Dissection of the 66 000-Dalton Subunit of the Acetylcholine Receptor†

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ABSTRACT: The 66 000-dalton or δ subunit of the acetylcholine receptor from *Torpedo marmorata* was covalently labeled in the presence of carbamoylcholine by 5-azido[^{3}H]trimethisoquin (5-A[^{3}H]T), a photoaffinity derivative of the local anesthetic trimethisoquin. After the attack of purified receptor with increasing concentrations of trypsin, the δ chain successively yielded fragments with apparent molecular weights of 50 000 (distinct from the β subunit and referred to as the 50 000-bis fragment), 49 000, and 47 000. With nondenatured (sodium cholate solubilized or membrane-bound) receptor, the 47 000-dalton fragment was not sensitive to trypsin and contained all of the covalent 5-A[^{3}H]T label. This fragment was still glycosylated and had the same amino acid N terminus, valine, as the intact δ chain. A specific in vitro phosphorylation site of the δ subunit was located between the 49 000- and 50 000-dalton trypsin cleavage fragment and most likely is exposed to the cytoplasmic side of the membrane. A 16 000-dalton fragment of the δ chain was identified, which carries a disulfide bond (or bonds) capable of cross-linking nonreduced receptor 9S monomers into 12S dimers. This fragment did not remain associated with the receptor molecule after trypsin treatment.

Convergent biochemical, immunological, and pharmacological studies concerning the regulation of ion transport by acetylcholine (Heidmann & Changeux, 1978; Fambrough, 1979; Raftery et al., 1979; Lindstrøm, 1979; Karlin, 1980) have led to the proposal that the elementary functional unit involved in this process is, at least for *Torpedo* species, composed of four distinct subunits (apparent molecular weights of 40 000 (α), 50 000 (β), 57 000 (γ), and 66 000 (δ)) with 2:1:1:1 stoichiometry (Reynolds & Karlin, 1978; Lindstrøm et al., 1979a; Raftery et al., 1980). The structure of these individual subunits has been probed with antibodies (Fuchs, 1979; Tzartos & Lindstrøm, 1980) and by N-terminal sequence analysis (Devillers-Thiéry et al., 1979; Hunkapiller et al., 1979; Raftery et al., 1980). Important primary structural homologies exist between the four subunits (Raftery et al., 1980; Tzartos & Lindstøm, 1980) which, nevertheless, exhibit strikingly different reactivities toward a variety of ligands.

The α chain is labeled by affinity reagents specific for the AcChR[1] site [review by Karlin (1980) and Karlin et al. (1979)]. The δ chain can be covalently labeled by 5-A[^{3}H]T (Oswald et al., 1980; Saitoh et al., 1980), a photoaffinity derivative of the noncompetitive blocking agent trimethisoquin (Waksman et al., 1980). This labeling is enhanced by the cholinergic agonist carbamoylcholine and selectively blocked by histrionicotoxin, a toxin thought to interfere directly with ion translocation through the acetylcholine ionophore (Eldefrawi et al., 1980). No definite function for the β and γ chains yet is known, but cross-linking experiments show that these subunits are topographically related to the α chain (Karlin et al., 1979; Witzemann & Raftery, 1980a,b). The α, β, γ, and δ chains traverse the membrane (Wennogle & Changeux, 1980; Strader & Raftery, 1980).

The receptor macromolecule exists as a 500 000-dalton dimer (H form) in solutions of nondenaturing detergents under nonreducing conditions (Reynolds & Karlin, 1978). Since in the dimer the δ chain exists as a disulfide-bonded 132 000-dalton unit (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977, 1979), this chain is very likely responsible for the dimerization. No physiological role of this dimerization is known.

Phosphorylation of the AcChR (Teichberg et al., 1977; Gordon et al., 1977; Saitoh & Changeux, 1980) and other membrane proteins (Greengard, 1978) has been postulated as a possible regulatory mechanism for ion translocation (Greengard, 1978) and/or aggregation and stabilization (Saitoh et al., 1979) of the receptor in the subsynaptic membrane during development (Changeux, 1979). Interestingly the δ polypeptide is one of the AcChR subunits phosphorylated in vitro (Gordon et al., 1977; Saitoh & Changeux, 1980). Because of the possible role of the δ subunit in ion transport, we have undertaken an analysis of the transmembrane orientation and structure of this chain.

Materials and Methods

AcChR-rich membranes from *Torpedo marmorata* were purified as described by Saitoh et al. (1980), in a buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 0.5 μg/mL pepstatin, and 5 units/mL zymophren (buffer I). Membranes isolated in the presence of *N*-ethylmaleimide (NEM) were prepared in the same way, except that homogenization of the electric organ was done in the presence of 5 mM NEM and that 5 mM sodium phosphate (pH 7.2) replaced the Tris-HCl buffer in buffer I. Membranes were treated at pH 11 as described in Neubig et al. (1979).

