Evidence for the Extramembranous Location of the Putative Amphipathic Helix of Acetylcholine Receptor†

Brian P. Dwyer
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
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ABSTRACT: Evidence has been obtained demonstrating that the peptides GVKYIAE and AIKYIAE found in the potential amphipathic helices of the α and β subunits, respectively, of acetylcholine receptor are not buried in the membrane. The peptide KYIAE was synthesized, and polyclonal antibodies were prepared against a conjugate of bovine serum albumin and synthetic peptide. An immunoadsorbent capable of binding and subsequently releasing peptides ending with the sequence -YIAE was produced by attaching these specific antibodies to agarose. Native acetylcholine receptor was labeled with pyridoxal phosphate and $Na[^3H]BH_4$. The labeled protein was stripped of phospholipid and digested with the protease from *Staphylococcus aureus* strain V8. The digest was submitted to immunoadsorption to isolate the labeled indigenous peptides. As a control, α and β polypeptides prepared by gel filtration of a solution of acetylcholine receptor in detergent were stripped of detergent and labeled with pyridoxal phosphate and $Na[^3H]BH_4$ in the presence of 8 M urea. The labeled α and β polypeptides were digested and submitted to immunoadsorption. The specific radioactivities of the indigenous peptides from the α and β subunits labeled under native and denaturing conditions were nearly equal. In similar experiments using isethionyl (2′,4′-dinitrophenyl)-3-aminopropionimidate as the labeling agent, the indigenous peptides from native and denatured receptor were also labeled to the same extent. Since these peptides are labeled to the same extent whether or not the protein is denatured, they cannot be buried in the membrane.

Nicotinic acetylcholine receptor, found in the postsynaptic membranes of the electric organs of *Torpedo californica*, is the most widely studied membrane-spanning protein capable of forming a channel for inorganic ions. Upon binding of acetylcholine, the channel opens, allowing net currents of Na^+ and K^+ to flow across the plasma membrane in the direction of their respective electrochemical gradients. The receptor is constructed of four unique polypeptides designated α, β, γ, and δ according to electrophoretic mobility and has the stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978). The structure obtained from image construction is that of a pentamer with a pseudo-5-fold rotational axis of symmetry (Brisson & Unwin, 1985). It has been assumed that the ion channel lies along this axis.

The sequences of the four polypeptides from which acetylcholine receptor is composed have been deduced from their respective cDNAs, and they are homologous (Noda et al., 1983b). This homology demonstrates that they have evolved from a common ancestor and have the same topology in their native conformation. Plots of the distribution of hydropathy over these sequences reveal four hydrophobic segments in each of them, which are long enough and hydrophobic enough to be candidates for spanning the membrane. In addition, a fifth region of possible functional significance has been identified in the sequences of all four polypeptides. In the carboxy terminal third of each polypeptide, between the hydrophobic sequences M3 and M4 (Noda et al., 1983b), is a sequence of amino acids that displays the pattern of hydropathy expected of an amphipathic helix (Finer-Moore & Stroud, 1984). Two very similar models (Guy, 1984; Finer-Moore & Stroud, 1984) have been proposed in which five of these segments whose sequences are those expected of amphipathic helices, one from each of the subunits, form the ion channel, in a fashion similar to the staves in a barrel. There is no direct experimental evidence, however, to support either of these models. In fact, several results suggest that these models are incorrect. Using site-directed mutagenesis, Mishina et al. (1985) replaced the entire sequence of the potential amphipathic helix in the α subunit of acetylcholine receptor with only two serine residues and observed that the mutant receptor bound carbamylcholine with normal affinity. This observation by itself should disprove the hypothesis that these sequences span the membrane. If two entire membrane-spanning segments had been removed from acetylcholine receptor, it does not seem possible that the protein would have been able to assume its native structure and be capable of binding agonist. The authors of the publication in which these results were presented, however, reached the conclusion that their evidence was consistent with the transmembrane disposition of these sequences in the unaltered protein. When Tobimatsu et al. (1987) replaced the amphipathic helix region of the α subunit with a transmembrane segment from VSV glycoprotein or from interleukin II receptor, no detectable currents were measured for the mutant acetylcholine receptors in the presence of agonist. Nevertheless, deletion of all or part of the amphipathic helix region of the α subunit diminishes but does not abolish channel activity. Recently, Roth et al. (1987) prepared, by limited proteolytic digestion, a form of acetylcholine receptor that also seems to be missing entirely the sequence from the α subunit whose pattern is that of an amphipathic helix. No details of the effect of this degradation on the function of the protein or its structure were, however, presented.

