Extracytoplasmic Disposition of Lysine β165 of Acetylcholine Receptor†

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ABSTRACT: The location, with respect to the membrane, of Lys 165 in the folded β polypeptide of native nicotinic acetylcholine receptor has been determined by site-directed immunochemistry. Sealed, right-side-out vesicles rich in acetylcholine receptor were modified with pyridoxal phosphate and sodium [^{3}H]-borohydride. Saponin was added to one portion of the vesicles to make them permeable to the pyridoxal phosphate and sodium borohydride; the other portion was modified in the absence of saponin. Both samples were then exhaustively succinylated and digested with trypsin and thermolysin to produce the peptide LDAKGER, which contains Lys β165. The digests were passed over an immunoadsorbent specific for peptides with the sequence LDAXGER, where X represents any modified or unmodified amino acid, and specifically bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. The eluates were submitted to high-pressure liquid chromatography, and two peptides, N^{ϵ}-phospho[^{3}H]pyridoxalLDAKGER and N^{ϵ}-succinylLDAKGER, modified at the ε amino group of lysine with pyridoxal phosphate and sodium [^{3}H]-borohydride or succinic anhydride, respectively, were identified by comparison to standards. The relative specific radioactivity of N^{ϵ}-phospho[^{3}H]pyridoxalLDAKGER modified in the presence or absence of saponin, respectively, was 0.9 ± 0.4. The incorporation of phospho[^{3}H]pyridoxyl groups into Lys α380, a residue located on the cytoplasmic surface of acetylcholine receptor, was also monitored. The relative specific radioactivity of the peptide that contains the modified Lys α380, N^{ϵ}-phospho[^{3}H]pyridoxalGVKYIAE, increased 3.6-fold when the modification was performed in the presence of saponin. This result verifies that the vesicles used in these experiments were sealed and right-side-out. Because the incorporation of [^{3}H]pyridoxyl groups into Lys β165 is the same in the presence or absence of saponin, Lys β165 must have been located on the outside surface of the sealed, right-side-out vesicles, and therefore on the extracytoplasmic surface of native acetylcholine receptor.

Acetylcholine receptor is the paradigm of a superfamily of proteins that form ligand-gated channels for ions. This protein, found at the vertebrate neuromuscular junction and serendipitously in the electric organ of electric rays, forms a cation-selective channel that opens and closes in response to the binding of acetylcholine. When the channel opens, sodium ions and potassium ions flow through it in the directions of their respective gradients of concentration and cause local depolarization at the membrane. To understand how acetylcholine receptor accomplishes the regulated opening and closing of a channel selective for cations across a membrane, it will be necessary to gain an understanding of the structure of the protein.

Four polypeptides, designated α, β, γ, and δ in order of their electrophoretic mobilities (Weill, 1974), combine with a stoichiometry of $\alpha_2\beta\gamma\delta$ to form acetylcholine receptor (Reynolds & Karlin, 1978). The amino acid sequence of each of the polypeptides has been determined from the respective complementary DNA (Noda et al., 1982, 1983a,b; Claudio et al., 1983), and pairwise comparisons of the primary sequences have revealed that the polypeptides are all homologous to each other (Raftery et al., 1980). Therefore, each properly folded polypeptide has the same tertiary structure. Profiles of hydropathy (Kyte & Doolittle, 1982) for the polypeptides have identified four hydrophobic segments (M1–M4) in each polypeptide in homologous locations. Each of these segments is at least 20 amino acids long (Claudio et al., 1983; DeVillers-Thiery et al., 1983; Noda et al., 1983a), and it has been proposed that each spans the membrane as an α-helix in the native protein. Other methods of structure prediction had led to the conclusion that a fifth segment, located between M3 and M4, spanned the membrane as an amphipathic α-helix (Guy, 1984; Finer-Moore & Stroud, 1984), but Dwyer (1988) showed that Lys α380, which is in the middle of this putative amphipathic α-helix, is fully exposed to the aqueous solution on the surface of the protein. Experimental results have led to as yet unchallenged proposals that other segments may also span the bilayer (Criado et al., 1985a; Pedersen et al., 1990).

Electron crystallographic studies of membranes rich in acetylcholine receptor have provided structural information for this protein (Brisson & Unwin, 1984; Toyoshima & Unwin, 1990; Unwin, 1993). The five polypeptides, each folded into the common tertiary structure, are arranged around a 5-fold rotational axis of pseudosymmetry and form a pentagonal cylinder 12.5 nm long with a diameter of 7 nm. A cylindrical depression, 8 nm deep and 2.5 nm in diameter, at the center of the pentameter is visible on the extracytoplasmic side of the protein, and a shallow depression, on the cytoplasmic side. It is believed that the channel for cations lies on the rotational axis of pseudosymmetry.

An aspect of the structure of native acetylcholine receptor that has received considerable attention is the assignment of regions of the folded polypeptides to one side of the membrane or the other. On the extracytoplasmic surface of the protein are located the main immunogenic region (α67–α76 of the amino acid sequence of the α polypeptide from human muscle; Tzartos et al., 1988); the site of *N*-glycosylation present in each of the subunits, (Asn 141 of the α-polypeptide and its

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homologues in the other polypeptides; Kellaris & Ware, 1989); and the binding sites for acetylcholine (Sine & Taylor, 1980; Cash & Hess, 1980) formed in part by a vicinal disulfide (Cys α192–α193; Kellaris & Ware, 1989) that can be modified, along with Tyr α190, by affinity labels (Kao & Karlin, 1986; Dennis et al., 1988; Abramson et al., 1989; Galzi et al. 1991). On the cytoplasmic surface of the protein are located sites of *in vivo* phosphorylation that include Ser γ353 and its homologue Ser δ361 (Yee & Huganir, 1987; Schroeder et al., 1991). The common topology of the folded polypeptides has been further defined by several less unambiguous observations. Photoactivated hydrophobic probes have been incorporated into the protein to identify those regions that might be exposed to the hydrocarbon of the bilayer (Blanton & Cohen, 1992). Several investigations have used immunoglobulins specific for defined segments in the amino acid sequences of the denatured polypeptides as probes to indicate the cytoplasmic or extracytoplasmic location of the epitopes in native acetylcholine receptor (Criado et al., 1985a,b; La Rochelle et al., 1985; Lindstrom et al., 1984; Ratnam et al., 1986a,b; Pedersen et al., 1990; Young et al., 1985). Fusion proteins between truncated portions of acetylcholine receptor and prolactin have been used to infer the topology of the full length, properly folded polypeptides (Chavez & Hall, 1992). Some of the results from these latter experiments have been contradictory.

Results have been presented suggesting that at least two segments of the common fold of the polypeptides, located between the position of the glycosylation site at Asn 141 in the α-subunit and its homologues in the other subunits and the position of the extracytoplasmic, vicinal disulfide between Cys 192 and Cys 193 in the α-subunit, span the membrane. There have been two reports (Criado et al., 1985a; Pedersen et al., 1990) reaching the conclusion that epitopes between Lys α155 and Lys α179 are only accessible to specific immunoglobulins when those immunoglobulins have access to the cytoplasmic surface of the α-subunit of native acetylcholine receptor. These conclusions implied that one membrane-spanning segment must lie between the glycosylation site at Asn α141 and Thr α154 and another between Lys α179 and the sites of covalent modification by affinity labels from the extracytoplasmic surface at Tyr α190 and Cys α192. Neither of these putative membrane-spanning segments, however, seemed to be either long enough or hydrophobic enough to produce a membrane-spanning α-helix. More recent experimental results from Chavez & Hall (1991) have contradicted the conclusion that this region has a cytoplasmic location, but the suggestion, based on the most recent maps of electron scattering density of acetylcholine receptor (Unwin, 1993), that most of the mass of the membrane-spanning portion of acetylcholine receptor could be a β-barrel made the possibility that this region of the polypeptide may contain segments that span the membrane more provocative.

The topological disposition of this region of the polypeptide was determined by applying site-directed immunochemistry (Kyte et al., 1987) to identify the location of Lys 165 of the β-subunit of acetylcholine receptor, the homologue of Glu α161, which is in the center of the putative cytoplasmic segment. The results demonstrate that this residue is located on the extracytoplasmic surface of the protein.