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[1] Abbreviations used: AcChR, acetylcholine receptor; 5-A[^{3}H]T, 5-azido[^{3}H]trimethisoquin; MPTA, 4-(*N*-maleimido)phenyltrimethylammonium; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; dansyl, 8-dimethylamino-1-naphthalenesulfonate.

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Solubilization of the membranes was performed with 3% sodium cholate.

Solubilized receptor (with trace amounts of α-[^{125}I]bungarotoxin) was purified by sedimentation (5–20% sucrose gradients in 1% sodium cholate) at 4 °C for 13 h at 36 000 rpm in a Beckman SW 56 rotor. Gradients were collected in 4-drop fractions from the bottom of the tube. Buffer I or buffer II [0.4 M NaCl, 3.0 mM EDTA, and 50 mM sodium phosphate (pH 7.5)] was used in the sucrose gradient, as noted in the text.

Labeling of membranes with 5-A[^{3}H]T was performed in the presence of 10^{-4} M carbamoylcholine as previously described (Oswald et al., 1980; Saitoh et al., 1980). Phosphorylation of membranes (1 μM in α-bungarotoxin sites) was performed in 30 mM $MnCl_2$, 0.15 M KF, 2×10^{-7} M ATP supplemented with 0.2 μCi of γ-[^{32}P]ATP, 125 mM Tris-HCl (pH 6.8), and 4 mM ouabain for 30 min at 37 °C, and membranes were washed twice with 50 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and EGTA.

$NaDodSO_4$–polyacrylamide gel electrophoresis was done by a variation of the Laemmli system in one (Sobel et al., 1977) and two (Hamilton et al., 1977) dimensions. For the latter, the first dimension was performed in tube gel without β-mercaptoethanol and the second after equilibration of the tube gel with 5% β-mercaptoethanol for 30 min at 37 °C. Apparent molecular weights were determined relative to standards (Pharmacia). Samples were dissolved in Tris–$NaDodSO_4$ buffer at room temperature and were run without heating. Fluorography using preflashed Kodak X-omat film and counting of individual bands on gels after solubilization (Saitoh & Changeux, 1980) were done by using standard procedures.

Determination of α-bungarotoxin sites was performed by using the DEAE filter paper assay (Klett et al., 1973) and N-terminal analysis of subunits purified by $NaDodSO_4$–polyacrylamide gel electrophoresis (Devillers-Thiéry et al., 1979) by the dansyl procedure (Gray, 1967). Membranes were washed, when noted, by pelleting at top speed in a Beckman airfuge for 1 min. Trypsin (3 times crystallized from Worthington Biochemical Corp. TRTPCK) solutions were prepared immediately before use in either 0.05 mM sodium phosphate (pH 7.5) or 0.1 M Tris-HCl (pH 7.5) buffer (see figure legends). Trypsin cleavages were carried out at room temperature at a 5 μM concentration of α-bungarotoxin sites.

N-Ethylmaleimide was from Schwartz Bioresearch, diisopropyl fluorophosphate (DFP) and *Escherichia coli* alkaline phosphatase were from Sigma Chemical Co., ^{125}I-labeled α-bungarotoxin was from New England Nuclear, and γ-[^{32}P]ATP was from Amersham. All other chemicals were of the highest purity available.

Results

(A) 5-A[^{3}H]T Labeling of δ Chain of AcChR Receptor. As described previously (Oswald et al., 1980; Saitoh et al., 1980), 5-A[^{3}H]T selectively labels the δ chain of the membrane-bound AcChR in the presence of cholinergic agonists, and this labeling is blocked by histrionicotoxin. We took advantage of this specificity to follow the fate of the δ chain during the attack of the nondenatured (sodium cholate solubilized) receptor by trypsin. When analysis by fluorography was performed after $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1), trypsin progressively cleaved the δ chain into fragments of molecular weights 50 000, 49 000, and 47 000.

The same series of fragments was found with AcChR-rich membranes after treatment at pH 11 or sonication. We will refer to the 50 000-dalton fragment originating from the δ chain as the 50 000-bis fragment to avoid confusion with the original 50 000-dalton (β) subunit of the native receptor. The 47 000-dalton fragment kept all (typically 94%) of the 5-A-[^{3}H]T label originally present on the δ chain.