From the results of experiments using monoclonal antibodies coupled to colloidal gold, two groups of investigators independently concluded that the region of the sequences of the polypeptides of acetylcholine receptor between M3 and the potential amphipathic helix is located on the cytoplasmic side of the membrane (La Rochelle et al., 1985; Ratnam et al., 1986b). In addition, the region of the sequence of the β

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polypeptide between the potential amphipathic helix and the hydrophobic sequence M4 has also been assigned to the cytoplasmic side (Ratnam et al., 1986a). If both of these assignments were true, then both ends of the potential amphipathic helix would be located on the cytoplasmic surface of acetylcholine receptor, and this would require that the potential amphipathic helix proper be located entirely on the cytoplasmic surface. The results of Ratnam et al. (1986a) concerning the region between the potential amphipathic helix and the hydrophobic sequence M4 are, however, equivocal. The conclusion that this segment was located on the cytoplasmic surface was based on a result in which an antibody bound a peptide 40 residues in length from the same region of a polyacrylamide gel containing a peptide which was found by antibody that recognizes the carboxy terminus of the β polypeptide; however, no definitive demonstration that those were the same peptide was made. Furthermore, the titers of antigen recognized by the antibodies in these experiments were very low, an observation which suggests that the sites at which colloidal gold was attached were denatured forms of the β polypeptide. In a more recent experiment (Roth et al., 1987), an antibody raised against a synthetic peptide, whose sequence was from the middle of the potential amphipathic helix of the α polypeptide, was shown to recognize as antigen with a high titer acetylcholine receptor dissolved in 0.1% Emulphogene. If the structure of the receptor in this region of its sequence retained its native conformation after the protein had been dissolved in the solution of detergent, these results would suggest that the potential amphipathic helix was fully exposed on the surface of the native protein.

Some attention has been focused on the hydrophobic segment M2 as a possible channel-forming sequence since it contains a serine residue that becomes labeled with two different noncompetitive antagonists, chlorpromazine and (triphenylmethyl)phosphonium, which are believed to be channel blockers (Giraudat et al., 1986; Oberthür et al., 1986). The M2 region and the region between M2 and M3 have been implicated in modulation of the ion transport rate in the acetylcholine receptor channel (Imoto et al., 1986; Tobimatsu et al., 1986).

Rather than using them as probes of the structure of the native protein, antibodies directed against short peptides from the sequence of a particular polypeptide can be used to isolate rapidly peptides with those sequences from a proteolytic digest of the polypeptide. This procedure has been combined with the labeling of sealed vesicles with impermeant reagents to demonstrate that Lys_{501} of the α polypeptide of sodium and potassium ion activated adenosine triphosphatase is located on the cytoplasmic surface of the enzyme (Kyte et al., 1988). In the experiments described here, antibodies to a short peptide from the middle of the potential amphipathic helices in the sequences of the α and β polypeptides of acetylcholine receptor were used to isolate this peptide rapidly from a digest produced with the proteolytic enzyme from *Staphylococcus aureus* strain V8. It has been demonstrated that the lysyl residues at the centers of the potential amphipathic helices of the α and β subunits could be readily labeled in native acetylcholine receptor with anionic electrophiles. This result indicates that the regions in the sequences of the α and β polypeptides, whose patterns of hydropathy are those of amphipathic helices, are fully exposed on the surface of the native protein when it is embedded in the membrane and that they do not span the membrane.

Experimental Procedures

Materials. Torpedo californica was purchased from Pacific Biomarine, Torrance, CA. Electroplax from freshly sacrificed specimens was immediately frozen in $N_2(l)$ and stored at −70 °C. Amino acid derivatives, protected with *tert*-butoxycarbonyl (BOC)[1] groups at their α-amino nitrogens, were purchased from Bachem and pyridoxal phosphate, leucine aminopeptidase, and Sepharose 4B from Sigma. Ammonium sulfate, enzyme grade, was purchased from Schwarz Mann, protease from *S. aureus* strain V8 from Miles Scientific, bovine serum albumin from Miles Diagnostics, Freund's adjuvant from Difco, carboxypeptidase Y from Worthington, and Ecolite from Westchem. Biogel A-1.5m, Affigel-10, Aminex A-5, and chloromethylstyrene (Biobeads S-X1) were from Bio-Rad. Cyanogen bromide was from MCB, ninhydrin from Pierce, and glutaraldehyde (20% solution in H_2O) from ICN. These compounds were used directly. Urea was purchased from Mallinckrodt and recrystallized from 50% ethanol; sodium dodecyl sulfate ($NaDodSO_4$) was purchased from Calbiochem and recrystallized from 95% ethanol; pyridine and dimethylformamide were redistilled after refluxing with phthalic anhydride; trifluoroacetic acid was redistilled after the addition of a small amount of H_2O; $Na[^3H]BH_4$ was obtained from New England Nuclear Corp. as a dry solid. Sodium hydroxide (10 mM) was added, and the solution was stored at −70 °C. Isethionyl (2′,4′-dinitrophenyl)-3-aminopropionimidate (INDAP) was synthesized exactly as described by Denny and Roberts (1982).

Analytical Methods. High-performance liquid chromatography was performed on a system composed of two Waters M6000A pumps, a Waters 680 automatic gradient controller, a Waters U6K injector, a Waters 440 absorbance detector fitted with an extended wavelength module operating at 229 nm, and a Waters μBondapak C-18 column (0.46 × 25 cm). Amino acid analysis was performed on a Beckman 118 amino acid analyzer. Samples were spiked with norleucine and then hydrolyzed in 6 N HCl for 24 h at 110 °C. Radioactivity was counted with a Beckman LS-233 liquid scintillation counter. The cocktail used for liquid scintillation counting was Ecolite. Protein was determined by the method of Lowry et al. (1951) or by amino acid analysis. Electrophoresis on 8% polyacrylamide gels in 0.1% $NaDodSO_4$ was performed as described by Weber and Osborn (1969).