EXPERIMENTAL PROCEDURES

Materials. Electroplaque from *Torpedo californica* was purchased from Winkler Enterprises, San Pedro, CA. The N^{α}-(9-fluorenylmethyloxycarbonyl) amino acids (N^{α}-FMOC amino acids)[1] and the *p*-alkoxybenzyl resin were obtained from either Bachem, Inc. or Calbiochem-Novabiochem Corp. Other chemicals purchased from Calbiochem-Novabiochem Corp. were dithiothreitol, iodoacetamide, saponin, sodium dodecyl sulfate ($NaDodSO_4$),[1] and thermolysin. Sodium dodecyl sulfate was recrystallized from 95% ethanol (Burgess 1969). Amberlite XAD4, bovine serum albumin, α-bungarotoxin, carboxypeptidase B, carboxypeptidase P, carboxypeptidase Y, iodoacetic acid, leucine aminopeptidase, methylene blue, phenylmethanesulfonyl fluoride, pyridoxal phosphate, Sephadex G25, Sephadex G75, sodium cyanoborohydride, and succinic anhydride were purchased from Sigma Chemical Co. α-Bungarotoxin was reductively methylated according to the procedure of Rice and Means (1971) to a specific radioactivity of $(1–2) \times 10^5$ cpm nmol^{-1}. Succinic anhydride was recrystallized from chloroform, and iodoacetic acid was recrystallized from petroleum ether. Trifluoroacetic acid (TFA)[1] was purchased from Halocarbon Products Corp.; ninhydrin and *N*-[(*m*-maleimidobenzoyl)hydroxy]sulfosuccinimide (sulfo-MBS)[1] were purchased from Pierce Chemical Co.; and sodium [^{3}H]borohydride (100–1000 mCi mmol^{-1}) was purchased from NEN Research Products, Du Pont Co.; Affigel 10, Affigel 102, and Biogel A1.5m were purchased from Bio-Rad Laboratories, Inc.; Freund's adjuvant was purchased from Difco Laboratories, Corp.; Ecolume was purchased from ICN Biochemicals, Inc.; trypsin that had been treated with *N*-(*p*-tolylsulfonyl)-L-phenylalanyl chloromethyl ketone and the proteinase from *Staphylococcus aureus* strain V8 were purchased from Worthington Biochemical Corp.; diisopropylcarbodiimide, ethanedithiol, hydrindantin, 1-hydroxybenzotriazole, *N*-methylpyrrolidinone, and piperidine were purchased from Aldrich Chemical Co. Unless otherwise noted, chemicals were used directly.

Synthesis of Peptides. Peptides were synthesized by conventional, solid-phase methods for FMOC amino acids (Stewart & Young, 1984). The first amino acid, N^{α}-FMOC-N^{δ}-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)-L-arginine, was attached to *p*-alkoxybenzyl resin with dicyclohexylcarbodiimide. To produce the peptide HALDAKGER, the following L-amino acids were added in order: N^{α}-FMOC-L-glutamic acid O^{γ}-butyl ester, N^{α}-FMOC-glycine, N^{α}-FMOC-N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine, N^{α}-FMOC-L-alanine, N^{α}-FMOC-L-aspartic acid O^{β}-butyl ester, N^{α}-FMOC-L-leucine, N^{α}-FMOC-L-alanine, and N^{α}-FMOC-N^{im}-(triphenylmethyl)-L-histidine. The peptide LDACGER was synthesized by adding in order the following L-amino acids to arginine-coupled *p*-alkoxybenzyl resin: N^{α}-FMOC-L-glutamic acid O^{γ}-butyl ester, N^{α}-FMOC-glycine, N^{α}-FMOC-*S*-(triphenylmethyl)-L-cysteine, N^{α}-FMOC-L-alanine, N^{α}-FMOC-L-aspartic acid O^{β}-butyl ester, N^{α}-FMOC-L-leucine. For each peptide, each amino acid was added to the elongating chain as a preformed hydroxybenzotriazole ester. The product was cleaved from the resin in 90:5:5 trifluoroacetic acid:ethanedithiol:anisole, and the TFA was removed under reduced pressure. For each peptide, the residue remaining after removal of TFA was dissolved in 10% acetic acid and extracted with diethyl ether. The aqueous phase was lyophilized, and the resulting crude product was purified by reverse-phase,

[1] Abbreviations: FMOC, 9-fluorenylmethyloxycarbonyl; $NaDodSO_4$, sodium dodecyl sulfate; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; sulfo-MBS, *N*-[(*m*-maleimidobenzoyl)hydroxy]-sulfosuccinimide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; PBS, 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4; Tris, tris-*N*-(hydroxymethyl)aminomethane; sodium diatrizoate, sodium 3,5-bis(acetylamino)-2,4,6-triiodobenzoate; C_{18}, octadecylsilyl silica gel; succ, succinyl; pyr, phosphopyridoxyl.

high-pressure liquid chromatography (HPLC).[1] The purified peptide HALDAKGER migrated on HPLC as a single peak the amino acid composition of which, after acid hydrolysis, was $H_{1.0}A_{1.9}L_{1.0}D_{1.0}K_{1.0}G_{1.0}E_{1.0}R_{1.1}$. Leucine aminopeptidase digestion and carboxypeptidase Y digestion of the peptide gave the compositions $H_{1.0}A_{2.0}L_{1.0}D_{0.9}K_{1.0}G_{1.0}E_{1.0}R_{1.0}$ and $E_{0.6}R_{0.9}$, respectively. The crude synthetic product of the peptide LDACGER gave two major peaks (with retention times of 18 and 21 min) on HPLC. Reduction of this mixture with dithiothreitol prior to chromatography produced a single peak that corresponded to the peptide from the crude mixture that had exhibited the shorter retention time (18 min). Acid hydrolysis, leucine aminopeptidase digestion, and carboxypeptidase digestion of the material in this peak gave the following respective compositions: $L_{1.0}D_{0.9}A_{0.9}G_{0.9}E_{1.0}R_{1.3}$, $L_{0.9}D_{0.3}A_{0.3}G_{0.3}E_{0.4}R_{0.2}$, and $E_{0.1}R_{1.2}$. The cysteine content of this peptide was quantified by reacting it with iodoacetic acid prior to acid hydrolysis and analysis.

The cysteine in the peptide LDACGER was assayed as the *S*-(carboxymethyl) derivative following modification with iodoacetic acid (Gurd, 1967; Hirs, 1967). Dithiothreitol (to 0.4 mM) was added to a solution purged with Ar containing 16 nmol of LDACGER in 0.2 mL of 0.1 M sodium bicarbonate, pH 8.1. After 5 min at room temperature under Ar, iodoacetic acid (2.5 mM) was added, and the mixture was allowed to react for 10 min at room temperature. The solution was then mixed with an equal volume of 12 M hydrochloric acid and hydrolyzed. The resulting *S*-(carboxymethyl)cysteine was identified by amino acid analysis, and the yield was 0.7 nmol (nmol of peptide)$^{-1}$.

Modification of Synthetic HALDAKGER with Succinic Anhydride. Solid succinic anhydride (to 10 mM) was added to a solution of purified, synthetic HALDAKGER (0.2 μmol in 1 mL) in 0.1 M sodium phosphate, pH 8.0, to a final concentration of 10 mM. The pH was maintained at 8.0 during the reaction by small additions of 12.5 M sodium hydroxide. The reaction was allowed to proceed at room temperature until all of the solid succinic anhydride had dissolved (about 30 min) after which solid hydroxylamine hydrochloride was added to 1 M. The pH was raised to 10 with 12.5 M sodium hydroxide. After 1 h, the pH was lowered with acetic acid to pH 3, and the modified peptide was purified from the labeling reagents on reverse-phase HPLC. The modified peptide was digested with thermolysin (0.1 mg mL^{-1}) for 2 h at 37 °C to remove the amino-terminal succinylhistidylalanine and repurified on HPLC.

Modification of Synthetic HALDAKGER with Pyridoxal Phosphate and Sodium Cyanoborohydride. Phosphopyridoxyl peptide was produced by a method similar to that of Thibault (1993). Synthetic HALDAKGER (0.6 mM) and pyridoxal phosphate (1.5 M) were dissolved in 0.1 M sodium phosphate, and the pH was adjusted to pH 8.0. All subsequent steps were performed in dim light. After 30 min, sodium cyanoborohydride was added to a final concentration of 1.5 mM and allowed to react for 30 min before acetic acid was added to adjust the solution to pH 5. The reaction mixture was submitted to HPLC to separate the modified peptide from the reagents. Several peaks eluted near the position of the unmodified peptide, and they were pooled and dried. This material was digested with thermolysin (0.1 mg mL^{-1}) to remove the amino-terminal phosphopyridoxylhistidylalanine, and the product was purified on HPLC.

Preparation of Immunoadsorbents. A haptenic conjugate of the peptide LDACGER and bovine serum albumin (BSA)[1] was used to produce polyclonal, antipeptide immunoglobulins in rabbits. The peptide LDACGER was coupled to BSA through its cysteinyl thiol with the bifunctional cross-linking reagent sulfo-MBS. This reagent is a water soluble analogue of *N*-[(*m*-maleimidobenzoyl)hydroxy]succinimide, described by Kitagawa and Aikawa (1976), which has been used to prepare protein immunoconjugates (Liu et al., 1979). Bovine serum albumin (5 mg in 0.2 mL) and sulfo-MBS (19 mM) were dissolved in 0.1 M sodium phosphate, pH 6.5, and allowed to react for 30 min at room temperature. The modified BSA was separated from the reagents on a column of Sephadex G25 (1.5 cm × 14 cm) in 0.1 M sodium phosphate, pH 6.5. The fractions from the column that eluted at the position of BSA were pooled (4 mL), and EDTA was added to the solution (to 5 mM) before solid LDACGER (2.5 μmol) was added. The reaction proceeded overnight at room temperature, and the conjugate between BSA and the peptide was purified on a column of Sephadex G75 (1.5 cm × 14 cm). A portion of the product was submitted to amino acid analysis following acid hydrolysis. The extent of coupling was determined to be 8 nmol of peptide to every nmol of serum albumin by amino acid analysis. This conjugate was used to produce polyclonal, antipeptide immunoglobulins in white New Zealand rabbits. A 1:1 suspension of the conjugate (0.5 mg mL^{-1}) and Freund's complete adjuvant was injected subcutaneously and intramuscularly for the initial immunization. Subsequent injections were made with a suspension of the conjugate in Freund's incomplete adjuvant.