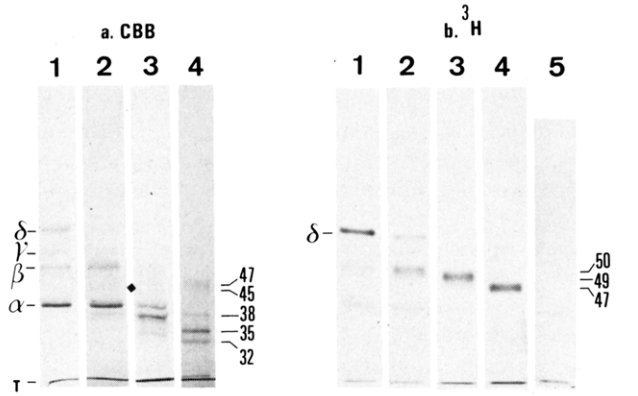

FIGURE 1: Effect of trypsin on 5-A[^{3}H]T-labeled receptor. AcChR from 5-A[^{3}H]T-labeled membranes was purified in buffer II containing 1% sodium cholate (Materials and Methods), dialyzed against the same buffer, and treated with increasing concentrations of trypsin for 20 min: (1) 0, (2) 3.6×10^{-4}, (3) 1.4×10^{-3}, and (4) 2.3×10^{-2} mg/mL. The reaction was stopped by adding DFP to 5 mM. 15 min later, samples were dissolved in $NaDodSO_4$ sample buffer containing β-mercaptoethanol (5%), and $NaDodSO_4$–polyacrylamide gel electrophoresis was performed (Materials and Methods): (a) a Coomassie brilliant blue (CBB) stained gel and (b) a fluorogram (^{3}H) of a parallel gel. In (b-5) the 5-A[^{3}H]T labeling was done in the presence of 10^{-5} histrionicotoxin. Molecular weights are expressed $\times 10^{-3}$. The diamond indicates a weakly stained fragment of apparent molecular weight 45 000. (T) stands for tracking dye front. Counts seen at the bottom of gel b-4 represent a minor population of counts derived nonspecifically from the top of the gel, from the gel itself, and, to a lesser extent, from the δ and α chains.

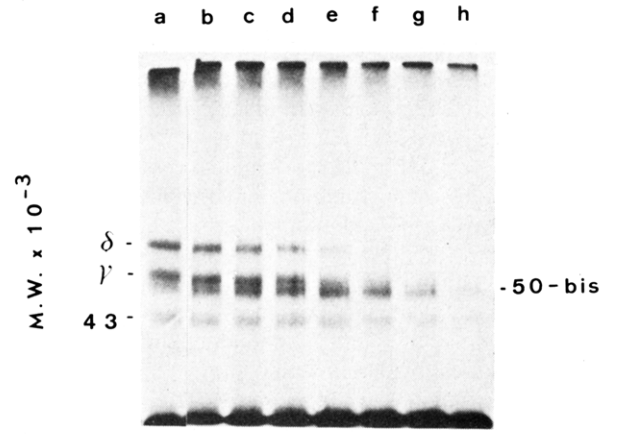

FIGURE 2: Effect of trypsin on receptor-phosphorylated subunits. AcChR-rich membranes were phosphorylated and then treated at pH 11 (Materials and Methods), solubilized in 3% sodium cholate, diluted to 1% sodium cholate and 0.1 M Tris-HCl (pH 7.5), and incubated with trypsin for 15 min at the concentrations indicated. Trypsin treatment was terminated and $NaDodSO_4$–polyacrylamide gel electrophoresis performed as described in Figure 1. An autoradiogram of the dried gel is presented. Trypsin concentrations were (a) 0, (b) 5×10^{-5}, (c) 1×10^{-4}, (d) 2×10^{-4}, (e) 4×10^{-4}, (f) 8×10^{-4}, (g) 1.6×10^{-3}, and (h) 3.2×10^{-3} mg/mL.

(B) In Vitro Phosphorylation of Receptor-Rich Membrane Fragments. In vitro phosphorylation of the AcChR has been investigated in detail with receptor-rich membranes purified under conditions which minimize proteolytic degradation (Saitoh et al., 1980). With these membranes, radioactive phosphate was incorporated into chains of apparent molecular weights 66 000 (δ), 57 000 (γ), and 43 000 (Figure 2a). The α chain was never labeled by this method.

For localization of the ^{32}P label on the δ chain, membranes were phosphorylated, solubilized in sodium cholate, and treated with trypsin. As seen in Figure 2b–f, the δ chain yielded a 50 000-dalton fragment (the 50 000-bis fragment) which conserved the ^{32}P radioactivity (see below) of the original δ subunit. However, after a more extensive trypsin treatment which converted the 50 000-bis fragment into a 49 000-dalton fragment, all the radioactivity was lost (Figure 2g,h). Evidently, a specific phosphate site exists on the δ chain between the cleavage sites yielding the 50 000-bis and the 49 000-dalton fragments. No phosphorylated fragments of other receptor subunits were detected.