Preparation of Membranes Containing Acetylcholine Receptor. Fragments of membrane, rich in acetylcholine receptor, were prepared essentially as described by Elliot et al. (1980) with the following modifications. Slightly thawed tissue (100–120 g) was diced, and an equal volume of 0.4 M NaCl, 5 mM EDTA, 5 mM iodoacetamide, 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 blender 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 Ti 50.2 rotor (Beckman Corp.) at 16000 rpm for 60 min. Subsequent centrifugations were performed in this Ti 50.2 rotor. The 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 the final concentrations of 30% sucrose, 0.4 M NaCl, 1 mM EDTA, 0.2% NaN_3, and 10 mM sodium phosphate (pH 7.4). The suspension was

[1] Abbreviations: IDNAP, isethionyl *N*-(2′,4′-dinitrophenyl)-3-aminopropionimidate; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; BOC, *tert*-butoxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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, 0.2% NaN_3, and 20 mM sodium phosphate (pH 7.4). The gradient was overlain with 1.5 mL of this buffer with no sucrose and 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 were stored at −70 °C overnight.

On the second day, the suspensions were thawed, diluted, 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 resultant mixture was carefully adjusted to pH 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 resultant mixtures were carefully adjusted to pH 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. The yield was approximately 10 mg of purified acetylcholine receptor (110 g of electroplax), as determined by total amino acid analysis of the final product.

Synthesis of Peptide. The peptide KYIAE was synthesized by solid-phase methods (Merrifield et al., 1982) on [[[4-(oxymethyl)phenyl]acetamido]methyl]copoly(styrene–divinylbenzene) resin as the solid support. The first amino acid, glutamic acid, was attached to (aminomethyl)poly(styrene) as N^{α}-BOC-O^{γ}-benzyl-O^{α}-[4-(carboxymethyl)benzyl]-L-glutamic acid (Tam et al., 1979) by activation with dicyclohexylcarbodiimide in dimethylformamide (Merrifield, 1982). The following protected amino acids were used in the following order: N^{α}-BOC-L-alanine, N^{α}-BOC-L-isoleucine, N^{α}-BOC-*O*-benzyl-L-tyrosine, and N^{α}-BOC-N^{ϵ}-(benzoxycarbonyl)-L-lysine. The peptide was cleaved from the resin with liquid anhydrous HF, washed in anhydrous diethyl ether and ethyl acetate, extracted in 5% acetic acid in water, and lyophilized.

The crude peptide was dissolved in 60% acetic acid in water and applied to a column (0.9 × 10 cm) of Aminex A-5 cation-exchange resin. The column was eluted with a linear gradient beginning with 0.05 M pyridinium acetate (pH 2.5) and ending with 2.0 M pyridinium acetate (pH 5.0) (Degen & Kyte, 1978). The total volume of the gradient was 300 mL. Distribution of peptide was determined with alkaline ninhydrin (Hirs et al., 1956).

Preparation of Radioactive Peptide Labeled with IDNAP. Radioactive synthetic peptide was produced by the method of Rice and Means (1971). Formaldehyde, prepared from paraformaldehyde, was added to a solution of the synthetic peptide [5 mg in 400 μL of 0.2 M sodium borate (pH 9.0)] to a final concentration of 0.08 M. The resulting formimine was reduced by the addition of 60 μL of 0.13 M $Na[^3H]BH_4$ followed by four additions (60 μL each) of 0.13 M $NaBH_4$ at 30-min intervals. The solution was dried under vacuum, and the radioactive peptide was purified by high-pressure liquid chromatography (HPLC). The specific radioactivity was determined to be 7500 cpm $nmol^{-1}$.

Synthetic peptide was also labeled with IDNAP. To a solution of peptide [23 mg in 2.0 mL of 50 mM sodium borate (pH 8.0)] was added 60 mg of IDNAP. The pH was adjusted to 8.0, and the reaction was allowed to proceed for 2 h at room temperature. The labeled peptide was then purified by ion-exchange chromatography.

Preparation of Specific Antibodies. A conjugate of the synthetic peptide KYIAE and bovine serum albumin was prepared (Walter et al., 1980). Peptide (7.4 mg) was dissolved in a solution of bovine serum albumin (26 mg) in 2.0 mL of 0.1 M sodium phosphate (pH 7.5). Glutaraldehyde (1 mL, 20 mM) was added with stirring and allowed to react for 30 min at room temperature. The solution was then dialyzed against phosphate-buffered saline (PBS), 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate (pH 7.4).

Polyclonal antibodies were raised in white New Zealand rabbits. An emulsion (1:1) of haptenic conjugate (1 mg/mL in PBS) and complete Freund's adjuvant was injected into the lymph nodes of the hind legs and foot pads. Eight weeks later, the booster doses were injected subcutaneously into the back.

