Assessment of the Number of Free Cysteines and Isolation and Identification of Cystine-Containing Peptides from Acetylcholine Receptor†

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Appendix: Reversible Modification of Cysteine with Cyanogen Bromide

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ABSTRACT: The number of free cysteines in each polypeptide of acetylcholine receptor from the electric organ of *Torpedo californica* has been assessed by alkylating the native protein with *N*-ethylmaleimide and iodoacetamide during homogenization of the tissue and alkylating the polypeptides with *N*-ethylmaleimide as they were unfolded in solutions of dodecyl sulfate. The cysteines unavailable for alkylation could be accounted for as specific cystines, connecting positions in the amino acid sequences of the individual polypeptides. Unreduced, alkylated polypeptides of acetylcholine receptor were digested with thermolysin or trypsin. Cystine-containing peptides in the chromatograms of the digests were identified electrochemically by the use of a dual gold/mercury electrode. Three thermolytic peptides and three tryptic peptides have been isolated from these digests and shown to contain intact cystines that were originally present in the native protein. The majority of these peptides contained an intact, intramolecular cystine connecting two cysteines in locations homologous to cysteines 128 and 142 from the α polypeptide. Each of these cystines from each of the polypeptides of acetylcholine receptor was isolated in at least one peptide, respectively. Each of these cystine-containing peptides also contained glucosamine. It can be concluded that each asparagine in the sequence Asn-Cys-Thr/Ser, which occurs in the respective, homologous location in every polypeptide, is glycosylated even though a cystine sits between the asparagine and the threonine or serine. In addition, the existence of the cystine connecting the adjacent cysteines, α192 and α193, in the α subunit of acetylcholine receptor [Kao, P. N., & Karlin, A. (1986) *J. Biol. Chem. 261*, 8085–8088] has been confirmed.

Acetylcholine receptor is a transmembrane glycoprotein of approximate molecular weight 250000 (Noda et al., 1983c) located in the postsynaptic membranes of vertebrates. It is made up of four subunits (α, β, γ, and δ) that are homologous to each other in their amino acid sequences (Noda et al., 1983c) and that are arranged in a pseudosymmetric pentamer of the composition $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1978; Brisson & Unwin, 1985). Binding of acetylcholine to the α subunit leads to the transient opening of a pore, selectively permeable to cations, through the receptor (Adams et al., 1980), and ultimately to muscle contraction. Though the primary sequences of the four polypeptides from various species are known (Noda et al., 1982, 1983a–c; Claudio et al., 1983; Merlie et al., 1983; Devillers-Thiery et al., 1983; Takai et al., 1984; Nef et al., 1984; La Polla et al., 1984; Tanabe et al., 1984; Shibahara et al., 1985; Kubo et al., 1985; Yu et al., 1986; Isenberg et al., 1986; Boulter et al., 1986), very little is known about their tertiary structure when folded to form their respective subunits. We were interested in determining the disposition of the cystines in acetylcholine receptor as a means of identifying juxtapositions in the tertiary structures of the subunits.

By affinity labeling acetylcholine receptor with an analogue of acetylcholine, [4-(*N*-maleimido)benzyl]tri[^{3}H]methylammonium iodide ([^{3}H]MBTA),[1] Karlin (1969) demonstrated that a readily reducible cystine exists within 1 nm of the binding site for acetylcholine. In a later study, Kao et al. (1984) were able to isolate a cyanogen bromide fragment, identified as residues α179–α207, from acetylcholine receptor of *Torpedo californica*, into which [^{3}H]MBTA had become incorporated after mild reduction of native acetylcholine receptor with dithiothreitol. Automated Edman degradation of this fragment showed that either cysteine α192 or cysteine α193, or both, contribute to at least half of the cystine near the binding site.

In a later study, Kao and Karlin (1986) presented several results bearing on the disposition of the cystines within the α subunit of native acetylcholine receptor from *T. californica*. When the labeling with [^{3}H]MBTA after mild reduction was performed in the presence of *N*-[^{14}C]ethylmaleimide, to capture the other half of the cystine whose opening had presented cysteine α192 or cysteine α193 to the [^{3}H]MBTA, only the one cyanogen bromide fragment, α179–α207, was labeled with either *N*-[^{14}C]ethylmaleimide or [^{3}H]MBTA. When this

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[1] Abbreviations: [^{3}H]MBTA, [4-(*N*-maleimido)benzyl]tri[^{3}H]-methylammonium iodide; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; TPBS, 0.05% (v/v) Tween 20, 0.9% sodium chloride, and 10 mM sodium phosphate, pH 7.5; PTC, phenylthiocarbamyl; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane.

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cyanogen bromide fragment was isolated from the α subunit of unreduced and unlabeled receptor, it was shown that the incorporation of [^{14}C]iodoacetamide into this purified cyanogen bromide fragment, which contains cysteines α192 and α193 as its only cysteines, occurred only after exposure to dithiothreitol. A second cyanogen bromide fragment was isolated from the unreduced α subunit and identified as residues α118–α144, the sequence containing cysteines α128 and α142. It, too, incorporated [^{14}C]iodoacetamide only after exposure to dithiothreitol. From these results, Kao and Karlin (1986) concluded that cysteines α192 and α193 together form a vicinal cystine within 1 nm of the binding site for acetylcholine and that a separate, distinct cystine exists between cysteines α128 and α142.

We have isolated six peptides from acetylcholine receptor with their cystines intact and have identified them by their amino acid sequences. In addition, the number of free cysteines in acetylcholine receptor has been assessed.

Experimental Procedures

Materials. Sodium dodecyl sulfate ($NaDodSO_4$)[1] was recrystallized from 95% ethanol. Sodium perchlorate ($NaClO_4$) was recrystallized from dioxane. Iodoacetamide and *o*-phthalaldehyde were recrystallized from *n*-heptane. Trifluoroacetic acid, phenyl isothiocyanate, triethylamine, and 88% formic acid were redistilled before use. 1-Propanol and 2-propanol of a purity suitable for high-pressure liquid chromatography (HPLC)[1] were from Burdick & Jackson; *N*-ethylmaleimide was from Kodak Biochemicals; [^{3}H]sodium borohydride was from New England Nuclear; leucine aminopeptidase, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES),[1] diaminobenzidine tetrakis(hydrochloride), ovalbumin, and thermolysin were from Sigma Chemical Co.; tris(hydroxymethyl)aminomethane (Tris)[1] was from Mallinckrodt; biotinylated concanavalin A, avidin DH, and biotinylated horseradish peroxidase were from Vector Laboratories Inc.; nitrocellulose paper (0.2 μm) was from Schleicher & Schuell; bovine serum albumin was from Miles Diagnostics; acetonitrile suitable for HPLC was from Fisher Chemical Corp.; and trypsin treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone was from Worthington Biochemical Corp.

Purification of Acetylcholine Receptor. Acetylcholine receptor was purified by the method of Elliot et al. (1980) with the following modifications. Electric organs obtained from freshly killed *T. californica* were frozen in liquid nitrogen and stored at −70 °C until use. Slightly thawed tissue (100–120 g) was diced, and an equal volume of 0.4 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA),[1] 5 mM iodoacetamide, 5 mM *N*-ethylmaleimide, 3 mM phenylmethanesulfonyl fluoride, and 10 mM sodium phosphate, pH 7.8, at 4 °C, was added. The tissue was fragmented at high speed in a commerical blender (Waring Corp.) for 4 min, and the homogenate was centrifuged in a GSA rotor (Sorvall Corp.) at 5000 rpm for 10 min at 4 °C. The supernatant was filtered through four layers of cheesecloth and pelleted by centrifugation in a 50.2 Ti rotor (Beckman Corp.) at 16000 rpm for 60 min. All further centrifugations were performed in this 50.2 Ti rotor. Pellets were resuspended in 0.8 M NaCl, 2 mM EDTA, 0.04% NaN_3, and 20 mM sodium phosphate, pH 7.4, and sucrose and water were added to produce final concentrations of 30% sucrose, 0.4 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 10 mM sodium phosphate, pH 7.4. The suspension was homogenized, and portions of this suspension (7.5 mL) were layered onto a discontinuous gradient of 3.75 mL of 50% sucrose (w/w), 6 mL of 39% sucrose (w/w), and 6.75 mL of 35% sucrose (w/w), each prepared in 0.4 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 20 mM sodium phosphate, pH 7.4. The gradient was overlaid with 1.5 mL of this buffer containing no sucrose, and it was centrifuged for 60 min at 45000 rpm. Bands at the interface between 35% and 30% sucrose (band 2) and at the interface between 39% and 35% sucrose (band 3) were collected separately, an equal volume of the above buffer was added to each, and the samples were homogenized. These homogenates were centrifuged at 30000 rpm for 60 min, and the resulting pellets were resuspended in 0.4 M NaCl, 0.25 M sucrose, 1 mM EDTA, and 10 mM sodium phosphate, pH 7.4, and stored at −70 °C overnight.

On the second day, the suspensions were thawed, diluted, and homogenized with 10 mM TES, pH 5.5, and centrifuged at 30000 rpm for 60 min. The pellets were collected and homogenized in 1 mM TES, and the pH was carefully adjusted to 11.0 with 0.25 M NaOH. The suspension was stirred at 4 °C for 60 min and centrifuged at 18000 rpm for 45 min. Hard and soft pellets were collected separately and resuspended in water, and the pH was carefully adjusted to 11.0. The suspensions were centrifuged at 18000 rpm for 45 min, and final pellets were resuspended in a small volume of 0.25 M sucrose, 1 mM EDTA, and 30 mM histidinium chloride, pH 7.4, for storage at −70 °C.

Purity of the resulting membranes enriched in acetylcholine receptor was determined by electrophoresis. Both the hard and soft pellets from band 2 and the hard pellet from band 3 typically gave the cleanest preparations. Yield was approximately 10 mg of purified acetylcholine receptor (110 g of electroplax)$^{-1}$, as determined by total amino acid analysis of the final product.

