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The 43-Kilodalton Protein of *Torpedo* Nicotinic Postsynaptic Membranes: Purification and Determination of Primary Structure†

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ABSTRACT: The primary structure of the 43-kilodalton peripheral membrane protein (43-kDa protein) of *Torpedo* nicotinic postsynaptic membrane has been determined. The 43-kDa protein, which was isolated by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis, has an amino terminus resistant to Edman degradation, while the sequence at the carboxyl terminus is Tyr-Val. An amino acid sequence of 405 residues was obtained by NH_2-terminal sequence analysis of complementary peptides generated by digestion with trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease, and endoproteinase Lys-C, as well as by chemical cleavage at methionine. This sequence of molecular mass 45 618 daltons lacks the amino terminus but extends to the carboxyl terminus of the 43-kDa protein. Unusual structural features of the 43-kDa protein include two regions of ~80 residues, each containing 10% cysteine, as well as stretches predicted to exist as amphipathic α-helices. Other than the group blocking the amino terminus, no evidence was found for posttranslational modification of amino acids. The 43-kDa protein may represent a novel protein family because a computer search of this sequence with the National Biomedical Research Foundation data base (Release 12.0) did not reveal any significant homology to known protein sequences.

In highly purified postsynaptic membranes isolated from *Torpedo* electric tissue, the nicotinic acetylcholine receptor (AChR)[1] constitutes as much as 40% of the membrane protein. The predominant nonreceptor protein is a protein of molecular mass 43 000 (43-kDa protein or ν_1) that is a peripheral protein on the cytoplasmic surface (Neubig et al., 1979; Wennogle & Changeux, 1980; Elliott et al., 1980; St. John et al., 1982). By biochemical and immunological criteria, this protein is distinct from actin and creatine phosphokinase, major constituents of the electrocyte cytoplasm that are also characterized by M_r ~43 000 and that are present in variable amounts in preparations of postsynaptic membranes (Gysin

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; 43-kDa protein, the basic, membrane-bound 43-kilodalton protein of *Torpedo* postsynaptic membranes; LIS, lithium diiodosalicylate; IAA, iodoacetamide; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; [^{14}C]CAM-43K, [^{14}C](carboxamidomethyl)-43-kDa protein; DFP, diisopropyl phosphorofluoridate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; TPCK, N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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et al., 1981, 1983; Porter & Froehner, 1983; Perryman et al., 1985). When present in *Torpedo* postsynaptic membranes, these proteins have been referred to as ν_2 (creatine phosphokinase) and ν_3 (actin), in contrast to 43-kDa protein (ν_1).

The 43-kDa protein has no direct role in the control of membrane permeability by acetylcholine, since it can be removed from *Torpedo* membranes by alkaline extraction without modification of the ligand binding or ion transport properties of AChRs (Neubig et al., 1979). Indirect evidence suggests that this protein may be involved in the regulation of distribution of AChRs on the cell surface [for a review, see Froehner (1986)]. First evidence for such a role was the observation that in membranes depleted of 43-kDa protein by alkaline extraction, the rotational and translational mobilities of AChRs are greatly enhanced (Cartaud et al., 1981; Rousselet et al., 1981) and the AChR has increased thermal lability (Saitoh et al., 1979). Immunocytochemical studies using monoclonal antibodies establish that in the electrocyte plasma membrane the distribution of 43-kDa protein is coextensive with AChR (Sealock et al., 1984), and in *Torpedo* electric organ and skeletal muscle, 43-kDa protein and AChRs are present in approximately equal concentration (LaRochelle & Froehner, 1986). An immunologically related protein is present at the vertebrate neuromuscular junction (Froehner et al., 1981; Froehner, 1984) and associated with clusters of AChRs in cultured muscle cells (Burden, 1985; Peng & Froehner, 1985). In *Torpedo* membranes, the 43-kDa protein is in close proximity to AChRs, since it can be cross-linked to the AChR β-subunit (Burden et al., 1983), and results of freeze–fracture immunoelectron microscopy also provide evidence for a direct association of 43-kDa protein with AChR (Bridgman et al., 1987). While 43-kDa protein may link receptors to the cytoskeleton by acting as a binding site for actin (Walker et al., 1984), isolated 43-kDa protein has been reported to bind with high affinity to liposomes of various compositions, an association disrupted by alkaline pH but not by salt (Porter & Froehner, 1985).

As yet, there is no simple biochemical assay for the 43-kDa protein. Its presence at the nicotinic synapse was originally defined by the appearance of a band of M_r 43 000 on a SDS–polyacrylamide gel of AChR-rich membranes (Sobel et al., 1978), and this criterion is still the most straightforward method of detection. Little is known about the molecular characteristics of this polypeptide other than that it is a basic protein (Gysin et al., 1981; Porter & Froehner, 1983) which can be labeled by sulfhydryl reagents (Sobel et al., 1978; Hamilton et al., 1979) and will form large insoluble aggregates at pH 7.4 (Sobel et al., 1978; Porter & Froehner, 1985). We have pursued an investigation into the biochemical properties of the 43-kDa protein as an aid to understanding its functional significance, and we provide a first report on the primary structure of almost the entire molecule.

Experimental Procedures

Preparation of AChR-Rich Membranes and Solubilization of 43-kDa Protein. Membranes were isolated from electric organ of *Torpedo californica* (Marinus, Inc., Westchester, CA) according to the procedure of Sobel et al. (1977) with the modifications described previously (Pedersen et al., 1986). The final membrane suspensions in 36% sucrose–0.02% NaN_3 were stored at −80 °C under argon and contained 0.8–1.9 nmol of ACh binding sites per milligram of protein as measured by a direct [^{3}H]acetylcholine binding assay (Boyd & Cohen, 1980). The 43-kDa protein (as well as other peripheral proteins) was extracted from the membranes by exposure to pH 11 for 1 h at 4 °C according to the method of Neubig et al. (1979), except that membrane suspensions contained ~10 mg of protein/mL. After removal of the membranes by centrifugation, the extract was adjusted to pH 8 by addition of 1 N HCl. For extraction by lithium diiodosalicylate (LIS) (Elliott et al., 1980; Porter & Froehner, 1983), membranes were suspended at ~15 mg of protein/mL in 10 mM Tris, pH 8.1. A $^1/_9$th volume of 0.1 M LIS in 10 mM Tris, pH 8.1, was added, the suspension was incubated on ice for 60 min, and the membranes were then removed by centrifugation for 40 min at 100000*g*.

Reaction of AChR-Rich Membranes and Alkaline Extract with [^{14}C]Iodoacetamide (IAA). Purified AChR-rich membranes (6 mg of protein in 0.4 mL of 20 mM sodium phosphate, pH 8) were incubated with 1 mM dithiothreitol (DTT) for 60 min at 25 °C. This suspension was combined with an equal volume of 2 M Tris, pH 8, containing 20 mM IAA and 0.6 mM [^{14}C]IAA (ICN, Irvine, CA; 17.9 μCi/μmol). After incubation for 1 h in the dark, the reaction mixture was centrifuged for 40 min at 100000*g*, the supernatant containing excess reagents was removed, and the membrane pellet was dissolved in sample buffer for electrophoresis. As an alternative protocol to maximize incorporation of radioactivity into 43-kDa protein, membranes suspended in 1 M Tris, pH 8, were incubated with 1 mM DTT for 60 min and then reacted for 1 h with 3 mM [^{14}C]IAA (17.9 μCi/μmol). After that time, carboxamidomethylation was completed by addition of nonradioactive IAA to a final concentration of 8 mM (45-min reaction). Membranes were then prepared for electrophoresis as described above. Freshly prepared alkaline extracts were reacted with [^{14}C]IAA by similar protocols. For maximal incorporation of radioactivity, concentrated extract (4.5 mg of protein in 0.5 mL) was reduced with 3 mM DTT and then added to solid [^{14}C]IAA (final concentration 3 mM). After 60 min, carboxamidomethylation was completed by addition of nonradioactive IAA (final concentration 10 mM in 1 M Tris, pH 8). Protein was then separated from excess reagents by passage over a 3-mL column of Sephadex G-50 equilibrated in 50 mM NH_4HCO_3–0.5% Lubrol-PX (Pierce). Protein eluting in the void volume was then dissolved in sample buffer for electrophoresis.

Gel Electrophoresis and Immunoblotting. Proteins were separated by SDS–polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970), with modifications introduced to optimize the resolution of the 43-kDa protein from AChR α-subunit, creatine kinase, and actin. Polyacrylamide gels were prepared from stock solutions containing 30% acrylamide–1.2% *N,N'*-methylenebis(acrylamide), and the electrode buffer was composed of 0.05 M Tris, 0.38 M glycine, and 0.15% SDS with 0.2 mM thioglycolate included to prevent oxidation of proteins during electrophoresis (Hunkapiller et al., 1983). To isolate 43-kDa protein, the separating and stacking gels were of 8% and 4% acrylamide, respectively, while to isolate proteolytic fragments of the 43-kDa protein, the separating gel contained 12% acrylamide. Gels were stained with Coomassie Blue or with silver (Merril et al., 1981). Gels containing labeled proteins were prepared for fluorography after Coomassie Blue staining by soaking them in water and then in 1 M sodium salicylate for 30 min. The gels were dried and exposed to Kodak XAR-5 film at −90 °C.

Two-dimensional gel electrophoresis was accomplished essentially as described by O'Farrell (1975) except that the isoelectric focusing gels were not prerun before the samples were loaded. Sample buffer was composed of 9.95 M urea, 2% Nonidet P-40, 100 mM DTT, 0.02% SDS, 0.1% Triton

X-100, and 2% ampholines (LKB, Bromma, Sweden). Ampholines were comprised of three parts pH 3.5–10, one part pH 5–7, and one part pH 7–9.

For immunoblot experiments, membranes or purified 43-kDa protein was subjected to one- or two-dimensional gel electrophoresis, and then the gel was incubated for 1 h in 25 mM sodium phosphate, pH 6.5. A replica of the gel was prepared by electrophoretic transfer (2.5 h at 175 mA) of the proteins to nitrocellulose paper in the same buffer. After incubation of the replica in 10 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 0.05% Tween 20 (blot buffer) for 30 min, it was incubated for 2 h with antibody (diluted appropriately into blot buffer containing 1% bovine serum albumin), washed 3 times with blot buffer, and then incubated for at least 1 h with biotinylated anti-mouse or -rabbit IgG, 1:200 in blot buffer. After another wash in blot buffer, the replica was incubated in Vectastain ABC reagent (Vector Labs, Burlingame, CA) for 30 min, washed in phosphate-buffered saline 3 times, and reacted with 3,3′-diaminobenzidine for 30–60 s. Primary antibodies used were a mouse monoclonal specific for *Torpedo* 43-kDa protein (Mab 2316A) whose preparation and characterization have been described (Bridgman et al., 1987), a mouse anti-actin monoclonal (Amersham, N.350), and a rabbit serum against human muscle creatine kinase (Research Plus, Bayonne, NJ).