The ^{32}P radioactivity in the 50 000-bis band did not result from the fragmentation of the phosphorylated γ chain, which forms instead a (nonphosphorylated) fragment of 45 000 daltons (Saitoh et al., 1980) after trypsin treatment (Figure 1a). The maximum quantity of [^{32}P]phosphate associated with the 50 000-bis band after trypsin treatment always approached the quantity lost from the δ chain (up to 70% of the radioactivity in the δ chain was found in the 50 000-bis fragment). Whether all the phosphate from the δ chain was conserved in the 50 000-bis fragment is difficult to determine, since this fragment was rapidly degraded to the 49 000-dalton one. In addition, we have analyzed these samples by using gel systems capable of resolving peptides of apparent molecular weights as small as 12 000. No phosphate label was found in small fragments (such as a 16 000-dalton chain, see below) that would correspond to portions of the δ chain lost after trypsin treatment.

Since after mild proteolytic attack little or no change in the sedimentation constant of the L form of the receptor was observed, the possibility was considered [see Raftery et al. (1979)] that in the proteolyzed molecule all of the peptide fragments remain associated. Accordingly, phosphorylated receptor was purified by sucrose gradient centrifugation (see Materials and Methods) under conditions which do not denature the receptor but which remove lipids and nonreceptor proteins. After this treatment, 30.5% (±0.5%, two experiments) of the total ^{32}P radioactivity was recovered in the δ chain (as analyzed after $NaDodSO_4$–polyacrylamide gel electrophoresis) and 26% (±7) in the γ chain. This purified receptor was then incubated with 0.1 mg/mL trypsin for 1 h and passed through a second sucrose gradient. The normal pattern of protease degradation fragments was found [Figure 1, Wennogle & Changeux (1980)]. The analysis of the gradient revealed that 80.9% (±7.8, three experiments) of the ^{32}P was found in the supernatant and, therefore, no longer belonged to the receptor protein (8.2 ± 3.5% of the ^{32}P remained with the receptor). Thus, the fragments of the receptor carrying the in vitro sites of phosphorylation did not remain bound to the receptor protein complex after trypsinization and purification.

The highly purified trypsin used here did not appear to contain phosphatases, because the loss of phosphate from purified receptor polypeptide chains was inhibited completely by excess soybean trypsin inhibitor. Furthermore, the disappearance of ^{32}P-labeled fragments occurred at the same time as the disappearance of the 50 000-bis and the appearance of the 49 000-dalton peptide fragments stained by Coomassie brilliant blue.

(*C*) *Localization of an Intermolecular Disulfide Bond on δ Chain.* In the native nonreduced receptor, the δ chain exists as a dimer due to an intermolecular disulfide cross-link (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977, 1979). We tested whether trypsin degradation had any influence on the cross-linking of the δ subunits. $NaDodSO_4$–polyacrylamide gel electrophoresis of 5-A[^{3}H]T-labeled δ chain showed that it existed primarily (77%) as a dimer of apparent molecular weight 132 000 (δ–δ). When this receptor was treated with increasing concentrations of trypsin, several successive degradation products were observed (Figure 3A). The 50 000-bis, 49 000-, and 47 000-dalton fragments, seen in gels run with reducing agents (Figure 1), were also observed in the absence of these agents. They, therefore, do not contain intermolecular disulfide bonds. This bond (bonds) must be carried on the part of the molecule (16 000 daltons) distal to the trypsin cleavage that yields the 50 000-bis fragments. This interpretation is supported by the observation of an 82 000-dalton fragment labeled by 5-A[^{3}H]T which is observed upon mild trypsinization but is subsequently lost at higher trypsin concentrations (parts 2–4 of Figure 3A). This band was demonstrated by two-dimensional gel electrophoresis to consist of an intact 66 000-dalton chain disulfide bonded to a 16 000-dalton peptide (Figure 4a). Thus, the 82 000-dalton band seems to be the result of a single cleavage of the 132 000-dalton protein which yields a free 50 000-dalton fragment (50 000-bis), labeled with 5-A[^{3}H]T, and an 82 000-dalton fragment, also labeled with 5-A[^{3}H]T, which consists of an intact 66 000-dalton (δ) subunit and a 16 000-dalton peptide from another δ subunit. A second cleavage of this 82 000-dalton fragment should produce a 32 000-dalton, disulfide-cross-linked dimer of the 16 000-dalton chain, in addition to a 50 000-bis chain. We have seen fragments in the 32 000-dalton range on $NaDodSO_4$–polyacrylamide gels of trypsinized receptor run in the absence of reducing agents. However, since these fragments are not labeled by 5-A[^{3}H]T, we cannot be sure of their identity. A summary of these results is presented in Figure 4b.