Binding of Toxin. Determination of nanomoles of binding sites for α-toxin (mg of receptor)$^{-1}$ was done by the method of Kohanski et al. (1977) with the following modifications. α-Toxin from *Naja naja siamensis* (Biotoxins Inc., St. Cloud, FL), purified by ion-exchange chromatography, was reductively alkylated by the method of Rice and Means (1971), except that NaB_3H_4 was used rather than [^{14}C]formaldehyde. Specific radioactivity was 1.5×10^7 cpm (mg of toxin)$^{-1}$ as determined by amino acid analysis. [^{3}H]-α-Toxin (6.7×10^{-11} mol) was added to a sample of acetylcholine receptor (approximately 5×10^{-11} mol of binding sites) suspended in 275 μL of 0.4 M NaCl, 1 mM EDTA, and 10 mM sodium phosphate, pH 7.2. Binding was allowed to occur over 10 min at room temperature. Cholate was added to 1%, and the sample was dialyzed against 0.01% Triton X-100, 0.02% NaN_3, and 1 mM sodium phosphate, pH 7.2. The dialyzed sample was applied to a column packed with 1.25 mL of (carboxymethyl)cellulose (Whatman CM-52) equilibrated with the same buffer. Columns were eluted with 1.25–1.6 mL of the same buffer, and the effluent, containing the complex between [^{3}H]-α-toxin and acetylcholine receptor, was submitted to liquid scintillation counting. The addition of cholate (maximum effect at 1%) was necessary to maximize the yield of the complex. This may be due to the presence of sealed, inside-out vesicles in our preparations. Levels of binding were 3–4 nmol of toxin (mg of protein)$^{-1}$.

Electrophoresis. Membranes rich in acetylcholine receptor were submitted to electrophoresis on slab gels ($0.15 \times 10 \times 14$ cm) or tube gels (12×0.8 cm) of polyacrylamide cast from 10% acrylamide in 0.1% $NaDodSO_4$ with a stacking gel of 2% polyacrylamide (Laemmli, 1970). Purified membranes produced the four heavily stained polypeptides (α, β, γ, and δ) and δ_2 dimer depending on whether or not the samples had been reduced. Two other components, one of high molecular

weight (approximately 1000 residues in length) and the other being the ν subunit (Porter & Froehner, 1983), were usually observed.

Isolation of Denatured Polypeptides from the Subunits. A suspension of acetylcholine receptor (approximately 30 mg) was brought to 5 mM in *N*-ethylmaleimide, and $NaDodSO_4$ was added to a final 3-fold excess by weight over protein. The *N*-ethylmaleimide was added first to ensure alkylation of all free sulfhydryls as they became exposed upon unfolding of the protein (Cecil, 1963). After 30 min at 37 °C, the sample was lyophilized, brought up in 4 mL of water, and applied to a column (2.6 × 90 cm) of agarose A5m (Bio-Rad Corp.) equilibrated with 0.1% $NaDodSO_4$ and 40 mM Tris sulfate, pH 8.0 (Kyte, 1972). Protein was eluted at a flow rate of 7 mL h^{-1}, and the distribution of the α, β, γ, δ, and δ_2 polypeptides over the elution profile was determined electrophoretically. The fractions containing the desired polypeptides were pooled and concentrated by lyophilization. The dodecyl sulfate was removed from the polypeptides exactly as described by Nicholas (1984).

Thermolytic Digestion. Thermolysin (1 mg mL^{-1}) in 50 mM ammonium bicarbonate, pH 8.0, was made up immediately before use and a portion added to the protein stripped of its dodecyl sulfate and dialyzed into the same buffer. Thermolysin was added at a 1:100 ratio of thermolysin:acetylcholine receptor, and the solution was incubated at 37 °C.

The degree of thermolytic digestion was monitored by the digestion of a portion of the material with leucine aminopeptidase. The nanomoles of isoleucine, leucine, phenylalanine, and valine released by leucine aminopeptidase from a small sample were compared to the total nanomoles of these four amino acids as determined by total amino acid analysis on a sample of equal volume. Specifically, two aliquot parts were removed from the digest. After norleucine was added as an internal standard, one of these was lyophilized and hydrolyzed in 300 μL of 6 M HCl for 20 min at 155 °C under nitrogen. To the other, leucine aminopeptidase at 1 unit (4 mg of protein)$^{-1}$ was added along with 900 μL of 2.5 mM $MgCl_2$, 50 mM sodium phosphate, and 50 mM sodium borate, pH 8.5, and digestion was carried out at 37 °C. At 30 and 60 min, respectively, a portion was removed, norleucine was added, and the sample was lyophilized. Samples were then run on a Model 118C amino acid analyzer (Beckman Corp.).

Digestion was continued by adding a second equal portion of thermolysin if this was indicated by the results of the assay with leucine aminopeptidase. Digestion was terminated by lyophilization.

Tryptic Digestion. Trypsin (1 mg mL^{-1}) in 50 mM ammonium bicarbonate, pH 8.0, was made up immediately before use and a portion added to the protein stripped of its dodecyl sulfate and dialyzed into the same buffer. Trypsin was added at a 1:100 ratio of trypsin:acetylcholine receptor. The solution was incubated at 37 °C. After 6 h, another aliquot of trypsin was added and the solution was incubated overnight.

To assess how far the digestion had proceeded, the nanomoles of lysine and arginine released by carboxypeptidase B from a small sample of the digest were compared to the total nanomoles of these two amino acids determined by total amino acid analysis on a sample of equal volume. Specifically, two equal portions were removed from the digest. To one, norleucine was added, and the sample was lyophilized and hydrolyzed in 300 μL of 6 M HCl for 20 min at 155 °C under nitrogen. The other was digested with carboxypeptidase B at 1 unit (4 μg)$^{-1}$, which was added along with 300 μL of 100 mM *N*-ethylmorpholine, pH 8.5, and digestion was carried out at 37 °C. At 30 and 60 min, respectively, a portion of the digest was removed, norleucine was added, and the sample was lyophilized. All samples were then run on a Model 118C amino acid analyzer.

Molecular-Exclusion Chromatography on Fractogel TSK HW40s. Thermolytic or tryptic peptides were dissolved in 500 μL of 88% formic acid. The resulting slightly cloudy solution was applied to a column (0.9 × 50 cm) of Fractogel TSK HW40s (Supelco Co.) equilibrated in 95% ethanol/88% formic acid (4:1) (Nicholas, 1984). Peptides were eluted with this solvent at a flow rate of 7.5 mL h^{-1}.

High-Pressure Liquid Chromatography. Fractions from the column of HW40s chromatographic medium were lyophilized, redissolved in 88% formic acid, and submitted to HPLC on a system consisting of the following components from Waters Associates: two Model M6000A pumps with pulse dampener, a Model 600 automatic gradient controller, and a Model 400 UV detector equipped with an extended wavelength module operating at 229 nm. The effluent from the detector was directed into an electrochemical cell designed for detection of disulfides with the following components from Bioanalytical Systems Inc.: two Model LC-4B amperometric controllers, a dual gold/mercury electrode with a collection port, and a Model RE3 reference electrode. Samples were dissolved in 50–100 μL of 88% formic acid, purged of oxygen under a stream of argon, and injected onto a Vydac reverse-phase C_4 column (0.46 × 25 cm), a Brownlee Labs reverse-phase C_8 column (0.46 × 25 cm), an Altex reverse-phase ultrasphere ODS (C_{18}) column (0.46 × 25 cm), or an Altex reverse-phase C_3 column (0.46 × 7.5 cm).

To detect cystine with the electrochemical cell, a reduction (−1.00 V) was carried out at the first electrode and an oxidation (+0.156 V) was carried out at the second electrode. Current at the second electrode was monitored at 20 or 50 nA full scale. To exclude oxygen, buffers were kept under a constant stream of helium and only stainless steel tubing was used. The electrodes were turned off until the solvent breakthrough had occurred to improve performance. Only a very small percentage of the disulfides in the effluent are reduced or oxidized at the electrode surface, and this permits the collection of peptides from the electrochemical flow cell with their cystines essentially intact.

High-pressure liquid chromatography of phenylthiocarbamyl (PTC)[1] amino acids and PTC amino sugars was done on a Waters Novapak C_{18} column at a constant temperature of 52 °C. A hydrolysate that had been reacted with phenylisothiocyanate (Heinrickson & Meredith, 1984) was dissolved in 100 μL of 22% acetonitrile (v/v) and injected onto the column. It was eluted from the column with use of a linear gradient formed from 0.14 M sodium acetate and 0.5 mL L^{-1} triethylamine, pH 6.36 (solvent A), and 60% acetonitrile in water (solvent B). The linear gradient was between 95% solvent A and 5% solvent B and 48% solvent A and 52% solvent B over 30 min (Bidlingmeyer et al., 1984). On this chromatographic system, the PTC derivative of glucosamine elutes after PTC-glutamic acid and before PTC-serine.

Analysis for Glucosamine. Samples to be assayed for glucosamine were hydrolyzed for 6 h in 4 M HCl at 100 °C under reduced pressure. Hydrolysates were then modified with phenylisothiocyanate according to the method of Heinrikson and Meredith (1984).

Amino Acid Analyses. Amino acid analyses were performed on samples hydrolyzed at 110 °C for 24 h in 6 M HCl. For analyses of polypeptides separated by electrophoresis on cylindrical polyacrylamide gels, stained bands were sliced from

the gels and hydrolyzed directly. All samples were evacuated with three cycles of freezing and thawing under a vacuum of 20–80 μmHg. Norleucine was used an an internal standard in all samples. Hydrolysates were run on a Model 118C amino acid analyzer (Beckman Corp.). To separate *S*-succinylcysteine from aspartic acid, the elution regime designed by the manufacturer for hydrolysates of collagen was used. Amino acids in the effluent from the cation-exchange column of the amino acid analyzer were monitored with the standard ninhydrin regime or by mixing the effluent flow (0.6 mL min^{-1}) with a flow of 6 mM *o*-phthalaldehyde, 70 mM 2-mercaptoethanol, 0.18% Brij 35, and 0.4 M sodium borate, pH 10.5 (0.3 mL min^{-1}) and following fluorescence (Benson & Hare, 1975) in the effluent stream with a Gilson Model FL-1A flow spectrophotometer. This method of fluorometric detection was modified to enable detection of cystine eluting as the intact disulfide from the cation-exchange column. The modification consisted of continuous reduction (with 2-mercaptoethanol) and alkylation (with iodoacetic acid) of the effluent from the chromatographic column before reaction with the *o*-phthalaldehyde.[2] This adaptation permits the detection of 0.1 nmol of cystine.

Sequencing. Manual, dansyl–Edman sequencing was performed in the presence of $NaDodSO_4$ by the method of Weiner et al. (1972). For automated sequencing, the sample to be sequenced was dissolved in 88% formic acid and submitted to Edman degradation on a Model 470A protein sequencer (Applied Biosystems Inc.) according to published methods (Hewick et al., 1981). Triethylamine (2 mM) was added to the aqueous solution of the on-line Model 120 phenylthiohydantoin (PTH)[1] analyzer (Applied Biosystems Inc.) in order to stabilize the retention times of PTH-histidine and PTH-arginine.