Purification of 43-kDa Protein. 43-kDa protein was usually isolated by electroelution from polyacrylamide slab gels. Alkaline or LIS extracts enriched in 43-kDa protein were prepared from 15 mg of AChR-rich membranes, dissolved in sample buffer (final volume 1 mL), and loaded onto a slab gel (14 × 16 cm, 1.5 mm thick). Electrophoresis was performed at 8 mA overnight, and the polypeptides were then visualized by brief (20–30 min) staining with Coomassie Blue (0.37% Coomassie Blue R–30% 2-propanol–10% acetic acid) and destaining in methanol–acetic acid (16.5%:5%) until bands were visible. The band containing 43-kDa protein was excised from the gel and soaked in water for ~2 h with several changes, and then the protein was eluted from the gel slice by the method of Hunkapiller et al. (1983) with modifications described previously (Pedersen et al., 1986) using an ISCO Model 1750 electrophoretic concentrator (ISCO, Lincoln, NE). The concentrated sample was precipitated by −20 °C acetone to remove most of the SDS and collected by centrifugation at 12500*g*, and the pellet was then resuspended in 0.125 mL of water. Typically, 0.2 mg of 43-kDa protein was recovered from 1.5 mg of alkaline or LIS extract. Preparative slab gel electrophoresis was also used to isolate [^{14}C](carboxamidomethyl)-43-kDa protein ([^{14}C]CAM-43K) from both alkaline extracts and membranes after reaction with [^{14}C]IAA.

For several experiments, 43-kDa protein was isolated from alkaline extracts by tube gel electrophoresis using a preparative gel cell (Savant, Hicksville, NY; Model PAG15WC). Extract (1–2 mg of protein) dissolved in sample buffer (1 mL final volume) was electrophoresed through a cylindrical gel (1.5-cm diameter) containing a 2-cm stacking gel and an 8-cm separating gel. Electrophoresis was carried out overnight at 15 mA, and proteins eluting from the bottom of the gel were collected as 1-mL fractions in 10 mM Tris, pH 8.1. Aliquots of each fraction were analyzed by SDS–PAGE, and those fractions containing 43-kDa protein alone were pooled and concentrated by vacuum centrifugation (Savant Speed Vac concentrator). About 0.15 mg of 43-kDa protein was recovered from 1.5 mg of extract.

Care was required in the handling and storage of purified 43-kDa protein to avoid substantial losses as a result of surface adsorption. 43-kDa protein was stored in polyethylene tubes or in siliconized glass tubes. Significant losses of protein occurred during efforts to concentrate the sample or to remove excess reagents by dialysis. Without the substantial quantities of SDS remaining with 43-kDa protein after electroelution, acetone precipitation caused the protein to adsorb strongly to glass.

Enzymatic Digestions. For limited proteolysis, 43-kDa protein (isolated by tube gel electrophoresis, 1 mg/mL) was treated with *Staphylococcus aureus* V8 protease (Worthington, Freehold, NJ) at 25 °C for 9 h in 0.125 M Tris, pH 6.8, and 0.1% SDS at an enzyme to substrate ratio of 1:100. Digestion was terminated by addition of diisopropyl phosphorofluoridate (DFP) to 1 mM, and the generated peptides were isolated by electroelution after separation on a 12% polyacrylamide slab gel.

For more complete digests, [^{14}C]CAM-43K was treated with V8 protease, endoproteinase Lys-C (Boehringer-Mannheim, Indianapolis, IN), TPCK–trypsin (Worthington), or TLCK–chymotrypsin (Sigma Chemical), with all digestions terminated by the addition of DFP to 1 mM. [^{14}C]CAM-43K (240 μg) was concentrated to 50 μL by vacuum centrifugation, diluted 10-fold with 0.1 M NH_4HCO_3 containing 0.1% Lubrol-PX, and then incubated at 37 °C with either 15 μg of TPCK–trypsin for 2.5 h or 8 μg of TLCK–chymotrypsin for 1.5 h. [^{14}C]CAM-43K (240 μg in 0.22 mL) was treated with endoproteinase Lys-C (4 units in 0.4 mL of 25 mM Tris, pH 8.6, 1 mM EDTA, and 0.1% SDS) for 24 h at 25 °C. [^{14}C]CAM-43K (60 μg) was treated with 2 μg of V8 protease for 18 h at 37 °C in 0.3 mL of 0.1 M sodium phosphate, pH 7.8. All digests were separated by reverse-phase HPLC using a Waters (Milford, MA) system consisting of a Model 680 gradient controller, two Model 510 pumps, and a Model 440Z UV detector. Columns used include Vydac (Hesperia, CA) C_4 (214TP54) and C_{18} (218TP54) and Baker (Phillipsburg, NJ) cyanopropyl. Samples were eluted with linear water–acetonitrile (Baker Chemical, HPLC grade) gradients containing trifluoroacetic acid (TFA, Pierce Chemical). Solvent A was 0.1% TFA in water, and solvent B was 0.09% TFA in acetonitrile. Unless otherwise noted, fractions of 0.5 mL were collected in siliconized tubes, and radioactivity was monitored by liquid scintillation counting of 10-μL aliquots of each fraction. For each digest, fractions containing peptides of interest were pooled and concentrated to ~0.1 mL by vacuum centrifugation. Concentrated samples were transferred to a clean siliconized glass tube, and the original tubes were rinsed with 1 mL of water containing 0.3 mg of SDS. The rinse was also concentrated to ~0.1 mL and then added to the original sample. In the absence of added SDS, recovery of hydrophobic peptides was poor, while 0.3 mg of SDS did not interfere with subsequent use of samples for sequencing.

Cyanogen Bromide Digest. [^{14}C]CAM-43K (240 μg) was evaporated to dryness and dissolved in 0.3 mL of freshly prepared 1 M cyanogen bromide in 88% formic acid. The tube was flushed with argon, stoppered, and incubated at 4 °C for 12 h in the dark. After addition of 0.3 mL of water, excess reagents were removed by vacuum centrifugation. The sample was concentrated to 50 μL, 0.2 mL of water was added, the volume was reduced again, and the process was repeated twice. The sample was then dissolved in 0.3 mL of water containing 0.1% SDS and 0.8% formic acid. Insoluble material was removed by centrifugation at 12000*g* for 30 s, and the digest was then separated by HPLC.

Determination of Carboxyl Terminus. The amino acids of the carboxyl terminus of 43-kDa protein were identified by

analysis of amino acids released by reaction with carboxypeptidase Y (Hayashi, 1977). Aliquots of 43-kDa protein (2 μg) were treated at 23 °C for 2–24 h with 0.1 μg of carboxypeptidase Y (Boehringer) in 0.03 mL of 50 mM ammonium acetate, 1 mM EDTA, pH 6.4, 0.1% SDS, and 0.001% pepstatin. The reaction was terminated by addition of DFP to 1 mM. Reaction mixtures were subjected to amino acid analysis to identify released amino acids.

Amino Acid Analysis. The amino acid composition of acid hydrolysates of 43-kDa protein and of carboxypeptidase digests was determined by reversed-phase HPLC after derivatization with phenyl isothiocyanate (Bidlingmeyer et al., 1984). Phenylthiocarbamyl derivatives of amino acids were resolved at 38 °C on an Altex Ultrasphere ODS-PTH column (4.6 × 250 mm). Samples (~2 μg) were hydrolyzed for 24, 48, and 72 h. Cysteine (as cysteic acid) and methionine (as methionine sulfone) were determined after oxidation with performic acid (Glazer et al., 1975).

Microsequencing. Edman degradation of peptides was performed on an Applied Biosystems (ABI, Foster City, CA) Model 470A protein sequencer. Peptides were immobilized on TFA-treated glass fiber filters with 1.5 mg of Biobrene Plus. All chemicals were purchased from ABI, and the standard degradation run (03RPTH) was used. Phenylthiohydantoin (PTH)–amino acids were analyzed with the Applied Biosystems Model 120A PTH analyzer utilizing a 50-μL sample loop, the standard 220 × 2 mm C_{18} column at 55 °C, and the recommended gradient with slight modifications. (Carboxamidomethyl)cysteine eluted with glutamate and (carboxymethyl)cysteine with glutamine, so for peptides isolated from [^{14}C]CAM-43K, the presence of cysteine was confirmed by liquid scintillation counting of aliquots of PTH–amino acid for each cycle. Cycle yields for PTH–amino acids were estimated from observed peak heights.

Protein Determination. Protein was determined by using the amido black dye binding assay of Schaffner and Weissman (1973).

Results

Preparations of *Torpedo* postsynaptic membranes can contain significant quantities of creatine phosphokinase (EC 2.7.3.2) (ν_2; Barrantes et al., 1983a; Gysin et al., 1983) and skeletal muscle actin (ν_3; Gysin et al., 1983; Porter & Froehner, 1983), as well as 43-kDa protein. We wished to determine whether those cytosol proteins were present in our preparations and whether they could be separated from 43-kDa protein in a one-dimensional SDS–polyacrylamide gel system (Figure 1). Highly purified AChR-rich membranes contain (Figure 1A), in addition to the four AChR subunits, a single major band of M_r 43 000 whose relative mobility (R_f) is 0.64 and a minor component with an R_f of 0.62 (indicated by the star in Figure 1A). A cytosol fraction, i.e., the supernatant after centrifugation of electric organ homogenate at 100000*g* for 60 min, has higher levels of this protein which migrates behind the M_r 43 000 band, as well as large amounts of a protein moving ahead of it with an R_f of 0.67 (arrowhead) (Figure 1B). There is only a faint suggestion of the M_r 43 000 band in this fraction. When nitrocellulose replicas of a sample containing both AChR-rich membranes and cytosol proteins (Figure 1C) were probed with antibodies specific for each protein (Figure 1D–H), 43-kDa protein (R_f = 0.64) was found to be well resolved both from creatine phosphokinase (R_f = 0.67) and from actin (R_f = 0.62). As judged by immunoblotting, in our preparation of AChR-rich membranes, creatine kinase was undetectable while the band of R_f = 0.62 (M_r ~45 000) was actin.

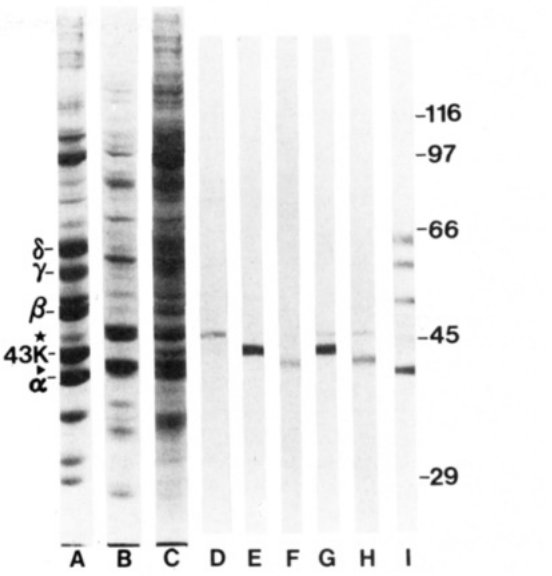

FIGURE 1: Resolution of *Torpedo* 43-kDa protein (ν_1) from creatine kinase (ν_2) and actin (ν_3) by one-dimensional SDS–PAGE. Samples of AChR-rich membranes (A), electric organ cytosol proteins (B), or an equal mixture of both (C–I) were resolved by SDS–PAGE and stained with Coomassie Blue (A–C) or transferred to nitrocellulose (D–I). The nitrocellulose replicas were probed with the following: (D) a mouse monoclonal against actin (ascites diluted 1:2000); (E) monoclonal 2316A against *Torpedo* 43-kDa protein (ascites diluted 1:1000); (F) rabbit serum against human muscle creatine kinase (3:1000 dilution); (G) anti-actin and anti-43 kDa; (H) anti-actin and anti-creatine kinase; (I) mouse monoclonal 2579B recognizing all receptor subunits. Migration of molecular weight marker proteins is indicated on the right for β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). AChR subunits and the 43-kDa protein are identified on the left.