(*D*) *Factors Which Control the Sedimentation Value of the Receptor.* Having localized the intermolecular disulfide bond on a 16 000-dalton fragment of the δ chain, we performed experiments to test the effect of trypsin cleavage on the sedimentation value of the receptor. For these experiments, membranes were purified and solubilized in the presence of 5 mM NEM to prevent artificial changes in the sulfhydryl groups of membrane proteins (Materials and Methods). All manipulations were then performed in the absence of reducing agents. Purified receptor was treated with various concentrations of trypsin and analyzed by velocity sedimentation in sucrose gradients. With intact receptors, more than 70% was present in the 12S H form. After trypsin cleavage, this dimer was converted into a 9S L form (Figure 3B).

Details of this experiment are presented in Figure 3C. Here, except for a population of trypsin-insensitive dimers, the disappearance of the 132 000-dalton chain (analyzed by counting the band on $NaDodSO_4$–polyacrylamide gels) correlates with the disappearance of the H species seen in the sucrose gradients. Most likely, only one δ chain is present per purified receptor monomer (Karlin, 1980), and only this chain is cross-linked by an intermolecular disulfide bond to give the 132 000-dalton chain. Clearly, this disulfide bond is sufficient to cross-link the receptor. Furthermore, the 16 000-dalton chain, which carries this disulfide bond, does not remain associated with the receptor after trypsinization under these conditions.

When we began this work, receptor-rich membranes were isolated without regard to possible oxidation during purification. In this case, a large amount of β chain contained intermolecular disulfide cross-links, and much larger trypsin concentrations were needed to convert receptor dimer to mo-

