7.6	7.2	7.4	27	34	38	42
Glu	10.3	9.7	11.2	11.2	10.7	35	50	58	60
Pro	5.7	6.0	6.9	6.3	6.4	21	31	33	36
Gly	4.9	5.4	6.5	6.5	6.3	19	28	34	35
Ala	5.2	4.4	6.2	5.5	6.3	16	27	29	30
Cys	1.3	0.9	0.8	1.2	1.0	3	4	6	6
Val	7.5	7.4	7.1	6.1	6.0	26	31	32	34
Met	1.9	2.8	1.5	1.4	1.7	10	7	7	10
Ile	7.6	7.8	5.9	6.8	6.7	28	26	35	38
Leu	9.3	8.5	9.1	9.5	9.4	30	40	50	53
Tyr	3.8	3.7	3.3	3.6	3.6	13	15	19	20
Phe	5.0	4.5	4.1	4.4	4.4	16	18	23	25
His	2.6	3.1	2.2	2.2	2.2	11	10	11	12
Lys	5.7	5.9	5.2	5.5	5.2	21	23	29	29
Arg	4.1	3.8	4.1	4.1	4.1	14	19	21	23
Trp	1.2	1.2	1.0	1.6	2.2	4	4	8	12
carbohydrate[d]									
mannose	1.7								
glucose	0.17								
galactose	0.33								
N-acetylneuraminic acid	0.9	0.56	trace	trace	0.7	2			3
N-acetylneuraminic acid	~0.1								

[a] Determined as described under Methods. The composition of the purified receptor is the average of seven different determinations and includes corrections for time-dependent destruction or appearance of residues as described in the text. The standard deviation for each residue was less than 0.15 mol %. [b] The subunit compositions are the average of four 24-h hydrolysis determinations. Subunit cysteine and tryptophan values are from a single determination. The average standard deviation for the subunit values is 0.3 mol %/residue. [c] Values computed by assuming molecular weights of 40 000, 50 000, 60 000, and 65 000 for subunits 1–4, respectively, before correction for the carbohydrate contribution to the molecular weights. [d] Expressed as residues of carbohydrate per 100 amino acid residues.

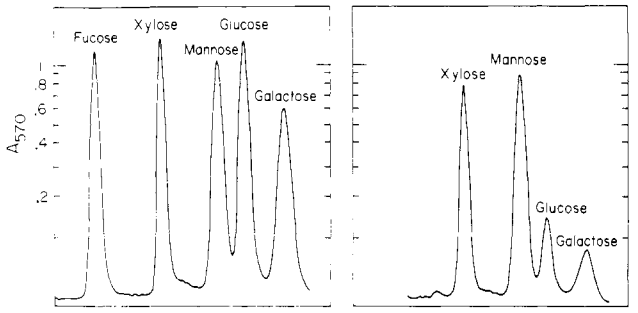

FIGURE 2: Determination of neutral sugars by ion-exchange chromatography (Heller & Raftery, 1976). (Left) Standards (20 nmol each) were applied to an Aminex A5 resin equilibrated in 75% ethanol. The column was attached to a Beckman Model 120C analyzer and was developed with 75% (w/w) ethanol. Quantitation of eluted sugars was at 570 nm after reaction with tetrazolium blue (Mopper & Degens, 1972). (Right) AcCh receptor samples (0.2–0.5 mg) were dialyzed against H_2O for 24 h and then hydrolyzed under reduced pressure with 1 N HCl for 1–2 h. Xylose (20 nmol) was included as internal standard. After hydrolysis, the HCl was removed under vacuum, and the samples were dissolved in 75% ethanol and centrifuged to remove protein before application to the resin.