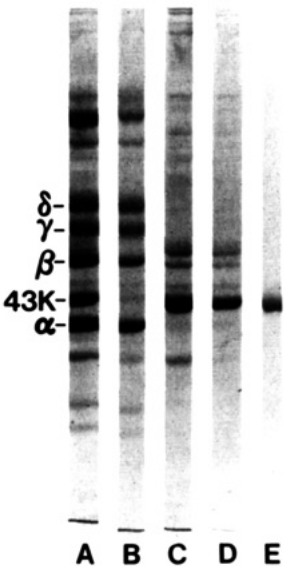

FIGURE 2: Purification of 43-kDa protein from *Torpedo* postsynaptic membranes. Polypeptide composition determined by SDS–PAGE of the following: (A) AChR-rich membranes (25 μg) containing 2.2 μmol of AcCh sites/g of protein; (B) membranes after alkaline extraction according to procedure of Neubig et al. (1979); (C) corresponding alkaline extract; (D) LIS extract (from a separate experiment); (E) 43-kDa protein (2 μg) purified from a LIS extract by preparative slab gel electrophoresis. The gel was stained with Coomassie Blue.

Purification of 43-kDa Protein. In agreement with previous results, 43-kDa protein was extracted from AChR-rich membranes after incubation of membrane suspensions for 60 min at pH 11 (Figure 2C) or with 10 mM LIS buffered at pH 8

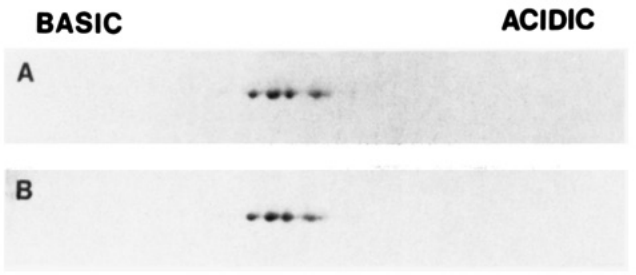

FIGURE 3: Characterization of purified 43-kDa protein by two-dimensional gel electrophoresis. (Panel A) Coomassie Blue stain. (Panel B) Immunoblot of a parallel gel incubated with monoclonal antibody 2316A (culture supernatant diluted 1:30) and processed as described under Experimental Procedures. Only the center portion of the gel and immunoblot is shown, since top and bottom portions contained no stained or immunoreactive material. 43-kDa protein was purified by preparative slab gel electrophoresis from an LIS extract; 2.6 μg of protein was used for panel A and 0.8 μg for panel B.

(Figure 2D). 43-kDa protein comprises ~80% of the protein in these extracts, which also contain most of the actin present in membrane suspensions. The 43-kDa protein isolated from such extracts by preparative SDS–PAGE appeared as a single band on a one-dimensional gel (Figure 2E).

The purity of the isolated 43-kDa protein was characterized by two-dimensional polyacrylamide gel electrophoresis. The 43-kDa protein in AChR-rich membranes has been shown to be composed of isoelectric variants (p*I* = 7.0–8.0), which have identical peptide maps and are recognized by the same monoclonal antibodies (Gysin et al., 1981; Porter & Froehner, 1983; Froehner, 1984). 43-kDa protein in AChR-rich membranes prepared as described under Experimental Procedures displays similar isoelectric variants, and a monoclonal antibody (2316A) raised to purified 43-kDa protein recognizes these variants (Bridgman et al., 1987). The 43-kDa protein isolated by preparative SDS–polyacrylamide gel electrophoresis was also characterized by isoelectric variants (p*I* = 7.0–7.6) (Figure 3A), and each was recognized by monoclonal antibody 2316A (Figure 3B). Thus, as judged by two-dimensional gel electrophoresis and immunoblotting, *Torpedo* 43-kDa protein can be isolated by one-dimensional SDS–PAGE. Typically, 200–300 μg of 43-kDa protein was obtained from 12 mg of AChR-rich membranes. The amino acid composition of 43-kDa protein isolated by this procedure (Table I) is similar to that reported by Gysin et al. (1983) for the protein isolated by two-dimensional polyacrylamide gel electrophoresis.

Isolation of [^{14}C]CAM-43K Protein. To monitor the recoveries of 43-kDa protein and its proteolytic fragments, it was important to prepare a radiolabeled derivative of the protein. 43-kDa protein in *Torpedo* AChR-rich membranes contains free sulfhydryls that react readily with IAA (Sobel et al., 1978) or *N*-ethylmaleimide (Hamilton et al., 1979). Because we found that SDS-purified 43-kDa protein reacted with low yield with [^{14}C]IAA, [^{14}C]CAM-43K was isolated by preparative slab gel electrophoresis from AChR membranes after reaction with [^{14}C]IAA as described under Experimental Procedures. 43-kDa protein was not isolated from alkaline or LIS extracts of radiolabeled membranes because these procedures extracted [^{14}C]CAM-43K from membranes only in low yield. The reason for this is not known, but similar properties were reported after reaction of membranes with *N*-ethylmaleimide (Barrantes et al., 1983b) as well as after iodination catalyzed by chloramine-T or lactoperoxidase (Porter & Froehner, 1985).

In four preparations, isolated [^{14}C]CAM-43K contained 25 ± 5 nmol of IAA/nmol of protein (where 43 μg of protein was equated to 1 nmol). This value was 40% higher than the number of cysteines determined by amino acid analysis (Table I, performic acid oxidation). Since it was possible that under the reaction conditions used IAA reacted with other amino acids such as methionine or histidine, a 24-h acid hydrolysate of [^{14}C]CAM-43K was separated into its constituent amino acids on an amino acid analyzer, and aliquots of all fractions were counted in a scintillation counter. Radioactivity was found almost exclusively (>93%) with the (carboxymethyl)-cysteine derivative.

Table I: Comparison of Amino Acid Composition of 43-kDa Protein Determined by Acid Hydrolyses and Sequence Analyses of Peptides

		sequence	
	acid hydrolysis[a] (mol %)	mol %	no. of residues
Asp/Asn	7.7	4.4/3.0	18/12
Thr	3.1[b]	2.5	10
Ser	5.3[b]	4.9	20
Glu/Gln	14.2	8.2/5.4	33/22
Pro	2.3	2.2	9
Gly	8.3	7.4	30
Ala	9.1	7.6	38
Cys	4.3[c]	5.7	23
Val	4.1	4.4	18
Met	4.5[c]	3.7	15
Ile	2.9	2.7	11
Leu	12.8	12.6	51
Tyr	3.5	4.0	16
Phe	3.1	3.2	13
His	2.7	3.2	13
Lys	6.3	6.4	26
Arg	6.5	5.9	24
Trp		0.7	3
total			405

[a] Average of two determinations, values between runs differ by less then 10%. [b] Ser and Thr determined by extrapolation to zero time from timed hydrolyses of 24, 48, and 72 h. [c] Cys and Met determined as cysteic acid and methionine sulfone after performic acid oxidation.

The purity of [^{14}C]CAM-43K was monitored by one- and two-dimensional gel electrophoresis by comparison of the patterns revealed by Coomassie stain and by fluorography (data not shown). On a one-dimensional gel, [^{14}C]CAM-43K appears as a single band similar to that shown in Figure 2E, with its mobility retarded slightly relative to the unmodified protein. The multiple isoelectric variants of [^{14}C]CAM-43K were slightly more basic than their unalkylated counterparts. In several preparations of [^{14}C]CAM-43K isolated from AChR-rich membranes, low levels of AChR α-subunit were detected by an α-subunit-specific monoclonal antibody (data not shown) as well as during sequence analysis. AChR α-subunit was not detected in preparations of [^{14}C]CAM-43K isolated after alkylation of freshly prepared alkaline extracts.

Sequencing of the NH_2-Terminus of 43-kDa Protein and of Peptides V8–28 and V8–18. Although little is known about the domain structure of 43-kDa protein, Froehner (1984) reported that digestion by *S. aureus* V8 protease of 43-kDa protein in LIS extracts produced two large, stable fragments of M_r ~28 000 (V8–28) and ~18 000 (V8–18) that were each recognized by separate classes of monoclonal antibodies. Digestion of purified 43-kDa protein also produced similar fragments (Figure 4B,C), although the peptides formed were further degraded upon longer digestion or increased concentration of enzyme. V8–28 and V8–18 were purified by electroelution from polyacrylamide gel slices (Figure 4D,E), yielding 15 μg of V8–28 and 10 μg of V8–18 isolated from 175 μg of 43-kDa protein. When characterized by two-dimensional gel electrophoresis, V8–28 was more acidic than 43-kDa protein and characterized by multiple isoelectric variants (p*I* = 6.0–6.4), while V8–18 (p*I* = 7.6–8.0) was slightly more basic than 43-kDa protein (data not shown).

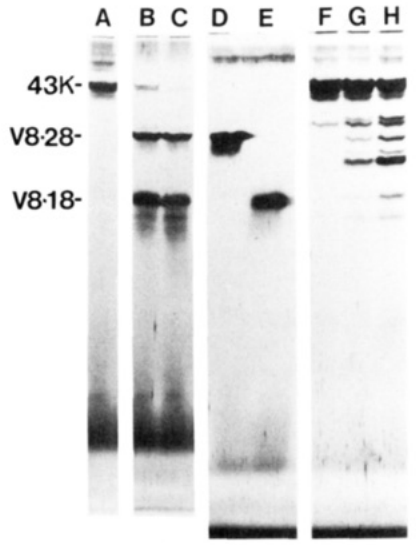

FIGURE 4: Digestion pattern of 43-kDa protein by *S. aureus* V8 protease (B, C) and by trypsin (F–H). 43-kDa protein was incubated with V8 protease at 25 °C for 9 h in 0.125 M Tris, pH 6.8, and 0.1% SDS or with trypsin at 38 °C for 4 h in 0.1 M NH_4HCO_3. Reaction products were resolved on a 12% polyacrylamide slab gel and visualized by silver stain. 43-kDa protein (1.5 μg) in the absence of protease (lane A) or after digestion with 0.08 μg (lane B) or 0.16 μg (lane C) of V8 protease. A larger sample of 43-kDa protein (170 μg) was digested with V8 protease (6 μg), and proteolytic fragments of M_r 28 000 (V8–28) and 18 000 (V8–18) were isolated by electroelution after separation on a 12% polyacrylamide slab gel: lane D, V8–28; lane E, V8–18. 43-kDa protein (1.5 μg) was digested with 0.1 μg (F), 0.4 μg (G), or 0.8 μg (H) of trypsin. Indicated on the left is the nomenclature for the predominant proteolytic fragments produced by *S. aureus* V8 protease.

Monoclonal antibody 2316A recognized V8–28 but not V8–18.