An immunoadsorbent for immunoglobulins that recognize the sequence LDAXGER, where X is any modified or unmodified amino acid, was constructed by coupling the peptide LDACGER to a solid support. Amino alkyl resin, Affigel 102 (3 mL packed volume), was activated with 15 μmol of sulfo-MBS for 30 min in 0.01 M sodium phosphate buffer, pH 7.0. The resin was washed three times with 0.1 M sodium phosphate, pH 6.5. After the addition of 5 mM ethylenediaminetetraacetic acid (EDTA),[1] LDACGER (15 μmol) was added to a slurry of resin in the same buffer. The coupling proceeded at room temperature overnight. Uncoupled peptide was removed by washing the resin. A portion of the washed resin was removed and digested with carboxypeptidases P (5 units) and B (3.5 units) for 2 h at 37 °C, and the released amino acids were submitted to amino acid analysis. The peptide LDACGER was coupled to resin to the extent of 180 nmol of carboxypeptidase-accessible peptide (3 mL of packed resin)$^{-1}$.

Antiserum was either used fresh or mixed with an equal volume of saturated ammonium sulfate and stored at 4 °C until needed. Ammonium sulfate was removed by dialysis against phosphate-buffered saline (PBS),[1] which contained 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4. In either case, the antiserum or the reconstituted antiserum was passed over a column that contained the immunoadsorbent made from LDACGER. Nonspecifically bound proteins were washed away with PBS, and specifically bound proteins were eluted with 0.1 M sodium phosphate, pH 2.5. The acid eluates were neutralized immediately with a solution of the free base of tris-*N*-(hydroxymethyl)aminomethane (Tris)[1] and dialyzed into PBS. The solution of immunoglobulins was concentrated in a Centricon 30 microconcentrator (Amicon) to a final volume of 1.5 mL and was added to 1 mL of cross-linked carboxymethyl agarose activated as an *N*-hydroxysuccinimide ester (Affigel 10) in 0.1 M sodium *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonate, pH 7. The solution was agitated at 4 °C overnight. The capacity of this immunoadsorbent to bind the peptide

LDACGER was determined by adding an excess of this synthetic peptide, washing away the unbound peptide with PBS, and eluting the specifically bound peptide with 0.1 M sodium phosphate, pH 2.5. The acid eluates were submitted to HPLC, and the peptide was quantified by amino acid analysis after acid hydrolysis. This immunoadsorbent had the capacity to bind 2.5 nmol of peptide. This immunoadsorbent also bound the synthetic peptides LDAK(succ)GER and LDAK(pyr)GER and released them when eluted with 0.1 M sodium phosphate, pH 2.5. It was used to purify peptides with the sequence LDAK(J)GER from tryptic and thermolytic digests of modified acetylcholine receptor, where J refers to any modification.

The immunoadsorbent specific for peptides that have the carboxy-terminal sequence –YIAE was that produced by Dwyer (1988).

Preparation of Sealed, Right-Side-Out Vesicles and Open Membranes. Sealed, right-side-out vesicles and open membranes were prepared according to the method of Dwyer (1991) except that iodoacetamide (5 mM) and phenylmethanesulfonyl fluoride (3 mM) were included and the 0.1% 2-mercaptoethanol was eliminated from the initial buffer for the homogenization. Sealed, right-side-out vesicles are the fraction of membranes that float on a gradient (10% to 22%) of sodium 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (sodium diatrizoate),[1] and open membranes are the fraction that sink through a gradient of sodium diatrizoate when the gradient is spun at 25 000 rpm in a Beckman SW27 rotor for 20 h. Each preparation of vesicles and membranes was assayed for concentration of protein and for its ability to bind α-bungarotoxin. Concentration of protein was determined by the method of Lowry et al. (1951). The assay for α-bungarotoxin binding was performed as described by Dwyer (1991) except that the membranes were pelleted at 35 000 rpm in a Beckman Ti 50 rotor for 35 min.

Modification of Membrane-Bound Acetylcholine Receptor with Succinic Anhydride. Open membranes or sealed vesicles (26 mg of protein in 2 mL) were suspended in 8 M urea, 0.25 M sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0, after making the solutions 0.4% in saponin. Solid succinic anhydride was added in three portions (final concentration 0.6 M), and the pH was maintained at pH 8 by small additions of 12.5 M sodium hydroxide as necessary. After 30 min, hydroxylamine hydrochloride was added to 1 M, and the pH was raised to pH 10 with 12.5 M sodium hydroxide. After 1 h, the solution was dialyzed against 0.05 M sodium bicarbonate, pH 7.8, to remove excess reagents. The suspensions of succinylated membranes were digested with trypsin and thermolysin and passed over the immunoadsorbent specific for the sequence LDAXGER.

Modification of Open Membranes and Sealed, Right-Side-Out Vesicles with Pyridoxal Phosphate. Open membranes (26 mg of protein in 2 mL) were modified in a saturated solution of pyridoxal phosphate. Solid pyridoxal phosphate (to a final concentration of 1.5 M) was added to a solution of membranes in 0.25 M sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, and the pH was adjusted to 8 with 12.5 M sodium hydroxide. After 30 min at room temperature, 1 equiv of sodium cyanoborohydride was added, and the reaction was allowed to proceed for 30 min. The pH was then lowered to pH 6 with 12 M hydrochloric acid, and excess reagents were removed by dialysis against 0.05 M ammonium bicarbonate, pH 7.8. The protein was digested with trypsin and thermolysin and passed over the immunoadsorbent specific for the sequence LDAXGER.

Sealed, right-side-out vesicles were modified by the method of Ohkawa and Webster (1981) as described by Dwyer (1991). Briefly, sealed, right-side-out vesicles in 0.25 M sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0, were split into two portions (each conatined 26 mg of protein in 2 mL). To one portion was added saponin to 0.4%, the other did not contain saponin. Pyridoxal phosphate (12 mM) was added to each portion for 20 min at room temperature. Sodium [^{3}H]borohydride was then added (12.5 mCi, 35 nmol) in 0.1 mL of 0.01 M sodium hydroxide, and the reaction proceeded for 30 min at room temperature. The sample modified in the presence of saponin was dialyzed against 0.25 M sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0. The sample modified in the absence of saponin was reapplied to a gradient of sodium diatrizoate and submitted to centrifugation at 25 000 rpm in a Beckman SW 27 rotor for 20 h. The band of vesicles that floated on the gradient was collected, diluted with 0.25 M sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0, and pelleted at 30 000 rpm in a Beckman Ti 45 rotor for 30 min. The phospho[^{3}H]pyridoxylated vesicles were either modified with succinic anhydride or used to prepare protein free of phospholipid.

Preparation of Modified Protein Free of Phospholipid. Modified vesicles were dissolved with $NaDodSO_4$ by adding 5–6 mg of detergent for every mg of protein, and the solution was applied to a gel filtration column (2.6 cm × 90 cm) of Biogel A1.5m. The column was equilibrated with 0.1% $NaDodSO_4$, 40 mM Tris sulfate, pH 8. The effluent was screened for polypeptides of acetylcholine receptor by electrophoresis in 0.1% $NaDodSO_4$ on 10% polyacrylamide gels (Laemmli, 1970), and the fractions containing the α- and β-polypeptides of acetylcholine receptor were pooled and lyophilized. The complex between dodecyl sulfate and protein was dissolved in water and made 8 M in urea, and the dodecyl sulfate was removed by the method of Weber and Kuter (1971) as modified by Nicholas (1984). The polypeptides free from phospholipid were dialyzed into 0.05 M ammonium bicarbonate, pH 7.8, and digested with the proteinase from *S. aureus* strain V8. The digest was then applied to the immunoadsorbent specific for peptides with the carboxy-terminal sequence –YIAE.

Enzymatic Digestions. The digestion of peptides with leucine aminopeptidase (0.07 unit mL^{-1}) was performed in 2.5 mM $MgCl_2$, 0.1 M Tris sulfate, pH 8.6, at 37 °C for 4 h. The digestion of peptides with carboxypeptidase Y (25 units mL^{-1}) was performed in 0.1 M pyridinium acetate, pH 5.5, at 37 °C for 4 h. Modified peptides were digested with thermolysin (0.1 mg mL^{-1}) in 20 mM $CaCl_2$, 0.05 M ammonium bicarbonate, pH 7.8 at 37 °C for 2 h (succinyl peptide) or overnight (phosphopyridoxyl peptide). Peptides were digested with subtilisin (16 μg mL^{-1}) in 0.05 M ammonium bicarbonate, pH 7.8, at 37 °C for 1 h. Modified proteins in membranes were digested simultaneously with trypsin (0.3 mg mL^{-1}) and thermolysin (0.2 mg mL^{-1}) in 1 mM $CaCl_2$, 0.05 M ammonium bicarbonate, pH 7.8, at room temperature for 1–2 days. Polypeptides stripped of phospholipid were digested with the proteinase from *S. aureus* strain V8 (0.02 mg mL^{-1}) in 0.05 M ammonium bicarbonate, pH 7.8, at 37 °C for 1 day.