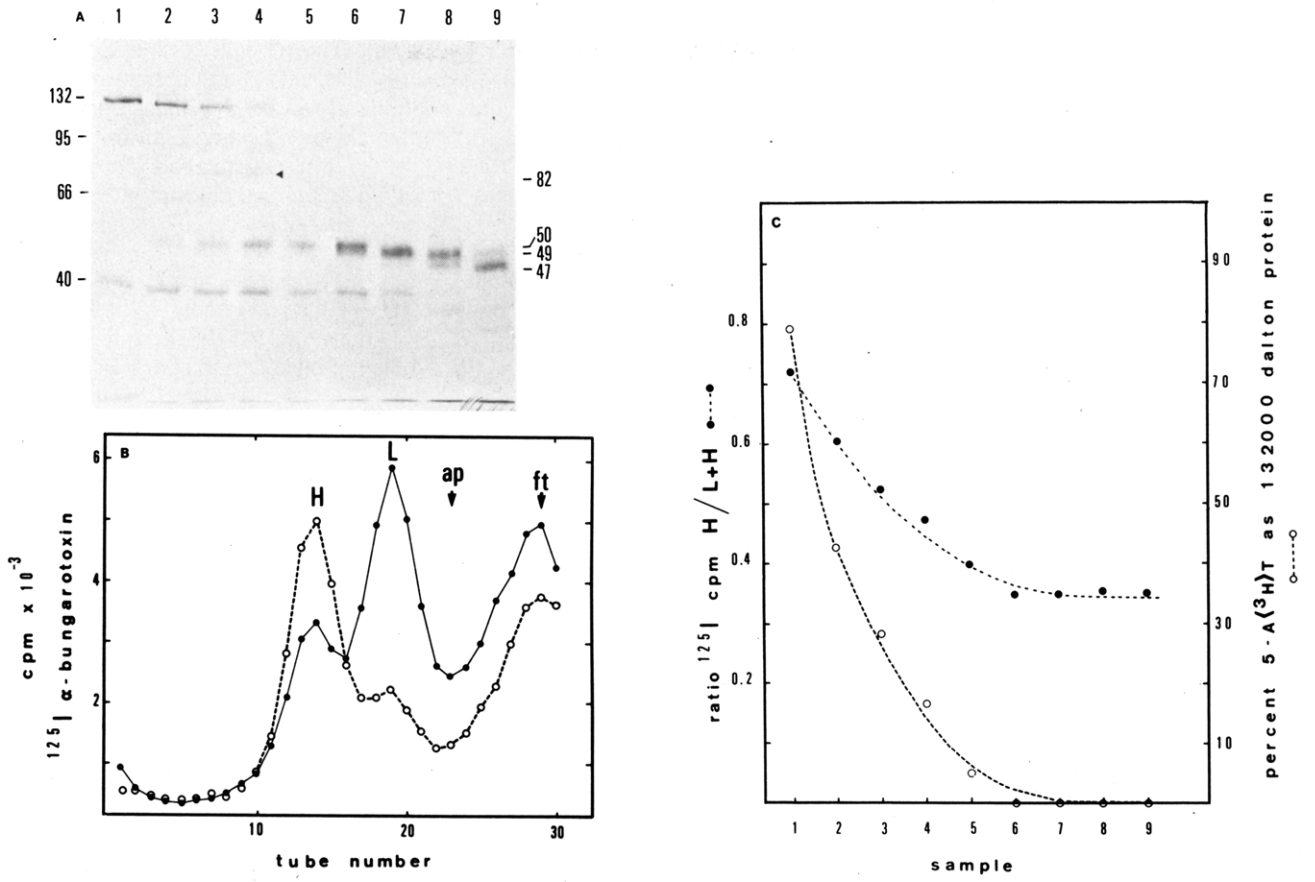

FIGURE 3: Loss of receptor dimer and 66 000-dalton disulfide bond. AcChR-rich membranes were purified in the presence of NEM, labeled with 5-A[^{3}H]T, solubilized in 3% cholate, and diluted into buffer II containing 1% sodium cholate (Materials and Methods). Aliquots were treated with trypsin for 20 min by using the concentrations indicated in (A). The reaction was stopped with DFP. (A) $NaDodSO_4$–polyacrylamide gel analysis. A fluorogram of a gel run in the absence of reducing reagents is shown. Trypsin concentrations were (1) 0, (2) 1×10^{-4}, (3) 2×10^{-4}, (4) 4×10^{-4}, (5) 8×10^{-4}, (6) 1.6×10^{-3}, (7) 3.2×10^{-3}, (8) 6.4×10^{-3}, and (9) 1.3×10^{-2} mg/mL. The diamond indicates the 82 000-dalton protein fragment. A small amount of 5-AT label is found nonspecifically bound to the α subunit. (B) Sedimentation analysis. 1 pmol of each sample was incubated with an equal concentration of α-[^{125}I]bungarotoxin in 100 μL of buffer I (1% sodium cholate and 0.1% BSA) and then separated by sedimentation in a sucrose gradient. Samples also included 6 μg of alkaline phosphatase (ap). Gradients are presented from digestions with 0 (○) and 8.0×10^{-4} (●) mg/mL trypsin (ft = free toxin). (C) Parallel loss of H form and 132 000-dalton protein. The band of 132 000 daltons in the polyacrylamide gel of (A) was cut out and counted. The control point (1) is listed as 79%, since another 21% of the radioactivity was in the δ monomer. In parallel, the radioactivity of α-bungarotoxin associated with the H peak in the corresponding sucrose gradients (B) was determined. The point at the trough between the H + L forms was counted with the form that was the major species in all cases. Reproducibility of this latter analysis, after running of two parallel gradients of the same sample and counting, is within 7%.

nomer than to cleave the δ subunit. The fraction (35%) of receptor H form seen in Figure 3C that remains resistant to trypsin digestion may be due to secondary cross-linking of the β chain during membrane preparation.

(*E*) *N-Terminal Analysis of the δ Chain.* The N-terminal amino acids of the δ chain and its 47 000-dalton trypsin cleavage fragment were determined as their dansyl derivatives (Gray, 1967) after purification of the chains by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis (Devillers-Thiéry et al., 1979). In both cases, an N-terminal valine residue was found [see Raftery et al. (1980)]. This finding indicates that trypsin very likely cleaved the 66 000-dalton chain progressively from near its C terminal to yield the 50 000-bis, 49 000-, and 47 000-dalton fragments. The 16 000-dalton fragment, carrying the intermolecular disulfide bond, may thus be the C-terminal fragment of the molecule.