Samples (~1 nmol) of 43-kDa protein, V8–28, and V8–18 were subjected to automated Edman degradation. No amino acids were released for 43-kDa protein or V8–28, but the sequence of the first 15 amino acids of V8–18 was identified as Ser-Met-Lys-Ile-Ala-Leu-Gln-X-Gln-Asp-X-Pro-Leu-Glu-Ala. The amino acids at cycles 8 and 11 could not be identified in the sequence runs using two independently isolated samples of V8–18. These results indicated that V8-28 probably originated at the amino terminus of 43-kDa protein, while V8–18 was generated by an internal cleavage. The group blocking the amino terminus of 43-kDa protein has not been identified. Amino-terminal glutamine can become cyclized under acid conditions to pyroglutamate (Smyth et al., 1963) which is resistant to Edman degradation. The presence of this residue is unlikely, however, because 43-kDa protein was resistant to Edman degradation after incubation with the pyroglutamate aminopeptidase.

General Strategy of Sequence Analysis. Conditions could not be found to generate V8–28 and V8–18 in sufficient quantities to use those fragments as a starting material for further proteolytic degradation. Rather, [^{14}C]CAM-43K was digested with a variety of proteases and CNBr. Peptides were isolated from these digests by reversed-phase HPLC and subjected to automated Edman degradation. Carboxypeptidase Y was used to identify the COOH-terminal amino acids of 43-kDa protein. The overall strategy of the analyses of the sequence is presented in Figure 5, and the cycle yields for peptide sequences crucial for the proof of structure are shown in Table II. The NH_2-terminus which is blocked has not been identified, but a sequence of 405 amino acid (45 618 daltons) has been determined. Thirty-four peptides generated by trypsin cleavage (T1–T34) were aligned by overlapping them with fragments resulting from cleavage of [^{14}C]CAM 43K by chymotrypsin (C1–C17), *S. aureus* V8 protease (V1–V8), endoproteinase Lys-C (L1–L7), and cyanogen bromide (CN1–CN7). The peptides generated by each type of digestion are numbered sequentially from the amino terminus.

Cleavage of the Protein, Peptide Isolation, and Sequence Assignments. After isolation by preparative SDS–PAGE, 43-kDa protein in bicarbonate buffer was surprisingly resistant to trypsin (Figure 4F–H) or chymotrypsin (data not shown). However, upon addition of 0.1% Lubrol-PX to the digestion buffer, 43-kDa protein or [^{14}C]CAM-43K was readily digested by low amounts of these enzymes. When fractionated by reversed-phase HPLC on a C_4 column and eluted with a gradient of acetonitrile, both tryptic (Figure 6A) and chymotryptic (Figure 6B) digests contained numerous peaks. For both digests, material not retained on the C_4 column was collected and rechromatographed on a C_{18} column with an acetonitrile gradient (Figure 7, tryptic digest). To produce cleavages at glutamate, [^{14}C]CAM-43K was digested with *S. aureus* V8 protease in phosphate buffer, pH 7.8, and the digest was also fractionated on a C_4 column (Figure 8). Endoproteinase Lys-C, which cleaves at the carboxy-terminal side of lysine (Jekel et al., 1983), retains activity and specificity in SDS. As judged by SDS–PAGE, the enzyme digested [^{14}C]CAM-43K effectively in 0.1% SDS–25 mM Tris buffer. However, the digestion products were not well resolved on a C_4 column, and better resolution was attained by use of a cyanopropyl column (Figure 9). Even with that column, only a limited number of peaks were resolved. Similarly, cleavage of [^{14}C]CAM-43K with CNBr in 88% formic acid produced peptides only partially resolved on a C_4 column (Figure 6C).

Fractions corresponding to the HPLC peaks were pooled and analyzed by NH_2-terminal sequencing without further purification. Of the 34 peptides from the tryptic digest, 28 were present in HPLC peaks containing only a single predominant peptide as judged by microsequencing; i.e., a secondary sequence, when detected, was present at a concentration less than 20% that of the primary sequence. Three HPLC peaks each contained two peptides in approximately equal amount (Figure 6A, T17 and T29 in fractions 70–71; Figure 7, T1 and T30 in fractions 12–13, T22 and T26 in fractions 8–9). The sequence T29 was assigned once T17 was recognized by comparison to sequences of peptides V4 and V5 from the *S. aureus* digest. The sequences of T1, T22, T26, and T30 were determined after T1 was separated from T30 (and T22 from T26) by repurification on a C_{18} column equilibrated in 10 mM acetate adjusted to pH 6.5 with triethylamine. Peptides were readily eluted and resolved with a linear gradient from 2% to 20% acetonitrile. Digestion with trypsin appeared quite complete, since only three sequenced peptides (T5, T21, T34) contained internal arginine or lysine.

Forty-five peaks from the HPLC fractionation of a chymotryptic digest were characterized by microsequencing. Of the 17 peptides important for the proof of structure, 3 (C6, C10, and C15) were identified as unique peptides within the HPLC peaks, and 10 were present within peaks each containing 2 sequences. For six of these (C4 and C8, C6′ and C17, and C7 and C11), one of the two sequences predominated, and assignment of major and minor sequences was unambiguous. Peptides C1 and C14 were present at a ratio of 3:2; glycine was the only amino acid released at cycle 5, and the amount released indicated contributions from both peptides. Peptides C3 and C13 were present at a ratio of 2:3 in fractions 168–171. Arginine in cycle 1 and glycine in cycle 4 were identified as amino acids common to both sequences.