Binding of Concanavalin A. Binding of concanavalin A was performed by using the biotin–avidin–horseradish peroxidase system (Hsu et al., 1981) for blotting protein (Vector Laboratories). The purified peptide was dissolved in 5 μL of 88% formic acid and pipetted onto 0.2-μm nitrocellulose paper. Ovalbumin (1 μL at 2 mg mL^{-1} in 88% formic acid) and bovine serum albumin (1 μL at 2 mg mL^{-1} in 88% formic acid) were pipetted separately onto the same membrane. Ovalbumin, which is glycosylated and binds concanavalin A, and bovine serum albumin, which is not glycosylated and does not bind concanavalin A, were used as internal monitors. The membrane was immersed in 0.05% (v/v) Tween 20, 0.9% sodium chloride, and 10 mM sodium phosphate, pH 7.5 (TPBS),[1] and incubated for 30 min with gentle agitation. The membrane was then incubated for 60 min in 4 mL of TPBS containing 50 μg of biotinylated concanavalin A and washed three times over 30 min in TPBS without concanavalin A. The substoichiometric complex between avidin and biotinylated horseradish peroxidase was prepared by adding 50 μL each of solutions of avidin DH (300 μM in sites) and biotinylated horseradish peroxidase (75 μM in biotin) to 10 mL of TPBS and allowing the solution to stand for 30 min. Incubation of the membrane in this solution for 30 min with gentle agitation was followed by three washes with 0.9% sodium chloride and 100 mM sodium phosphate, pH 7.5, over 30 min. Substrate for the peroxidase was prepared by adding 1 mL of a 5 mg mL^{-1} solution of diaminobenzidine tetrahydrochloride in 0.1 M Tris, pH 7.2, to a solution containing 5 μL of 30% hydrogen peroxide, 50 μL of 8% $NiCl_2$,and 9 mL of water. The membrane was developed in the solution of diaminobenzidine until maximum color intensity had been reached, usually between 1 and 5 min.

[2] J. Kyte and S. Smith, personal communication.

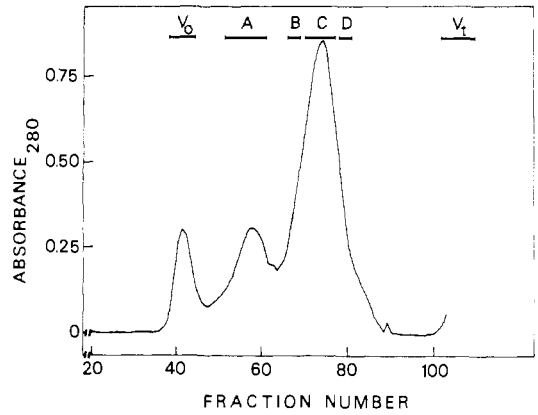

FIGURE 1: Molecular-exclusion chromatography of acetylcholine receptor. A solution of *N*-ethylmaleimide, dissolved in ethanol, was added to a final concentration of 5 mM to 30 mg of acetylcholine receptor suspended in 0.25 M sucrose, 30 mM histidinium chloride, and 1 mM EDTA, pH 7.1. A 3-fold excess of $NaDodSO_4$ over protein was added, and the solution was incubated at 37 °C for 30 min and lyophilized. The sample was dissolved in 4 mL of water and loaded onto a column of Bio-Gel A5m (2.6 × 90 cm) equilibrated in 0.1% $NaDodSO_4$ and 40 mM Tris–sulfate, pH 8.0, and the polypeptides eluted at a flow rate of 7 mL h^{-1}. The positions of the void (V_0) and included (V_t) volumes are indicated. The approximate elution positions of the polypeptides are indicated by pools A–D as follows: contaminant of apparent length 1000 residues and δ_2 dimer (pool A); α, β, γ, and δ_2 polypeptides (pool B); α, β, and γ polypeptides (pool C); and α polypeptide (pool D).

Results

Total Number of Cysteines in Each Polypeptide of Native Acetylcholine Receptor. Acetylcholine receptor (30 mg, 100 nmol), purified by a modification of the method of Elliot et al. (1980), in the presence of iodoacetamide to prevent proteolytic degradation and *N*-ethylmaleimide to prevent disulfide interchange, was brought to 5 mM *N*-ethylmaleimide and dissolved by adding an appropriate amount of a concentrated solution of $NaDodSO_4$ (3 mg of $NaDodSO_4$ for every milligram of protein). This sample was submitted to molecular-exclusion chromatography on a column of agarose A5m (Figure 1). The fractions between pools A and B (Figure 1) contain significant amounts of the α, β, and γ polypeptides and the covalent dimer of the δ polypeptide (δ_2). A sample (100 μg) was removed from a pool of these fractions, and the polypeptides were separated by gel electrophoresis on a cylindrical gel of 10% polyacrylamide. The α, β, γ, and δ_2 polypeptides, identified by their electrophoretic mobilities, were sliced from the stained gel and subjected to hydrolysis for 24 h at 110 °C. Cysteines present in native acetylcholine receptor in vivo should end up as *S*-succinylcysteine or *S*-(carboxymethyl)cysteine in the hydrolysates of the various polypeptides, because all free sulfhydryls should have been alkylated with either *N*-ethylmaleimide or iodoacetamide during the various steps in the procedures used to purify the polypeptides. A quantitative determination of these two derivatives of cysteine should indicate the minimum number of cysteines in each of the subunits in the native protein.

S-(*N*-Ethylsuccinimid-2-yl)-L-cysteine was synthesized by the method of Smyth et al. (1960) and submitted to hydrolysis in acid and to amino acid analysis with a buffer system designed for the separation of amino acids derived from collagen on the Model 118C amino acid analyzer. This system cleanly separates *S*-succinylcysteine and aspartic acid. The retention times[3] were 0 min for cysteic acid, 20 min for *S*-succinyl-

Table I: Content of *S*-Succinylcysteine in Hydrolysates of the Polypeptides of Acetylcholine Receptor[a]

	α polypeptide		β polypeptide		γ polypeptide		δ_2 polypeptide	
amino acid	nmol in hydrolysate	nmol of polypeptide[b]	nmol in hydrolysate	nmol of polypeptide[b]	nmol in hydrolysate	nmol of polypeptide[b]	nmol in hydrolysate	nmol of polypeptide[b]
Asx	2.3	0.056	1.6	0.033	2.6	0.052	2.6	0.048
Thr	1.6	0.054	1.0	0.033	1.4	0.054	1.2	0.046
Ser	1.8	0.057	1.2	0.036	1.6	0.056	1.6	0.046
Glx	2.2	0.060	1.7	0.042	3.0	0.053	2.0	0.046
Ala	1.1	0.080	1.1	0.042	1.4	0.062	1.3	0.054
Val	2.3	0.057	1.5	0.036	1.9	0.056	1.4	0.041
		0.061[d]		0.037		0.056		0.047
S-succinyl-Cys[c]		0.12 nmol		0.10 nmol		0.25 nmol		0.09 nmol

[a] The four polypeptides of acetylcholine receptor that had been alkylated with iodoacetamide and *N*-ethylmaleimide during homogenization of the tissue and *N*-ethylmaleimide during unfolding of the protein in a solution of dodecyl sulfate were separated by electrophoresis and hydrolyzed for total amino acid analysis. The nanomoles of selected amino acids from each sample are tabulated. [b] Calculated from the nanomoles of the particular amino acid and the amino acid composition of the polypeptide. Each value is the nanomoles of the polypeptide contributing to the sample actually submitted to amino acid analysis. [c] Amount of *S*-succinylcysteine in each sample submitted to amino acid analysis. [d] Mean value.

cysteine, and 27 min for aspartic acid.

Hydrolysates of the slices containing the α, β, γ, and δ_2 polypeptides, respectively, were submitted to analysis on this chromatographic system (Table I). From these analyses it can be calculated that there were 2.0 nmol of *S*-succinylcysteine for each 1 nmol of the α polypeptide, 2.7 nmol for each 1 nmol of the β polypeptide, 4.5 nmol for each 1 nmol of the γ polypeptide, and 1.9 nmol for each 1 nmol of the δ polypeptide. Only the hydrolysate from the γ polypeptide contained *S*-(carboxymethyl)cysteine. There were 2.4 nmol of *S*-(carboxymethyl)cysteine (nmol of γ polypeptide)$^{-1}$. The experiment was repeated, and 2.6 nmol of *S*-succinylcysteine was found for each 1 nmol of the α polypeptide; 3.2 nmol, for each 1 nmol of the β polypeptide; 3.8 nmol, for each 1 nmol of the γ polypeptide; and 0.8 nmol, for each 1 nmol of the δ polypeptide. In this second assessment, the hydrolysate of the γ polypeptide contained 1.5 nmol of *S*-(carboxymethyl)cysteine (nmol of polypeptide)$^{-1}$.

Digestion and Molecular-Exclusion Chromatography. The fractions from the column of agarose A5m containing the α, β, and γ polypeptides, which were found to be within pool C (Figure 1) by electrophoresis, were combined and lyophilized. A second sample of acetylcholine receptor (also 30 mg) was run identically to the first, and the respective pool containing the α, β, and γ polypeptides was combined with the first pool. The polypeptides in the combined pools (12 mg by amino acid analysis) were then stripped of dodecyl sulfate by the method of Weber and Kuter (1971) as modified by Sharkey (1983) and digested at 37 °C with 10 μg of thermolysin (mg of protein)$^{-1}$. The extent of digestion was determined by treatment with leucine aminopeptidase. After 5 h of thermolytic digestion, the release of valine, isoleucine, leucine, and phenylalanine by leucine aminopeptidase was 39%, 49%, 32%, and 32%, respectively, of the total amount of each of these amino acids in the sample. A second addition of thermolysin (10 μg mg^{-1} for 10 h) only increased these percentages to 41%, 56%, 31%, and 45%, respectively. The lack of a significant increase indicated that maximum digestion had been reached. The digest was lyophilized, redissolved in 88% formic acid, and submitted to molecular-exclusion chromatography on a column of Fractogel TSK HW40s equilibrated and eluted with 95% ethanol/88% formic acid (4:1 v/v). The distribution of eluted peptides was determined by reading the ultraviolet absorption of each fraction at 280 nm and by amino acid analysis (Figure 2). Cystine was found in fractions 20 and 21 [140 nmol of cystine (350 μmol of total amino acids)$^{-1}$] and fractions 22 and 23 [15 nmol of cystine (7 μmol of total amino acids)$^{-1}$].