Immunoadsorption. Digests of protein were passed over the immunoadsorbents for peptides, and nonspecifically bound peptides were washed away with 0.05 M ammonium bicarbonate, pH 7.8. Specifically bound peptides were eluted with

0.1 M sodium phosphate, pH 2.5. The immunoadsorbents were washed and stored in PBS.

High-Pressure Liquid Chromatography. Synthetic peptides and acid eluates from the immunoadsorbents were submitted to reverse-phase HPLC on octadecylsilyl silica gel (C_{18}).[1] The column (Vydac, 0.46 cm × 25 cm) was eluted with a mobile phase of 0.05% TFA in water that was developed at a rate that increased 1% min^{-1} in 0.02% TFA in acetonitrile from 0 to 40 min (Mahoney & Hermodson, 1980). The effluent from the column was continuously monitored for absorbance (λ = 229 nm or λ = 325 nm) and collected in fractions of 1 min. The system for HPLC was a Spectra-Physics Chromatograph SP8100 with a variable-wavelength Spectra-Physics Spectra 100 UV–vis detector.

Amino Acid Analysis and Determination of Radioactivity. Acid hydrolysis of peptides and protein was performed in 6 M hydrochloric acid, under vacuum, at 155 °C for 40 min. The amino acid analyzer was a modular system composed of a Spectra-Physics autosampler SP8875, Spectra-Physics ternary HPLC pump SP8800, and a Pickering sodium cation-exchange analytical column with postcolumn derivatization of amino acids with ninhydrin. Radioactivity was determined by dissolving samples in Ecolume liquid scintillation cocktail and assaying them with a Beckman LS-1701 scintillation counter.

RESULTS

Modification of Synthetic HALDAKGER. In the β-chain of acetylcholine receptor, the sequence surrounding Lys 165 is ...QHALDAKGEREVK.... Digestion of this region of the β-polypeptide with thermolysin and trypsin when lysine is modified (indicated by a J) should produce the peptide that includes Lys β165, LDAK(J)GER. Peptides with this sequence and modified appropriately at lysine were synthesized and used as standards to identify peptides isolated from digests of membranes rich in acetylcholine receptor.

The peptide HALDAKGER was modified with either succinic anhydride (Klotz, 1967) or pyridoxal phosphate and sodium cyanoborohydride (Thibault, 1993) and then digested with thermolysin to produce the standards. The products of these reactions are LDAK(succ)GER and LDAK(pyr)GER, where succinyl (succ)[1] or phosphopyridoxyl (pyr)[1] designate the modification. Succinylation of synthetic HALDAKGER produced a peptide that eluted during HPLC at a later retention time than the unmodified peptide. The product purified by HPLC was digested with thermolysin to remove the amino-terminal succinylhistidylalanine and generate a new, unmodified amino terminus. This product was then purified on HPLC. The resulting peak had a longer retention time (19 min) than that of unmodified LDAKGER (16 min), and the amino acid composition of this product after acid hydrolysis was $L_{0.8}D_{1.0}A_{1.3}K_{0.8}G_{1.2}E_{0.9}R_{1.1}$. Lysine is detected following acid hydrolysis since the modification with succinic anhydride produces an amide.

The phosphopyridoxyl peptide was produced by reacting synthetic HALDAKGER with 1.5 M pyridoxal phosphate to form the imine, followed by reduction with sodium cyanoborohydride to produce the stable secondary amine. The product of these reactions was purified by reverse-phase HPLC, digested with thermolysin, and repurified by HPLC. The composition of the purified, modified peptide after acid hydrolysis was $L_{1.0}D_{0.9}A_{1.1}K_{0.15}G_{1.0}E_{1.0}R_{1.0}$. The presence of a single alanine in the composition confirms that digestion with thermolysin removed the amino-terminal phosphopyridoxylhistidylalanine. Also, the low yield of lysine indicates that while the retention time of this peptide on HPLC (16 min) is indistinguishable from that of unmodified LDAKGER (16 min), the lysine has been modified in a manner partially stable to acid hydrolysis. The peptide also has a maximum of absorbance at 325 nm, a characteristic of pyridoxamines (Kirtley & Koshland, 1972). These observations indicate that lysine has been modified as the N^{ϵ}-phosphopyridoxyl derivative in the peptide designated LDAK(pyr)GER.

Preparation of Sealed Vesicles and Open Membranes. The topological disposition of Lys β165 was investigated using a method developed in our laboratory (Kyte et al., 1987). This method relies on the ability to produce sealed, oriented vesicles containing the membrane-spanning protein of interest, the ability to modify the amino acid of interest in that protein with reagents impermeant to the vesicles, and the ability to isolate peptides containing that modified amino acid from a digest of the protein.

In each preparation of sealed, right-side-out vesicles and open membranes used in these experiments, the total acetylcholine receptor content was determined by an assay for the binding of α-bungarotoxin. The toxin binds only to the extracytoplasmic surface of acetylcholine receptor and is used to assess the percentage of receptor molecules in these respective preparations of membranes whose extracytoplasmic surfaces are accessible to large proteins or impermeant reagents. The binding of toxin can also be used to quantify the amount of total acetylcholine receptor present in a preparation. To determine the percentage of acetylcholine receptor molecules in sealed vesicles that are oriented right-side-out, a binding assay is performed in the presence and absence of saponin. Froehner (1981) and St. John et al. (1982) showed that the binding of α-bungarotoxin to acetylcholine receptor is not affected by the presence of saponin. Prior to exposing the sealed, right-side-out vesicles used in these experiments to saponin, [^{3}H]methyl α-bungarotoxin was bound at 60–80% of the maximum level obtained by opening the vesicles with saponin. In the open membranes that sank through sodium diatrizoate, 95% of the total [^{3}H]methyl α-bungarotoxin binding sites found in the presence of saponin were accessible to toxin in the absence of saponin.

In the sealed, right-side-out vesicles, the concentration of [^{3}H]methyl α-bungarotoxin binding sites was usually 1 nmol (5 mg of protein)$^{-1}$, and in the open membranes the concentration was usually 1 nmol (2 mg of protein)$^{-1}$. In each experiment, either with sealed, right-side-out vesicles or with open membranes, 26 mg of total protein was usually used for each sample. This corresponds to 3 or 8 nmol of acetylcholine receptor, respectively.

Isolation of the Peptide LDAK(succ)GER from Succinylated Acetylcholine Receptor. Open membranes were succinylated in the presence of 8 M urea, dialyzed to remove reagents, and then digested directly without isolating the polypeptides from the membrane. This strategy was prompted by the observation of Moore et al. (1989) that the region of the β-polypeptide surrounding Lys β165 could be digested into small peptides even while the protein was still in the membrane. To block digestion of the polypeptide with trypsin at Lys β165 itself, the protein was succinylated (Klotz, 1967). After modification of the lysines, the membranes were digested simultaneously with trypsin and thermolysin, and this digest was passed over the immunoadsorbent (capacity 2.5 nmol). Bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5, and analyzed by HPLC. The high-pressure liquid chromatogram showed a peak of absorbance (λ = 229 nm) with the retention time (19 min) of the synthetic standard,

LDAK(succ)GER. The material in this peak was collected, and following acid hydrolysis its composition was determined to be $L_{0.9}D_{1.2}A_{0.8}K_{1.0}G_{1.2}E_{1.1}R_{0.9}$. The yield of peptide was 1.3 nmol. Since this peptide was bound by an immunoadsorbent specific for the sequence LDAXGER and since it had the amino acid composition and the retention time on HPLC of synthetic LDAK(succ)GER, it could be concluded that it was LDAK(succ)GER cut from the succinylated β-polypeptide of the acetylcholine receptor in these open membranes.

Isolation of the Peptide LDAK(pyr)GER from Open Membranes Modified with Pyridoxal Phosphate and Sodium Cyanoborohydride. To determine if Lys β165 could be modified with the impermeant reagent pyridoxal phosphate, open membranes (26 mg of protein) were modified with a saturated solution (1.5 M) of pyridoxal phosphate and the product subsequently reduced with sodium cyanoborohydride. Under these conditions, the maximum possible extent of reaction between Lys β165 and pyridoxal phosphate should occur. The membranes were dialyzed to remove excess reagents and digested with thermolysin and trypsin. The products of digestion were passed over the immunoadsorbent specific for LDAXGER (capacity 2.5 nmol), and the acid eluates were analyzed by HPLC. The high-pressure liquid chromatogram contained a single peak of absorbance (λ = 229 nm) with a retention time (16 min) corresponding to that of synthetic LDAK(pyr)GER. The material in the peak isolated from open membranes and an equivalent sample of the synthetic standard were submitted separately to amino acid analysis (yield of 0.3 nmol), and each sample gave the same composition; however, in both samples the relative amounts of glycine, aspartate, and glutamate were elevated (by 140–200%). Since the peptide isolated from the digest of the open membranes was bound by an immunoadsorbent specific for LDAXGER and since it had the same retention time and amino acid composition as synthetic LDAK(pyr)-GER, it could be concluded that this peptide isolated from digested, open membranes was LDAK(pyr)GER cut from the pyridoxylated β-polypeptide of the acetylcholine receptor in the open membranes.