Table IV: Hydrophobicities and Discrimination Function Values for Several Membrane Proteins[a]

protein	$H\Phi_{av}$ (cal)	DF
AcCh receptor[b]	1184	0.37
subunit 1	1182	0.38
subunit 2	1119	0.29
subunit 3	1140	0.31
subunit 4	1140	0.31
AcCh esterase[c]	1059	0.25
cytochrome *c* oxidase[d]	1185	0.45
subunit 1	1185	0.51
subunit 2	1011	0.25
subunit 4	1033	0.16
subunit 6	1029	0.14
(Na^+-K^+)ATPase[e]	1272	0.42
large subunit	1248	0.42
small subunit	1309	0.44
rhodopsin (bovine)[f]	1208	0.51
purple membrane protein[g]	1247	0.56
nonmembrane proteins[h]	996 ± 98	0.16 ± 0.11
membrane proteins[h]	1197 ± 98	0.52 ± 0.11

[a] Average hydrophobicities ($H\Phi_{av}$) are computed from published amino acid composition data (omitting tryptophan) according to Bigelow (1967) and expressed in calories. Discriminant function values are computed as described by Barrantes (1975) as DF + (−0.345) [Σ(charged residues)/Σ(hydrophobic residues)] + 0.0006($H\Phi_{av}$). [b] This study. [c] Taylor et al., 1974. [d] Poyton & Schatz, 1975. [e] Kyte, 1972. [f] DeGrip et al., 1973. [g] Oesterhelt & Stoeckenius, 1971. [h] Barrantes, 1975.

Further support for the notion that the receptor is a glycoprotein stems from preliminary experiments indicating that each receptor molecule carries a small number of sialic acid groups.

A calculation of the polarity of the purified receptor and of its constituent subunits by the method of Capaldi & Vanderkooi (1972) indicated that the receptor was not significantly different from typical water-soluble proteins. In general, however, calculation of the average hydrophobicity (Bigelow, 1967) for a protein, based on the kind and distribution of amino acids and their relative hydrophobicities (Tanford, 1962), appeared to be a more accurate index of the polarity. The results of such a calculation for the receptor and its subunits and for several other membrane proteins are given in Table IV, with average hydrophobicity values ($H\Phi_{av}$) for membrane and nonmembrane proteins being taken from Barrantes (1973). These calculations indicate that the receptor

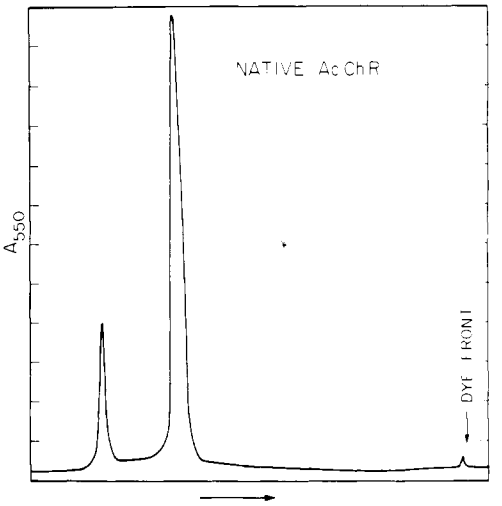

FIGURE 3: Gel electrophoresis of purified AcChR on 4% acrylamide and 0.14% bis(acrylamide) gels with 0.1% sodium cholate. Receptor protein (5–15 μg) was applied in a 25-μL volume (including 0.1% cholate, glycerol, and bromophenol blue) and electrophoresed at 2–3 mA/gel for 5 h. Reservoir buffers contained 0.1 M Tris–acetate, pH 7.4, with 0.1% cholate. Staining and destaining were performed as described under Methods.

and each of its subunits, none of which are soluble without detergent, can best be classified as intrinsic membrane for hydrophobic proteins. On the other hand, the AcChE from the same *Torpedo* species (Taylor et al., 1974), which was solubilized without the use of detergents, gave an average hydrophobicity more typical of nonmembrane or water-soluble proteins.

Further refinement of this type of analysis has been described by Barrantes (1975). This has been extended to include compositions of the subunits of several membrane proteins for which calculated values are given in Table IV. By use of such criteria, the AcChR is a hydrophobic protein composed of subunits with generally similar hydrophobic parameters. On the other hand, those subunits of cytochrome *c* oxidase from yeast for which amino acid compositions have been reported (Poyton & Schatz, 1975) varied from hydrophilic to hydrophobic depending on the subunit (Table IV). No such variation was observed among the AcChR subunits.