For the purification of polyclonal antibodies specific for the carboxy terminal sequence of the synthetic peptide KYIAE, an affinity column was constructed. Affigel-10 (1.2 mL) was shaken with the peptide (10 mg) dissolved in 0.1 M sodium *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonate, pH 7.5 at 4 °C for 24 h. The amount of accessible peptide covalently attached to the resin was determined by digestion with carboxypeptidase Y. Washed peptide resin was diluted to 10 mL with 0.05 M pyridinium acetate, pH 5.5. A portion of this slurry (400 μL) was treated with carboxypeptidase Y (25 μg) at 37 °C for 5 h. The amount of glutamic acid released was determined by amino acid analysis.

Fresh antiserum was applied to a column (4.5 × 0.5 cm) of the peptide resin. The column was then washed with PBS, and adsorbed antibodies were eluted with 0.2 M glycinium chloride, pH 2.5. The eluate was dialyzed against PBS, and the purified antibodies were precipitated with an equal volume of saturated $(NH_4)_2SO_4$. The purified antibodies were then covalently attached to Sepharose 4B, exactly as described by March et al. (1974), to produce the immunoadsorbent.

Labeling of Membranes Rich in Acetylcholine Receptor with Pyridoxal Phosphate and $Na[^3H]BH_4$. Membranes rich in acetylcholine receptor were labeled by the method of Ohkawa and Webster (1981) as modified by Kyte et al. (1987) in 6 mM pyridoxal phosphate and 3 mM $Na[^3H]BH_4$. The membranes were allowed to react with pyridoxal phosphate for 15 min and then with the $Na[^3H]BH_4$ for 20 min. The labeled protein was then stripped of phospholipid by gel filtration in a solution of $NaDodSO_4$. Pelleted, membrane-bound protein was dissolved by adding $NaDodSO_4$ to a weight ratio of 40 g (g of protein)$^{-1}$. The sample was submitted to gel filtration on a column (2.5 × 50 cm) of Biogel A-1.5m equilibrated with 0.1% $NaDodSO_4$ in 0.04 M Tris–sulfate at pH 8.0. The fractions containing the α and β polypeptides of acetylcholine receptor, as determined by electrophoresis on polyacrylamide gels, were pooled and lyophilized.

Labeling of Denatured Acetylcholine Receptor with Pyridoxal Phosphate and $Na[^3H]BH_4$. Phospholipid-free α and β polypeptides, obtained by gel filtration in 0.1% $NaDodSO_4$ on Biogel A-1.5m, were dialyzed against 0.1% $NaDodSO_4$ in 0.06 M sodium borate at pH 8.0. The method of Weber and Kuter (1971) as modified by Sharkey (1983) was used to remove the $DodSO_4$. The stripped protein was then labeled with pyridoxal phosphate and $Na[^3H]BH_4$ in the presence of 8 M urea.

Labeling of Membranes Rich in Acetylcholine Receptor and Denatured α and β Polypeptides with IDNAP. To a suspension of membranes in 0.25 M sucrose and 0.06 M sodium borate (pH 8.0) was added IDNAP to 30 mM. The pH of the suspension was adjusted to 8.0 with NaOH, and the reaction was allowed to proceed for 5 h at room temperature. α and β polypeptides, free of phospholipid and stripped of $DodSO_4$, were labeled with IDNAP in 8 M urea under the same conditions.

Enzymatic Digestions. All samples of acetylcholine receptor labeled in its native conformation in the membrane were dissolved in a solution of $NaDodSO_4$. The α and β polypeptides were separated from other proteins and phospholipid by gel filtration, and the $DodSO_4$ was then removed while the polypeptides were dissolved in 8 M urea. All labeled polypeptides were dialyzed from urea in 0.1 M sodium phosphate (pH 7.8) and digested with the proteolytic enzyme from *S. aureus* strain V8 (Drapeau, 1972) at a concentration of 50–125 μg mL^{-1} for 18 h at 37 °C.

Immunoadsorption. Digests of labeled protein were applied to the immunoadsorbent, which was then washed with PBS. The bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. Fractions that contained eluted radioactive peptides were submitted directly either to HPLC or to liquid scintillation counting. As a control, synthetic peptide was added to a sample of digest in an excess (20-fold) over sites on the immunoadsorbent. It was assumed that under these conditions none of the indigenous peptides would be bound by the immunoadsorbent.

Results

Immunoadsorbent. The sequences from the α and β polypeptides of acetylcholine receptor whose patterns of hydropathy are those of an amphipathic helix (Finer-Moore & Stroud, 1984) comprise residues 371–388 and 400–417, respectively. The two sequences are -DVKSAIEGVKYIAEHMKS- and -DLKEAVEAIKYIAEQLES- (Noda et al., 1982, 1983a). Lys_{380} and Lys_{409}, respectively, which are found in the centers of these regions, are the targets of these experiments. For the rapid and specific purification of the indigenous peptides GVKYIAE and AIKYIAE, produced by the proteolytic enzyme from *S. aureus* strain V8 from the α and β polypeptides, respectively, an immunoadsorbent was constructed.