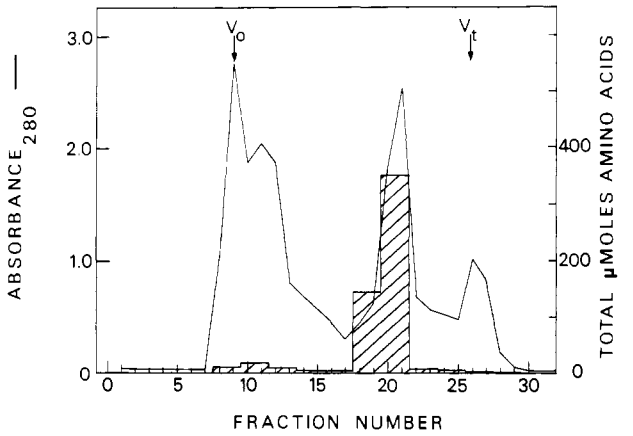

FIGURE 2: Molecular-exclusion chromatography of thermolytic peptides from acetylcholine receptor. A pool containing the α, β, and γ polypeptides of acetylcholine receptor (pool C, Figure 1) was submitted to thermolytic digestion, lyophilized, and dissolved in 500 μL of 88% formic acid. The sample was applied to a column of Fractogel HW40s (0.9 × 50 cm) equilibrated in 95% ethanol/88% formic acid (4:1 v/v). The peptides were eluted at a flow rate of 7.5 mL h^{-1}, and fractions of 1.25 mL were collected. Small portions from pairs of adjacent fractions were combined to make a total of 12 pools to be submitted to total amino acid analysis, the results of which are shown as the hatched bars in the figure. The positions of the void (V_0) and included (V_t) volumes are indicated.

Cystine-Containing Peptide α192–α198. Fractions 21 and 22 from the molecular-exclusion chromatogram (Figure 2) were submitted separately to HPLC on the C_4 reverse-phase column developed with a linear gradient between 0.1 M $NaClO_4$ and 1-propanol. The effluent was monitored simultaneously by the absorption at 229 nm and the electrochemical current through a dual gold/mercury electrode (Allison & Shoup, 1983). The first electrode was set at −1.00 V to reduce cystines, and the second electrode was set at +0.156 V to oxidize the cysteines produced by the first. Current passing through the second electrode was recorded. For both fractions, one major peak of electrochemically active peptide was seen (Figure 3A,B). The electrochemical signal was not observed when the reducing electrode was grounded (Figure 3C), behavior consistent with that expected from a peptide containing an intact disulfide. The electrochemically active peptides were collected as indicated by the brackets in Figure 3 and resubmitted to HPLC on a Brownlee Labs C_8 reverse-phase column

[3] Retention time is the difference between the time at which a retarded component is recorded on the chromatogram and the time at which an unretarded component is recorded on the chromatogram.

Table II: Amino Acid Analyses of Isolated Cystine-Containing Peptides[a]

amino acid	α192–α198[b] first	α192–α198[b] second	(γ123–γ131)–(γ137–γ144)	(δ125–δ134)–(δ139–δ145)	α126–α145	(β126–β131)–(β132–β147)	γ126–γ147
Asx	1.7 (1)	1.4 (1)	2.6 (2)	2.5 (3)	1.9 (2)	2.0 (2)	2.3 (2)
Thr	1.3 (1)	1.3 (1)	1.0 (1)	<0.1 (0)	1.6 (2)	1.5 (2)	1.8 (2)
Ser	0.4 (0)	0.6 (0)	1.6 (2)	2.2 (3)	1.2 (1)	2.3 (2)	2.1 (2)
Glx	0.6 (0)	0.8 (0)	1.5 (1)	1.3 (1)	2.8 (3)	1.4 (1)	1.3 (1)
Gly	0.2 (0)	0.7 (0)	7.7 (0)	0.4 (0)	1.2 (0)	1.3 (0)	0.8 (0)
Ala	0.2 (0)	0.5 (0)	1.7 (1)	0.1 (0)	0.7 (0)	0.7 (0)	1.1 (1)
Cys[c]	0.4 (1)	0.8 (1)	0.2 (1)	1.0 (1)	0.3 (1)	0.8 (1)	0.6 (1)
Val	0.2 (0)	0.5 (0)	0.5 (0)	1.6 (1)	1.1 (1)	1.5 (2)	1.9 (2)
Met	<0.1 (0)	<0.1 (0)	<0.1 (0)	<0.1 (0)	0.5 (1)	1.0 (2)	0.2 (0)
Ile	<0.1 (0)	<0.1 (0)	1.4 (2)	1.8 (2)	1.2 (2)	0.8 (1)	1.2 (1)
Leu	0.3 (0)	0.1 (0)	1.2 (1)	0.4 (0)	0.5 (0)	0.3 (0)	1.3 (1)
Tyr	0.7 (1)	0.5 (1)	0.7 (1)	0.3 (0)	0.7 (1)	0.7 (1)	0.8 (1)
Phe	<0.1 (0)	0.2 (0)	1.2 (1)	1.8 (2)	1.5 (2)	2.1 (3)	2.5 (3)
His	<0.1 (0)	<0.1 (0)	<0.1 (0)	0.2 (0)	1.0 (1)	<0.1 (0)	0.2 (0)
Lys	<0.1 (0)	0.2 (0)	0.4 (0)	0.7 (0)	2.2 (1)	5.0 (2)	1.2 (0)
Arg	<0.1 (0)	0.1 (0)	0.7 (1)	0.8 (1)	0.3 (0)	0.1 (0)	1.0 (1)

[a] Peptides were hydrolyzed at 110 °C for 24 h in 6 M HCl, and amino acids were detected with *o*-phthalaldehyde. Reported numbers are normalized to the theoretical amino acid composition of the peptides on the basis of the amino acid sequences of the polypeptides. Numbers in parentheses are the theoretical compositions of each peptide. [b] Cystine-containing peptide α192–α198 was isolated twice and submitted each time to amino acid analysis, respectively. [c] Cystine.

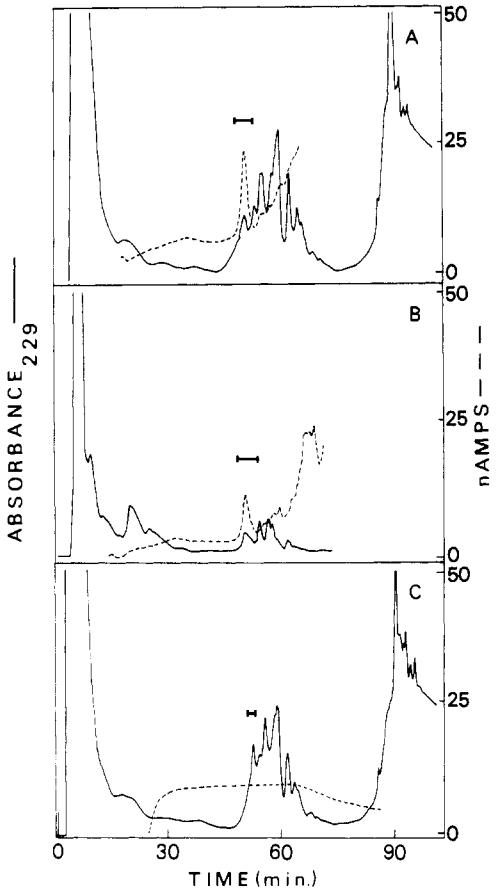

FIGURE 3: Purification of peptide α192–α198 by HPLC on C_4 reverse-phase chromatographic medium. Portions of fractions from the molecular-exclusion chromatogram (Figure 2) were dissolved in 50–75 μL of 88% formic acid, purged of oxygen, and injected onto a Vydac C_4 column (0.46 × 25 cm) equilibrated in 0.1 M $NaClO_4$. The samples were eluted with a linear gradient between solvent A (0.1 M $NaClO_4$) and solvent B (1-propanol) as follows: 0–60 min, 0–40% B, 0.5 mL min^{-1}; and 60–90 min, 40–60% B, 0.8 mL min^{-1}. Absorbance at 229 nm was monitored at 0.5 absorbance unit full scale, and electrochemical detection was performed at a setting of 50 nA full scale. At approximately 35% 1-propanol the electrochemical detector displayed a steady increase in signal accompanied by severe noise. (A) Portion from fraction 21 of Figure 2. (B) Portion from fraction 22 of Figure 2. (C) Portion from fraction 21 as in panel A but with the reducing electrode grounded. The samples designated by brackets were collected for further purification.

developed with a linear gradient between 0.1% trifluoroacetic acid and 60% 2-propanol/0.1% trifluoroacetic acid (Figure 4). The electrochemically active peaks from these chromatograms were collected as indicated by the brackets and resubmitted to HPLC on an Altex C_{18} reverse-phase column developed with a linear gradient between 0.1% trifluoroacetic acid and 60% 2-propanol/0.1% trifluoroacetic acid (Figure 5). Only one electrochemically active peak was seen in each chromatogram, and in the final step each peak appeared chromatographically homogeneous. Because the electrochemically active peptides purified in this series of chromatographic steps originally came from adjacent fractions in the molecular-exclusion chromatogram (Figure 2) and behaved very similarly in the later chromatographic steps (Figures 3–5), the two peaks indicated by the brackets in Figure 5 were pooled at this stage.

A portion of this pooled material was analyzed by manual, dansyl Edman sequencing. No amino terminus or second amino acid was observed, but *O*-dansyltyrosine did appear clearly. A portion was submitted to amino acid analysis, monitoring the effluent from the cation-exchange column by the *o*-phthalalaldehyde procedure (Benson & Hare, 1975), modified by detect cystine.[2] Cystine was present in the hydrolysate, but its amount was less than that of aspartic acid or threonine (Table II). A portion of the pool was sequenced on an automated, gas-phase sequencer (Figure 6A), and the sequence obtained corresponded to that of residues 192–198 from the α subunit (Figure 7A).

With correction for analytical losses and the intentional division of intermediate samples, the yield of the final peptide, calculated from the picomoles of PTH-proline appearing in the third cycle of the sequencing (Figure 6A) and the nanomoles of α polypeptide in the pool from molecular-exclusion chromatography (Figure 1), was approximately 25%. This yield is based on the picomoles of PTH amino acid actually emerging from the sequencer and the original nanomoles of α polypeptide present before digestion. Considering the number of steps between these two points, this is a remarkably high yield. It indicates that all of the α polypeptides in the native acetylcholine receptor contain a cystine connecting positions 192 and 193 in their sequences.