Table II: Amino Acid Sequence Data for *Torpedo* 43-kDa Protein

Position/ Residue	Peptide, (cycle), yield (pmol)			
	T1			
1 Gln	(1) 136	C1		
2 Gln	(2) 166	(1) 47		
3 Ile	(3) 200	(2) 70		
4 Glu	(4) 190	(3) 89		
5 Lys	(5) 80	(4) 80	L1	
6 Gly		(5) 42	(1)110	
7 Leu		(6) 63	(2)127	
8 Gln		(7) 26	(3) 78	
9 Leu		(8) 52	(4)198	
10 Tyr		(9) 17	(5) 84	
11 Gln			(6) 75	
12 Ala			(7)126	
13 Asn			(8) 74	
14 Glu			(9) 60	
15 Thr			(10) 25	
16 Gly			(11) 46	
17 Lys	T3		(12) 33	
18 Ala	(1)1364		(13) 76	
19 Leu	(2)1431		(14) 76	
20 Glu	(3)1243	V1	(15) 68	
21 Ile	(4)1125	(1) 52	(16) 42	
22 Trp	(5) 365	(2)	(17)	
23 Gln	(6) 785	(3) 27	(18) 19	
24 Gln	(7) 697	(4) 31	(19) 27	
25 Val	(8) 807	(5) 21	(20) 12	
26 Val	(9) 829	(6) 25	(21) 31	
27 Glu	(10) 584	(7) 39	(22) 38	
28 Arg	(11) 68	(8) 7	(23) 13	T4
29 Ser		(9) 4		(1)396
30 Thr		(10)		(2)249
31 Glu		(11) 17		(3)773
32 Leu		(12) 17		(4)550
33 Pro		(13) 26		(5)498
34 Gly		(14) 7		(6)395
35 Arg		(15) 3		(7) 38
36 Phe		(16) 6		
37 Arg	T5	(17) 6		
38 Ala	(1) 420	(18)		
39 Leu	(2) 340	(19) 9		
40 Gly	(3) 209	(20) 7		
41 Cys	(4) 273			
42 Leu	(5) 318			
43 Ile	(6) 165			
44 Thr	(7) 81			
45 Ala	(8) 178			
46 His	(9) 66			
47 Ser	(10) 43			
48 Glu	(11) 146			
49 Met	(12) 102			
50 Gly	(13) 89			
51 Lys	(14) 54			
52 Tyr	(15) 89			
53 Glu	(16) 100			
54 Asp	(17) 64			
55 Met	(18) 53	CN1		
56 Leu	(19) 53	(1)463		
57 Arg	(20) 8	(2) 84		
58 Phe		(3)360	C4	
59 Ala		(4)450	(1)201	
60 Val		(5)311	(2)182	
61 Ala		(6)326	(3)264	
62 Gln		(7)192	(4)180	
63 Ser		(8) 94	(5) 40	
64 Glu		(9)239	(6)199	
65 Ala		(10)212	(7)138	
66 Ala		(11)275	(8)148	
67 Arg		(12) 67	(9) 26	
68 Gln		(13) 81	(10) 88	
69 Met			(11)118	
70 Gly			(12) 81	
71 Asp			(13) 60	
72 Pro			(14) 58	
73 Glu			(15) 61	
74 Arg		T8	(16) 19	
75 Val		(1)648	(17) 42	
76 Thr		(2)264	(18) 15	
77 Glu	V2	(3)506	(19) 43	
78 Ala	(1) 267	(4)530	(20) 34	
79 Tyr	(2) 179	(5)436	(21) 18	
80 Leu	(3) 220	(6)429		
81 Asn	(4) 133	(7)272		
82 Leu	(5) 192	(8)346		
83 Ala	(6) 171	(9)286		
84 Arg	(7) 49	(10) 23		
85 Gly	(8) 87			
86 His	(9) 27			
87 Glu	(10) 74			
88 Lys	(11) 41	T9		
89 Leu	(12) 52	(1)253		
90 Cys	(13) 56	(2)184		
91 Glu	(14) 48	(3)205		
92 Phe		(4)170		
93 Ser		(5) 32		
94 Glu	V3	(6)101		
95 Ala	(1) 79	(7) 92		
96 Val	(2) 35	(8) 81		
97 Ala	(3) 42	(9)106		
98 Tyr	(4) 24	(10) 77		
99 Cys	(5) 31	(11) 64		
100 Arg	(6) 12	(12) 9	T10	
101 Thr	(7) 7		(1)151	
102 Cys	(8) 32		(2)301	
103 Leu	(9) 23		(3)295	
104 Gly	(10) 22		(4)201	
105 Ala	(11) 18		(5)238	
106 Glu	(12) 18		(6)149	
107 Gly	(13) 15		(7)119	
108 Gly	(14) 18		(8)109	
109 Pro	(15) 8		(9) 84	
110 Leu	(16) 12		(10) 80	
111 Arg	(17) 4	T11	(11) 6	
112 Leu	(18) 14	(1)235		
113 Gln	(19) 5	(2)192		
114 Phe	(20) 6	(3)214		
115 Asn	(21) 4	(4)113		
116 Gly		(5)113		
117 Gln		(6)159		
118 Val		(7)114		
119 Cys		(8)110		
120 Leu		(9) 85	C6	
121 Ser		(10) 21	(1)100	
122 Met	CN2	(11) 68	(2)220	
123 Gly	(1) 424	(12) 53	(3)131	
124 Asn	(2) 279	(13) 43	(4)118	
125 Ala	(3) 320	(14) 48	(5)137	
126 Phe	(4) 339	(15) 46	(6)124	
127 Leu	(5) 336	(16) 57	(7)101	
128 Gly	(6) 184	(17) 34	(8) 65	
129 Leu	(7) 272	(18) 36	(9) 90	
130 Ser	(8) 50	(19) 7	(10) 20	
131 Ala	(9) 179	(20) 26	(11) 55	
132 Phe	(10) 167	(21) 22	(12) 60	
133 Gln	(11) 133	(22) 15	(13) 37	
134 Lys	(12)102	(23) 4	(14) 10	
135 Ala	(13) 98		(15) 41	
136 Leu	(14)118		(16) 37	
137 Glu	(15) 90		(17) 28	
138 Cys	(16) 80		(18) 32	
139 Phe	(17) 83		(19) 25	
140 Glu	(18) 71		(20) 23	
141 Lys	(19) 45		(21) 3	
142 Ala	(20) 32		(22) 17	
143 Leu	(21) 73		(23) 16	
144 Arg	(22) 28		(24) 10	T13
145 Tyr	(23) 61	C7	(25) 7	(1)539
146 Ala	(24) 40	(1)329		(2)491
147 His	(25)	(2) 55		(3)122
148 Gly	(26) 18	(3)112		(4)205
149 Asn	(27) 11	(4) 86		(5)201
150 Asp	(28) 17	(5) 97		(6)248
151 Asp	(29) 23	(6) 95		(7)242
152 Lys	(30) 5	(7) 83	L2	(8)117
153 Met		(8) 73	(1)263	
154 Leu		(9) 93	(2)232	
155 Glu		(10) 72	(3)170	
156 Cys		(11) 98	(4)163	
157 Arg		(12) 26	(5) 50	
158 Val		(13) 43	(6)154	
159 Cys		(14) 41	(7)116	
160 Cys		(15) 27	(8)126	
161 Ser			(9)124	
162 Leu			(10)108	
163 Gly			(11) 77	
164 Ala			(12) 82	
165 Phe			(13) 87	
166 Tyr	C9		(14) 69	
167 Val	(1)872		(15) 62	
168 Gln	(2)569		(16) 55	
169 Leu	(3)560		(17) 68	
170 Lys	(4)649		(18) 49	
171 Asp	(5)869		(19) 39	
172 Tyr	(6)605		(20) 41	
173 Glu	(7)620	V4	(21) 28	
174 Lys	(8)360	(1)297	(22) 20	
175 Ala	(9)569	(2)321		
176 Leu	(10)209	(3)272		
177 Phe	(11) 45	(4)238		
178 Phe		(5)237		
179 Pro		(6)185		
180 Cys		(7)192		
181 Lys		(8)135	T17	
182 Ser		(9) 20	(1)413	
183 Ala		(10)100	(2)877	
184 Glu	V5	(11) 28	(3)806	
185 Leu	(1)145		(4)692	
186 Val	(2) 94		(5)489	
187 Ala	(3) 96		(6)574	
188 Asp	(4) 78		(7)603	
189 Tyr	(5) 58		(8)516	
190 Gly	(6) 39		(9)300	
191 Arg	(7) 19	T18	(10)123	
192 Gly	(8) 25	(1)589		
193 Trp	(9)	(2)117		
194 Ser	(10) 5	(3)134		
195 Leu	(11) 13	(4)299		
196 Lys	(12) 5	(5)141		
197 Tyr	(13) 6			
198 Lys	(14) 6		L3	
199 Ala	(15) 6		(1)137	
200 Met	(16) 3		(2)104	
201 Ser	(17)5		(3) 29	
202 Arg			(4) 22	
203 Tyr			(5) 55	
204 His			(6) 16	
205 Met			(7) 51	
206 Ala			(8) 58	
207 Ala			(9) 62	
208 Ala			(10) 59	
209 Tyr			(11) 32	
210 Arg			(12) 16	
211 Lys			(13) 26	
212 Leu			(14) 40	
213 Gly			(15) 46	
214 Arg	T20		(16) 24	
215 Met	(1)121		(17) 10	
216 Asp	(2) 75		(18) 13	
217 Asp	(3) 76			
218 Ala	(4) 52			
219 Met	(5) 43			
220 Glu	(6) 62			
221 Cys	(7) 77			
222 Cys	(8) 83			
223 Glu	(9) 53			
224 Glu	(10) 81	V6		
225 Ser	(11) 11	(1) 39		
226 Met	(12) 14	(2) 76	CN4	
227 Lys	(13) 9	(3) 61	(1)384	T21
228 Ile		(4) 71	(2)463	(1)833
229 Ala		(5) 71	(3)453	(2)797
230 Leu		(6) 64	(4)508	(3)658
231 Gln		(7) 54	(5)325	(4)488
232 His		(8) 9	(6)150	(5)198
233 Gln		(9) 38	(7)266	(6)442
234 Asp		(10) 32	(8)301	(7)379
235 Arg		(11) 12	(9)250	(8)208
236 Pro		(12) 23	(10)209	(9)313
237 Leu		(13) 31	(11)306	(10)350
238 Gln		(14) 18	(12)164	(11)216
239 Ala		(15) 20	(13)182	(12)300
240 Leu		(16) 24	(14)220	(13)272
241 Cys		(17) 15	(15)171	(14)176
242 Leu		(18) 18	(16)240	(15)271
243 Leu		(19) 16	(17)318	(16)269
244 Cys		(20) 12	(18)203	(17)172
245 Phe			(19)132	(18)197
246 Ala			(20)182	(19)176
247 Asp			(21)141	(20)130
248 Ile			(22)100	(21)138
249 His			(23) 27	(22) 50
250 Arg			(24) 50	(23) 27
251 His			(25) 27	
252 Arg	T22		(26) 46	
253 Ser	(1)323		(27) 15	
254 Asp	(2)536		(28) 61	
255 Ile	(3)596		(29) 45	
256 Gly	(4)438		(30) 46	
257 Lys	(5)358	L5	(31) 68	
258 Ala		(1)500	(32) 56	
259 Leu		(2)465	(33) 76	
260 Pro		(3)319	(34) 25	
261 Arg		(4) 86	(35) 23	
262 Tyr		(5)276	(36) 39	
263 Glu		(6)239	(37) 60	
264 Ser		(7) 41	(38)	
265 Ser		(8) 52	(39)5	
266 Leu		(9)184		
267 Asn		(10)103		
268 Ile		(11)150		
269 Met		(12)152		
270 Thr		(13) 30		
271 Glu		(14) 87		
272 Ile		(15)110		
273 Gly		(16) 82		
274 Asn		(17) 55		
275 Arg		(18) 38		
276 Leu		(19)104		
277 Gly		(20) 60		
278 Gln		(21) 65		
279 Ala		(22) 63		
280 His		(23) 17		
281 Val		(24) 40		
282 Leu		(25) 54		
283 Leu		(26) 79		
284 Asn		(27) 22		
285 Ile		(28) 31		
286 Ala		(29) 33		
287 Lys		(30) 20	T25	
288 Cys		(31) 11	(1)297	
289 Trp		(32)	(2)143	
290 Met	CN6	(33) 10	(3)388	
291 Thr	(1) 94		(4) 99	
292 Glu	(2)354		(5)214	
293 Lys	(3)239	T26	(6)179	
294 Lys	(4)285	(1)274		
295 Leu	(5)300	(2)378		
296 Asp	(6)213	(3)263		
297 Lys	(7)186	(4)160	L6	
298 Thr	(8) 65		(1)108	
299 Leu	(9)200		(2)400	
300 Gly	(10)145		(3)267	
301 Val	(11)156		(4)224	
302 Val	(12)150		(5)226	
303 Gln	(13)174		(6)216	
304 Lys	(14)107		(7)140	
305 Ala	(15)149		(8)217	
306 Glu	(16)188		(9)118	
307 Glu	(17)201		(10)142	
308 Leu	(18)266		(11)163	
309 Ala	(19) 94		(12)163	
310 Asp	(20) 77		(13) 86	
311 Ala	(21)105		(14)119	
312 Val	(22) 74		(15) 97	
313 Gly	(23) 56		(16) 73	
314 Asn	(24) 45		(17) 39	
315 Lys	(25) 63	T27	(18) 40	
316 Leu	(26)106	(1) 252	(19) 69	
317 Val	(27) 56	(2) 137	(20) 39	
318 Leu	(28)119	(3) 115	(21) 71	
319 Leu	(29)120	(4) 125	(22) 86	
320 Lys	(30) 55	(5) 62	(23) 26	T28
321 Ala	(31) 54		(24) 32	(1)444
322 His	(32)		(25) 11	(2) 85
323 Cys	(33) 38		(26) 23	(3)243
324 Leu	(34)102		(27) 35	(4)341
325 Tyr	(35) 21		(28) 17	(5)279
326 Glu	(36) 43		(29) 24	(6)245
327 Thr	(37) 10		(30) 3	(7) 69
328 Ile			(31) 13	(8)261
329 Tyr	C13		(32) 13	(9)198
330 Arg	(1) 56	T29		(10) 16
331 Glu	(2)133	(1)1185	V8	
332 Met	(3)157	(2)1252	(1)240	
333 Gly	(4)119	(3) 771	(2)210	
334 Ser	(5) 51	(4) 330	(3) 63	
335 Asp	(6) 72	(5) 727	(4) 94	
336 Gln	(7) 70	(6) 480	(5) 91	
337 Leu	(8) 96	(7) 576	(6)106	
338 Leu	(9)110	(8) 697	(7)133	
339 Arg	(10) 43	(9) 88	(8) 24	T30
340 Asp	(11) 61		(9) 68	(1)395
341 His	(12) 19		(10) 11	(2)102
342 Val	(13) 52		(11) 54	(3)271
343 Val	(14) 72		(12) 71	(4)277
344 Lys	(15)	T31	(13) 52	(5)158
345 Phe	(16) 27	(1) 396	(14) 51	
346 His	(17) 7	(2) 97	(15) 11	
347 Glu	(18) 34	(3) 324		
348 Cys	(19) 39	(4) 343		
349 Met	(20) 14	(5) 325		
350 Glu	(21) 30	(6) 254		
351 Asp	(22) 10	(7) 174		
352 Met	(23)	(8) 243	CN7	
353 Glu	(24) 27	(9) 185	(1)391	
354 Leu	(25) 8	(10) 233	(2)402	
355 Tyr		(11) 180	(3)296	
356 Cys		(12) 171	(4)263	
357 Gly		(13) 157	(5)203	
358 Leu		(14) 172	(6)280	
359 Cys		(15) 154	(7)210	
360 Gly		(16) 112	(8)169	
361 Glu		(17) 121	(9)197	
362 Ser		(18) 22	(10) 46	
363 Ile		(19) 84	(11)142	
364 Gly		(20) 63	(12) 94	
365 Asp		(21) 49	(13) 92	
366 Gln		(22) 54	(14) 80	
367 Asn		(23) 43	(15) 77	
368 Ser		(24) 13	(16) 26	
369 Gln		(25) 36	(17) 60	
370 Leu		(26) 38	(18) 61	
371 Gln		(27) 34	(19) 58	
372 Ala		(28) 26	(20) 45	
373 Leu		(29) 28	(21) 64	
374 Pro		(30) 18	(22) 42	
375 Cys		(31) 17	(23) 31	
376 Ser		(32) 3	(24) 7	
377 His		(33) 3	(25) 5	
378 Leu		(34) 11	(26) 20	
379 Phe	C15	(35) 6	(27) 11	
380 His	(1) 75	(36) 2	(28) 7	
381 Leu	(2)169	(37) 9	(29) 19	
382 Lys	(3)182	(38) 1	(30) 4	T32
383 Cys	(4)130		(31) 11	(1)242
384 Leu	(5) 36		(32) 19	(2)294
385 Gln				(3)181
386 Thr				(4) 81
387 Asp				(5)164
388 Gly				(6)101
389 Asn				(7)109
390 Arg	T33			(8) 19
391 Gly	(1)130			
392 Cys	(2)116			
393 Pro	(3)115			
394 Asn	(4) 75	C17		
395 Cys	(5) 74	(1) 69		
396 Lys	(6) 67	(2) 97		
397 Arg		(3) 40	T34	
398 Ser		(4) 24	(1)429	
399 Ser		(5) 21	(2)371	
400 Val		(6) 51	(3)570	
401 Lys		(7) 47	(4)629	
402 Pro		(8) 47	(5)516	
403 Gly		(9) 35	(6)323	
404 Tyr		(10) 31	(7)453	
405 Val		(11) 13	(8)190	