The peptide KYIAE was synthesized by solid-phase methods (Merrifield et al., 1982). The synthetic peptide, after purification by cation-exchange chromatography (Degen & Kyte, 1978), was demonstrated to be homogeneous by HPLC. The amino acid composition of the synthetic peptide, obtained by hydrolysis in acid, was $K_{0.98}Y_{1.09}I_{0.97}A_{0.98}E_{0.99}$. Leucine aminopeptidase and carboxypeptidase Y digestions gave the compositions $K_{0.95}Y_{1.02}I_{0.99}A_{1.02}E_{1.01}$ and $E_{1.00}A_{0.75}$, respectively.

The synthetic peptide was conjugated to bovine serum albumin with glutaraldehyde (Walter et al., 1980). It was determined by amino acid analysis that 17 nmol of peptide had been covalently attached to every nanomole of bovine serum albumin. This conjugate was used to immunize rabbits. In this way polyconal antibodies against the carboxy terminal sequence -YIAE were produced. Antibodies specific for this sequence were purified by affinity chromatography on a column constructed from the purified synthetic peptide KYIAE coupled to the solid phase through its lysyl residue and its amino terminus. The concentration of accessible peptide molecules covalently attached to the solid phase of this peptide resin was determined by carboxypeptidase Y digestion to be 0.38 μmol (mL of gel)$^{-1}$. Tritiated synthetic peptide was applied to the immunoadsorbent both in the absence and in the presence of an excess of unlabeled peptide. Whereas 98% of the labeled peptide was bound by the immunoadsorbent and 74% was subsequently released in the absence of synthetic peptide, most of the labeled peptide (75% of the radioactivity recovered) was not bound by the immunoadsorbent in the presence of the unlabeled peptide at a 20-fold excess over sites on the immunoadsorbent. These results indicate that the immunoadsorbent is specific for peptides containing, as their carboxy terminus, -YIAE. Similar results were obtained with synthetic peptide that had first been amidinated with isethionyl *N*-(2′,5′-dinitrophenyl)-3-aminopropionimidate (IDNAP).

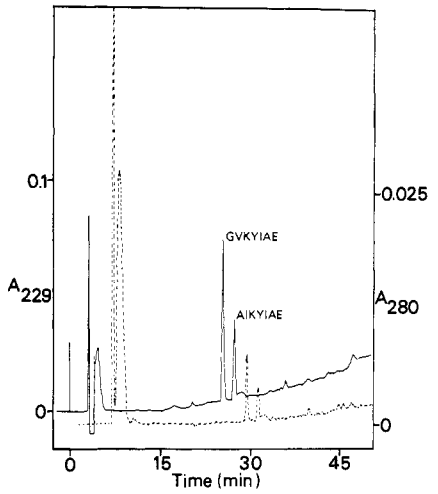

FIGURE 1: High-pressure liquid chromatography of the acid eluate from the immunoadsorbent. α and β polypeptides from unlabeled acetylcholine receptor free of phospholipid and $DodSO_4$ were digested with proteolytic enzyme from *S. aureus* strain V8 and submitted to immunoadsorption. The immunoadsorbent was washed with PBS and eluted with 0.1 M sodium phosphate, pH 2.5. The eluate (1.0 mL) was applied to a μBondpak C-18 column equilibrated in 0.1% trifluoroacetic acid in H_2O (solvent A). The column was developed with a linear gradient of 0.1% trifluoroacetic acid in neat acetonitrile (solvent B) at a rate of 1% increase of solvent B min^{-1}. The peptides were collected, and their identifies were confirmed by amino acid analysis. The solid line represents A_{229} and the dashed line represents A_{280}.

The immunoadsorbent was used to purify the indigenous peptides GVKYIAE and AIKYIAE from the α and β polypeptides of acetylcholine receptor. Acetylcholine receptor, dissolved in a solution of $NaDodSO_4$, was submitted to gel filtration. Fractions containing the α and β polypeptides separated from the phospholipid were pooled, and the $DodSO_4$ was removed. The polypeptides were then digested with the proteolytic enzyme from *S. aureus* strain V8, and the digest was applied to the immunoadsorbent. When the peptides that had been bound and released from the immunoadsorbent were submitted to HPLC, only two peaks of absorbance were observed (Figure 1). Amino acid analysis identified these two peptides as GVKYIAE and AIKYIAE, which are the two peptides that should have been released upon digestion of the appropriate regions in the sequences of the α and β polypeptides. The compositions of the two peptides were $G_{1.6}V_{1.1}K_{1.5}Y_{0.5}I_{0.9}A_{1.1}E_{1.0}$ and $A_{1.9}I_{1.6}K_{1.1}Y_{1.0}E_{1.1}$. It is of interest to note that the ratio in which the two peptides were obtained (Figure 1) is consistent with the subunit stoichiometry $\alpha_2\beta$.

