Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2631–2634, May 1984
Biochemistry

Molecular weight and structural nonequivalence of the mature α subunits of *Torpedo californica* acetylcholine receptor

(N-glycosylation/sequence analysis/peptide maps/affinity labeling/post-translational modification)

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Communicated by John D. Roberts, December 27, 1983

ABSTRACT A discrepancy of about 20% exists between the molecular weight of the α subunit of *Torpedo californica* electroplax acetylcholine receptor as determined by gel electrophoresis of the mature protein (M_r 40,000 ± 2000) and by nucleotide sequence analysis of cDNA (M_r ≈50,000). We demonstrate by amino acid sequence analysis that post-translational processing does not occur and that the mature subunit has a M_r of ≈50,000. The functional acetylcholine receptor contains two copies of this α subunit in addition to one each of related β, γ, and δ subunits. The binding sites for cholinergic ligands that are located on the α subunits have been shown to be nonequivalent. Amino acid sequence analysis of peptides obtained by proteolytic cleavage of the α subunit reveals that N-asparagine glycosylation at a single site (residue 141) occurs to a different extent in the two copies of this polypeptide in the mature protein and provides an explanation for nonequivalence of their binding sites.

The nicotinic acetylcholine receptor (AcChoR) from *Torpedo* and *Electrophorus* electric organ and from mammalian skeletal muscle is formed by four different, homologous proteins, commonly referred to as α, β, γ, and δ (1). They presumably arose from a single ancestral gene (2), are associated in the AcChoR molecule with a stoichiometry of $\alpha_2\beta\gamma\delta$, and upon $NaDodSO_4$ gel electrophoresis they have apparent M_rs ranging between 40,000 and 65,000 (1). The complete amino acid sequence of precursors of all *Torpedo* subunits has been deduced from the corresponding nucleic acid clones obtained by recombinant DNA technology (3–8).

Each α subunit contains a high-affinity binding site for cholinergic ligands and possibly for α-bungarotoxin (1). These two sites are not equivalent. After reduction of a disulfide bridge close to the binding sites, they both can be covalently labeled by cholinergic agents such as bromoacetylcholine (9, 10) and one of them is more susceptible to such labeling (9), so that for many years it was believed that only one site on each AcChoR molecule could be labeled (11). The nonequivalence of the two sites can be explained by their different microenvironments since each α chain must be flanked by different subunits. However, the two α subunits themselves could be biochemically different, in spite of the identical primary structure of their precursors (3, 4, 8) and of the mature proteins (2). Another open question regards the exact molecular weight of the mature α subunit. The sequences deduced from its precursors consistently yielded a M_r of ≈50,000 both for *Torpedo californica* (4) and *Torpedo marmorata* AcChoR (8). This predicted value is considerably larger than the apparent molecular weight of the α subunit calculated from its behavior upon $NaDodSO_4$ gel electrophoresis, which is between M_r 38,000 and M_r 44,000 depending on the gel system used (1). The possibility of a post-translational cleavage of a carboxyl-terminal peptide has been suggested (4), supported by the existence in this region of paired basic residues, which generally represent sites of proteolytic processing (12). To answer these questions, we have directly investigated the amino acid sequence of critical segments of the mature α subunits of *T. californica* AcChoR.

MATERIALS AND METHODS

Purification of the α Subunit. Membrane-bound AcChoR was isolated from *T. californica* electric organ by subcellular fractionation (13) followed by pH 11 extraction (13, 14). The membrane fragments (25–50 mg of protein) were incubated in 1.5% $NaDodSO_4$/5% glycerol/2.5% mercaptoethanol for 2 min at 90°C to achieve complete dissociation of the subunit. The sample was made 0.02% in bromophenol blue/2% in sodium thioglycolate and loaded on a preparative slab gel, according to Laemmli (15), containing 8.75% polyacrylamide. The running gel was 0.5 cm thick, 13 cm long, and 26 cm wide; the spacer gel was 1.5 cm long. The gels were run for ≈20 hr at 60 mA, stained with Coomassie blue for 6 hr, and destained for 24 hr as described (16). The stained protein bands were cut and stored frozen. The AcChoR subunits were recovered by electroelution and were electrodesalted (16). The $NaDodSO_4$ used in the buffers for electroelution and desalting had been recrystallized twice from hot ethanol. The purity and the integrity of the isolated subunit were checked by $NaDodSO_4$/polyacrylamide gel electrophoresis. The protein bands were visualized by Coomassie blue staining or by Bio-Rad silver staining (Fig. 1*A*). The purity of the isolated α subunit was also checked by amino-terminal amino acid sequence analysis. The preparations of α subunit used showed a single band at the expected molecular weight upon $NaDodSO_4$ gel electrophoresis (Fig. 1*A*) and contained the expected amino-terminal sequence of the α subunit with occasional trace amounts (<3%) of contaminating sequences originating from degradation products of the higher molecular weight subunits.