Determination of the Location of Lysine β165 in Intact Acetylcholine Receptor. Samples of sealed, right-side-out vesicles were modified with 12 mM pyridoxal phosphate and sodium [^{3}H]borohydride and then with succinic anhydride. In each experiment, one sample was treated with 0.4% saponin prior to labeling to allow the reagents access to both the interior and exterior surfaces of the vesicles, and the other sample was modified in the absence of saponin to restrict the reaction to the exterior surfaces of the vesicles. At this concentration of pyridoxal phosphate only a small proportion of each lysine should be modified (Kyte et al., 1987), and this modification can be identified by the stable incorporation of tritium to form the N^{ϵ}-phospho[^{3}H]pyridoxyl derivative (Ohkawa & Webster, 1981). After labeling the vesicles, the sample exposed to saponin was dialyzed to remove excess reagents, and the other sample was applied a second time to a gradient of sodium diatrizoate. This step was performed to remove any vesicles that may have been broken at any step prior to or during modification with pyridoxal phosphate. These samples were suspended in 8 M urea, treated with 0.4% saponin, and succinylated with excess succinic anhydride to modify all the remaining lysines. Succinylation of Lys β165 permits the isolation, following digestion, of the peptide LDAK(succ)GER. This peptide can be used as an internal standard that accounts for differences in overall yield between the two samples during the isolation of LDAK([^{3}H]pyr)-GER. After dialysis of both samples, trypsin (0.3 mg mL^{-1}) and thermolysin (0.2 mg mL^{-1}) were added, and digestion proceeded for 1–2 days. Each digest was then passed over the immunoadsorbent specific for LDAXGER and eluted with low pH. Acid eluates were submitted to HPLC (Figure 1). Half of each fraction from the HPLC was submitted to liquid scintillation counting. For both the sample labeled in the presence (Figure 1B, lower panel) and that labeled in the absence (Figure 1C, lower panel) of saponin, the chromatogram contains a predominant peak of absorbance (peak b) of the same retention time (19 min) as the succinylated standard, LDAK(succ)GER (Figure 1A). As expected, very little absorbance was detected at the position where the product of phosphopyridoxylation, LDAK(pyr)GER (peak a, Figure 1A), should elute. Three peaks of radioactivity were present in both samples (Figures 1B and 1C, upper panels). The retention

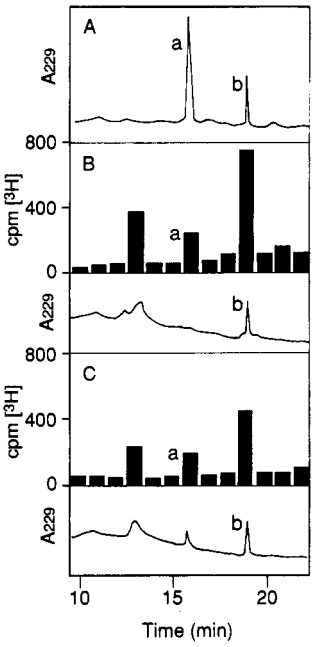

FIGURE 1: Incorporation of phospho[^{3}H]pyridoxyl groups into Lys β165 of acetylcholine receptor in sealed, right-side-out vesicles. Two portions of sealed, right-side-out vesicles, each containing 52 mg of protein in 4 mL, were modified in the presence or absence of 0.4% saponin, respectively, with 12 mM pyridoxal phosphate and Na-[^{3}H]BH_4 (12.5 mCi, 35 nmol) for 20 min at room temperature. After refloating the sealed vesicles, both samples were split in half, and half of each was then transferred to 0.4% saponin and 8 M urea, modified with 0.6 M succinic anhydride, and dialyzed. The succinylated protein was digested with trypsin (0.3 mg mL^{-1}) and thermolysin (0.2 mg mL^{-1}), and these digests were passed over the immunoadsorbent specific for the sequence LDAXGER (2.5-nmol capacity). Unbound peptides were washed away with PBS, and bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. The acid fractions were submitted to analytical reverse-phase HPLC. The column of C_{18} chromatographic medium (0.46 cm × 25 cm) was run in 0.05% TFA in water and developed with a gradient of slope 1% acetonitrile min^{-1}, from 0 to 40 min, produced with 0.02% TFA in neat acetonitrile. The effluent from the column was continuously monitored for absorbance at 229 nm and collected in fractions of 1 min. Half of each fraction was assayed by liquid scintillation. (A) Chromatogram of a mixture of the synthetic peptides LDAK(pyr)GER (peak a) and LDAK-(succ)GER (peak b). (B) Results of an experiment in which the vesicles were modified in the presence of saponin. (C) Results of an experiment performed in the absence of saponin. In both B and C, the high-pressure liquid chromatogram of the acid eluate from the immunoadsorbent (lower panels) and a bar graph representing the counts of tritium from the fractions are presented (upper panels). Peaks of radioactivity with retention times (16 min) identical to the standard peptide LDAK(pyr)GER are marked a, and peaks of absorbance with retention times (19 min) identical to the standard peptide LDAK(succ)GER are marked b.

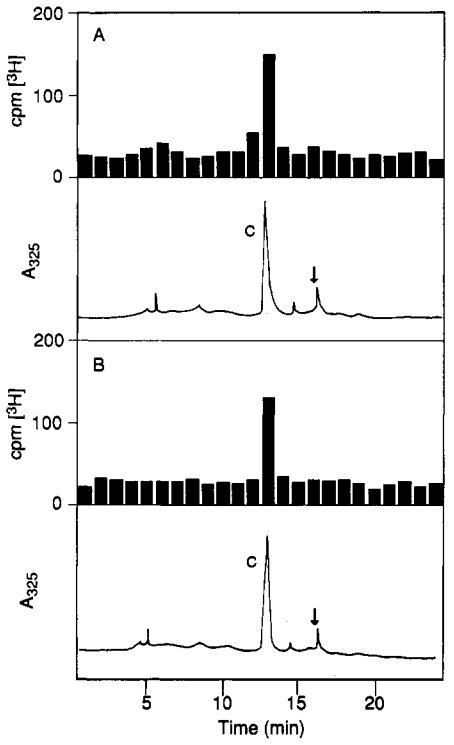

FIGURE 2: Digestion of LDAK([^{3}H]pyr)GER with subtilisin. Half of each of the fractions from the two chromatograms shown in Figure 1B,C that contained LDAK([^{3}H]pyr)GER (peaks a, retention times 16 min) was mixed separately with synthetic LDAK(pyr)GER (3 nmol) and evaporated to dryness. Subtilisin (16 μg mL^{-1}) was added in 0.05 M ammonium bicarbonate, pH 7.8, and the digestion proceeded for 1 h at 37 °C. Each of the two samples were then submitted to analytical reverse-phase HPLC under the same conditions as those used for the samples in Figure 1. The effluent from the column was continuously monitored for absorbance at 325 nm (lower panels) and was collected in fractions of 1 min. Each fraction was submitted to liquid scintillation (upper panels). (A) Results for the sample from vesicles modified in the presence of saponin. (B) Results for the sample from vesicles modified in the absence of saponin. In both chromatograms, peak c contains LDAK(pyr), the product of the digestion, while the arrow indicates the retention time (16 min) of LDAK(pyr)GER before digestion with subtilisin.

time (16 min) of one of these peaks (peak a) coincides with that of the phosphopyridoxylated standard, LDAK(pyr)GER (peak a, Figure 1A).

To analyze this peak of radioactivity further, half of this fraction was mixed with 3 nmol of synthetic LDAK(pyr)-GER and digested with subtilisin. If the tritium is bound to the peptide LDAK([^{3}H]pyr)GER, then the pattern of absorbance following proteolysis for the synthetic, standard peptide and the pattern of radioactivity from the peptide isolated from modified acetylcholine receptor should be identical. The products of digestion with subtilisin were separated by HPLC, and each fraction was assayed for radioactivity. The chromatogram was monitored for absorbance at a wavelength of 325 nm to identify those peptides that contain lysylphosphopyridoxamine. In theory, all of the radioactivity should coelute only with peptides containing N^{ϵ}-phosphopyridoxyllysine, since tritium was incorporated into this modified residue. Each of the chromatograms (Figure 2) shows one peak of absorbance and one peak of radioactivity, and they coelute with a retention time of 13 min. In a separate experiment, synthetic LDAK(pyr)GER was digested with subtilisin, and the product that absorbed at 325 nm (retention time 13 min) was identified as LDAK(pyr) by acid hydrolysis followed by amino acid analysis. The composition obtained for this peptide was $L_{1.0}D_{1.2}A_{1.1}$; in addition, since this peptide also absorbs at 325 nm, it must contain lysylpyridoxamine. Under these conditions for digestion with subtilisin, nearly complete conversion of synthetic LDAK(pyr)GER to LDAK-(pyr) is achieved. These results verify that the peak of radioactivity eluting with the retention time of synthetic LDAK(pyr)GER (16 min) is the peptide LDAK([^{3}H]pyr)-GER.