Sucrose Velocity Gradient Centrifugation. Samples of the purified receptor protein were centrifuged in a 5–20% sucrose gradient containing 10 mM sodium phosphate, pH 7.4, and 0.1% Triton X-100. It was found that the receptor was resolved into two distinct components, centered around 9.5 S and 13 S (Raftery et al., 1972), the latter corresponding approximately to a dimer of the 9.5S peak. These two components were composed of identical subunits as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions. Also, specific activities, as judged by the amount of $[^{125}I]$-α-BuTx bound per mg of protein, were identical for the two peaks (9–10 nmol of α-BuTx/mg of protein), and the amino acid compositions of each were identical within experimental error. The relative amounts of protein in each peak varied depending on the preparation and the conditions used.

Gel Electrophoresis of Purified AcChR under Nondenaturing Conditions. Two components (Figure 3) were observed in 4% polyacrylamide gels in 0.1% sodium cholate with running buffers of pH 7.4, 8.2, or 8.9. Protein from each peak obtained from sucrose gradients (9.4 S and 13.7 S) was electrophoresed on these gels and the faster migrating electrophoretic band was found to correspond to the 9.5S sucrose gradient peak

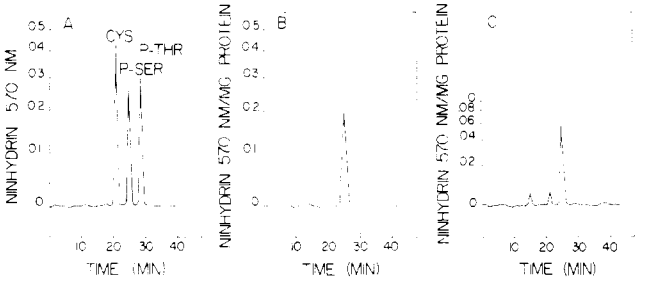

FIGURE 4: Resolution of acidic amino acids with 0.2 M sodium citrate, pH 1.7. (A) Mixture of standard cysteic acid, phosphoserine, and phosphothreonine. (B) Mild acid hydrolysate of β-casein. (C) Mild acid hydrolysate of purified AcChR. Typically mild acid hydrolysates equivalent to 1 mg of AcChR or more were analyzed for P-Ser. The minimum detection limit for P-Ser was below one residue per AcChR with this quantity of material.

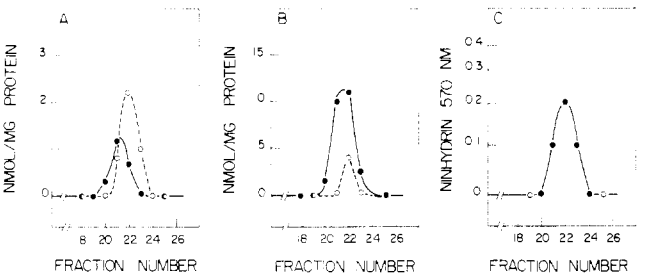

FIGURE 5: Hydrolysis of fractions containing phosphoserine. Serine (●) and glutamic acid (○) contents of fractions surrounding and including phosphoserine following isolation and hydrolysis in 6 N HCl. (A) AcChR. (B) β-Casein. (C) Control using phosphoserine.

while the slower band corresponded to the 13S peak. Thus, the 13S peak appeared to be a stable molecular entity rather than an associating–dissociating species or a detergent-induced artifact.

Analysis of O-Phosphoserine Residues. Hydrolysis of β-casein was found to give maximal recovery of P-Ser when carried out in 4 N HCl at 110 °C for 6 h. When purified AcChR was hydrolyzed under these conditions and analyzed for amino acids as described under Methods, a peak corresponding to that of standard P-Ser was observed. Conceivably, other amino acids, such as cysteic acid derived from cysteine or cystine during hydrolysis, could also be present. Therefore, the buffer system described by Cohen-Solal & Glimcher (1977) was adopted to further resolve such components. Upon such analysis a peak corresponding to P-Ser was observed in addition to two other components subsequently identified as cysteic acid and acidic peptides (Figure 4C). It is worthwhile to note here that P-Thr was absent from the profile.

To confirm the identity and examine the homogeneity of the peak corresponding to P-Ser when the pH 1.7 system was used, fractions were collected from a Dowex-50 column chromatograph of a mild acid hydrolysate of the AcChR. Such fractions were subjected to complete acid hydrolysis. A profile of the resulting amino acids of the peak fractions is shown in Figure 5.

