THE AMINO-TERMINAL SEQUENCE OF THE 40 000 MOLECULAR WEIGHT SUBUNIT OF THE ACETYLCHOLINE RECEPTOR PROTEIN FROM *TORPEDO MARMORATA*

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1. Introduction

The membrane receptor for the neurotransmitter acetylcholine (ACh) is now a well characterized protein that has been isolated and purified in milligram quantities from fish electric organ and vertebrate skeletal muscle, in a state that binds cholinergic (nicotinic) ligands (reviewed [1]). Although its exact quaternary structure is still a matter of controversy, general agreement exists on two major issues:

(1) The 9 S species found after extraction by non-denaturating detergents in the presence of a reducing agent has mol. wt ~250 000 [2–6] and dissociates into smaller subunits in the presence of sodium dodecylsulphate (SDS) [7–16];

(2) The predominant subunit has app. mol. wt ~40 000 upon electrophoresis in SDS–polyacrylamide gels. This 40 k subunit can be affinity-labelled by 4-(*N*-maleimido)-phenyl-[^{3}H]-trimethylammonium ([^{3}H]MPTA), a reagent known to bind covalently to a cysteinyl residue present in, or in the close vicinity of, the ACh binding site after reduction of the molecule by dithiothreitol [17–19]. The 40 k subunit, therefore, carries the ACh-receptor site and appears to be the most critical component of the ACh receptor protein.

Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine

In this letter, we present the amino-terminal sequence of 20 amino acids for the 40 k subunit purified from the electric organ of *Torpedo marmorata*.

2. Material and methods

2.1. *Purification of the 40 k subunit of the ACh-receptor protein from* Torpedo marmorata

ACh-receptor-rich membrane fragments were prepared by the method in [14], slightly modified as follows. P_3 pellets were resuspended with a conical all-glass homogenizer in a minimal volume of solution 1: twice-distilled water containing 0.1 mM phenylmethylsulfonyl fluoride and 0.02% NaN_3. Solid sucrose was added to a final density of 36%; 35 ml of the solution were layered on a 30 ml 41% sucrose cushion in a 65 ml poly-carbonate tube and centrifuged in a Beckman 45 Ti rotor at 45 000 rev./min for 160 min. The upper phase of the discontinuous gradient was discarded, then the dense layer on top of the 41% sucrose cushion was collected, diluted 3 times with solution 1 and centrifuged at 45 000 rev./min in a 45 Ti rotor for 30 min.

After resuspension the ACh-receptor-rich membrane fragments were labelled with [^{3}H]MPTA (spec. act. 5 Ci/mmol) by the method in [17] as described [20]. Labelled membrane fragments were subsequently carboxymethylated in the presence of iodoacetamide. Membrane fragments were collected by centrifugation in a Beckman 45 Ti rotor at 45 000 rev./min for 30 min.

Pellets were resuspended in solution 1 to 10–15 mg

protein/ml final conc.; 5 ml portions were frozen and kept at −70°C until use.

Before use, a 5 ml portion was diluted with 18.5% sucrose solution to yield 0.5 mg protein/ml, adjusted to pH 11 with NaOH as in [21] and allowed to stand at room temperature for 45–60 min. It was then layered over a 20 ml 26% sucrose cushion adjusted to pH 11 in a 100 ml polyallomer tube and centrifuged in a Beckman 45 Ti rotor at 45 000 rev./min for 90 min. The supernatant contained the 43 k subunit and some other proteins that, under these conditions, were released from the membrane fragments.

Pellets were resuspended in a minimal volume of solution 1 and homogenized with a conical all-glass homogenizer. The suspension was adjusted to 12 ml final vol. containing 0.15 M Tris (pH 8.5) 4% SDS, 25 mM dithiothreitol, 15% sucrose (w/v) and some bromophenol blue as a marker, then applied to 4 polyacrylamide gels (each gel was 2 mm thick, 200 mm high, 320 mm wide with only 1 large slot). Running and stacking gels contained, respectively, 10% and 5% acrylamide and both contained 0.13% *N,N'*-methylenebisacrylamide in the presence of 0.1% SDS. Electrophoresis was carried out at a constant power of 1 W for 17 h.