Because of the high yield of aspartic acid on amino acid analysis (Table II), we examined the possibility that one of

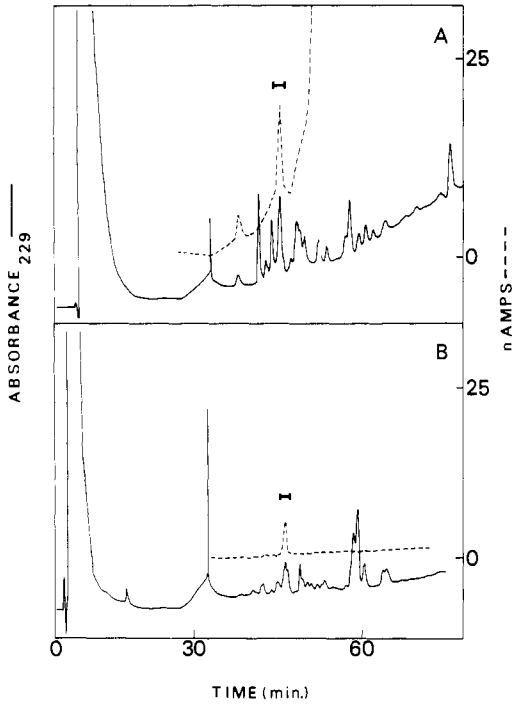

FIGURE 4: Purification of peptide α192–α198 by HPLC on C_8 reverse-phase chromatographic medium. The samples designated by the brackets in Figure 3 were dissolved in 50–75 μL of 88% formic acid, purged of oxygen, and injected onto a Brownlee Labs C_8 column (0.46 × 25 cm) equilibrated in 0.1% trifluoroacetic acid. The columns were eluted with linear gradients between 0.1% trifluoroacetic acid and 60% 2-propanol/0.1% trifluoroacetic acid over 80 min. Flow rate was 0.5 mL min^{-1}, absorbance at 229 nm was monitored at 0.5 absorbance unit full scale, and electrochemical detection was performed at a setting of 50 nA full scale. (A) Sample from chromatogram in Figure 3A. (B) Sample from chromatogram in Figure 3B. The samples designated by brackets were collected for further purification.

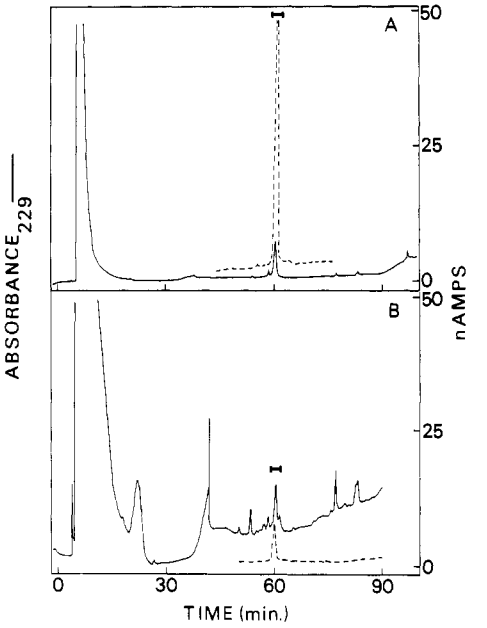

FIGURE 5: Purification of peptide α192–α198 by HPLC on C_{18} reverse-phase chromatographic medium. The samples designated by brackets in Figure 4 were dissolved in 50 μL of 88% formic acid, purged of oxygen, and injected onto an Altex C_{18} column (0.46 × 25 cm) equilibrated in 0.1% trifluoroacetic acid. The samples were eluted with a linear gradient between solvent A (0.1% trifluoroacetic acid) and solvent B (60% 2-propanol/0.1% trifluoroacetic acid) as follows: 0–70 min, 0–52% B, 0.5 mL min^{-1}, and 70–80 min, 52–100% B, 0.7 mL min^{-1}. Electrochemical detection was performed at a setting of 50 nA full scale. (A) The chromatography of the sample designated by the bracket in Figure 4A was followed by its absorbance at 229 nm monitored at 0.2 absorbance unit full scale. (B) The chromatography of the sample designated by the bracket in Figure 4B was followed by its absorbance at 229 nm monitored at 0.05 absorbance unit full scale.

the amino acids in the isolated peptide had been a free cysteine in the native receptor in vivo, which had become *S*-succinylcysteine during the isolation of the α polypeptide and the hydrolysis of the thermolytic peptide, and that it was now coeluting with aspartic acid upon amino acid analysis. The peptide α192–α198 was prepared again and submitted to hydrolysis, and the hydrolysate was run on the chromatographic system designed to separate the hydrolysates of collagen, the effluent of which was monitored with *o*-phthalaldehyde. No *S*-succinylcysteine [<0.05 nmol (nmol of Asx)$^{-1}$] was seen. A duplicate hydrolysate was run on the normal chromatographic system monitored with *o*-phthalaldehyde and modified to detect cystine. This second run displayed a higher yield of cystine than the first, though tyrosine was still low (Table II).

Cystine, which elutes between alanine and valine from the cation-exchange column used for amino acid analysis, remains as the disulfide during acid hydrolysis and cation-exchange chromatography. Therefore, its presence in the chromatogram is direct proof that the peptide hydrolyzed contained an intact disulfide. The fact that thermolysin recognized cysteine 192 as a hydrophobic amino acid and hydrolyzed the preceding peptide bond is also consistent with its involvement in a disulfide. The lack of results from the manual, dansyl–Edman sequencing, with the exception of *O*-dansyltyrosine, is consistent with the assignment of the first two residues of the peptide as half-cystines.

Cystine-Containing Peptide (γ123–γ131)–(γ137–γ144). Fraction 20 from the molecular-exclusion chromatogram (Figure 2) was submitted to HPLC on a Vydac C_4 reverse-phase column developed with a linear gradient between 0.1 M $NaClO_4$ and 1-propanol (0.5 mL min^{-1} and 120 min for a gradient to 60% 1-propanol). Several peaks of electrochemically active peptide were seen. One of these (eluting at 46% 1-propanol) was resubmitted to chromatography on the same C_4 reverse-phase column developed instead with a linear gradient between 0.1% trifluoroacetic acid and 60% 2-propanol/0.1% trifluoroacetic acid (0.5 mL min^{-1} and 90 min for the complete gradient). The only electrochemically active peak on this chromatogram (eluting at 32% 2-propanol), which coincided with a peak of absorbance at 229 nm, was collected and assumed to be a pure peptide. It gave two dansyl amino termini (Phe and Ile) and two other dansyl amino acids on the first cycle of manual, dansyl–Edman sequencing (Asx and Tyr). A portion of this pool was then sequenced on an automated gas-phase sequencer. The results observed (Figure 6B) clearly indicated that two peptides were present. The clarity of the sequence observed, the results from manual, dansyl–Edman sequencing, the equimolarity of the amino acids appearing in the first two steps of sequencing (Figure 6B), the chromatographic homogeneity of the peptide, and its electrochemical activity demonstrate that the isolated product consists of peptides comprising residues 123–131 and 137–144 from the γ subunit of acetylcholine receptor (Figure 7B) that remain linked by a disulfide bond between their respective cysteines. All amino acids in the two peptides were unambiguously identified with the exception of asparagine 141, which is a candidate for glycosylation, and cysteines 128 and 142, the phenylthiohydantoins of which appeared to migrate as PTH-2-aminoacrylate.

With correction for analytical losses and the intentional division of intermediate samples, the yield of the final peptide,