Following complete acid hydrolysis, serine was obtained from those AcChR fractions found at the elution position of P-Ser upon mild hydrolysis. The peak fraction corresponding to P-Ser found to give serine upon complete acid hydrolysis was free of any other amino acids. Several fractions following the peak, however, were found to contain glutamic acid following complete acid hydrolysis. This was also observed for β-casein. The glutamic acid (Figure 5) was possibly derived from small peptides of *O*-phosphoserine and glutamic acid resulting from incomplete hydrolysis of the protein, as has been shown for β-casein (Levene & Hill, 1933) where phosphoserine residues are closely associated with glutamic acid and glutamine residues (Ribadeau-Dumas et al., 1972). However, we cannot rule out other possibilities such as pyroglutamic acid, which should not bind to the resin during the first chromatography and would therefore be a likely contaminant. It would be converted, at least partially, to glutamic acid during the second acid hydrolysis step. Table V summarizes the analyses of purified AcChR for P-Ser content. Purified AcChR subunits were also analyzed, and it was shown (Table VI) that P-Ser was a component of all four polypeptides, with the 65 000-dalton species containing the highest proportion of the modified amino acid.

Table V: Recovery of Phosphoserine from Mild Acid Hydrolysis of β-Casein and AcChR. Estimation of the Number of Residues per AcChR Molecule

protein	phosphoserine found at pH 1.7[a]	no. of P-Ser/ molecule
β-casein I	0.76 (31.4%)	5.0[b]
β-casein II	0.79 (32.5%)	5.0
AcChR I	0.084	5.9
AcChR II	0.079	5.6
AcChR III	0.096	6.8
AcChR IV	0.087	6.1

[a] Values given in residues per 100 amino acid residues. [b] The number of phosphoserine residues given for β-casein is from Ribadeau-Dumas et al. (1972). The number of phosphoserine residues per AcChR was estimated by assuming a molecular weight of 270 000 and that the percent recovery of phosphoserine for the AcChR hydrolysate was equal to the average of that of β-casein. Roman numerals correspond to different experiments. Acetylcholine receptor purified from organs of different *Torpedo* was used in each of the four experiments. AcChR III and IV included 100 mM NaF throughout the preparation to inhibit phosphotases. Purification of AcChR in buffer containing NaF (10 mM Na_3PO_4, pH 7.4, 100 mM NaF, 300 mM NaCl, 5 mM EDTA, and 0.02% NaN_3) was carried out with an α-BuTx–Sepharose 2B resin. The resin, prepared according to a previously described procedure for cyanogen bromide activated agarose gels (Porath et al., 1973), contained 1.7×10^{-8} mol of α-BuTx binding site/g (dry weight) of resin. Purification of AcChR with the toxin resin was achieved by a batch method with an amount of resin equivalent to a twofold excess of the number of α-BuTx binding sites as compared to that of the AcChR of the Triton extract. AcChR extracted in 2% Triton was allowed to bind to α-BuTx resin by incubating at 4 °C overnight. Unbound material was removed by washing extensively on a sintered funnel with the same NaF buffer that was used for preparation. Bound AcChR was eluted from the resin by incubating in the same buffer containing 6 M urea and 1% $NaDodSO_4$ at 4 °C overnight. The mixture was then centrifuged at 5000 rpm for 15 min, and the supernatant was electrodialyzed through 20 mA of current in 2 L of 50 mM NH_4HCO_3 overnight to remove $NaDodSO_4$, followed by further dialysis in H_2O. The purified receptor was lyophilized before it was used for $NaDodSO_4$–polyacrylamide gel electrophoresis and amino acid analyses.

Table VI: Phosphoserine Content of AcChR Subunits

AcChR subunit	phosphoserine found at pH 1.7[a]	no. of P-Ser/ subunit
40 000	0.072 ± 0.007	0.75 ± 0.07
50 000	0.050 ± 0.011	0.65 ± 0.16
60 000	0.137 ± 0.006	2.18 ± 0.08
65 000	0.288 ± 0.021	4.90 ± 0.36

[a] Values given in residues per 100 amino acid residues. AcChR subunits were purified by preparative slab gel electrophoresis from purified AcChR as described under Methods. Values were obtained from two subunit preparations by using organs from different *Torpedo*. The number of P-Ser residues per subunit was estimated by assuming that the percent of recovery of phosphoserine for the subunit hydrolysate was equal to the average of that of β-casein.