After electrophoresis, small marker strips on each side of the gels and in the middle of the gels were cut, fixed, stained and destained [14] and allowed to regain their initial size in a concentrated solution of ethanol. The stained strips and the rest of the unfixed gel were then aligned and a band containing the 40 k subunit cut out and eluted out by electroelution in 0.025 M Tris, 0.2 M glycine, 0.1% SDS. The eluate containing the 40 k subunit was passed through a millipore filter (0.45 μm) to remove small gel particles, concentrated by lyophilization, dialyzed against distilled water to remove Tris, glycine and the detergent and lyophilized to dryness. The yield of the elution was close to 100% as monitored by the elution of ^{125}I-labelled 40 k subunit in a control experiment.

2.2. *Sequence analysis*

The purified 40 k subunit of the ACh-receptor was analyzed by automated Edman degradation in a Beckman sequencer 890C. Two types of additive were used to prevent losses of material: succinylated polyornithine and polybrene. As recommended [22], glycyl–glycine was added to avoid partial blocking by polybrene contaminants. The degradation program was essentially that in [22] which uses 0.33 M Quadrol, 5% phenylisothiocyanate with simple or double cleavage. Conversion of the phenylthiazolinone derivatives was achieved with 20% trifluoroacetic acid. Repetitive degradation yields over the first 20 steps were > 95%.

The phenylthiohydantoin (PTH) derivatives were identified by high pressure liquid chromatography on a Waters instrument equipped with a C18 microbondapak column using a sodium acetate–methanol gradient as in [23]. Thin-layer chromatography on micropolyamide sheets [24] was used as a second method of identification. Finally the PTH-amino acid derivatives were back-hydrolyzed in HI at 150°C in vacuum sealed tubes [25], followed by amino acid analysis on a Durrum 500C instrument.

3. Results

3.1. *Purification of the 40 k subunit*

In fig.1 are presented the results of the main steps of purification of the 40 k subunit from a suspension of ACh-receptor-rich membrane fragments prepared from *Torpedo marmorata* electric organ. The fractions were submitted to SDS–polyacrylamide gel electrophoresis and stained by Coomassie blue (fig.1a); a densitometric scan of the final product of the purification is shown in fig.1b.

ACh-receptor-rich membrane fragments were purified on a discontinuous gradient (slot 2) from a homogenate of fresh electric organ following the procedure in section 2.1. The membrane fragments were then treated at pH 11 for 1 h as in [21]. After this treatment 5–10% of the 43 k subunit remained associated with the membrane fragments as shown in slot 3 of fig.1a but several other bands were removed. In a routine large scale purification, 45 mg protein from alkaline-treated membranes were applied to 4 large SDS–polyacrylamide gels and run in parallel. After electrophoresis, the portions of the gels containing the 40 k subunit were cut out following the procedure in section 2.1 and submitted to electroelution in Tris–glycine–SDS buffer. The resulting solution was filtered through a millipore filter to remove small particles of gel and concentrated to dryness by lyophilization; the lyophilized residue was