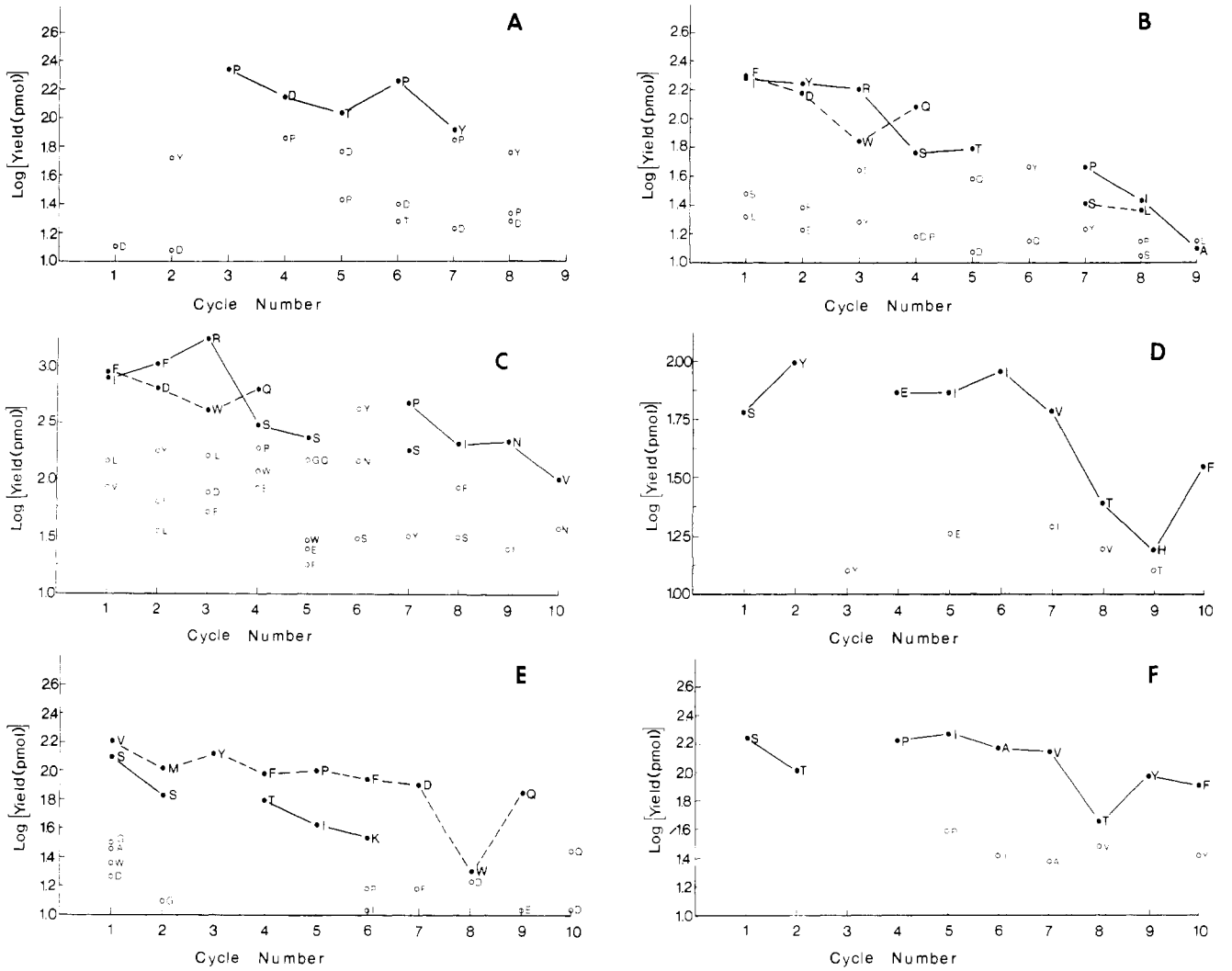

FIGURE 6: Results from sequencing the cystine-containing peptides. The logarithms of the yields in picomoles of every PTH-amino acid observed on a particular cycle of the automated sequencing are plotted. The amino acids assigned as those from the peptide and those expected at a particular cycle are in boldface. (A) Results from sequencing the peptide assigned as $\alpha 192$–$\alpha 198$; (B) results from sequencing the peptide assigned as ($\gamma 123$–$\gamma 131$)–($\gamma 137$–$\gamma 144$); (C) results from sequencing the peptide assigned as ($\delta 125$–$\delta 134$)–($\delta 139$–$\delta 145$); (D) results from sequencing the peptide assigned as $\alpha 126$–$\alpha 145$; (E) results from sequencing the peptide assigned as ($\beta 126$–$\beta 131$)–($\beta 132$–$\beta 147$); (F) results from sequencing the peptide assigned as $\gamma 126$–$\gamma 147$.

calculated from the picomoles of PTH-amino acids appearing in the first cycle of the sequencing (Figure 6B) and the nanomoles of γ polypeptide in the pool from molecular-exclusion chromatography (Figure 1), was approximately 15%.

Because all four subunits of acetylcholine receptor are known to be glycosylated (Lindstrom et al., 1979) and the isolated peptide contains the sequence Asn-X-Ser designating glycosylation (Schachter & Roseman, 1980), we again prepared the peptide, identified it by gas-phase sequencing, and determined if it was indeed glycosylated. A portion of the peptide was subjected to hydrolysis at 100 °C in 4 M HCl for 6 h, and the hydrolysate was modified with phenyl isothiocyanate following the method of Heinrikson and Meredith (1984). The PTC derivatives were separated by reverse-phase HPLC on a Waters Novapak C_{18} column (Bidlingmeyer et al., 1984). An equal portion was submitted to amino acid analysis (Table II), and the effluent from the cation-exchange column was monitored by the *o*-phthalaldehyde procedure (Benson & Hare, 1975). Each sample contained norleucine as an internal standard. Using both of these methods, we found 1.2 nmol of glucosamine (nmol of peptide)$^{-1}$. A third portion of this peptide was spotted onto nitrocellulose paper, and its ability to bind concanavalin A was assessed by peroxidase staining in a coupled assay. Both the peptide and ovalbumin gave positive results, while bovine serum albumin did not.

Cystine-Containing Peptide ($\delta 125$–$\delta 134$)–($\delta 139$–$\delta 145$). Fractions containing the δ_2 dimer from the agarose A5m column (pool A, Figure 1), obtained from two additional runs (each of 30 mg) identical with that shown, were combined and lyophilized. The polypeptides (8 mg by amino acid analysis) were stripped of dodecyl sulfate and digested with thermolysin. The digest was lyophilized, redissolved in 88% formic acid, and submitted to molecular-exclusion chromatography on the column of Fractogel TSK HW40s. The distribution of eluted peptides was determined by reading the ultraviolet absorption of each fraction at 280 nm, and cystine was located by amino acid analysis. A pool of fractions from this chromatogram, which had relative mobilities equivalent to fractions 16–18 of the chromatogram of Figure 2 and which contained 20 nmol of cystine (75 μmol of total amino acids)$^{-1}$, was chosen for further purification. They were submitted to HPLC on a C_4 reverse-phase column developed with a linear gradient between 0.1 M chloroacetic acid and 1-propanol (0.7 mL min^{-1} and 60 min for a gradient to 60% 1-propanol). Several peaks of electrochemically active peptide were observed. The same electrochemically active peaks, eluting after approximately 42 min (42% 1-propanol) on several chromatograms, were pooled,

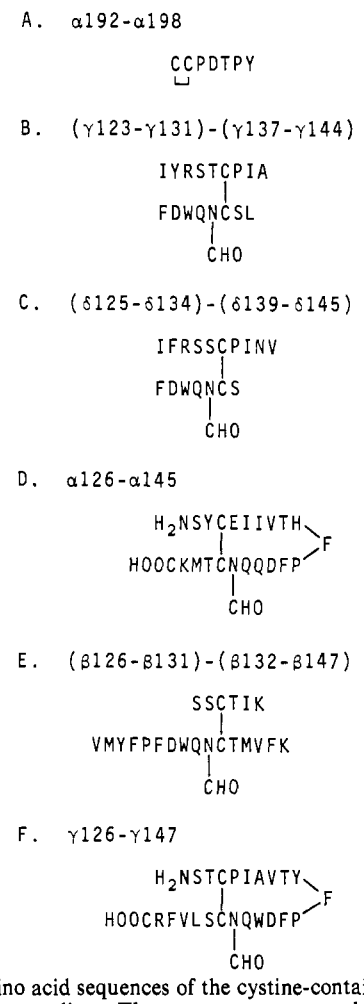

FIGURE 7: Amino acid sequences of the cystine-containing peptides isolated in these studies. The sequences presented are from the complete amino acid sequences of the respective polypeptides, and their amino termini and carboxy termini were assigned on the basis of the observed sequences and the specificities of the proteolytic enzymes used to produce them. Cystine is indicated based on the observed amino acid compositions.

and this pool was resubmitted to HPLC on the same C_4 column developed instead with a linear gradient between 0.1% TFA and 1-propanol (0.7 mL min^{-1} and 60 min for a gradient to 60% 1-propanol). Several peaks absorbing at 229 nm were seen, only one of which gave a positive electrochemical signal (eluting at 38% 1-propanol). The electrochemically active peak was collected and resubmitted to HPLC on a C_8 column developed with a linear gradient between 0.1% trifluoroacetic acid and 60% 2-propanol in 0.1% trifluoroacetic acid (0.7 mL min^{-1} and 60 min for the complete gradient). One major and two minor peaks were seen, all three of which absorbed at 229 nm and gave positive electrochemical signals.

The major peak was submitted to amino acid analysis (Table II), gas-phase sequencing (Figure 6C), analysis for glucosamine, and binding of concanavalin A. The sequence obtained corresponded to that of residues 125–134 from the δ subunit (Figure 7C) connected by an intact disulfide bond to residues 139–145, also from the δ subunit. The peptide-bound concanavalin A and was found to have 2 nmol of glucosamine (nmol of peptide)$^{-1}$. Yield was approximately 10%.

Cystine-Containing Peptides α126–α145 and (β126–β131)–(β132–β147). A pool equivalent to pool C of Figure 1, containing α, β, and γ polypeptides, was again prepared, and the resulting protein (14 mg) was stripped of dodecyl sulfate and digested with two additions of 10 μg of trypsin (mg of protein)$^{-1}$. Treatment of a portion of the product with carboxypeptidase B indicated essentially complete digestion. The digest was lyophilized, dissolved in 1 mL of ethanol/88% formic acid (4:1) containing 0.2 mg of tryptophan, and submitted to molecular-exclusion chromatography on a column of Fractogel TSK HW40s. The distribution of eluted peptides was determined by reading the ultraviolet absorption of each fraction at 280 nm and by amino acid analysis. One of the fractions from this chromatogram, which had a relative mobility equivalent to fraction 17 of the chromatogram in Figure 2 and which was one of three fractions that together contained 60 nmol of cystine (7 μmol of total amino acids)$^{-1}$, was chosen for further purification. It was submitted to HPLC on the C_4 column developed with a linear gradient between 0.1 M chloroacetic acid and 1-propanol (0.6 mL min^{-1} and 90 min for a gradient to 70% 1-propanol). Two major and two minor peaks of electrochemically positive peptide were observed, along with several peaks of absorbance at 229 nm that were not electrochemically active.

The first major, electrochemically active peak, eluting at approximately 51 min (40% 1-propanol), was collected and resubmitted to chromatographic separation on an Altex C_3 column eluted with a linear gradient developed between 50 mM ammonium acetate and 1-propanol (0.7 mL min^{-1} and 90 min for a gradient to 60% 1-propanol). A single electrochemically active peak was seen that coincided with a peak of absorbance at 229 nm. This peptide was collected and submitted to amino acid analysis (Table II), gas-phase sequencing (Figure 6D), analysis for glucosamine, and binding of concanavalin A. It was identified as the tryptic peptide α126–α145 (Figure 7D) containing an intact cystine. The peptide was found to bind concanavalin A and had 3.2 nmol of glucosamine (nmol of peptide)$^{-1}$. Yield was approximately 5%.

One of the minor, electrochemically active peaks from the C_4 column, eluting at approximately 53 min (41% 1-propanol), was collected and combined with the same peak from an identical high-pressure liquid chromatogram of the fraction from the HW40s TSK column having a relative mobility equivalent to that of fraction 18 (Figure 2). The combined fractions were resubmitted to chromatographic separation on an Altex C_3 column eluted with a linear gradient developed between 50 mM ammonium acetate and 1-propanol (0.7 mL min^{-1} and 90 min for a gradient to 60% 1-propanol). A single electrochemically active peak was seen that coincided with a peak of absorbance at 229 nm. This peptide was collected and submitted to amino acid analysis (Table II), gas-phase sequencing (Figure 6E), analysis for glucosamine, and binding of concanavalin A. It was identified as the tryptic peptide β126–β131 connected by an intact disulfide bond to the tryptic peptide β132–β147 (Figure 7E). The peptide was found to bind concanavalin A and had 4 nmol of glucosamine (nmol of peptide)$^{-1}$. Yield was approximately 10%.