Labeling with Pyridoxal Phosphate. One of the reagents chosen to label the lysines found in the potential amphipathic helices of the α and β subunits is pyridoxal phosphate. Lysine reacts with pyridoxal phosphate to form an imine. Subsequent reduction of the imine with $Na[^3H]BH_4$ results in formation of the stable secondary pyridoxamine and incorporation of nonexchangeable tritium on the former aldehyde carbon of

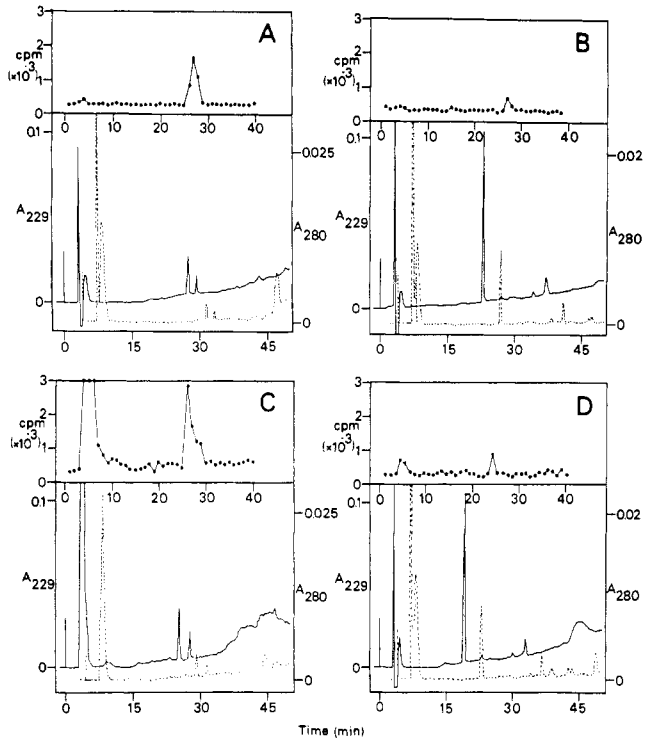

FIGURE 2: High-pressure liquid chromatography of eluates from the immunoadsorption of proteolytic digests of the α and β polypeptides of acetylcholine receptor labeled with pyridoxal phosphate and Na[^{3}H]BH_4. Digests either from acetylcholine receptor labeled in its native form embedded in the membrane (A and B) or from α and β polypeptides labeled as denatured polymers in 8 M urea (C and D) were submitted to immunoadsorption on a column (4.5 × 0.5 cm) of immunoadsorbent with 14-nmol capacity. Equivalent samples were prepared without (A and C) or with (B and D) the addition of a large excess of unlabeled synthetic peptide KYIAE (280 nmol). Each of the acidic eluates from the immunoadsorbent were then submitted to HPLC as in Figure 2. The effluent from the chromatographic column was collected in fractions, and the radioactivity in the fractions was determined by liquid scintillation. The solid line represents A_{229} and the dashed line represents A_{280}.

the pyridoxal. Although the combination of pyridoxal phosphate and Na[^{3}H]BH_4 was chosen as a nonspecific label, some selectivity in this modification has been reported (Greenwall et al., 1973; Rippa et al., 1967) when pyridoxal phosphate was used at much lower concentrations than those used here (6 mM).

When a digest of the α and β polypeptides from acetylcholine receptor, labeled in its native conformation in intact membranes with pyridoxal phosphate and Na[^{3}H]BH_4, was applied to the immunoadsorbent, radioactivity was bound by the column and could be eluted with acid. When the eluate was submitted to HPLC (Figure 2A), the radioactivity was found to cochromatograph with two peptides that had retention times equal to those of the indigenous peptides from unlabeled acetylcholine receptor (Figure 1). When the same digest was applied to the immunoadsorbent in the presence of an excess of the nonradioactive synthetic peptide KYIAE, eluted with acid, and submitted to HPLC (Figure 2B), no labeled peptides were found, and no unlabeled peptides recognizable with retention times of the indigenous peptides were seen. The only absorbance seen was that the of synthetic peptide. On the basis of this control experiment, the radioactive peptides bound by the immunoadsorbent and released by acid had been bound specifically.

A pool containing the denatured α and β polypeptides of acetylcholine receptor was labeled with pyridoxal phosphate and Na[^{3}H]BH_4 in the presence of 8 M urea. When a portion of the digest of these labeled α and β polypeptides was applied to the immunoabsorbent in the absence of synthetic peptide, radioactivity again was eluted with acid. The eluate was submitted to HPLC (Figure 2C), and as with peptides from acetylcholine receptor labeled in native intact membranes, radioactivity was found to cochromatograph with the indigenous peptides. The results of the control (Figure 2D), in which excess synthetic peptide had been added before immunoadsorption, indicated that these radioactive peptides had been specifically bound by the immunoadsorbent.