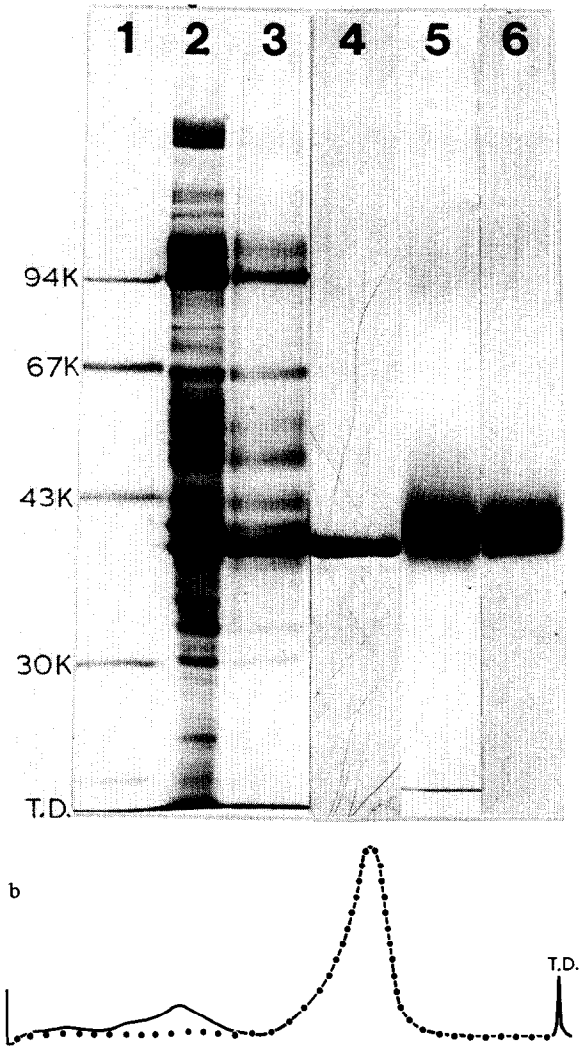

Fig.1. Large scale purification of the 40 k subunit. Analysis of the product of each step of the purification by SDS–polyacrylamide gel electrophoresis. (a) Samples were subjected to electrophoresis on a 10% polyacrylamide gel in the presence of 0.1% SDS according to [29]: slots 1–3, 5, Coomassie blue staining; Slots 4 and 6, fluorography [30] of slots 3 and 5, respectively, after 22 h exposure at −80°C using a Royal X-Omat film. Slot 1 molecular weight markers: phosphorylase *b* 94 k; bovine serum albumin 67 k; chick ovalbumin 43 k; carbonic anhydrase 30 k; trypsin inhibitor 20 k. Slot 2 ACh-receptor-rich membranes after centrifugation on a discontinuous sucrose gradient and labelling with 4-(*N*-maleimido)-phenyl-[^{3}H]trimethylammonium. Slots 3, 4 4-(*N*-maleimido)-phenyl-[^{3}H]trimethylammonium-labelled membranes treated at alkaline pH. Slots 5, 6 4-(*N*-maleimido)-phenyl-[^{3}H]trimethyl-ammonium-purified 40 k subunit. T.D., tracking dye. (b) Densitometric scans of slots 5, 6 of fig.1a. Solid line is the scan of slot 5; dots correspond to the scan of slot 6.

taken up in a minimal volume of water, dialyzed extensively against twice-distilled water and lyophilized to dryness again. The purified material (1.5 mg) gives a single band by SDS–polyacrylamide gel electrophoresis (slot 5 of fig.1a, fig.1b).

3.2. *Identification of the 40 k subunit by affinity labelling*

The suspension of ACh-receptor-rich membrane fragments was labelled with [^{3}H]MPTA, under conditions where this affinity reagent binds covalently to a cysteinyl residue present in or in the vicinity of the ACh binding site [18,26]. In agreement with [19], fluorography revealed that only the 40 k band became radioactive. After treatment at pH 11, the 40 k band was still the only one labelled (fig.1a, slot 4). At the end of the purification (fig.1a, slots 5, 6, fig.1b), the band corresponding to the 40 k component appears more diffuse than when observed at the beginning of the purification. However, comparison of the fluorogram and of the Coomassie blue-stained gel revealed that radioactivity and protein coincided. This was particularly clear when the densitometric scans of the final product of the purification were superimposed (fig.1b). In other words, all the protein present in the purified product was labelled with [^{3}H]MPTA and therefore was the 40 k subunit of the ACh-receptor.

It has been shown [27] by two dimensional gel analysis of ACh-receptor-rich membranes that the 40 k subunit can be resolved in a number of discrete components having slightly different isoelectric points, but the same apparent molecular weight. All were labelled by [^{3}H]MPTA. In other words, the material present in the 40 k band given by one-dimensional gel electrophoresis belongs exclusively to the subunit which carries the ACh-receptor-site. Sequence data (see below) support this conclusion.

3.3. *Amino acid sequence determination of the 40 k subunit of the ACh-receptor protein*

The amino-terminal sequence of the purified 40 k subunit was determined in 4 independent sequenator automated Edman degradations performed on 4 different preparations. Amounts ranging from 5–30 nmol protein were effectively degraded with initial degradation yields as high as 70–80%.