Another of the peaks of radioactivity isolated from the digest of these modified vesicles had a retention time (19 min) similar to that of the succinylated peptide. This peak was routinely isolated from phospho[^{3}H]pyridoxylated, succinylated vesicles in both the absence and presence of saponin. Since this peak of radioactivity was bound by the immunoadsorbent specific for LDAXGER, it may contain that sequence or a portion of that sequence. It may also be a modified form of this peptide. To investigate this possibility, the fraction containing this peak of radioactivity was mixed with synthetic LDAK(pyr)GER (3 nmol), digested with subtilisin, and submitted to HPLC. In this digested sample, a peak of radioactivity coeluted with synthetic LDAK(pyr). This peak represented 20–35% of the total tritium in the sample. The remaining tritium was not affected by digestion with subtilisin under these reaction conditions. Therefore, it was assumed that a portion of the radioactivity present in this peak represents a modification of Lys β165, while the majority is of undetermined origin. The other peak of radioactivity present in the digest of protein and retained by the immunoadsorbent has a retention time (13 min) similar to that of LDAK(pyr). Digestion of this peak with subtilisin, however, causes the tritium to elute near the void volume of the HPLC column. This result indicated that the tritium in this sample is not attached to any identified peptide containing Lys β165.

The relative radioactivity of LDAK([^{3}H]pyr)GER was calculated by dividing the number of counts eluting at the retention time of synthetic LDAK(pyr)GER (16 min) in the sample modified in the presence of saponin by the number of counts eluting at the same position in a sample modified in the absence of saponin. This ratio does not account for differences in either the yield of protein throughout the procedure or the efficiency of digestion in the two samples. These differences should, however, be reflected in the relative yield of succinyl peptide, LDAK(succ)GER, which could be estimated by dividing the area of the peak of succinyl peptide obtained from experiments performed in the presence of saponin by that obtained from experiments performed in the absence of saponin. Finally, the relative incorporation of tritium into LDAK([^{3}H]pyr)GER in the presence and absence of saponin [(cpm+)(area−)(cpm−)$^{-1}$(area+)$^{-1}$] was determined by dividing the relative radioactivity by the relative yield. It was assumed that this calculation provides an accurate measure of the relative degree of incorporation of phospho-[^{3}H]pyridoxyl groups into Lys β165 in the original, intact protein in the two samples. For the experiment depicted in Figure 1, the sum of the counts per minute of tritium for LDAK([^{3}H]pyr)GER in acid eluates from the immunoadsorbent were 230 and 190 cpm, and the sum of the peak areas for LDAK(succ)GER in acid eluates from the immunoadsorbent were 7.7 and 7.0 mm^2 in the presence and absence of saponin, respectively. The relative incorporation of phospho-[^{3}H]pyridoxyl groups into Lys β165 was 1.1 in this experiment.

The relative incorporations of phospho[^{3}H]pyridoxyl groups into Lys β165 from several identical experiments (Table 1) varied from 0.2 to 1.3 (0.9 ± 0.4). After subtilisin digestion of LDAK([^{3}H]pyr)GER, the relative radioactivity of the two peaks eluting at the position of LDAK([^{3}H]pyr) in the presence

Table 1: Relative Specific Radioactivity of LDAK([^{3}H]pyr)GER from Modified Vesicles[a]

expt	% srso[b]		+saponin	−saponin	+saponin/ −saponin	[(cpm+)(area−)]/ [(cpm−)(area+)]
1	77	cpm	115	96	1.2[d]	1.2[f]
		area[c]	1.2	1.1	1.0[e]	
2	77	cpm	573	761	0.75	0.20
		area	5.2	1.4	3.7	
3	75	cpm	375	255	1.5	0.66
		area	6.6	2.9	2.3	
4	61	cpm	320	153	2.1	0.87
		area	7.0	2.9	2.4	
5[g]	73	cpm	638	312	2.0	1.3
		area	13.6	8.8	1.5	
6[h]	68	cpm	229	187	1.2	1.1
		area	7.7	7.0	1.1	
7[i]		cpm	136	99	1.4	1.2
		area	6.9	5.6	1.2	

[a] The counts per minute of LDAK([^{3}H]pyr)GER and area of absorbance of LDAK(succ)GER were determined from experiments similar to those described in Figure 1. [b] The percent sealed, right-side-out vesicles is the ratio of the concentration of binding sites for α-bungarotoxin measured in the absence and presence of saponin multiplied by 100. [c] Area is given in millimeters^2 of chart paper. [d] Relative radioactivity. [e] Relative yield. [f] Relative incorporation. [g] In this experiment, the amount of vesicles used (52 mg) was twice that of other experiments. [h] The data presented in Figure 1 are from this experiment. [i] Half of the fraction from HPLC that contained LDAK([^{3}H]pyr)GER in experiment 6 was digested with subtilisin and rechromatographed. The counts per minute of tritium are those that coelute with LDAK(pyr) as shown in Figure 2. The areas are the areas of LDAK(succ)GER on the original chromatograms from which the samples for digestion were taken.

and absence of saponin was 1.4. This ratio was divided by 1.2 to reflect the relative yield of the succinylated peptide isolated from the immunoadsorbent in the original fraction that was digested. The relative incorporation was 1.2 and is in agreement with the other relative incorporations presented in Table 1.

Lysine α380 as a Monitor of the Sealed, Right-Side-Out Vesicles. Lysine α380 of acetylcholine receptor is surrounded by the sequence ...AIEGVKYIAEHM.... This lysine can be modified by pyridoxal phosphate and sodium [^{3}H]borohydride and isolated from digests of the protein by an immunoadsorbent. By modification of sealed, right-side-out vesicles with impermeant reagents, it has been shown that this residue is located on the inside surface of these vesicles (Dwyer, 1991). The modification of this lysine was used to demonstrate that the vesicles used in the present experiments were right-side-out and sealed. Suspensions of vesicles modified in the presence and absence of saponin, respectively (52 mg of protein in each sample), were each separated into two equal portions after dialysis or floatation on gradients of sodium diatrizoate, respectively. One portion of each of these two suspensions was used to determine the yield of modification at Lys β165 (experiment 6 of Table 1, described in Figure 1), and the other portion was used to determine the yield of modification at Lys α380. The vesicles in these latter two portions were dissolved separately with $NaDodSO_4$ and submitted to gel filtration to separate the polypeptides of acetylcholine receptor from the lipids (Dwyer, 1988). The portions of the eluates from the column that contained both the α- and β-polypeptides were pooled and lyophilized. The dodecyl sulfate was removed from these samples in the presence of 8 M urea. The urea was removed by dialysis into 0.05 M ammonium bicarbonate, pH 7.8, and the sample was digested with the proteinase from *S. aureus* strain V8. The digests were passed over the same immunoadsorbent used in the earlier experiments of Dwyer (1991), which recognizes peptides that contain the carboxy-terminal sequence −YIAE, and bound peptides were eluted

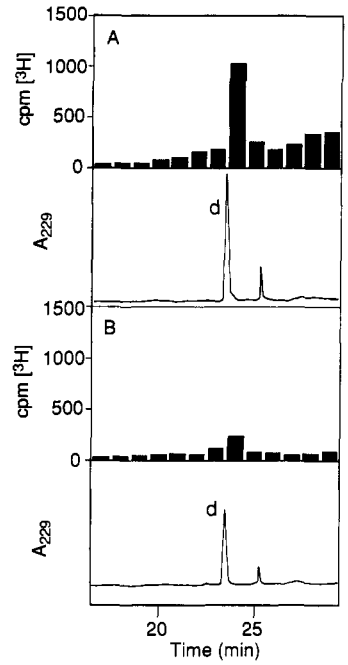

FIGURE 3: Incorporation of phospho[^{3}H]pyridoxyl groups into Lys α380 of acetylcholine receptor in sealed, right-side-out vesicles. The other half of each sample of vesicles labeled in the presence and absence of 0.4% saponin from the experiment described in Figure 1 was dissolved separately with $NaDodSO_4$ [5 mg (mg of protein)$^{-1}$], and the polypeptides of acetylcholine receptor were separated from lipid by gel filtration in 0.1% $NaDodSO_4$ (Dwyer, 1988). The fractions containing the α- and β-polypeptides of acetylcholine receptor were pooled, and the dodecyl sulfate was removed in 8 M urea (Nicholas, 1984). Modified, purified polypeptides, from which the detergent had been removed, were digested with the proteinase from *S. aureus* strain V8 and passed over an immunoadsorbent specific for the carboxy-terminal sequence −YIAE. The acid eluates were submitted to analytical reverse-phase HPLC, and the eluate was monitored continuously for absorbance at 229 nm (lower panels). The chromatographic column was developed in a manner identical to that described in Figure 1. Fractions of 1 min were collected and submitted to liquid scintillation counting (upper panels). (A) Result of the experiment that contained saponin during modification of vesicles with pyridoxal phosphate and Na[^{3}H]BH_4. (B) Result when saponin was absent during modification of vesicles. Peak d is the unmodified peptide GVKYIAE isolated from the digests.

with 0.1 M sodium phosphate, pH 2.5. The acid eluates were submitted to reverse-phase HPLC, and fractions of the effluent were subsequently submitted to liquid scintillation counting (Figure 3).