In very concentrated preparations, bands of M_r ≈80,000 and higher were occasionally observed, possibly dimers and higher degree polymers of the α subunits.

Cleavage of the α Subunit. Proteolytic peptides of the α subunit were obtained by digestion with *Staphylococcus aureus* V8 protease (Miles) as described by Cleveland *et al.* (17) and Froehner and Rafto (18). Peptide maps obtained with V8 protease were stained for carbohydrate according to Glossman and Neville (19) with the following modifications. After electrophoresis the gels were washed in 40% methanol/10% acetic acid for 90 min with three changes. After oxidation the gels were washed as above with 7% acetic acid.

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Abbreviation: AcChoR, acetylcholine receptor.
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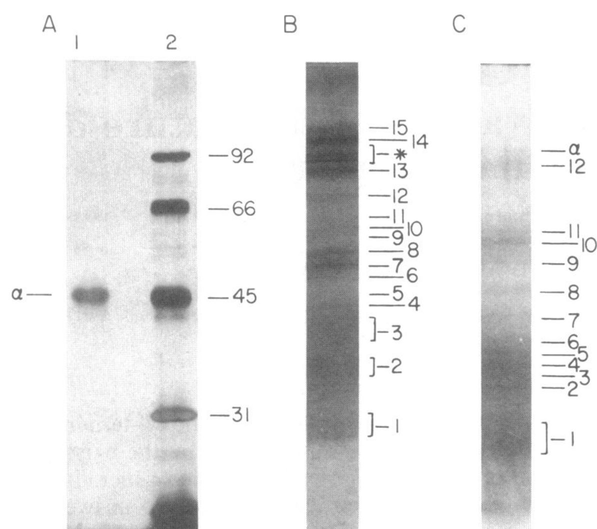

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of purified α subunit of *T. californica* AcChoR (*A*, lane 1) and of the peptides obtained by V8 protease hydrolysis (*B*) and CNBr cleavage at the tryptophanyl residues (*C*). Molecular weight standards are given as $M_r \times 10^{-3}$ in *A*, lane 2 (top to bottom): phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase. In *A* the gel contained 10% acrylamide; in *B* and *C* the gel contained an exponential gradient from 15% to 22% acrylamide. Details about the sequences of the various peptides are given in the text.

The gels were incubated in Schiff's reagent overnight at 4°C in the dark. The Schiff reagent was prepared according to Fairbanks *et al.* (20). Peptides stained with periodate–Schiff reagent were identified by comparison with identical samples stained with Coomassie blue or by direct Coomassie blue staining of the gel slabs previously stained for carbohydrates.

Amino-Terminal Amino Acid Sequence Analysis. The purified samples were lyophilized, dissolved in 30 μl of distilled water, and submitted to amino-terminal sequence analysis by automated Edman degradation on either a spinning cup (21) or a gas-phase (22) sequenator. Phenylthiohydantoin-derivatized amino acids were identified by HPLC on an IBM Cyano column. Details on identification of phenylthiohydantoin-derivatized amino acids and standard chromatograms have been described (23).

RESULTS

Enzymatic Cleavage. Cleavage of the α subunit with V8 protease gave peptide patterns very similar to those reported by others (18, 24), characteristic peptides appearing with increasing amounts of enzyme. Above an enzyme/substrate ratio of 1:10 (wt/wt), the pattern did not change by further increasing the protease concentration. In Fig. 1*B*, results of a typical V8 digest are shown. The peptides are numbered from 1 upwards—i.e., according to increasing size. The two peptides indicated with an asterisk are the two subunits of the V8 protease and peptide no. 15 is intact α subunit. The two peptides V8 no. 7 and V8 no. 8 of M_rs ≈17,000 and 19,000, respectively, were consistently present in roughly equal amounts. Even at a high enzyme/substrate ratio, they did not disappear nor was the one of higher molecular weight converted into the one of lower molecular weight. They correspond to the peptides VF and VE of Gullick *et al.* (24). During carbohydrate staining, peptide no. 7 appeared already after 1 hr of incubation in Schiff reagent, as described by Gullick *et al.* (24). By prolonging the incubation to 3–6 hr, also peptides nos. 8, 13, and 14 were stained, although the staining of peptide no. 8 never reached the intensity of no. 7. Even after overnight incubation, no staining of other peptides was detectable. The amino-terminal amino acid sequence of all of these peptides was determined. All of the peptides of M_r 26,000 and above (from V8 no. 9 to V8 no. 14) started from the amino terminus of the intact subunit. The two peptides V8 no. 7 and V8 no. 8 have the same amino terminus, starting at residue 46 (see Fig. 2). Peptide V8 no. 7 contained also a minor sequence (≈10%), starting at residue 52. Peptide V8 no. 3 (M_r ≈10,000) started at residue 339. Peptide V8 no. 2 (M_r ≈6000) started at the amino terminus. Peptide V8 no. 1 (M_r ≈3000) contained both the amino-terminal sequence and a second sequence starting at residue 339.