The major method of identification of the PTH-residues was high pressure liquid chromatography

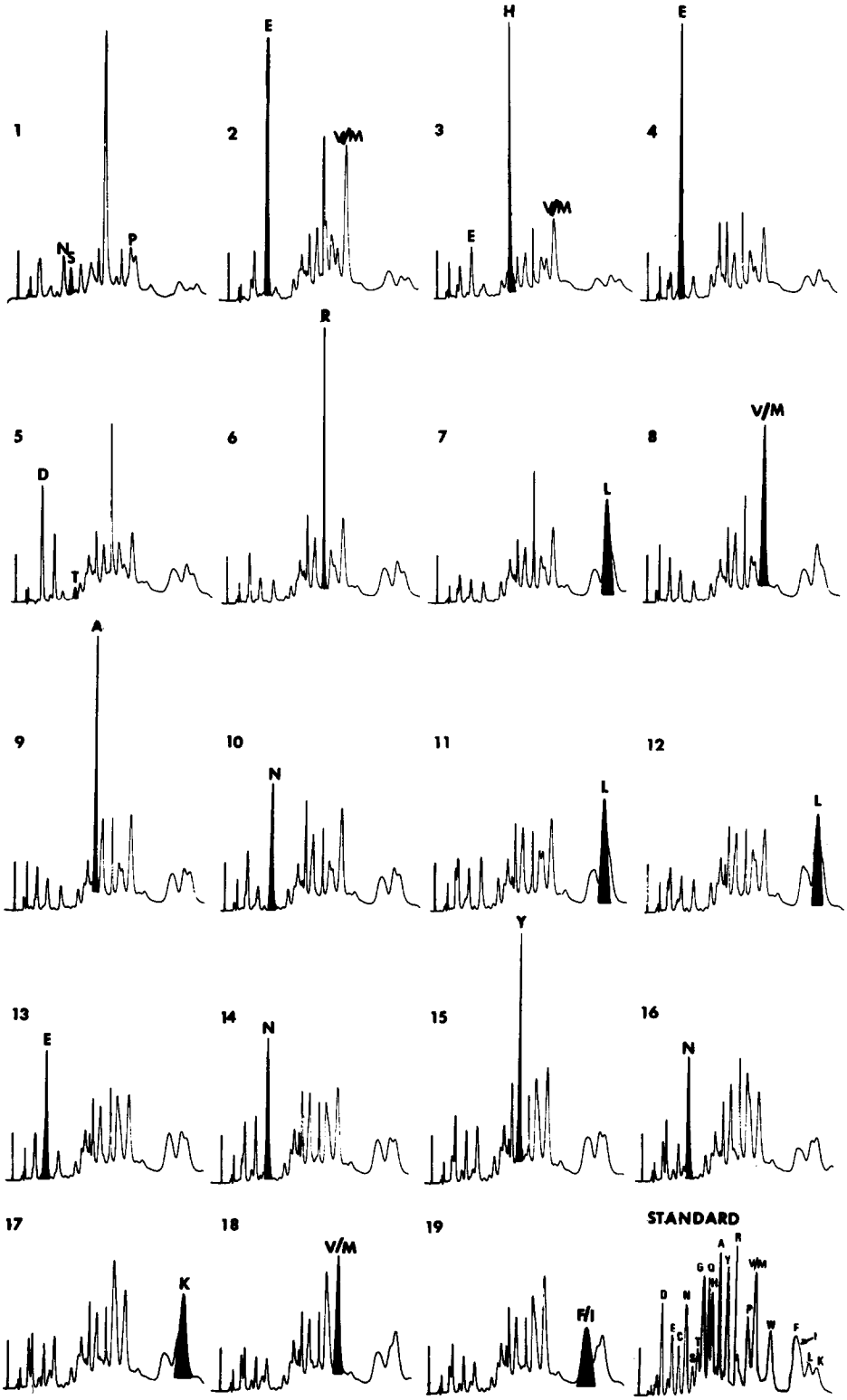
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Fig.2