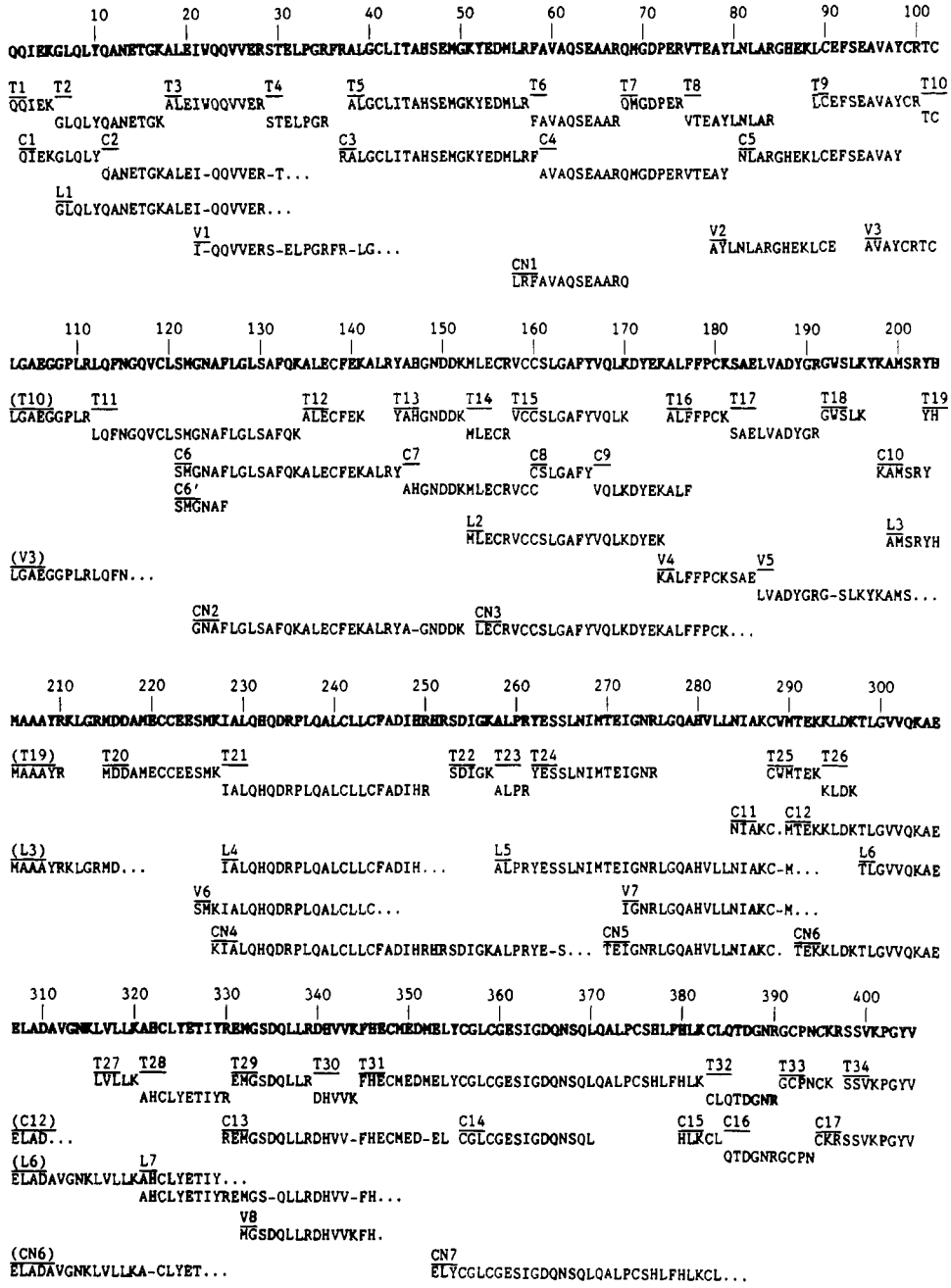

FIGURE 5: Summary of the proof of sequence of *Torpedo* 43-kDa protein. [^{14}C]CAM-43K was purified by preparative polyacrylamide gel electrophoresis as described in the text. Fragments were generated by digestion with trypsin (T), *S. aureus* V8 protease (V), endoproteinase Lys-C (L), chymotrypsin (C), and cyanogen bromide (CN). The digests were purified by reversed-phase HPLC, and the sequences of the peptides were determined by automated Edman degradation. Sequences of specific peptides (names are underlined) are given below the summary sequence (bold type). Within the sequences, dashes denote unidentified residues, and dots denote a long unidentified sequence. The numbering of the sequence is from the amino terminus of peptide T1 and not the amino terminus of 43-kDa protein which is blocked and has not been determined by microsequencing. As noted under Discussion, the amino terminus of 43-kDa protein probably extends six amino acids beyond T1. Digestion of 43-kDa protein with carboxypeptidase Y identifies a carboxyl terminus of Tyr-Val, the carboxyl terminus of T34, and the carboxyl terminus of the 405 amino acid sequence.

Assignment of amino acids to C3 was confirmed by comparison with the sequence of T5. Peptide C9 was the major sequence in fractions 132–135 but that peak also contained peptides C3 and C5, each present at about half the concentration of C9. Leucine and alanine released in cycles 3 and 9, respectively, were assigned to both C9 and C3, while glutamate and lysine of cycles 7 and 8 were assigned to both C9 and C5. These assignments were confirmed by comparison with the overlapping peptides L2 and T15 (for C9), T5 (for C3), and V2 (for C5). Peptide C12 was identified within fractions 182–185 that also contained a peptide from AChR α-subunit (beginning at glycine-73) as well as peptide C2 which was present as a secondary sequence. Lysine released at cycles 4 and 5 originated from both C12 and α-subunit, but the sequence of C12 was also confirmed in peptide CN6. The preparation of [^{14}C]CAM-43K used for the chymotryptic digest, which was isolated by slab gel electrophoresis of *Torpedo* membranes, was contaminated with AChR α-subunit as judged by immunoblot. Peptide C12 was the only chymotryptic peptide used for the proof of structure that eluted in a fraction also containing an AChR α-subunit peptide, although α-subunit peptides were identified in several other peaks, including fractions 112–114, 117–118, 124–125, and 191–192.

From the V8 digest (Figure 8), eight peptides were identified that provided important overlaps for the tryptic peptides. The

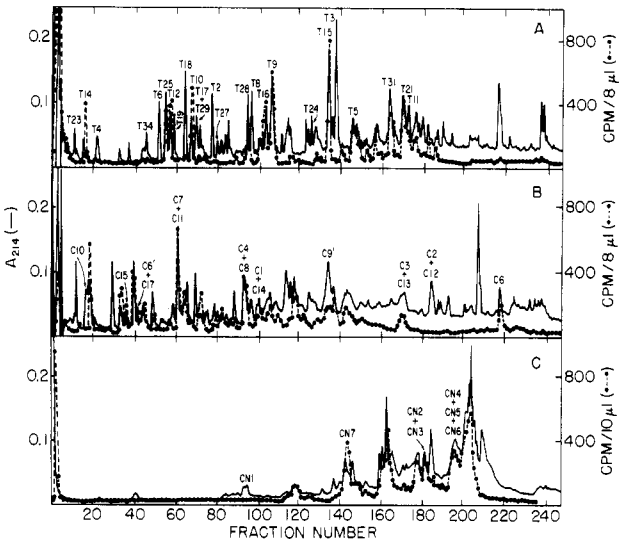

FIGURE 6: Reverse-phase separation of digests of [^{14}C]CAM-43K after cleavage with trypsin (A), chymotrypsin (B), or cyanogen bromide (C). Six-nanomole samples of [^{14}C]CAM-43K were digested as described under Experimental Procedures, and digests were resolved on a Vydac C_4 column with 0.1% TFA in water as solvent A and 0.09% TFA in acetonitrile in solvent B. The separations were monitored by measuring the absorbance at 214 nm (—) and by measuring the radioactivity (•••) in aliquots of each fraction. For the tryptic and chymotryptic digests, the flow rate was maintained at 1.0 mL/min, and 0.5-mL fractions were collected. After an initial wash with 5 mL of 5% solvent B, peptides were eluted with a linear gradient from 5% to 50% solvent B in 135 min. The breakthrough fraction from each gradient was collected and rechromatographed on a Vydac C_{18} column (Figure 7, for tryptic digest). Chymotryptic peptide C16 was isolated from this refractionation (data not shown). For the CNBr digest, after an initial wash with 10 mL of 10% solvent B, peptides were eluted with a linear gradient from 10% to 45% solvent B in 105 min. The peptides identified by the appropriate prefix T, C, or CN were then used for sequence analysis.

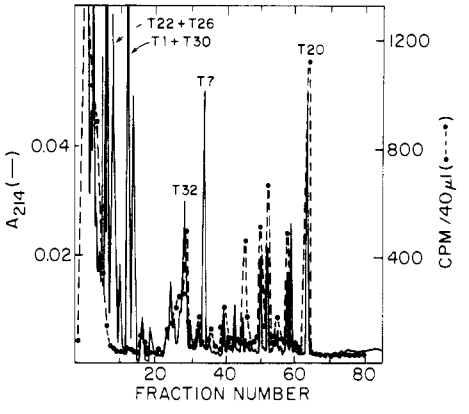

FIGURE 7: Rechromatography on a Vydac C_{18} column of material from the tryptic digest of [^{14}C]CAM-43K that was not retained on the C_4 column (Figure 6A). The C_{18} column was washed with 5 mL of 5% solvent B, and then peptides were eluted with a linear gradient from 5% to 65% solvent B in 120 min. The flow rate was 1 mL/min, and fractions of 0.5 mL were collected. Tryptic peptides T13 and T33 were isolated when material not retained on this column was rechromatographed on a C_{18} column with a gradient beginning at 2% solvent B.

sequences of six of the peptides were readily determined as the sole or primary sequence within a peak, while V1 and V6 were contained at a ratio of 1:2 in fractions 136–137. Arginine and histidine were both released in cycle 8; histidine was assigned to V6 by reference to peptides L4 and T21.