The specific radioactivities of acetylcholine receptor labeled under native and denaturing conditions were determined to be 0.008 nmol of tritium (nmol of lysine)$^{-1}$ and 0.012 nmol of tritium (nmol of lysine)$^{-1}$, respectively. The 1.5-fold difference between them may be due to steric effects on the reaction of pyridoxal phosphate with the protein. Under native conditions some lysyl residues may not be accessible to pyridoxal phosphate and Na[^{3}H]BH_4. From these values of specific radioactivity it is clear that in either the case of the native protein or the case of the denatured protein only a small percentage of each lysyl residue was being labeled with pyridoxal, the vast majority of each of these remaining unlabeled. With this in mind, it can be concluded that the peptides registered by their absorbance in Figure 2, parts A and C, were the unmodified indigenous peptides GVKYIAE and AIKYIAE. In this event, these peptides have acted as internal standards that have monitored the yields of the digestion, the purification by immunoadsorption, and the purification by HPLC. The counts per minute recovered upon HPLC divided by the areas in A min of the peptide peaks on the chromatograms are directly proportional to the nmol of tritium (nmol of original Lys_{380})$^{-1}$ or nmol of tritium (nmol of original Lys_{409})$^{-1}$ incorporated at these locations of the sequence in the original undigested, labeled protein or polypeptides. These values for acetylcholine receptor labeled in its native conformation in the membrane and for the α and β polypeptides labeled in 8 M urea are 2.0×10^4 cpm (A min)$^{-1}$ and 2.9×10^4 cpm (A min)$^{-1}$, respectively. The similarity of these two values demonstrates that these lysyl residues are as accessible to pyridoxal phosphate in the native receptor as they are in the unfolded peptides.

Labeling with IDNAP. The other reagent chosen for this study was IDNAP, whose reactive form is a zwitterionic imidate (Denny & Roberts, 1982). This imidate reacts with lysine to form a stable amidine. The dinitrophenyl ring that remains attached to the amidine permits detection at 360 nm.

The reaction of acetylcholine receptor (7 mg mL^{-1}) with IDNAP (30 mM) was studied. At various times samples were removed, the reaction was quenched with lysine, and the samples were submitted to polyacrylamide gel electrophoresis in 0.1% $NaDodSO_4$. The distribution of A_{360} from covalently attached dinitrophenyl across one of these gels prior to staining is presented in Figure 3. The distribution of A_{510} over the same gel after it has been stained for protein is superimposed. It can be seen that each of the polypeptides of acetylcholine receptor was modified by the reagent and that the incorporation was distributed fairly evenly over the four polypeptides relative to their mass ratios. Each of the gels from the different samples was scanned at 360 nm, stained, and scanned at 510 nm. When the A_{360}/A_{510} ratios were plotted against time, the reaction was determined to be complete after 5 h.

Membranes rich in acetylcholine receptor were labeled with IDNAP (30 mM) for 5 h. The labeled α and β polypeptides, isolated by gel filtration in 0.1% $NaDodSO_4$, were stripped of $NaDodSO_4$ and digested with the proteolytic enzyme of *S.*

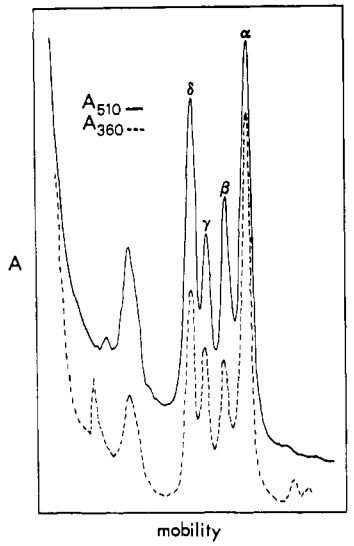

FIGURE 3: Distribution of the absorbance of dinitrophenyl across an 8% polyacrylamide gel on which modified acetylcholine receptor labeled in its native conformation with IDNAP (30 mM, pH 8, room temperature, 300 min) was submitted to electrophoresis (Weber & Osborn, 1969). The gel was scanned at 360 nm to follow the distribution of dinitrophenyl, stained with Coomassie Brilliant Blue, and scanned at 510 nm to follow the distribituion of protein.

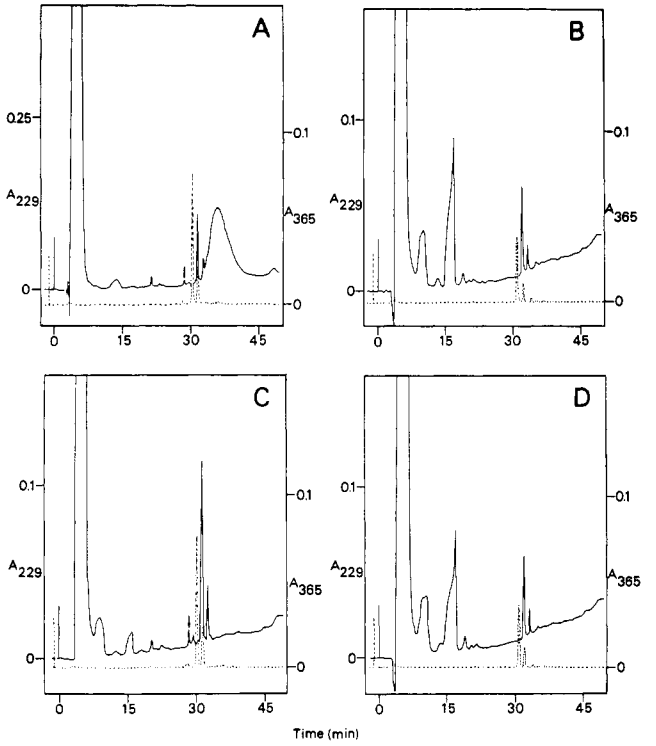

FIGURE 4: High-pressure liquid chromatography of eluates from immunoadsorption of digests of α and β polypeptides of acetylcholine receptor labeled with IDNAP. Digests either from acetylcholine receptor labeled with IDNAP in its native form (A and B) or from α and β polypeptides labeled with IDNAP in 8 M urea (C and D) were submitted to immunoadsorption on a column of immunoadsorbent (4.5 × 0.5 cm) with 14-nmol capacity. Equivalent samples were prepared without (A and C) or with (B and D) the addition of a large excess of unlabeled synthetic peptide (280 nmol). Each of the acidic eluates from the immunoadsorbent was then submitted to HPLC as in Figure 2. The solid line represents A_{229} and the dashed line represents A_{365}.