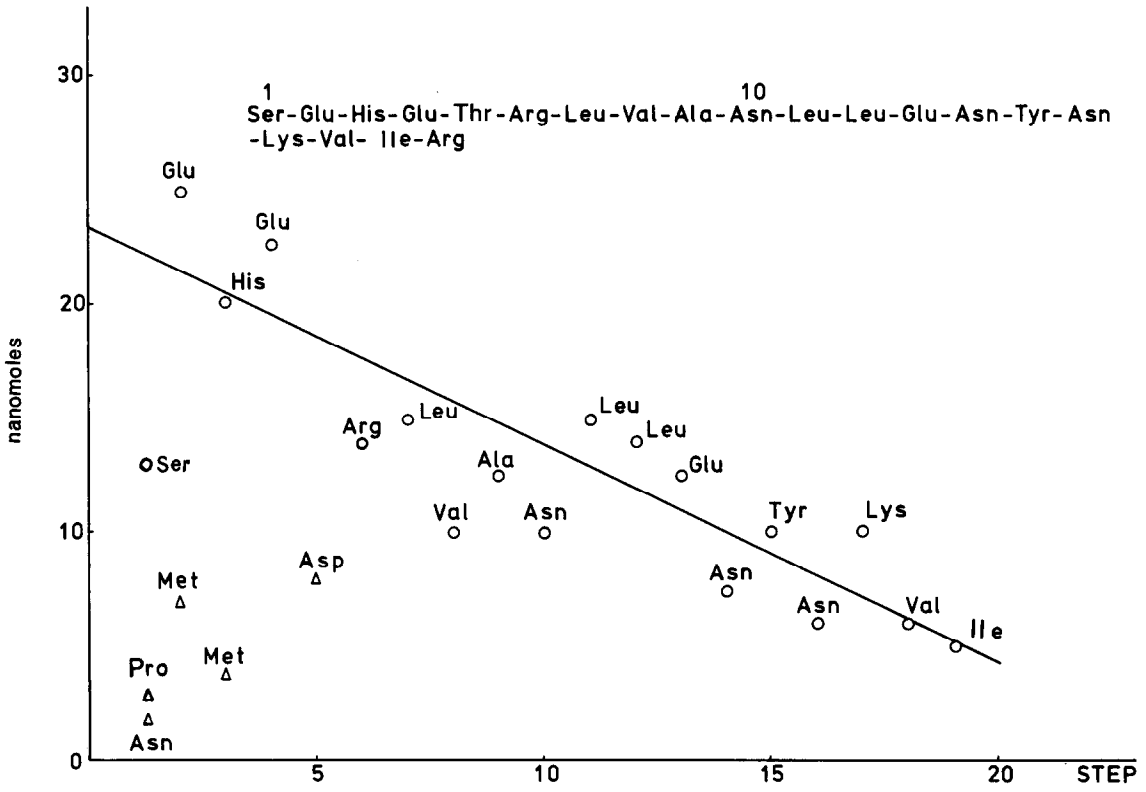

Fig.3. Yields of phenylthiohydantoin amino acids obtained at each step of the automated Edman degradation of the 40 k subunit of the acetylcholine receptor. Quantitative yields were computed by comparing the peak areas of the sample with the relevant standard PTH derivative. The proposed sequence is presented in the upper part of the figure. (○) Major component; (△) minor component. The serine residue at step 1 is assigned to the major sequence because of the known low yield generally obtained with this particular amino acid.

(HPLC) using a single gradient system developed in this laboratory that allows the separation of all polar and non-polar PTH residues except for the Val/Met doublet [23]. However, this doublet was easily resolved by the second method of identification: thin-layer chromatography (TLC) on polyamide sheets [24].

The results of the 4 runs clearly agree with each other. Analyses on HPLC of the first 19 steps of 1 representative degradation are shown in fig.2 along with the separation of a standard mixture of all 20 PTH derivatives. Yields for each step are shown in fig.3. A clear, unambiguous sequence, presented in fig.3 and table 1 was obtained. This sequence corresponds to 70–75% of the protein material introduced in the sequencer cup. A minor heterogeneity was noticed in the first 5 steps. From step 6 on however, only a single residue was identified at each step. Among the secondary residues observed for the first 5 steps, several were found repeatedly in the 4 runs and are given in table 1. In agreement with [28], the major amino acid obtained at the first degradation

Fig.2. HPLC analysis of the first 19 steps of the Edman degradation of the 40 k subunit of the ACh-receptor protein. Peaks of the major PTH residues present at each step have been shaded to allow a better perception of the area covered by each peak. The last analysis is a separation of a standard mixture of all 20 PTH residues. In this degradation 30 nmol material were loaded on the sequencer. Repetitive yield was 95%. Only 5% of the material was analyzed by HPLC. Full scale of the figure is 0.050 A_{254}.