Scheme I

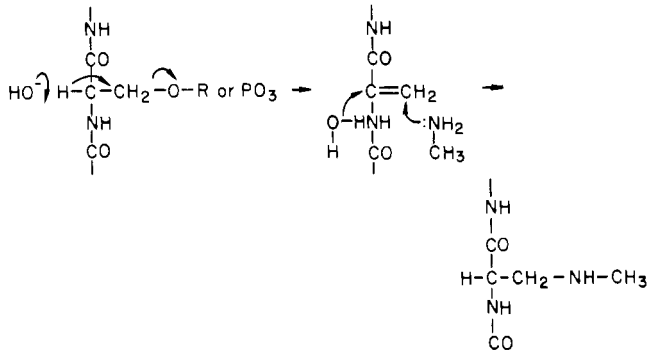

Table VII: Methylamine Reaction Standards

protein	O-substituted serine	cysteine	AMAP[a]	N
ox. BSA		6.30[b]	0.48 ± 0.03	2
β-casein	phosphoserine (2.4)[c]	0	2.4 ± 0.03	4
ox. BSM	glycosidylserine (2.8)[d]	1.4[e]	0.8	1
r.c. BSM	glycosidylserine (2.8)[d]	1.4[e]	1.2 ± 0.01	2

[a] Values in mole percent with standard deviations. ox. = oxidized; r.c. = reduced and carbamidomethylated; N = number of experiments. [b] Cysteine in hydrolysis of unmodified protein. [c] Ribadeau-Dumas et al., 1972. [d] Determined by sulfite reaction on r.c. material and calculated from dry weight. [e] Cysteine in hydrolysis of unmodified protein, calculated from dry weight.

Methylamine Modification. Purified AcChR was treated with CH_3NH_2 in base, a reaction which has been reported to convert phosphoserine residues to the acid stable α-amino-β-methylaminopropionic acid (AMAP) (Kolesnikova et al., 1974). Amino acid analysis at pH 1.7 of the modified AcChR revealed complete elimination of the peak corresponding to P-Ser; the identical result was observed for β-casein.

The CH_3NH_2 reaction was also used to quantitatively determine the amount of O-substituted serine residues. This reaction involves β-elimination in base to produce a dehydroalanine intermediate, followed by nucleophilic addition of CH_3NH_2 as designated in Scheme I. The product formed, AMAP, is stable to acid hydrolysis and can be separated on an amino acid analyzer (being eluted between histidine and ammonia) and was quantitated by comparison with a chemically synthesized standard.

Since cysteine can react, under the experimental conditions used, to give the same product as phosphoserine, proteins were either oxidized or reduced and carbamidomethylated before reaction. The results with standards are given in Table VII. By oxidation the reaction was minimized to yield an amount of AMAP equivalent to only 8% of the cysteine originally present in BSA. Quantitative reaction was shown with phosphoserine residues in β-casein, while fractional amounts of glycosylated serine residues in BSM were recovered.

The results of reaction of CH_3NH_2 in base with purified AcChR and its subunits are summarized in Table VIII. The receptor and all its subunits, with the possible exception of the 50 000-dalton polypeptide, were found to yield AMAP as product under these conditions.

Since phosphatidylserine is prevalent in *T. californica* membranes (Michaelson & Raftery, 1974), the reaction could conceivably have occurred with some tightly bound phosphatidylserine which had copurified with the AcChR. However, a control reaction with free phosphatidylserine (1000 nmol) showed no AMAP (detection limit ≃1 nmol).

Sulfite Reaction. In order to determine the extent of substituted threonine residues in the purified AcChR, reduced and carbamidomethylated samples were reacted in base with

Table VIII: Methylamine Reaction with AcChR

AcChR prepn	cysteine[b]	AMAP[a]	no. of AMAP/molecule	N
ox. whole	1.3	0.98 ± 0.20	22.1 ± 4.5	8
r.c. whole	1.3	1.1 ± 0.11	24.8 ± 2.5	2
ox. 65 000	1.0	0.67 ± 0.18	4.0 ± 1.1	2
ox. 60 000	1.2	0.72 ± 0.36	3.6 ± 1.8	3
ox. 50 000	0.8	0.22 ± 0.20	1.1 ± 1.0	2
ox. 40 000	0.9	0.64 ± 0.04	2.1 ± 0.1	2

[a] Values in mole percent with standard deviations. ox. = oxidized; r.c. = reduced and carbamidomethylated; N = number of experiments. [b] Cysteine present in unmodified protein.