Discussion

Studies concerning the transmembrane orientation of the individual subunits of the AcChR (Wennogle & Changeux, 1980) complement the primary sequence data (Devillers-Thiéry et al., 1979; Hunkapiller et al., 1979; Raftery et al., 1980) since they add information on the three-dimensional topology of the polypeptide chains and, in addition, may lead to an understanding of their function. In a previous report (Wennogle & Changeux, 1980) the AcChR-rich membranes, which most likely derive from the subsynaptic membrane of the electroplaque, were shown to reseal in vitro into right side out vesicles which appear strikingly insensitive to exogenously added trypsin. On the other hand, when these membranes were opened by sonication or pH 11 treatment, the addition of trypsin led to the degradation of the δ chain and other receptor subunits (Wennogle & Changeux, 1980). Since pH 11 treatment does not affect the binding and ion translocation properties of the receptor (Neubig et al., 1979) and opening of the membrane was reversible (Wennogle & Changeux, 1980), none of these treatments cause a denaturation of the membranes. Therefore, the sites of trypsin cleavage are normally exposed to the internal, cytoplasmic face of the membrane. Cleavage at the level of these sites yielded, in addition to other fragments, polypeptide fragments of 50 000-bis, 49 000, and 47 000 daltons. We have shown here that these fragments derive from the δ chain. In addition, a specific site of in vitro phosphorylation of the δ subunit is located between the 50 000-bis and 49 000-dalton cleavage sites. As a consequence, this phosphorylation site must also

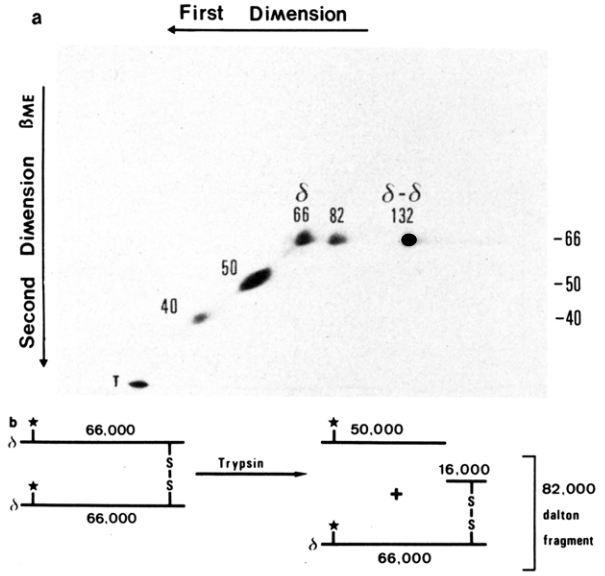

FIGURE 4: Identification of 82 000-dalton fragment of the 132 000-dalton band by two-dimensional gel electrophoresis. (a) Samples described in Figure 3 were analyzed by two-dimensional NaDodSO$_4$–polyacrylamide gel electrophoresis. The results of a fluorogram using a sample labeled with 5-A[^{3}H]T and treated with 4×10^{-4} mg/mL trypsin are presented. Peptides having no disulfide cross-links fall on the diagonal, whereas those cross-linked with disulfides fall off the diagonal. The molecular weights of the cross-linked species are indicated by migration in the first dimension, and the molecular weights following cleavage by β-mercaptoethanol are determined by migration in the second dimension. (b) This diagram outlines the cleavages yielding the 82 000-dalton fragment. The star indicates the hypothetical site of 5-AT labeling. (T = tracking dye front).

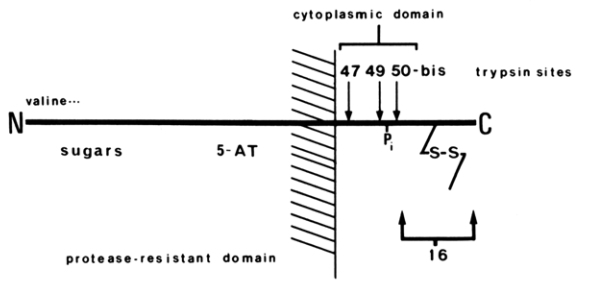

FIGURE 5: Model for transmembrane structure of the δ chain. The apparent molecular weights of trypsin fragments, defined by arrows, are expressed $\times 10^{-3}$.

be exposed to the membrane interior (Figure 5), making it susceptible to metabolic control by cytoplasmic effectors.

We further demonstrated that the cleavage of the δ chain, which gives the 50 000-bis fragment, results in the interconversion of the receptor from its H form to its L form. A 16 000-dalton (probably C-terminal) fragment of this chain carries the disulfide bond responsible for receptor dimerization. Yet the exact position of the disulfide bond on the 16 000-dalton fragment has not been determined. A preliminary map of the relative topology of these various sites on the δ subunit is shown in Figure 5.

The trypsin-resistant 47 000-dalton fragment of the δ chain still carries glycosyl moieties (Wennogle & Changeux, 1980). The reason for the high resistance to trypsin of the nondenatured receptor is not known. It might result from a rigid protein core structure, which renders the sensitive peptide bonds inaccessible to proteases. On the other hand, the lipid bilayer and/or a tightly bound annulus of detergent (after solubilization) may play a similar role.