The second major electrochemically active peak from the HPLC on the C_4 column was identified as the peptide γ126–γ147 (Figure 7F) by sequencing. It contained an intact cystine by amino acid analysis (Table II), and it was glycosylated. Yield was approximately 10%.

Discussion

The two results from the hydrolysates of each polypeptide isolated from acetylcholine receptor that had been homogenized in the presence of *N*-ethylmaleimide and iodoacetamide and dissolved with $NaDodSO_4$ in the presence of *N*-ethylmaleimide were averaged. There were 2.3 nmol of *S*-succinylcysteine for every 1 nmol of the α polypeptide, 3.0 nmol of *S*-succinylcysteine for every 1 nmol of the β polypeptide,

4.2 nmol of *S*-succinylcysteine for every 1 nmol of the γ polypeptide, and 1.4 nmol of *S*-succinylcysteine for every 1 nmol of the δ polypeptide. Only the hydrolysate of the γ polypeptide contained *S*-(carboxymethyl)cysteine, and it contained 2.0 nmol of *S*-(carboxymethyl)cysteine for every 1 nmol of the γ polypeptide. Within the amino acid sequences of the polypeptides of acetylcholine receptor there are 7 nmol of cysteine plus half-cystine in each 1 nmol of the α polypeptide, 5 nmol in each 1 nmol of the β polypeptide, 8 nmol in each 1 nmol of the γ polypeptide, and 6 nmol in each 1 nmol of the δ polypeptide (Noda et al., 1983c). Therefore, the results from the amino acid analyses and these amino acid compositions set upper limits of 2 intramolecular cystines in each α polypeptide, 1 intramolecular cystine in each β polypeptide, 1 intramolecular cystine in each γ polypeptide, and 4 cystines in each pair of δ polypeptides.

These values are consistent with the number of cystine-containing peptides actually isolated in these studies or identified previously, and presumably, all of the cystines in the acetylcholine receptor have been accounted for. Two cystine-containing peptides, α192–α198 and α126–α145, were isolated from the α polypeptide. One cystine-containing peptide, (β126–β131)–(β132–β147), was isolated from the β polypeptide. One cystine-containing peptide, (γ123–γ131)–(γ137–γ144), was isolated from the γ polypeptide. The covalent dimer between two δ polypeptides is cross-linked by at least one intermolecular cystine (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977), and a peptide containing a cystine between positions 130 and 144 in the amino acid sequence of the δ polypeptide was isolated. Together, these would account for at least 3 cystines in each pair of δ polypeptides. Though we were unable to isolate a peptide containing a cystine between two δ polypeptides, it has been deduced that there is a cystine connecting a pair of δ subunits through positions 500 in their amino acid sequences (Wennogle et al., 1981).

The remaining cysteines appear to exist as free sulfhydryls in the native receptor. Several of the cysteines within the amino acid sequence of each polypeptide fall within the hydrophobic segments M1, M2, M3, and M4 [as designated by Noda et al. (1983c)]. It is likely that these cysteines react with *N*-ethylmaleimide only during the unfolding of the protein when $NaDodSO_4$ is added in the presence of 5 mM *N*-ethylmaleimide. The γ subunit contains two cysteines that appear as *S*-(carboxymethyl)cysteine after hydrolysis of the protein. They must react with iodoacetamide during the initial homogenization of the electroplax as this is the only time the protein is exposed to this reagent. It is interesting to note that the γ subunit is unique in having two cysteines, cysteines γ416 and γ420, in the portion of its amino acid sequence that has the pattern of an amphiphatic α helix (Finer-Moore & Stroud, 1984). This segment does not lie within the membrane (Dwyer, 1988) and may lie along the surface of the native acetylcholine receptor, as do the amphipathic α helices in hemoglobin (Perutz et al., 1965). If this were the case, this portion of the amino acid sequence would be accessible to the buffer during the initial homogenization step, and these cysteines could be the two cysteines in the γ subunit that react with iodoacetamide.

The locations of the cysteines in the various subunits agree with the models (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983a) that place the large stretch of amino acid sequence between segments M3 and segments M4 in each subunit on the cytoplasmic side of the membrane. With the exception of the cystine responsible for the formation of the

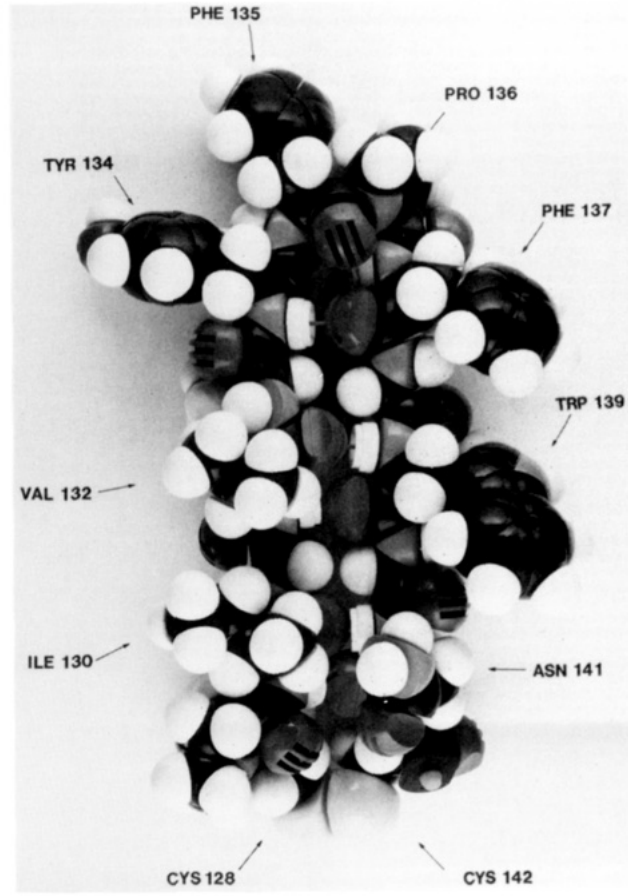

FIGURE 8: Hypothetical Corey–Pauling–Koltun model of residues γ128–γ142 from the γ subunit. The β hairpin shown is held together at its carboxy and amino termini by the disulfide between cysteines 128 and 142. Only the amino acids that are visible on the side exposed to the viewer are labeled. Proline 136 can be seen in the cis conformation in the third position of a β turn. Asparagine 141 is the glycosylation site.

δ dimer, all of the cystines lie in the amino acid sequences of each subunit to the amino-terminal side of the first hydrophobic domain, M1. The two cysteines in the region of the amino acid sequence of the γ subunit displaying the pattern of an amphipathic α helix, cysteines γ416 and γ420, could be located on the cytoplasmic side of the membrane because they are free cysteines in the native protein. The β subunit also contains a cysteine, cysteine β366, that falls in the sequence between M3 and M4. It also appears to be a free sulfhydryl in native acetylcholine receptor.

There are pairs of cysteines in the amino acid sequences of each of the subunits, cysteines 128 and 142 from the α subunit, cysteines 128 and 142 from the β subunit, cysteines 128 and 142 from the γ subunit, and cysteines 130 and 144 from the δ subunit, that are cystines in the native protein. These cystines are in homologous locations in each of the sequences [as aligned by Noda et al. (1983a)] and connect positions in the amino acid sequences that are always 13 residues apart. A completely conserved sequence, Phe-Pro-Phe-Asp, occurs midway between the two halves of these cystines in the amino acid sequences of all four subunits of acetylcholine receptor from *T. californica* (Noda et al., 1983a). It has been proposed that a β turn is present at each of these locations in the native protein and that these β turns are the tips of antiparallel β hairpins held together at their amino and carboxy termini by the disulfide bonds between the residues homologous to cysteines 128 and 142 (Noda et al., 1983a). Such a structure can be constructed, but only if the proline residue assumes the

third position in the turn (Figure 8). This is necessary to permit the two cysteines to approach closely enough to form a disulfide with the proper stereochemistry. Even though β turns with proline in the third position are rare, they have been observed, almost always with a cis conformation of the proline (Chou & Fasman, 1977), as shown in Figure 8. Because it is not possible for a disulfide to join neighboring strands of β structure (Richardson, 1981), the two half-cystines would have to terminate the β hairpin.

The most unexpected cystine in acetylcholine receptor is that connecting the adjacent positions 192 and 193 in the amino acid sequence of the α subunit. Even though an intramolecular cystine will form spontaneously in the dipeptide *N*-cysteinylcysteine (Wade et al., 1956) and indirect results have been presented suggesting that a vicinal cystine is present in transducin (Ovchinnikov et al., 1985), the possibility that a cystine can connect two adjacent positions in the amino acid sequence of a native protein has, until recently, been rejected on the grounds of the strain inherent in such a structure (Schulz & Schirmer, 1979). Because of this prejudice, rigorous proof must be assembled to conclude that a vicinal disulfide is present in a given protein. This requirement has been recently emphasized by experiments demonstrating that one of the two other cystines that had been reported to occur between two adjacent positions in the sequence of a protein was not a cystine after all.

It has been reported (Al-Saleh et al., 1987) that cysteines 322 and 323 in bovine rhodopsin were connected as a cystine. In these experiments, the free cysteines were first alkylated with [^{14}C]iodoacetic acid after the protein had been dissolved in a solution of $NaDodSO_4$. The protein was then reduced with 2-mercaptoethanol, and a second alkylation was performed with [^{3}H]iodoacetic acid. The peptide valine 318–alanine 348 was then isolated on the basis of its content of tritium and lack of ^{14}C, and it was found to release tritium only at the cycles from the sequencer corresponding to cysteines 322 and 323. In previous experiments (Mullen & Akhtar, 1983), it had been shown that a carboxy-terminal peptide of rhodopsin, produced by treatment with papain, that begins at lysine 312 or glutamine 313 can be released from the remainder of the protein without prior reduction. Because these two cysteines that incorporate tritium upon alkylation with [^{3}H]iodoacetic acid are the only ones occurring in the portion of the sequence after lysine 312, and because they are labeled only after treatment of the protein with 2-mercaptoethanol, it was concluded that these two immediately adjacent cysteines must be mutually involved in the formation of one cystine in each polypeptide of native rhodopsin.