Table IX: Sulfite Reaction for BSM and AcChR[a]

protein	Thr	Ser	decrease in Ser + Thr	cysteic acid + 2-amino-3-sulfonyl-butyric acid
BSM	7.7	8.2		
BSM + SO_3	4.6	5.4	5.7	6.3
AcChR	6.2	6.3		
AcChR + SO_3	5.4	4.9	3.2	2.3

[a] Values in mole percent. All samples were reduced and carbamidomethylated prior to reaction. Values for BSM calculated from dry weight. The color value of 2-amino-3-sulfonylbutyric acid was assumed to be the same as that of cysteic acid.

Table X: Sulfite Reaction. Effect on Carbohydrate Content of AcChR[a]

protein	mannose	glucose	glucosamine	galactose	total sugar loss
AcChR	1.70	0.15	1.25	0.27	
+SO_3	1.18	0.12	0.76	0.17	1.14
+SO_3	1.27	0.13	0.73	0.18	1.06

[a] Values given in residues of carbohydrate per 100 amino acid residues.

Table XI: O-Substitution of Serine and Threonine in AcChR[a]

O-substituted serine	O-substituted threonine	O-substituted Ser + Thr	P-Ser	base-labile sugar
22[b]	23[c]	38[d]	7[e]	24[d]

[a] Values given in residues per molecule by assuming a molecular weight for AcChR of 270 000. [b] Determined by methylamine reaction. [c] Determined by sodium borohydride reaction. [d] Determined by sulfite reaction. [e] Direct amino acid analysis.

Na_2SO_3. This reaction gives cysteic acid from O-substituted serine residues and 2-amino-3-sulfonylbutyric acid from O-substituted threonine residues, which comigrate when analyzed at both pH 3.2 and 1.8 (Simpson et al., 1972). Results of one such experiment are summarized in Table IX. As can be seen, the decrease in serine and threonine content in both BSM and AcChR roughly corresponds to the amount of cysteic acid and 2-amino-3-sulfonylbutyric acid produced. AcChR samples that had been treated in base with Na_2SO_3 were further analyzed for loss of neutral sugars to determine whether glycosidic bonds had been cleaved. Results from such analyses are given in Table X.

Reaction with Sodium Borohydride. Direct measurement of the number of O-substituted threonine residues in the purified AcChR was made by reaction with sodium borohydride following base catalyzed β-elimination. This reaction consists of elimination to yield dehydrobutyric acid, which is

then reduced to yield α-amino-*n*-butyric acid. The corresponding reaction sequence with serine yields alanine. The α-amino-*n*-butyric acid was separated on an amino acid analyzer and quantitated by comparison with a standard (Heller & Raftery, 1976). This reaction with purified AcChR yielded 1.0 × 0.15 mol % of α-amino-*n*-butyric acid (three experiments).

Discussion

The purified AcChR from *T. californica*, when isolated under conditions designed to minimize proteolytic degradation, existed as monomeric and dimeric forms. Each was a homogeneous protein species, as determined by gel electrophoresis and sucrose gradient centrifugation, and both species displayed identical subunit patterns on $NaDodSO_4$ electrophoresis. Four different subunits were observed with apparent molecular weights of 40, 50, 60, and 65 × 10^3 with the 40 000-dalton subunit being the major component.

From the results of the present study, the substantial variation in the number of subunits reported in the literature and their molecular weights may be ascribed to the effects of proteolytic degradation. When steps were taken to preclude proteolytic degradation, namely, the inclusion of millimolar concentrations of EDTA throughout the preparation, a well-resolved pattern of four polypeptide bands for *T. californica* (Figure 2) was observed (Raftery et al., 1974; Weill et al., 1974; Reed et al., 1975; Raftery et al., 1975; Karlin et al., 1975; Vandlen et al., 1976). In fact, the same four polypeptide species were found in several *Torpedo* species and in *Narcine brasiliensis* preparations when the AcChR was purified in the manner described here (Deutsch and Raftery, unpublished experiments). The AcChR from *E. electricus*, when isolated under these conditions, was composed of three different polypeptide bands (Weill et al., 1974; Deutsch and Raftery, unpublished experiments).