Finally, these results offer a plausible interpretation of the particular subunit pattern and properties reported for *Electrophorus* AcChR (Meunier et al., 1974; Karlin et al., 1976; Lindstøm et al., 1979b). The presence of three bands instead of four on NaDodSO$_4$ gels, that is, the absence of a typical δ subunit and concomitantly of the H form, is consistent with the interpretation that these properties result from a limited proteolytic attack of a receptor with the same subunit organization as *Torpedo* receptor [see Lindstrøm et al. (1980)].

Acknowledgments

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Steady-State Kinetics and Inhibition Studies of the Aldol Condensation Reaction Catalyzed by Bovine Liver and *Escherichia coli* 2-Keto-4-hydroxyglutarate Aldolase†

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ABSTRACT: Two sensitive assays, one which fluorometrically measures only the L isomer of 2-keto-4-hydroxyglutarate after decarboxylation to L-malate and the other which spectrophotometrically determines both enantiomers by reductive amination with glutamate dehydrogenase, are described. By use of these assays, the steady-state kinetics of the aldol condensation of pyruvate with glyoxylate, as catalyzed by 2-keto-4-hydroxyglutarate aldolase from either bovine liver or *Escherichia coli*, were studied as was the inhibition of this reaction by glyoxylate and other anions. For the *E. coli* aldolase, double-reciprocal plots are linear except at high (above 5 mM) glyoxylate concentrations; apparent K_m values increase with increasing concentrations of the fixed substrate. The data are consistent with an ordered reaction sequence. Inhibition by halides follows the lyotropic or Hofmeister series. Esters are not good inhibitors; mono-, di-, and tricarboxylic acids are increasingly inhibitory. Of the substrate analogues tested, hydroxypyruvate is the most potent inhibitor. Inhibition studies with citrate, acetaldehyde, and glyoxylate (all competitive inhibitors) suggest there are two domains at the active site—the Schiff base forming lysyl residue which interacts with carbonyl analogues (like acetaldehyde) and a center of positive charge which binds anions (like citrate). In contrast to the bacterial enzyme, liver 2-keto-4-hydroxyglutarate aldolase is inhibited in a competitive manner by much lower concentrations (0.1 mM or even lower) of glyoxylate. Many salts and some carboxylic acids activate the liver enzyme. Similarly, substrate analogues like 2-ketobutyrate and fluoropyruvate are mild activators; no effect is seen with acetaldehyde. Besides glyoxylate, only glyoxal, 2-ketoglutarate, and hydroxypyruvate inhibit the aldol condensation reaction. A uniform value of 1 is found for the number of inhibitor molecules bound per active site of either liver or *E. coli* 2-keto-4-hydroxyglutarate aldolase.

2-Keto-4-hydroxyglutarate aldolase (2-keto-4-hydroxyglutarate glyoxylate-lyase) catalyzes the dealdolization of kHOGlt[1] (kHOGlt ⇌ pyruvate + glyoxylate), a terminal step in the degradation of L-hydroxyproline by mammals (Adams & Goldstone, 1960a,b; Maitra & Dekker, 1963). It catalyzes an analogous reaction (2-keto-4-hydroxybutyrate ⇌ pyruvate + formaldehyde), albeit less effectively, in L-homoserine catabolism (Lane et al., 1971). This enzyme, prepared in highly purified or homogeneous form from either rat liver (Maitra & Dekker, 1964; Adams, 1971b), bovine liver (Dekker et al., 1975a), or *Escherichia coli* (Dekker et al., 1975b), is a Schiff-base mechanism (class I type) aldolase. When compared with other class I aldolases, kHOGlt-aldolase is uniquely atypical in that it (1) forms a Schiff-base intermediate not only between the ε-amino group of an active-site lysyl residue and kHOGlt or pyruvate (as required mechanistically) but *also* with glyoxylate (thereby forming a dead-end or "abortive" Schiff base) (Kobes & Dekker, 1971a; Nishihara & Dekker, 1972), (2) is completely and irreversibly inactivated by cyanide *only* in the presence of glyoxylate (or other low molecular weight aldehydes) with stable formation of an aminonitrile (Hansen et al., 1974), and (3) is bifunctional, effectively catalyzing the β-decarboxylation of oxaloacetate as well as the aldol cleavage or formation of kHOGlt (Kobes & Dekker, 1971b).

Rosso & Adams (1967), in describing kHOGlt-aldolase purified from rat liver, noted that kHOGlt formation is inhibited by glyoxylate. For examination of this specific observation together with the general kinetics of the reaction

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[1] Abbreviations used: kHOGlt, 2-keto-4-hydroxyglutarate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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