Ovchinnikov et al. (1988), however, isolated the same peptide, valine 318–alanine 348, under nonreducing conditions and demonstrated that the cysteines could not be involved in a cystine. While sequencing the peptide, they introduced radioactive PITC at cycle 5 (cysteine 322) and nonradioactive PITC at cycle 6 (cysteine 323). If these two cysteines were in a disulfide, the effluent from the sixth cycle should have been radioactive. Instead, the sequence was blank at cycle 5 and no release of radioactivity occurred at cycle 6. The peptide was then subjected to alkaline hydrolysis followed by acid methanolysis, and the methyl ester of palmitic acid was released. The investigators concluded that palmitic acid was blocking each of these cysteines as thioesters and preventing modification with alkylating agents. If this were the explanation, then the 2-mercaptoethanol used in the experiments of Al-Saleh et al. (1987) would have been simply catalyzing the deacylation of the cysteines nucleophilically, rather than reducing a cystine, as they assumed.

Kao and Karlin (1986) have concluded that there is a cystine between positions 192 and 193 in the amino acid sequence of the α subunit of acetylcholine receptor. This conclusion, however, was based only on the ability of a cyanogen bromide fragment containing this portion of the sequence to incorporate iodoacetic acid following exposure to dithiothreitol, the same criterion that failed in the case of rhodopsin. Furthermore, cyanylation, formylation, or adventitious oxidation of free sulfhydryls can occur during cyanogen bromide cleavage, and the thiocyanates, formates, or disulfides produced artifactually register as disulfides by the analytical method chosen (see the Appendix). Isolation of peptides containing this disulfide by a method avoiding these problems would provide less equivocal evidence for the presence of this disulfide in native acetylcholine receptor.

An additional problem with the experiments of Kao and Karlin (1986) was that thiols exposed during the unfolding of the polypeptides of acetylcholine receptor in solutions of $NaDodSO_4$ were not alkylated. Although the native protein had been alkylated with *N*-ethylmaleimide, this precaution is insufficient because *N*-ethylmaleimide only reacts with a minority of the cysteines in a native protein. The thiols then exposed upon unfolding of the protein could have catalyzed disulfide exchange before the peptides were isolated. This would result in identification of cystines not present in native acetylcholine receptor.

That such disulfide interchange occurs during purification of acetylcholine receptors has been documented. Two forms of acetylcholine receptor can be isolated by centrifugation on gradients of sucrose. They are referred to as the heavy form ($MW \cong 500000$) and the light form ($MW \cong 250000$). Chang and Bock (1977) and Suarez-Isla and Hucho (1977) found that the only difference between these two forms is the presence and absence, respectively, of an intermolecular cystine between the δ subunits of two $\alpha_2\beta\gamma\delta$ protomers. The inclusion of *N*-ethylmaleimide in the initial homogenization prevents reduction of this intersubunit cystine and results in a greater than 90% yield of the heavy form. In contrast, when *N*-ethylmaleimide is added after the initial homogenization, approximately 60% of the heavy form has already been reduced to the light form, presumably by endogenous thiols released from the cells as they are ruptured (Chang & Bock, 1978).

In a later study, Chang and Bock (1980) also reported that cysteines exposed when acetylcholine receptor is dissolved in solutions of Triton X-100 undergo partial oxidation to form intramolecular and intermolecular cystines. Evidence for loss of cysteines due to oxidation by contaminants in the Triton X-100 itself was also reported by Suarez-Isla and Hucho (1977). The fact that Kao and Karlin (1986) dissolved the acetylcholine receptor used in their studies in Triton X-100 could have caused cystines or cysteine sulfinates to form adventitiously, and these would have later been identified as cystines present in the native protein by the criteria they used to make these decisions.

Our present work confirms the earlier conclusion of Kao and Karlin (1986) that there is a vicinal disulfide between cysteines α192 and α193 in the α subunit of acetylcholine receptor. The experiments described here are unique from and complementary to those carried out by Kao and Karlin and yet lead to the same conclusion. Acetylcholine receptor was purified in the absence of Triton X-100 and in the presence of *N*-ethylmaleimide to prevent disulfide interchange. *N*-Ethylmaleimide was present when the protein was unfolded

to cap any free cysteines exposed at this step. We have observed cystine directly upon amino acid analysis of the purified peptide α192–α198. We have also shown that there is only one sequence for the purified, disulfide-containing peptide when this is determined by Edman degradation and that the mobility of this peptide coincides with that of a strong electrochemical signal, indicative of cystine, on three different chromatographic separations (Figures 3–5). We have excluded the possibility that one or both of the half-cystines, α192 or α193, occur in the native enzyme as a free sulfhydryl by showing the absence of *S*-succinylcysteine in a hydrolysate of the peptide. The only possibility that still remains, other than the vicinal disulfide, is that cysteines α192 and α193 are both modified so that they cannot react with *N*-ethylmaleimide when the protein is denatured and that at least one of them is participating in a mixed disulfide with cysteine derived from cystine in the serum. This seems unlikely.

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Registry No. Cysteine, 52-90-4; cystine, 56-89-3; glucosamine, 3416-24-8; cyanogen bromide, 506-68-3; L-cysteinyl-L-cysteine, 18048-87-8; iodoacetic acid, 64-69-7; dithiothreitol, 3483-12-3; L-cystinyl-L-cystine, 75816-11-4; *S,S'*-bis(carboxymethyl)-L-cysteinyl-L-cysteine, 119437-71-7; *S*-(carboxymethyl)cysteine, 638-23-3.

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Appendix: Reversible Modification of Cysteine with Cyanogen Bromide

Abstract

When a peptide containing cysteine, specifically the dipeptide L-cysteinyl-L-cysteine, is exposed to 0.2 M cyanogen bromide in 70% formic acid, the cysteine is quantitatively modified to products that will not react with iodoacetic acid. When these products are exposed to dithiothreitol, the cysteine is quantitatively regenerated and reacts quantitatively with iodoacetic acid to yield the carboxymethylated product. The ability of a cyanogen bromide fragment to incorporate iodoacetic acid only after exposure to a thiol such as dithiothreitol cannot be used as a criterion for the presence of a disulfide in the native protein.

Introduction

A criterion that is often used to define an intramolecular cystine within a single intact peptide is the ability of that peptide to incorporate an alkylating agent such as iodoacetic acid only after exposure to a thiol such as 2-mercaptoethanol or dithiothreitol. There are, however, modifications of cysteine other than participation in a cystine that can be reversed nucleophilically by treatment with a thiol and would be mistakenly assigned as participation in a cystine by this criterion. In particular, when the parent polypeptide has been cleaved with cyanogen bromide in 70% formic acid, a free cysteine in the original protein could become cyanylated, formylated, or turned into the sulfinic acid of cysteine. It is also possible for adventitious cystines to form under these conditions. Any of these modifications could be reversed by dithiothreitol.

Kao and Karlin (1986) isolated two cyanogen bromide fragments from the α polypeptide of acetylcholine receptor that comprised amino acids 118–144 and 179–207 in the amino acid sequence of this polypeptide. Both of these cyanogen bromide fragments incorporated ^{14}C from iodo[^{14}C]acetamide, but only after exposure to dithiothreitol. By this criterion they concluded that the native protein contained intramolecular cystines within these regions of its amino acid sequence.

The experiments described here, however, demonstrate that free cysteine, upon exposure to cyanogen bromide in 70% formic acid, is quantitatively and covalently modified to several products. These products cannot be alkylated by iodoacetic acid directly, but after exposure to dithiothreitol they covalently incorporate iodoacetic acid to yield quantitatively the carboxymethyl derivatives of the original cysteines.

Experimental Procedures

Materials. Iodoacetic acid was purchased from Aldrich Chem. Co.; tris(hydroxymethyl)aminomethane (Tris), from Fisher Chemical Co.; and cysteine, from Calbiochem Corp. Cyanogen bromide was purchased from Matheson, Coleman, and Bell, and the transparent, colorless crystals of the sublimed solid attached to the walls of the container were used. The bis dipeptide, L-cystinyl-L-cystine, was purchased from Research Plus Inc. The grams per mole of the solid bis dipeptide were calculated from the elemental analysis provided by the manufacturer. The initial solution of L-cystinyl-L-cystine prepared for the experiment was 4.58 mg dissolved in 1.00 mL of 30 mM Tris-HCl, pH 9.0.

Chromatography. Samples were prepared for chromatography by removing 20 μL from the experimental solution and adding it to the appropriate amount (0.5–1.0 mL) of a mixture of equal parts of 0.5 mM norleucine in 0.01 M HCl and 0.066 M sodium citrate adjusted to pH 2.2 with hydrochloric acid. The samples were run on a Beckman 118C amino acid analyzer using a 3-h, single column program (Beckman Instruments, 1975). The peptides eluting from the chromatographic column (0.6 × 34 cm) were detected and quantified by the standard, continuous-flow, postcolumn ninhydrin system associated with the instrument.

Results

The bis dipeptide L-cystinyl-L-cystine was dissolved at 10 mM in a solution of 30 mM Tris-HCl, pH 9.0, that had been sparged with argon. Dithiothreitol was added as the solid to a final concentration of 50 mM, and the solution was sealed under argon for 30 min at room temperature. A sample (calculated to be 100 nmol of amino termini) was then removed and submitted to chromatographic analysis on a Model 118C amino acid analyzer (Beckman Corp.). A peak emerged with a retention time (68 min) between those of norleucine (63 min) and tyrosine (73 min) (retention time is the difference between the time at which a retarded component is recorded on the chromatogram and the time at which an unretarded component is recorded on the chromatogram). If it is assumed that this peak represents L-cysteinyl-L-cysteine, its yield of ninhydrin color is only 2% that of glycine at 570 nm. Cysteine itself has a yield of ninhydrin color that is only 8% that of glycine. This fact and the fact that peptides typically produce colored products in lower yields than do amino acids after reaction with ninhydrin are consistent with the assignment of this peak as L-cysteinyl-L-cysteine.

A sample of the reduced dipeptide (calculated to be 10 μmol of amino termini) was removed, and solid iodoacetic acid was added to a final concentration of 200 mM. The pH was raised to 9, and the reaction was allowed to proceed for 30 min at room temperature. A portion of this sample (calculated to be 100 nmol of amino termini) was submitted to chromatographic analysis. The peak previously assigned to L-cysteinyl-L-cysteine was completely absent (<20%), and a new peak, not present on the previous analysis, appeared. Its retention time (13 min) was intermediate between those of serine (9 min) and glutamate (14 min). If it is assumed that the carboxymethylation was quantitative and that this peak represents *S*,*S*′-bis(carboxymethyl)-L-cysteinyl-L-cysteine, then its yield of ninhydrin color is 65% that of glycine. A similar increase in the yield of ninhydrin color is observed when cysteine (8% that of glycine) is converted to *S*-(carboxymethyl)cysteine