The four subunits did not appear to result from degradation from a larger polypeptide. Recently, we have demonstrated that antibodies produced against each subunit do not cross-react with the other subunits (Claudio & Raftery, 1977). The four subunits were observed in $NaDodSO_4$ gel electrophoresis profiles of purified membrane fragments or in homogenates of electric organs (Duguid & Raftery, 1973; Raftery et al., 1974; Reed et al., 1975) when the procedure of Fairbanks et al. (1971) was used. In the present study the same pattern was observed when denaturation was carried out at room temperature instead of 100 °C and/or in the presence of PMSF or DFP.

Sucrose gradient centrifugation of purified, undegraded receptor from *T. californica* revealed the presence of two bands during gel electrophoresis and of two receptor species with S values of 9.5 and 13, similar to those observed for detergent-solubilized *T. californica* AcChR (Raftery et al., 1972). The slower moving component on polyacrylamide gel electrophoresis corresponded to the 13S component on sucrose gradients and probably represented a dimeric form. These two species were identical with respect to their specific activities, amino acid compositions, subunit patterns on $NaDodSO_4$ gel electrophoresis, and [^{125}I]-α-BuTx binding kinetics (Lee and Raftery, unpublished experiments).

Recently Sobel et al. (1977) have claimed the purification of an AcChR preparation from *T. marmorata* electroplax with PMSF as an inhibitor of proteolysis. We have found this reagent to be vastly inferior to EDTA in this respect. This is not surprising in view of recent findings (James, 1978) concerning the short half-life of PMSF. Surprisingly, despite the reported differences in subunit composition, the S values reported for most preparations indicate a major component of S ~9. This is most likely explained as being the result of proteolytic nicking of the constituent subunits which do not, however, dissociate as small fragments until the complexes are denatured by $NaDodSO_4$. Such events would account for simplified $NaDodSO_4$ gel patterns observed in some preparations (Sobel et al., 1977).

The molecular weight of the receptor from *Torpedo* has been estimated by several methods. Gel filtration studies performed in the presence of Triton X-100 suggested a molecular weight around 500 000 (Raftery et al., 1972) or 450 000 (Heilbronn, 1975). On the other hand, sedimentation coefficients determined in sucrose gradients of detergent-dispersed unpurified *Torpedo* AcChR were similar to those of proteins of about 250 000 daltons (Raftery et al., 1972, 1974; Meunier et al., 1972). The apparent differences in molecular weight as determined by these methods have been a subject of discussion (Meunier et al., 1972; Tanford et al., 1974) and may be due to the effects of bound detergent or to an asymmetrical protein shape or to a combination of these effects. Using a different method, namely, membrane osmometry, Martinez-Carrion et al. (1976) determined a value of 270 000 ± 30 000. The molecular weight of the receptor can be determined by sedimentation equilibrium and sedimentation velocity measurements (Tanford et al., 1974); such measurements for receptor from aged *T. marmorata* electroplax indicated molecular weights of 5 × 10^5 to 1 × 10^6 (Carroll et al., 1973). Recently, a minimum molecular weight of 330 000 for *Torpedo* receptor was obtained from sedimentation equilibrium experiments (Edelstein et al., 1975), in close agreement with the value reported for solubilized eel receptor when similar methods (Meunier et al., 1972) were used. More recently still, a value of 250 000 daltons has been reported by using a similar method (Reynolds & Karlin, 1978). Using still a different method, namely, chemical cross-linking, Hucho et al. (1978) obtained a value for the molecular weight of *T. californica* AcChR of 270 000. Thus, at present it seems most reasonable that the minimal molecular weight of *Torpedo* AcChR is in the range of 250 000–270 000, as estimated by these different methods.