From each digest of the modified polypeptides, a peptide with the retention time (24 min) of GVKYIAE was isolated by the immunoadsorbent (Figure 3A,B, lower panel). A peak of tritium eluted in the fractions following those containing that peptide. This is the same relative position of tritium and absorbance observed by Dwyer (1991) for the peptides GVKYIAE and GVK([^{3}H]pyr)YIAE. When vesicles were labeled in the absence of saponin, a small amount of tritium was incorporated into this peptide containing Lys α380. When the vesicles were breached with saponin before being labeled, a larger amount of tritium was incorporated into the peptide. The sum of the amounts of tritium incorporated into this peptide in the presence of saponin from all of the fractions of the acid eluates from the immunoadsorbent was 1880 cpm, and in the absence of saponin, it was 260 cpm, so the relative radioactivity was 7.1. The sum of the areas of the peaks of absorbance under these two conditions were 27 and 14 mm^2, so the relative yield was 2.0. The relative radioactivity was divided by the relative yield to account for the difference in overall yields in the two samples, and the resulting relative

incorporation was 3.6. This observation verifies that the vesicles used in these experiments were sealed and in a right-side-out orientation.

DISCUSSION

Site-directed immunochemistry was first used to demonstrate that Lys 501 of the α-polypeptide of Na^+/K^+-transporting ATPase is located on the cytoplasmic surface of the native protein (Kyte et al., 1987). It has also been used to define the locations of Lys α380 and Lys γ486 in native acetylcholine receptor (Dwyer, 1991). Since the first report, the method of site-directed immunochemistry has incorporated several improvements that permit a more rigorous analysis of the target peptide in the last steps of the experiment (Thibault, 1993), and these strategies were used in the present determination of the disposition of Lys β165.

For the topological studies, it was necessary to have a population of sealed, oriented vesicles. The conclusion that the vesicles used here were sealed is based on the observation that treating them with saponin caused them to sink through the gradients of sodium diatrizoate. Saponin, a mixture of plant glycosides, creates holes in membranes (Bangham & Horne, 1962) that are large enough to allow ferritin to pass through (St. John et al., 1982). It has been shown (Dwyer, 1991) that treating a preparation of these vesicles with saponin causes nearly all of them to sink through a second gradient of sodium diatrizoate. When the vesicles are not treated with saponin, however, the majority (90%) of the binding sites for α-bungarotoxin float on a second gradient of sodium diatrizoate. The conclusion that the vesicles are right-side-out is based on the observation that a majority of binding sites for α-bungarotoxin are accessible in the absence of saponin. That the vesicles used in these experiments were sealed and right-side-out was confirmed by monitoring the incorporation of phospho[^{3}H]pyridoxyl groups into Lys α380 in the presence and absence of saponin. This residue is known to have a cytoplasmic disposition in intact, native acetylcholine receptor (Dwyer, 1991; Anand et al., 1993). The modification of Lys α380 with pyridoxal phosphate was followed in one of the same experiments in which the modification of Lys β165 was also determined. In the presence of saponin, the incorporation of phospho[^{3}H]pyridoxyl groups into Lys α380 increased by 3.6-fold relative to the incorporation in the absence of saponin (Figure 3). This confirms that the vesicles used in these experiments were sealed to impermeant reagents and that they were predominantly oriented right-side-out.

Quantification of both the amount of radioactive LDAK([^{3}H]pyr)GER and the amount of LDAK(succ)GER on the final chromatograms (Figure 1) allowed the relative extent of modification of Lys β165 in the original labeling of the native protein to be estimated. The quantification of LDAK(succ)GER was based on the areas of the peaks of absorbance in the effluent from high-pressure liquid chromatograms and permitted correction for differences in the yield of peptides between samples. The two peptides, LDAK([^{3}H]pyr)GER and LDAK(succ)GER, both modified at lysine, are separated by HPLC and therefore can be quantified independently. Since both peptides are produced by the same cleavages of the intact β-polypeptide with trypsin and thermolysin, it was assumed that the yield of LDAK(succ)GER was directly proportional to the yield of LDAK([^{3}H]pyr)GER in all samples, and thus the areas of absorbance for LDAK(succ)GER were used to calculate specific radioactivities.

Modification of vesicles with pyridoxal phosphate and sodium [^{3}H]borohydride followed by exhaustive succinylation produced three tritiated peptides that were recognized by the immunoadsorbent specific for the sequence LDAXGER. One of these peptides (peak a, Figure 1) was identified as LDAK([^{3}H]pyr)GER by comparison of its mobility to that of the standard peptide and its susceptibility to digestion by subtilisin (Figure 2). Another of these peaks had a retention time similar to that of the standard peptide LDAK(succ)GER. It is possible that this peak contains the peptide, LDAKGER, modified by both a phospho[^{3}H]pyridoxyl group and a succinyl group. The phospho[^{3}H]pyridoxyl group is presumably incorporated into the protein as lysylphospho[^{3}H]pyridoxamine, a secondary amine. Succinic anhydride could react, subsequently, with that secondary amine in addition to the intended reaction at lysyl primary amines. The resulting product would presumably be a peptide that contains phospho[^{3}H]pyridoxamine, a modification that does not affect the retention time of the peptide, and a succinyl group, a modification that causes the peptide to elute later on HPLC. Pyridoxal phosphate also contains a phenolic hydroxyl group that could react with succinic anhydride, but it is unlikely that this modification would be present in the isolated peptides, because following succinylation hydroxylamine was added to the membranes and the pH raised to eliminate succinate esters.

When saponin is added to the sealed, right-side-out vesicles, Lys β165 is modified to the same extent as it is when saponin is omitted during the modification (Table 1). The same result was obtained when the counts of tritium were isolated in either the peptide LDAK([^{3}H]pyr)GER or the peptide LDAK([^{3}H]-pyr). All of these results indicate that this lysine is equally accessible to a small, membrane-impermeant electrophile in samples where reagents are restricted to the exterior surface of vesicles or in samples where access to both surfaces of vesicles is ensured. Because the population of vesicles is sealed in a predominantly right-side-out orientation, Lys β165 must be located on the extracytoplasmic surface of acetylcholine receptor.

Chavez and Hall (1991) have reported observations of the synthesis and glycosylation of the α-polypeptide of murine acetylcholine receptor that contains the natural site or an introduced site for *N*-glycosylation by an *in vitro* translation system. It was shown that introduction of a site for glycosylation at Lys α154 (the homologue of Lys α155 in acetylcholine receptor from *T. californica*) produced a shift in the mobility of the translated polypeptide on polyacrylamide gels. This result was consistent with glycosylation at that site *in vitro*, but no direct evidence of glycosylation at this specific site was presented, nor was it shown that the translated mutant polypeptide had folded into its native configuration. If the shift in mobility of the α-polypeptide was a result of glycosylation at Lys α154 in the properly folded and properly inserted α-polypeptide, then these results suggest that this portion of the natural polypeptide is located on the extracytoplasmic surface of the protein, in agreement with the results presented here.

To either side of Lys β165, there are sites that are also known to be located on the extracytoplasmic surface of acetylcholine receptor. All of the subunits contain (Kellaris & Ware, 1989) a conserved cystine (Cys 128-Cys 142 in the β-polypeptide) and a conserved site of *N*-glycosylation (Asn 141 in the β-polypeptide), both of which must be extracytoplasmic. The acetylcholine binding sites on the α subunits, which are also extracytoplasmic, contain cystine α192–α193 (Kao & Karlin, 1986) and Tyr α190 (Dennis et al., 1988; Abramson et al., 1989; Galzi et al., 1991), both of which can be modified by affinity labels for acetylcholine. The four

polypeptides in acetylcholine receptor are homologous; therefore, they share a common tertiary structure. Because, in the α-polypeptide, the amino acids that create the binding site for acetylcholine are extracytoplasmic, the homologous amino acids in all of the other subunits are extracytoplasmic. If all of these positions within the common amino acid sequence, those occupied by Cys β128–β142, Lys β165, Tyr α190, and Cys α192–α193, are extracytoplasmic it is hard not to conclude that the entire region comprising them is extracytoplasmic in each of the subunits of the native protein. The most recent maps of electron scattering density for acetylcholine receptor calculated by image reconstruction from frozen specimens (Unwin, 1993) suggest that the binding site for acetylcholine is 3.0 nm from the extracytoplasmic surface of the bilayer. This assignment would fix Tyr α190 and Cys α192–α193 at a location quite distant from the membrane. The lengths of the intervening sequences between the extracytoplasmic residues at the positions of Cys α142, Lys β165, and Tyr α190 in the aligned sequences are 18 and 28 amino acids, respectively, in the shortest polypeptide, which is the α-subunit. The first is too short to span the membrane twice, and the second is too short to span the membrane twice and extend 3.0 nm into the synapse to form a portion of the acetylcholine binding site.