From the endoproteinase Lys-C digest (Figure 9), peptides L2–L6 were identified as unique peptides within HPLC peaks. Fractions 61–63 contained L1 as well as sequences beginning

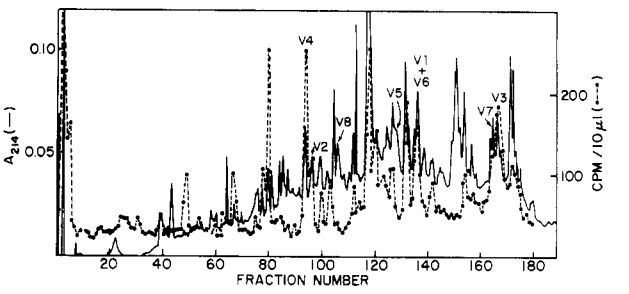

FIGURE 8: Reverse-phase separation of a digest of [^{14}C]CAM-43K after cleavage with *S. aureus* V8 protease. [^{14}C]CAM-43K (1.4 nmol) was digested as described under Experimental Procedures, and the digest was resolved on a C_4 column. Solvents A and B and flow rate were as in Figure 6. After an initial wash with 5 mL of 10% solvent B, peptides were eluted with a linear gradient from 10% to 50% solvent B in 80 min. Fractions were collected at 0.5-mL intervals. The peaks containing peptides V1–V8 used in the sequence determination are identified. As discussed in the text, fractions 136–137 contained peptides V1 and V6 in a 1:2 ratio. Fractions 164–165 contained peptides V7 and V3 at a 2:1 ratio, and fractions 166–167 contained peptides V3 and V7 at a 2:1 ratio.

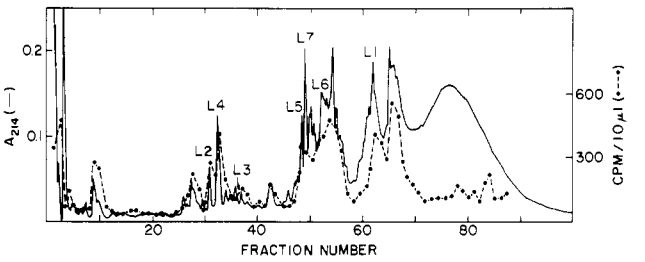

FIGURE 9: Reverse-phase separation of a digest of [^{14}C]CAM-43K after cleavage with endoproteinase Lys-C. [^{14}C]CAM-43K (6 nmol) was digested as described under Experimental Procedures, and the digest was resolved on a cyanopropyl column. Solvent A was 0.1% TFA in water; solvent B was 0.09% TFA in acetonitrile. After an initial wash with 5 mL of 10% solvent B, peptides were eluted with a linear gradient from 10% to 55% solvent B in 90 min. Fractions were collected at 1-mL (1-min) intervals. The peaks containing the peptides L1–L7 used in the sequence determination are identified. The peak containing L1 also contained sequences beginning at L5 and L6, while the peak containing L7 contained sequences beginning with the sequences of L2 and L5 (see text).

at L5 and L6, sequences identified in fractions 48–49, and fraction 54, respectively. Leucine in cycle 2 was common to all three sequences, while glutamine (cycle 6) and glutamate (cycle 9) were common to L1 and L6, and tyrosine (cycle 5) to L1 and L5. However, identification of L1 was confirmed by comparison to the sequence of T2. L7 was identified in fraction 50 that also contained peptides beginning at L2 and L5. Since the sequences of L2 and L5 were known, the sequence of L7 was determined by difference. Peptides L1, L3, L5, and L6 contained internal Lys, a result that indicates incomplete cleavage of 43-kDa protein by endoproteinase Lys-C. While this enzyme often cleaves all lysine bonds, similar partial cleavages have been reported when urokinase was digested in a buffer containing 0.1% SDS (Steffens et al., 1982).

From the HPLC fractionation of the CNBr digest (Figure 6C), 13 peaks were characterized by microsequencing. Hydrophilic peptides not retained on the column were not further characterized. Of the peptides important for the proof of structure, peptides CN1 and CN7 were identified as unique sequences, whereas CN2 and CN3 were present in fractions 181–183 at a ratio of 3:1 so that identification of these sequences was also unambiguous. Fractions 194–199 contained the sequences of peptides CN4, CN5, and CN6, and the structures of the three peptides could be determined only by reference to the tryptic, chymotryptic, and endoproteinase

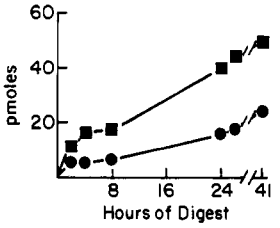

FIGURE 10: Identification of the carboxyl terminus of *Torpedo* 43-kDa protein: digestion with carboxypeptidase Y. Aliquots (2 μg, 50 pmol) of 43-kDa protein were incubated with 0.01 μg of carboxypeptidase Y at 23 °C for the indicated times. For two samples, a second aliquot of enzyme was added at 24 h, and incubation was continued for an additional 2 or 17 h. Digestion was terminated by addition of DFP to 1 mM, and free amino acids in the reaction mixture were identified after derivatization with phenyl isothiocyanate (see Experimental Procedures). Parallel control samples also analyzed included buffer blanks and enzyme in buffer. Only valine (■) and tyrosine (●) increased in a time-dependent manner above levels characteristic of the controls (5 pmol, for tyrosine and valine).

Lys-C peptides previously sequenced.

Alignment of Peptides. Peptide T1 is located close to the amino terminus of the 43-kDa protein, although its precise distance from the end is as yet unknown. Fragment C1 established that T2 and L1 follow directly on T1 while L1 allowed us to join T2 and T3. T3 identified at residue 22 the first of the 3 tryptophans contained in 43-kDa protein. V1 linked T3, T4, and T5. T4 was necessary to identify a threonine at residue 30. CN1 showed that C4 followed T5. V2, which overlapped with the last two amino acids of C4, was needed to link to T9. V3 overlapped both T9 and T11, and a substantial portion of T11 agreed with C6 and CN2. CN2 established that C7 followed directly on C6. C7 overlapped significantly with L2. C9 established that V4 followed L2 directly, while T17 established a similar ordering of V5 and V4. The second tryptophan at position 193 was identified in T18. L3 overlapped with V5 at its amino-terminal end and with T20 on the C-terminal end. The last three amino acids of T20 correspond to the first three of V6. Peptide V6 represents the beginning of V8–18 since its sequence agrees completely with that determined for V8–18. Peptides containing residues 228–244 were found in four separate digests of 43-kDa protein. CN4 showed not only that T21 followed T20 but also that L5 succeeded T22 directly. The last tryptophan at residue 289 was found in fragment T25. T26 and L6 were linked by C12 and CN6. L7, which overlapped with L6 for nine amino acids, established the ordering of the tryptic pieces T28–T31. It was present in a Lys-C HPLC peak with comparable amounts of L2 and L5 and could only be assigned a sequence because the primary structures of the other two peptides as well as T28–T31 had been clearly assigned previously. Asp-335 was identified in T29 and Lys-344 in T30. C13 was important in confirming that T31 directly followed T30. C15 linked T31 and T32 through a dipeptide overlap; C16 overlapped both T32 and T33, while C17 provides a dipeptide overlap with T33. As determined by timed digestion with carboxypeptidase Y (Figure 10), the carboxyl terminus of 43-kDa protein is tyrosine-valine, so we conclude that those residues at the carboxyl termini of T34/C17 are the carboxyl terminus of 43-kDa protein.

Discussion

In this report, we have characterized the primary structure of the *Torpedo* 43-kDa protein, the principal nonreceptor protein of the *Torpedo* nicotinic postsynaptic membrane. Because 43-kDa protein can comigrate with creatine kinase and actin on SDS–polyacrylamide gels, Gysin et al. (1983) concluded that it was necessary to use two-dimensional electrophoresis to isolate 43-kDa protein. However, by appropriate adjustment of the ratio of monomer acrylamide to bis(acrylamide), we have observed that it is possible to use a Laemmli buffer system to obtain excellent resolution in a one-dimensional gel between 43-kDa protein, the two cytosol proteins, and AChR α-subunit. With this modified gel system, 43-kDa protein or [^{14}C]CAM-43K can be readily purified from *Torpedo* membranes or from alkaline or LIS extracts of those membranes. The 43-kDa protein in the *Torpedo* membranes is characterized by isoelectric variants (pI = 7–8) (Gysin et al., 1981; Porter & Froehner, 1983), and we have found that 43-kDa protein isolated by SDS–PAGE from membranes, as well as from alkaline or LIS extracts, is also characterized by isoelectric variants. Porter and Froehner (1983) indicated that some of these charge variants might result artifactually from an interaction with SDS, but it remains possible that the charge heterogeneity results from in vivo modification of the protein.

In initial sequence experiments, 43-kDa protein was found to have an amino terminus resistant to Edman degradation. While 43-kDa protein can be cleaved by *S. aureus* V8 protease to generate two large fragments, V8–28 and V8–18 (Froehner, 1984), and we were able to isolate those peptides in sufficient quantity for microsequencing (see below), we were unable to identify conditions generating those large peptides as stable digestion products. Instead, intact 43-kDa protein was digested with proteases and CNBr to generate numerous small overlapping peptides necessary to determine the protein sequence.

The sequence of 43-kDa protein that has been determined comprises 405 amino acids extending to the carboxyl terminus and beginning with the tryptic peptide we denote T1. Our general sequencing strategy depended upon the direct identification of PTH–amino acids by microsequencing and not upon use of amino acid compositional analysis. With the HPLC system used to identify PTH–amino acids, derivatives of (carboxamidomethyl)cysteine and (carboxymethyl)cysteine coeluted with glutamate and glutamine, so cysteine was identified by released radioactivity. In initial sequencing experiments in the absence of on-line amino acid analysis, histidine and arginine were identified only rarely, while with on-line amino acid analysis, only identification of tryptophan was problematic. However, three tryptophans were identified unambiguously in the tryptic peptides T3, T18, and T25. While the PTH–amino acid was not detected in corresponding peptides from other digests, these peptides were sequenced at much lower levels than the tryptic peptides (Table II). With the exception of tryptophan and residues in five sequences (amino acids 36, 116–120, 211–214, 217–224, and 251–252), all the residues were identified in more than one peptide. However, sequence determination of peptides T11 (containing 116–120), L3 (containing 211–214), and T20 (containing 217–224) was unambiguous. Identification of Phe-36 and His-251–Arg-252 is most questionable since they were contained only within peptides V1 and CN4, respectively, peptide sequences determined from HPLC peaks also containing other peptides. Peptide alignments were made on the basis of overlapping peptides, and in most cases, peptides overlapped by more than two amino acids. Exceptions include dipeptide overlap between L3 and T20, C15 and T32, and T33 and C17. However T20 and T32 are preceded by appropriate basic residues, while peptide C17 could be generated by a less common cleavage at asparagine.

Of the 16 CNBr peptides predicted from the sequence, 10 were identified in the 13 peaks of the HPLC fractionation of

the CNBr digest (Figure 6C) that were sequenced. In addition to peptides CN1–CN7, peptides were identified beginning at Gly-70, Ala-206, and Gly-334 (Figure 6C, fractions 160–167, 93–95, and 160–162, respectively). The peptides not identified include five short, polar peptides (three to seven amino acids) probably not retained on the C_4 column, as well as a long peptide extending from the blocked amino terminus of the 43-kDa protein to Met-49.