The average hydrophobicity values for the receptor and its subunits or the more sensitive discriminant function analysis of Barrantes (1975) clearly showed the more nonpolar nature of this protein compared with typical globular proteins. An extension of the discriminant function analysis to the individual subunits of some other membrane proteins revealed some interesting correlations with known chemical reactivities of the same subunits. In the case of cytochrome *c* oxidase purified from Baker's yeast (Poyton & Schatz, 1975), subunit 1 of molecular weight 40 000 was very similar to integral membrane proteins while the other subunits were considerably more polar in composition. Poyton and Schatz's suggestion that subunit 1 might be buried in the lipid phase correlated well with these analyses. Furthermore, chemical labeling studies indicated that subunit 1 was almost completely inaccessible while subunit 2, with a somewhat greater polarity, was only partially accessible to protein-modifying reagents. On the other hand, subunits 4 and 6 were readily modified and were the most polar. From these results one could speculate that such calculations for the subunits of a protein might reveal some information concerning the membrane-associated topography of the molecule and this would be especially valuable for multisubunit protein systems to gain possible insight into their location and function.

A calculation of the discriminant function values from the reported compositions of the acetylcholine receptor from different preparations has been tabulated by Barrantes (1975); values ranging between 0.253 and 0.481 were obtained. This wide variation was probably due to differences in the purity and extent of proteolytic degradation of the various preparations; indeed, a comparative study of the compositions of AcCh receptors from various sources, purified under conditions which limited proteolytic degradation, revealed that all of these receptors had similar discriminant function values (Deutsch and Raftery, unpublished experiments).

The observed discriminant function value of 0.37 for the AcChR was intermediate between such values for membrane and nonmembrane proteins, indicating that a substantial part of the receptor molecule probably extends beyond the domain of the membrane or that the increased polarity might be due in part to the requirements for a hydrophilic channel through the receptor protein. There is some evidence that the receptor protein does extend a substantial distance outside the membrane; X-ray diffraction measurements on membrane fragments enriched in AcChR suggested a multilayer repeat of 110 Å between lipid bilayers, while protein-free bilayers have a typical spacing of 57 Å (Raftery et al., 1975; Ross et al., 1977). If we assume that the receptor must span the lipid bilayer in order to form an ion channel and if the repeat of 110 Å corresponds to the length of the receptor molecule, it appears that up to 53 Å of protein material could be outside the membrane and exposed to an aqueous environment, which would be compensated for by an increase in the polarity of the residues and a lower discriminant function. Recently, we have shown (Hartig & Raftery, 1977) that all four receptor subunits are available for lactoperoxidase-catalyzed iodination in membrane preparations and that all such subunits are accessible from the outside (synaptic) surface (Hartig and Raftery, unpublished experiments). This result is consistent with our finding that all four subunits are glycosylated.

We have obtained evidence for the presence of *O*-phosphoserine residues in the purified AcChR and in all subunits. It was also clear from the analyses of the receptor hydrolysate at pH 1.7 that *O*-phosphothreonine residues were not present.

Because of incomplete hydrolysis of and therefore only partial recovery of P-Ser in mild acid hydrolysis, it is difficult to accurately determine the number of residues per AcChR molecule. Nevertheless, if we assume that the rate of P-Ser liberation and stability in a 4 N HCl AcChR hydrolysis was similar to that of β-casein, we can make an estimate on the basis of the value obtained for β-casein, where the P-Ser content is known. Table V gives the values determined from four separate experiments of the number of *O*-phosphoserine residues per AcChR. When purified AcChR was treated with potato acid phosphatase at a concentration which dephosphorylated 75% of β-casein phosphoserine residues and then reacted with CH_3NH_2, the number of resulting AMAP was decreased by five residues per AcChR molecule. Inclusion of 100 mM NaF, a concentration that inhibits most phosphatases, throughout the preparation of AcChR (preparations III and IV) did not give a marked increase in the number of phosphoserine residues found per AcChR molecule. Provided that phosphatases in the *Torpedo* preparation are inhibited to a high degree, this suggests that these values are not a large underestimate of the true value.

We have also presented evidence concerning the total number of O-substituted serine and threonine residues in the AcChR. It should be noted that reaction in base with methylamine occurs not only with phosphoserine but with glycosylated serine residues as well (Scheme I). The data obtained following reaction with methylamine and sulfite suggest approximately 30 O-substituted serine residues per AcChR and point to their presence in all subunits. Since we report evidence for seven phosphoserine residues, there remain 23 O-substituted serine residues per AcChR, which we consider to be most likely joined in glycosidic linkage. In addition to these residues, sulfite reaction with the AcChR suggests there are approximately 20 O-substituted threonine residues per molecule.

Acknowledgments

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