Chemical Cleavage. The CNBr cleavage at the methionine residues yielded two relatively large peptides [of M_r 12,000 ("Met 1") and M_r 7000 ("Met 2")] plus a number of small peptides that were not separated by NaDodSO$_4$ gel electrophoresis and that were too hydrophobic to be separated with conventional HPLC methods. Met 1 starts at the amino terminus; Met 2 starts at residue 330 and overlaps the sequence obtained from peptide V8 no. 3.

To obtain further sequence data, peptide V8 no. 3, which starts at residue 339 and is likely to extend until the carboxyl terminus, was further cleaved at the methionine residues by using the CNBr method (25). The sequences present in the resulting peptide mixture were determined. Under the conditions used, cleavage occurred at all of the methionine residues predicted from the α-subunit precursor (residues 386, 404, and 415) and the three corresponding sequences were obtained (Met V8 no. 3, see Fig. 2) plus the sequence starting at residue 339—i.e., the amino terminus of the original V8 peptide. With this approach we obtained sequence data until residue 426—i.e., 11 residues before the carboxyl terminus predicted from the α-subunit precursor.

The CNBr cleavage at the tryptophan residues yielded a mixture of peptides that could be resolved by NaDodSO$_4$ gels (Fig. 1*B*). The amino-terminal sequence of the majority of these peptides was determined as indicated in Fig. 1*B*. Peptides of high molecular weight (between M_r 24,000 and M_r 28,000, Trp 9, Trp 10, and Trp 11) consistently started at the amino terminus of the α subunit. A peptide of M_r ≈11,000, Trp 4, started at residue 312 and overlapped peptides V8 no. 3, V8 no. 1, and Met 2. A protein band of M_r 9000, Trp 3, yielded three sequences, one starting at the amino terminus of the α subunit, one starting at residue 312 and overlapping the peptides Trp 4 and Met 2, and a third minor sequence starting at residue 87 and overlapping the sequence obtained for the peptides V8 no. 7 and V8 no. 8. Peptide Trp 8 yielded two sequences, a major one starting at residue 68 and a minor one starting at residue 61. Peptide Trp 7 yielded a sequence starting at residue 68. Both of these sequences overlapped those obtained for peptides V8 no. 7 and V8 no. 8.

The stretches of sequence obtained from all of the peptides discussed above, as well as from the V8 peptides, are shown in Fig. 2.

DISCUSSION

In Fig. 2 the stretches of amino acid sequence directly obtained from the native α subunit of *T. californica* are reported and compared with the published sequence of the α-subunit precursor (4). We obtained most of the hydrophilic parts of the α subunit—i.e., (*i*) the first ≈100 residues starting at the amino terminus, which form about half of the first hydrophylic domain, and (*ii*) the majority of the second hydrophylic domain (residues 312–397). Of the four hydrophobic domains present in the α subunit, only part of the last one [M4 (7)] was obtained. All of the peptides generated consistently did not contain the first three hydrophobic domains, which are next to each other along the α-subunit sequence (3, 4). It