Direct analysis of the carboxy terminus of 43-kDa protein by digestion with carboxypeptidase Y provides evidence that amino acids 404–405 (Tyr-Val) at the carboxy terminus of T34 are also the carboxy terminus of 43-kDa protein. The release of tyrosine was slow, a result consistent with the fact that penultimate glycine slows the release of the terminal amino acid (Hayashi, 1977). While the number of residues remaining unidentified at the amino terminus of 43-kDa protein is unknown, we suspect that it is limited to a single, short tryptic peptide. First, the calculated molecular weight of the 405 amino acid sequence (45 618) exceeds the molecular weight of 43-kDa protein estimated by SDS–PAGE. Also, from the extensive sequencing of peptides that has been completed, there are no peptides from any of the digests that cannot be assigned to the determined sequence. Although we have not listed all the tryptic peptides sequenced, there is only one dodecapeptide (residues 276–287) and two short peptides (residues 85–88 and 199–202) that have not been directly identified. In addition, by use of a synthetic oligonucleotide based upon amino acids 345–353 of the protein sequence, we have recently isolated and sequenced a cDNA clone encoding a substantial portion of *Torpedo* 43-kDa protein (Frail et al., 1987). This clone (43K.1) contains an open-reading frame encoding amino acids 1–382 of the amino acid sequence, as well as a possible amino-terminus extension of 30 amino acids. However, the only predicted methionine in that extension is located seven amino acids before Gln-1 of the protein sequence, and the sequence around that AUG matches the sequence for initiation codons. The sequence of 43-kDa protein predicted from the cDNA sequence confirms the directly determined sequence of 43-kDa protein, although cDNA clone 43K.1 does not extend to the carboxyl terminus of 43-kDa protein. The sequence of the amino terminus of 43-kDa protein predicted from the cDNA sequence would be a single tryptic peptide (Met-Gly-Gln-Asp-Gln-Thr-Lys) of six or seven amino acids depending on whether Met is removed. If Met is removed, the amino-terminus glycine is likely to be N-myristoylated, since the presence of Gly^1, Gln^2, and Thr^5 in the sequence matches the known specificity of protein *N*-myristoyltransferase (Towler et al., 1987). Experiments are currently under way to isolate that peptide and identify the group blocking its amino terminus.

When the sequence of 43-kDa protein was compared by use of the FASTP program (Lipman & Pearson, 1985) to the 4028 protein sequences currently in the data base of the National Biomedical Research Foundation (Release 12.0, 3/87), *Torpedo* 43-kDa protein was found to be a unique protein without significant homology to other known proteins. Thus, although 43-kDa protein has been reported to be a protein kinase (Gordon & Milfay, 1986), there is no homology to the family of protein kinases (Takio et al., 1986) or to *Torpedo* creatine kinase (Giraudat et al., 1984), and there is no evidence for a sequence within 43-kDa protein homologous to the segments within ATP binding domains of nucleotide binding phosphotransferases (Fry et al., 1986). In addition, there is no homology between 43-kDa protein and the recently sequenced erythrocyte protein 4.1 (Conboy et al., 1986), a protein that mediates interactions between integral membrane proteins and the proteins of the erythrocyte cytoskeleton. 43-kDa protein has been reported to be an actin binding protein (Walker et al., 1984), and a pentapeptide sequence (Lys-Leu-Asp-Lys-Thr, amino acids 294–298) is also found near the amino terminus of villin, an actin binding protein (Matsudaira et al., 1985). However, that sequence is not found in gelsolin, a related actin binding protein (Kwiatkowski et al., 1986), and there is no significant homology between 43-kDa protein and gelsolin or α-actinin (Baron et al., 1987).

Other than the group blocking the amino terminus, there is no evidence for posttranslational modifications of 43-kDa protein such as glycosylation, phosphorylation, or acylation. All released amino acids were directly identified, a result that rules out stoichiometric acylation of internal serine or glycosylation of Asn-13, a potential site for N-linked glycosylation. There are possible sites for phosphorylation by cyclic AMP dependent kinase (Ser-399; Krebs & Beavo, 1979) or by tyrosine kinase (Tyr-98, Tyr-189, Tyr-325; Hunter & Cooper, 1985), and phosphoamino acids would not remain stable under conditions used for Edman degradation. However, on the basis of the observed compositional analyses of 43-kDa protein after brief acid hydrolysis (Glazer et al., 1975; McCourt et al., 1985), there is less than 1 phosphoamino acid per mole of 43-kDa protein (data not shown).

Several aspects of the primary structure of 43-kDa protein suggest mechanisms concerning its mode of association with AChR or more generally with membrane lipid. After reaction of *Torpedo* membranes with sulfhydryl reagents (*N*-ethylmaleimide, iodoacetamide), 43-kDa protein can no longer be extracted by alkaline pH or LIS (Barrantes et al., 1983b). This suggests that cysteines are involved, and the cysteine content of 43-kDa protein is high (5.7 mol %). Of the 23 cysteines, 16 are concentrated in 2 regions of the molecule, constituting 10% of residues in amino acids 90–160 and 320–405. The high cysteine content of 43-kDa protein is unusual for cytoplasmic proteins in general and is in contrast to the low cysteine content of α-actinin (1 mol %; Baron et al., 1987) or band 4.1 (Conboy et al., 1986). Cysteine-rich domains have been associated with possible metal binding sites of proteins that bind to nucleic acids (Berg, 1986). In addition, protein kinase C, whose activity depends upon binding to phospholipid, contains 13 cysteines between residues 50 and 150 (Parker et al., 1986). The spacing of cysteines in kinase C beginning at Cys-115 ($CysX_2CysX_{16}CysCysX_6CysX_7Cys$) is similar to the distribution in 43-kDa protein after Cys-357 ($CysX_2CysX_{15}CysSerX_6CysX_8Cys$), and it will be important to determine whether these regions are important for interactions with phospholipids.

Analysis of the mean hydrophobicity and hydrophobic moment of amino acid sequences provides a means to identify within proteins α-helical segments that might be transmembrane or might interact directly with the membrane surface (Kyte & Doolittle, 1982; Eisenberg et al., 1984). Within the primary structure of 43-kDa protein, there are no stretches as long as 20 hydrophobic amino acids, i.e., the length characteristic of α-helical hydrophobic membrane spanning segments. However, residues 112–130 comprise a long stretch of hydrophobic and polar, uncharged amino acids, and as an α-helix, the polar residues would be clustered on one face. When the structure of 43-kDa proteins is analyzed according to the algorithm of Eisenberg et al. (1984), 3 stretches of 10–12 amino acids are identified that have average hydrophobicity (H) and hydrophobic moments (μ) characteristic of α-helices within known "surface-seeking" proteins (Table III). Also

Table III: Hydrophobicity and Hydrophobic Moment Analysis of 43-kDa Protein[a]

residues	$\langle H \rangle$	$\langle \mu_H \rangle$	classification
18–28	0.13	0.59	surface
36–46	0.35	0.42	surface
118–128	0.59	0.19	short hydrophobic
129–139	0.26	0.58	surface
158–168	0.49	0.17	short hydrophobic
236–246	0.61	0.10	short hydrophobic
276–286	0.50	0.18	short hydrophobic

[a] Mean hydrophobicity ($\langle H \rangle$) and mean hydrophobic moment ($\langle \mu_H \rangle$) were calculated for windows of 11 amino acids according to Eisenberg et al. (1984) using a normalized consensus sequence for amino acid hydrophobicity.

included in Table III are four stretches of amino acids that have hydrophobicity and hydrophobic moment parameters similar to the longer putative membrane spanning α-helices within bacteriorhodopsin (or M2 of AChR γ-subunit). While 43-kDa protein can be cross-linked to AChR β-subunit, any interaction that occurs between AChR and 43-kDa protein is disrupted by detergents such as Triton X-100 or cholate. It is tempting to speculate that hydrophobic interactions between 43-kDa protein and AChR are more important than ionic interactions.

Knowledge of the primary structure will permit the use of immunological techniques to identify within 43-kDa protein regions that are normally exposed when the protein is associated with the nicotinic postsynaptic membrane, as well as other regions that become accessible to antibodies only after 43-kDa protein is removed from the membrane. Monoclonal antibody 2316A, which recognizes an exposed epitope as judged by immunofluorescence of frozen sections of *Torpedo*, binds to V8–28, the large amino-terminus fragment of 43-kDa protein generated by *S. aureus* V8 protease. Froehner (1984) isolated a series of monoclonals to *Torpedo* 43-kDa protein and grouped them into five classes by reference to their binding to peptide fragments produced by V8 protease. One antibody (Mab 1201C), which also recognizes the mammalian counterpart of *Torpedo* 43-kDa protein, binds to an epitope contained within V8–18, the carboxyl third of 43-kDa protein beginning at Ser-225 of the protein sequence. By comparison of the binding of Mabs 2316A and 1201C to V8 proteolysis fragments of 43-kDa protein, the epitope recognized by 1201C can be further mapped to within ~3 kDa of the carboxyl terminus of 43-kDa protein. Experiments are in progress to identify exposed epitopes within 43-kDa protein in terms of the HPLC peptide map reported here and to define the topology of the protein by use of antibodies directed against synthetic peptides based upon sequences contained within 43-kDa protein.

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Electron Transfer from Cytochrome b_5 to Iron and Copper Complexes†

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ABSTRACT: The rates of electron transfer from the tryptic fragment of bovine liver cytochrome b_5 to $Fe^{III}NTA$, $Fe^{III}ATP$, $Cu^{II}NTA$, $Cu^{II}ATP$, and $Cu^{II}His$ have been measured by anaerobic stopped-flow techniques. The rates of reduction of the Fe(III) complexes are independent of ionic strength, enhanced at low pH, and slightly inhibited by $Zn^{II}NTA$. Saturation kinetics are observed with $Cu^{II}NTA$ ($k_{et} = 0.05\ s^{-1}$, $K = 8.6\ M^{-1}$), $Cu^{II}His$ ($k_{et} = 0.2\ s^{-1}$, $K = 2.6 \times 10^3\ M^{-1}$), and $Cu^{II}ATP$ ($k_{et} = 0.6\ s^{-1}$, $K = 4.5 \times 10^3\ M^{-1}$), thereby indicating that binding of Cu(II) to the protein occurs prior to electron transfer. ^1H NMR resonances of the three surface histidines and some neighboring residues have been assigned by two-dimensional NMR techniques. NMR titration experiments show unequivocally that $Cu^{II}NTA$ binds preferentially at a site near His-26 and Tyr-27.

It has been demonstrated that cytochrome b_5 is reduced by $Fe^{II}EDTA$[1] through direct transfer of an electron at the exposed heme edge (Reid & Mauk, 1982). A similar mechanism is employed in the reduction of $Fe^{III}NTA$ by myoglobin (Hegetschweiler et al., 1987). A very different pathway, however, is utilized in the oxidation of hemoglobin β chains by $Cu^{II}NTA$ (Rifkind, 1981). Subsequent to Cu(II) binding to Cys-93, an electron apparently is transferred a long distance from Fe(II) to Cu(II). The plausibility of a long-range mechanism for the $Cu^{II}NTA$ oxidation of hemoglobin has been strengthened by the observation of 10–25-Å electron transfers in fixed-distance experiments on several heme proteins (Mayo et al., 1986).

We have employed anaerobic stopped-flow techniques to measure the rates of electron transfer from the tryptic fragment of bovine liver cytochrome b_5 to $Fe^{III}NTA$, $Fe^{III}ATP$, $Cu^{II}NTA$, $Cu^{II}ATP$, and $Cu^{II}His$. The evidence reported here suggests that a long-range pathway is utilized in the b_5 to Cu(II) electron transfers.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid.

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