Table 1
Compilation of the data obtained by high pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC)

Step	HPLC	TLC	Proposed sequence
1	Ser, Pro, Asn	Ser	Ser, (Pro), (Asn)[a]
2	Glu, Val/Met	Glu, Met	Glu, (Met)
3	His, Val/Met	Met	His, (Met)
4	Glu	Glu	Glu
5	Thr, Asp	Asp	Thr (Asp)
6	Arg	Arg	Arg
7	Leu	Leu/Ile	Leu
8	Val/Met	Val	Val
9	Ala	Ala	Ala
10	Asn	Asn	Asn
11	Leu	Leu/Ile	Leu
12	Leu	Leu/Ile	Leu
13	Glu	Glu	Glu
14	Asn	Asn	Asn
15	Tyr	Tyr	Tyr
16	Asn	Asn	Asn
17	Lys	Lys	Lys
18	Val/Met	Val	Val
19	Phe/Ile	Leu/Ile	Ile
20	Arg	–	Arg

[a] Back hydrolysis of this step and subsequent amino acid analysis revealed the presence of alanine, aspartic acid and proline in the ratio 6 : 1 : 1. The alanine is the hydrolysis product of serine

Residues in brackets correspond to the minor components

step was serine. Two minor peaks were also present, corresponding to asparagine and proline. Step 2 yielded glutamic acid along with a small amount of methionine, also a minor component of the product of step 3. The major product of step 3 was histidine. Due to the presence of this type of residue some pre-cleavage of glutamic acid, which is the amino acid detected in the subsequent step, appeared also at step 3. Step 4 yielded only glutamic acid. Step 5 gave threonine and aspartic acid. This apparent heterogeneity could reflect either a variability of the amino-terminal sequence of the 40 k subunit or the presence of minor components that co-purify with the 40 k subunit and disappear after step 5.

4. Discussion

In this work we have established the sequence of the first 20 amino acid residues of the [^{3}H]MPTA-labelled subunit of the acetylcholine receptor. The data were obtained using amounts ranging from 200–1200 μg protein.

When a sequence determination is carried out on small amounts of material it becomes imperative to demonstrate that the data obtained concern the biologically active material. Two lines of evidence support the conclusion that we have indeed been sequencing the major subunit of the ACh-receptor protein.

1. All the purified 40 k material is labelled by MPTA.
2. After elution from the gel, this component is degraded with an absolute yield (step 2) of the automated Edman degradation which ranges from 70–80%, a value rarely exceeded with test proteins such as myoglobin or lysozyme. Even though the 40 k material gives after purification a rather diffuse band when analyzed by polyacrylamide gel electrophoresis in one dimension, it appears homogeneous as far as the major sequence is concerned. The apparent heterogeneity of molecular weight most likely results from the pH 11 treatment, since in earlier experiments where this step was omitted the 40 k band was sharper (data not shown). It is possible that the treatment at alkaline pH removes sugars from the protein moiety.

While different preparations of 40 k subunit were used for each of the 4 sequenator degradations, a number of minor residues were identified consistently in addition to the major sequence. They were at step 1 proline and asparagine, at steps 2 and 3 methionine, and at step 5, aspartic acid. Surprisingly, no additional residue was seen from step 6 on. One possibility is that an heterogeneity of the 40 k subunit of the ACh-receptor exists which manifests itself in a variability limited to the first 5 positions of the amino-terminal sequence. Another, and more likely, possibility is that the initial heterogeneity is due to a small contaminating peptide which is washed away after 5 steps. It would represent $< 30\%$ of the protein material introduced in the sequencer.

The first 20 residues of the 40 k subunit comprise a strikingly large proportion of both charged (3 acid, 4 basic amino acids) and hydrophobic residues (4 leucine or isoleucine, 2 valine, 2 asparagine).

In conclusion, the results presented here show that

the 40 000 mol. wt subunit of the ACh-receptor protein is a polypeptide chain which can be purified in sufficient quantities to be amenable to sequence determination.

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Note added in proof: In a fifth experiment none of the minor components were detected in the first five steps. They must therefore be considered as contaminants.

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