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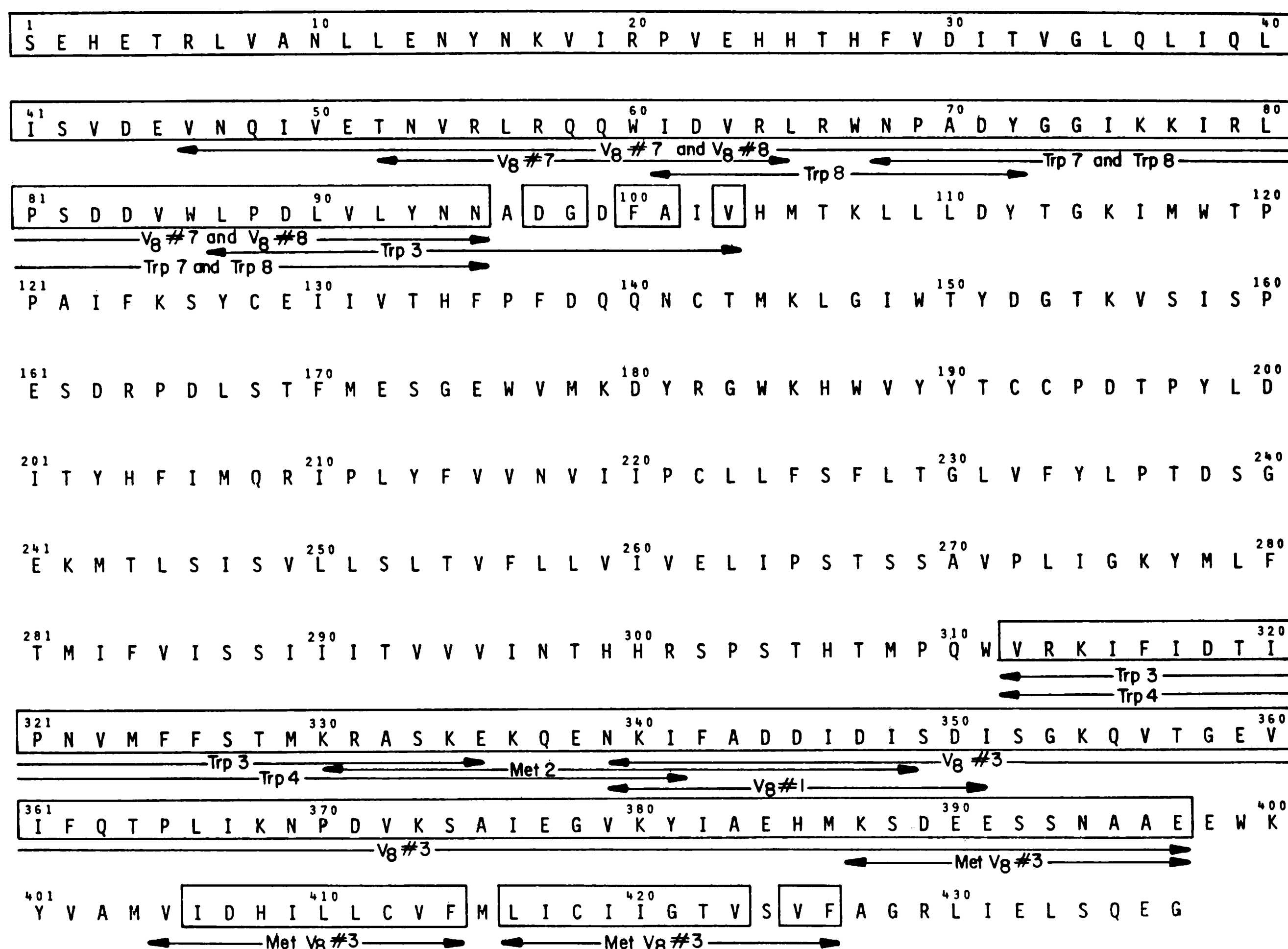

FIG. 2. Sequences of all of the peptides from the mature α subunit. Deduced sequence of the α-subunit precursor (3). The boxes indicate the stretches of sequence directly obtained from the mature α subunit. The sequences obtained from the single peptides are indicated by the arrows underneath the sequence.

is likely that after cleavage of the α subunit, the most hydrophobic peptides aggregate even in the presence of NaDodSO$_4$, and they do not enter the polyacrylamide gel used for separation.

The sequence we obtained extends until residue 426 of the α-subunit precursor. It is therefore likely that the mature α subunit retains all of the sequence of its precursor and eliminates the possibility of a post-translational cleavage with elimination of a large carboxyl-terminal peptide. This has been suggested (4, 8) to explain the disagreement between the predicted molecular weight of the α-subunit precursor and the molecular weight experimentally determined for the mature α subunit (1), and it was supported by the presence, at positions 330–331 and 313–314, of paired basic residues (K-R and R-K). Therefore, the molecular weight of the mature α subunits is much higher than the experimentally determined values [M_r ≈40,000 (1)] and must be close or identical to the value of M_r ≈50,000 calculated for its precursor (4). This discrepancy could be explained by unusually high NaDodSO$_4$ binding by the transmembrane, hydrophobic segments of the α subunit. In other animal species, a subunit of M_r ≈40,000–42,000 can be labeled with high affinity by cholinergic ligands (1, 26–28). This subunit has a primary sequence highly homologous to the *Torpedo* α subunit and is present in two copies in the receptor molecule (16, 29). It is likely that in all of the animal species, the molecular weight of such α subunits is much higher than experimentally determined. This is supported by two findings. (*i*) Electric eel AcChoR preparations were described containing, upon NaDodSO$_4$ gel electrophoresis, only polypeptides with M_rs 48,000, 54,000, and 60,000 (30). In such preparations the α subunit probably comigrated with a higher molecular weight subunit. In mammalian muscle AcChoR, an affinity reagent, maleimidobenzyltrimethylammonium iodide, labeled two polypeptides of M_rs 44,000 and 49,000, which had identical peptide maps (31). In these preparations one of the two α subunits must have run according to its real molecular weight. This also argues for a structural nonequivalence of the two α subunits in mammalian muscle (see below).