In the aligned sequences of the polypeptides of acetylcholine receptor, less than 15 amino acids to the amino-terminal side of Lys β165, there are two nucleophilic residues: Tyr α151 and Lys α155 (the homologues of Tyr β151 and Glu β155). Neither of these two amino acids could be labeled with any of several membrane-impermeant or membrane-permeant electrophiles (Dwyer, 1990). Tyrosine α151 could not be modified (<0.1% of the incorporation into Tyr α381) with diazotized *p*-[^{35}S]sulfanilic acid (Berg, 1969), a negatively charged, impermeant reagent, or even with [^{125}I]ICl (<2% of the incorporation into Tyr α381) in open, native membranes. Several attempts were made to modify Lys α155 with pyridoxal phosphate and sodium [^{3}H]borohydride. These reagents were incapable of modifying (<4% of the incorporation into Lys α380) Lys α155 reproducibly; only once, after alkaline extraction of the vesicles, was tritium incorporated into this residue. Another negatively charged reagent, 3-sulfo-*N*-([^{3}H]-acetylhydroxy)succinimide, was also used to modify vesicles, but no tritium (<20% of the incorporation into Lys α380) was found attached to Lys α155. Only [^{3}H]acetic anhydride was capable of modifying (50% of the incorporation into Lys α380) Lys α155 in native, membrane-bound acetylcholine receptor. This reagent, however, is not membrane impermeant and therefore could not be used to assess the sidedness of an amino acid with respect to the bilayer. The failure to modify either Tyr α151 or Lys α155 with a variety of electrophiles suggests that these two amino acids must be buried in the native structure of the protein.

Reports from two laboratories (Criado, 1985a; Pedersen, 1990) had suggested that a portion of the folded polypeptide of the common subunit of acetylcholine receptor containing the positions occupied by Lys α155 and Lys β165 was exposed to the cytoplasm. The first of these reports used a monoclonal immunoglobulin against a peptide that had a sequence corresponding to the segment α152–α167 of the α-polypeptide (homologous to β152–β177) to compete in binding to acetylcholine receptor with other monoclonal immunoglobulins. The side of the membrane to which these other competing immunoglobulins were bound had been previously determined by labeling them with colloidal gold and observing their disposition with respect to the bilayer in electron micrographs (Sargent et al., 1984). The monoclonal immunoglobulin against α152–α167 competed for binding with some of the immunoglobulins that were thought to bind to the cytoplasmic portion of the protein but did not compete for binding with any of the immunoglobulins against the extracellular, main immunogenic region of the protein. The other evidence for a cytoplasmic disposition of the region α152–α167 was that these same monoclonal immunoglobulins did not bind to native vesicles or to vesicles treated with saponin, but did bind to vesicles treated with lithium diiodosalicylate or at pH 11. Although exposing vesicles to saponin did provide access to the interior of the vesicles for other immunoglobulins binding to the cytoplasmic surface, this treatment was not sufficient to allow the monoclonal immunoglobulin against α152–α167 to bind. One possible explanation for these results is that this immunoglobulin binds only to a subpopulation of denatured protein that was produced by treating the membranes with lithium diiodosalicylate or high pH. A later report (Das & Lindstrom, 1991) seems to confirm this possibility. It indicated that this particular immunoglobulin did not bind well to native acetylcholine receptor, even though it bound very well to membranes denatured with KSCN or to polypeptides denatured with $NaDodSO_4$ on Western blots. Since the epitope for this monoclonal immunoglobulin contains Lys α155, which must be buried in the structure of the native protein (Dwyer, 1990), it seems that the most likely explanation for these results is that the monoclonal immunoglobulin against α152–α167 was bound by denatured protein on the surface of the vesicles.

A second report (Pedersen et al., 1990) had concluded that some portion of the α-polypeptide between α156 and α179 (homologous to β156–β189) was on the cytoplasmic surface of acetylcholine receptor. Electron micrographs were presented that showed two different, labeled monoclonal immunoglobulins specific for this sequence bound to the cytoplasmic surface of right-side-out vesicles. This paper also reported the results of a competition enzyme-linked immunoadsorbent assay (ELISA), in which acetylcholine receptor denatured with $NaDodSO_4$ and adsorbed to the wells competed with various populations of vesicles in the solution for binding to these immunoglobulins. Each of these immunoglobulins was bound very poorly by native vesicles or vesicles treated with saponin in competition with acetylcholine receptor denatured with $NaDodSO_4$, but alkaline extraction of the vesicles increased their ability to compete. The most effective competitor, however, was vesicles dissolved with $NaDodSO_4$. The conclusion that some portion of α156–α179 in the native α-subunit was located on the cytoplasmic face of the membrane relied on the assumption that the labeled immunoglobulins were bound by native protein in the vesicles rather than an unrepresentative subpopulation of denatured acetylcholine receptor. The results of the competition assay suggest that this may not have been a valid assumption.

The monoclonal immunoglobulins used earlier by Criado et al. (1985a) and Tzartos et al. (1986) were recently reassessed by a newly described method to investigate the transmembrane disposition of Ser α157 and Ser α162 (Ananad et al., 1993). A single copy or a double repeat of the epitopes for two of these monoclonal immunoglobulins was inserted genetically into the α-polypeptide at positions α157 or α162, and the fusion products were expressed in oocytes of *Xenopus laevis*. Two of the constructs, with insertions at the position of Ser α157, failed to be expressed in this system, as judged by α-bungarotoxin binding and recordings at voltage clamp. A mutant with an epitope of 8 amino acids inserted at the position of Ser α162 and one with an epitope of 15 amino acids inserted

at the position of Ser α157 did express acetylcholine receptor; however, the former mutant did not bind the immunoglobulins specific for the epitope, and the latter bound the immunoglobulin only weakly and only when acetylcholine receptor was dissolved in Triton X-100. This immunoglobulin did not bind to native oocytes, even in the presence of saponin. These experiments failed to locate this region of the folded polypeptides of native acetylcholine receptor, but they did illustrate the difficulty of assigning the topological disposition of this portion of the polypeptide with a macromolecule, such as an immunoglobulin, as a probe. Most of the results with these immunoglobulins suggest that, as the chemical modifications of Lys α155 and Tyr α151 have demonstrated (Dwyer, 1990), the epitopes are buried and inaccessible in the native protein.

An interesting feature of the most recent map of electron-scattering density obtained by image reconstruction is the conspicuous dearth of shafts of electron scattering density corresponding to membrane-spanning α-helices. At the resolution of this map, only one such shaft in each subunit is visible at the level of the bilayer. Surrounding a rotationally symmetric core formed from these five shafts, one from each subunit, was additional electron-scattering density at the level of the bilayer that does not display any structures corresponding to α-helices. Unwin (1993) has suggested that a continuous barrel of β-sheet, composed of perhaps 5–7 strands from each subunit, could account for this density.

This suggestion is a significant departure from previous proposals concerning the structure of acetylcholine receptor within the membrane. Hydropathic analysis of each of the four polypeptides of acetylcholine receptor originally suggested that each subunit contains four membrane-spanning segments, M1–M4, and it was proposed that each of these segments is an α-helix (Claudio et al., 1983; DeVillers-Thiery et al., 1983; Noda, 1983a). There is evidence that each of these segments do span the bilayer. Results from site-directed mutation (Imoto et al., 1986; Tobimatsu et al., 1987; Imoto et al., 1988; Leonard et al., 1988; Villarroel & Sakmann, 1992), with synthetic peptides in artificial membranes (Kersh et al., 1989; Oblatt-Montal et al., 1993a,b), and from chemical modification (Lauffer & Hucho, 1982; Oberthur et al., 1986; Hucho et al., 1986; Giraudat et al., 1989; Revah et al., 1990; Akabas et al., 1992; White & Cohen, 1992) have provided evidence that the hydrophobic segment M2 lines the channel for cations. Because Lys α380 is on the cytoplasmic surface of acetylcholine receptor and Lys γ486 is on the extracytoplasmic surface, some portion of the common folded polypeptide between these positions, probably M4, must span the membrane an odd number of times (Dwyer, 1991). Furthermore, hydrophobic probes can modify segments M1, M3, and M4 in the various subunits of acetylcholine receptor (Blanton & Cohen, 1992). Chavez & Hall (1992) made fusion proteins of truncated portions of acetylcholine receptor with a segment of prolactin. In these studies, the carboxy-terminal fragment of prolactin was fused downstream of each of the putative membrane-spanning sequences, M1, M2, M3, and M4. Prolactin fused to the carboxy-terminal side of M2 and M4 ended up on the extracytoplasmic surface of the membrane, and prolactin fused to the carboxy-terminal side of M1 and M3 ended up on the cytoplasmic surface. The obvious problem in interpreting results from these experiments is that the shorter fusion proteins studied contained only one, two, or three of the potential membrane-spanning sequences, respectively, fused to the amino-terminal side of a carboxy-terminal prolactin segment 142 amino acids in length. Because these three mutants contain, by design, only an incomplete copy of an acetylcholine receptor subunit, there is no reason to expect that these incomplete proteins would insert in the membrane with the same topology as the full-length protein. Nevertheless, until the most recent map of electron scattering density became available, it had been assumed that these hydrophobic regions of the polypeptides would span the membrane as α-helices. The possibility that some of the membrane-spanning portions of the protein might be in an extended β-conformation and the possibility that the total number of membrane-spanning segments could exceed four stresses the importance of obtaining further topological assignments.

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