aureus strain V8. The digest was applied to the immunoadsorbent, and the peptides bound and eluted were submitted to HPLC. Two peptides that adsorbed at 365 nm and had retention times different from the unlabeled indigenous peptides were observed (Figure 4A). Excess synthetic peptide added to the digest prior to immunoadsorption did not produce the same disappearance of the labeled peptides seen in the case with the peptides labeled with pyridoxal phosphate (Figure 4B). The presence of synthetic peptide did reduce, by ~50%, the amount of the labeled peptides bound and indicated specific binding. By amino acid analysis, however, these labeled peptides had the compositions $G_{1.27}V_{1.60}K_{0.76}Y_{0.17}I_{1.29}A_{1.23}E_{1.00}$ and $A_{1.61}I_{1.49}K_{0.42}Y_{0.73}E_{1.00}$, consistent with their identification as the modified indigenous peptides. When α and β polypeptides, labeled with IDNAP in the presence of 8 M urea, were digested and submitted to immunoadsorption followed by HPLC, similar results were obtained (Figure 4, parts C and D).

That the labeling had proceeded almost to completion at Lys_{380} from the α polypeptide and Lys_{410} from the β polypeptide in both native and denatured acetylcholine receptor was demonstrated by the fact that the amount of indigenous, unlabeled peptide (arrows in Figure 4, parts A and C) was only a small fraction, 0.13 and 0.07, respectively, of the corresponding labeled peptide present. These results demonstrate that both Lys_{380} from the α subunit and Lys_{409} from the β subunit are accessible enough in the native structure of acetylcholine receptor to be quantitatively modified by IDNAP.

Discussion

When they are present in the folded subunits of the native structure or in the polypeptides dissolved as random coils, Lys_{380} of the α polypeptide and Lys_{409} of the β polypeptide are labeled with pyridoxal phosphate and $Na[^3H]BH_4$ to nearly the same extent. The differences in yields of digestion, gel filtration, immunoadsorption, and HPLC for the samples from native and denatured preparations were corrected for by relating the counts per minute recovered to the yield of unlabeled peptide. It can be concluded that Lys_{380} and Lys_{409} have the same accessibility to pyridoxal phosphate in the aqueous phase under native or denatured conditions. A similar conclusion could be drawn from their ability to react in high yields, 87% and 93%, with IDNAP under either native or denatured conditions, respectively. If the potential amphipathic helices seen in the sequences were actually amphipathic helices that spanned the membrane in native acetylcholine receptor, it seems unlikely that lysine residues found at the centers of these sequences, which would end up at the center of the membrane, would be able to react with any polar electrophile. While these results could be explained by a model in which an equilibrium exists between a state where the amphipathic helices span the membrane and a state where the amphipathic helices are extramembranous (Guy & Hucho, 1987), such a conformational equilibrium is without precedent. The conclusion that follows from their ability to react is that the amphipathic helix does not span the membrane.

It could be argued that were the lysine residues within the amphipathic helices that did form the ion channel, they could be labeled by reagents that have entered the ion channel. The reagents used in this study, pyridoxal phosphate and IDNAP, were chosen because at pH 8.0 both should have been anionic and therefore should have been excluded from a channel specific for cations. The pyridinium nitrogen of pyridoxal has a pK_a of 6.2, and the phosphate group a pK_a of 4.1 and pK_{a2} of 8.7. The pK_a of the imidate of IDNAP was reported to be 5.7 (Denny & Roberts, 1982), and the isethionyl sulfonate is always anionic. As the actual labeling was done in the absence of agonist, there was no chance for the channel to be open during the labeling.

The results obtained from labeling with pyridoxal phosphate and with IDNAP are complementary. The labeling with pyridoxal phosphate was performed for 15 min followed by reduction with Na[^{3}H]BH_4 for 20 min. On the other hand, the labeling with IDNAP was performed at high concentration (30 mM) and for 5 h. Under these very different situations, low yield (1%) was seen with pyridoxal phosphate and Na[^{3}H]BH_4 and high yield (90%) with IDNAP. Yet, at both of these extremes, the lysyl residues in the potential amphipathic helices reacted to the same extent whether the protein was native or denatured. The possibility that the reaction performed in low yield is unrepresentative of the whole population of protein molecules is belied by the results of the stoichiometric reaction, and the possibility that the stoichiometric reaction disrupted the structure of the protein is belied by the results of the partial reaction.

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