The fact that the mature α subunit has the molecular weight predicted for its precursor lends further credence to the molecular weight predicted from the precursors of the other homologous subunits that are also different from the experimental determinations in the presence of NaDodSO$_4$ (β, M_r 53,681 vs. M_r ≈50,000; γ, M_r 56,000 vs. M_r ≈60,000; δ, M_r 57,565 vs. M_r ≈65,000). The four homologous subunits therefore have very similar molecular mass and the AcChoR complex has a M_r of 268,078. This value is significantly higher than the molecular weight experimentally obtained by ultracentrifugation [M_r 250,000 (32)] and agrees closely with a value of M_r 270,000 determined by membrane osmometry (33) and crosslinking (34).

We found that two peptides obtained by extensive cleavage with V8 protease, of very close apparent molecular weights (V8 no. 7 and V8 no. 8), have the same amino terminus. These peptides correspond to the peptides VF and VE of Gullick *et al.* (24), who demonstrated that peptide VE could be labeled by the cholinergic affinity ligand maleimidobenzyltrimethylammonium iodide and only peptide VF contained carbohydrates. At difference with these authors,

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we found that also peptide V8 no. 8 could be stained for carbohydrates, but to a minor extent and only with prolonged incubation in Schiff's reagent. This discrepancy can be reconciled by concluding that the two peptides differ in the extent of glycosylation and that peptide V8 no. 7 has a much higher carbohydrate content. Similar peptides have been demonstrated for mammalian muscle α subunits (35). Evidence has been presented that the α subunit of muscle AcChoR has a single N-asparagine-linked oligosaccharide chain (35). The only possible site of N-glycosidic linkage in the α subunit of *T. californica* is the asparagine residue at position 141 in the unique sequence Asn-Cys-Thr (4), which is contained in both peptides V8 no. 7 and V8 no. 8 and is more heavily glycosylated in one of them (peptide V8 no. 7). In addition, among the V8 peptides of higher molecular weight (from peptides V8 no. 9 to V8 no. 15), only two of them (peptides V8 no. 13 and V8 no. 14) can be stained for carbohydrates. Since all of them start at the amino terminus and contain the residue Asn-141, it is likely that they come from α subunits that differ in the extent of glycosylation and that the presence of sugars influences the sites of V8 cleavage. It seems therefore that the two mature α subunits differ in the extent of glycosylation and that the presence of sugars makes the disulfide bridge close to the high-affinity binding site less accessible to reducing agents and cholinergic affinity labels. This possibility is supported by the fact that the cysteine residue at position 142 is one of the two most likely candidates for being labeled by bromoacetylcholine and maleimidobenzyltrimethylammonium iodide (4). If so, the presence of a sugar moiety on Asn-141 of one α subunit could hinder the access to this cysteine and could explain the different susceptibility of the two α subunits to affinity labeling. The nonequivalence of the two α subunits both in *Torpedo* and in mammalian muscle is supported also by results obtained in studies of the binding of antibodies. It has been demonstrated that anti-*Torpedo* AcChoR antibodies can inhibit a maximum of half of the α-bungarotoxin binding sites (36), which are two for each AcChoR molecule and are believed to be at least partially formed by the two α subunits. More recently, similar findings have been obtained for antibody binding to mammalian muscle AcChoR, and it has been demonstrated that the inhibiting antibody was against a carbohydrate antigen, since its binding could be inhibited by sugars (37). All of these results strongly support the notion that the two α subunits differ in the extent of their glycosylation.

The fact that one of the two mature α subunits present in the AcChoR molecule is more heavily glycosylated raises interesting questions about the synthesis, assembly, and intracellular transport of such subunits. In this respect, the recent observation (38) that all four subunits of *Torpedo* AcChoR can form homopolyers during biosynthesis and transport may ultimately lead to understanding how the selective glycosylation we discuss here is achieved.

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