Reports

Acetylcholine Receptor: Complex of Homologous Subunits

Abstract. *The acetylcholine receptor from the electric ray* Torpedo californica *is composed of five subunits; two are identical and the other three are structurally related to them. Microsequence analysis of the four polypeptides demonstrates amino acid homology among the subunits. Further sequence analysis of both membrane-bound and Triton-solubilized, chromatographically purified receptor gave the stoichiometry of the four subunits (40,000:50,000:60,000:65,000 daltons) as 2:1:1:1, indicating that this protein is a pentameric complex with a molecular weight of 255,000 daltons. Genealogical analysis suggests that divergence from a common ancestral gene occurred early in the evolution of the receptor. This shared ancestry argues that each of the four subunits plays a functional role in the receptor's physiological action.*

The acetylcholine receptor (AcChR) is a prototype for many membrane-bound proteins that function in the nervous system by transiently altering the electrical properties of the cell membrane in response to chemical transmitters such as acetylcholine (AcCh) or to drugs. Molecular characterization of this receptor can serve as a model for interactions of small molecules with membrane-bound proteins and also for transport of ions across a biological membrane in response to these interactions. Here we give an account of new findings concerning the structure of the AcChR.

Two preparations from electric ray tissue (*Torpedo* and related species) are used for characterization of the receptor at the molecular level. (i) Detergent-solubilized AcChR purified by affinity chromatography has allowed study of the composition and structure of the protein (*1*). (ii) The AcChR is maintained in its native environment in membrane vesicles. These vesicles are purified by sucrose gradient centrifugation (*2*) followed by removal of peripheral membrane proteins (*3*). These membrane preparations contain no proteins other than the AcChR and are useful for studies in vitro of the mechanisms of AcCh binding and of receptor-associated channels for translocation of alkali metal cations.

Table 1. Properties of purified acetylcholine receptor from *Torpedo californica*.

	Structure	
Subunit composition	40 × 10^3, 50 × 10^3, 60 × 10^3, and 65 × 10^3 daltons (*10–13*)	
Subunit stoichiometry	2:1:1:1	
Molecular mass	270 ± 30 × 10^3 daltons (experimental) (*6*) 255 × 10^3 daltons (calculated)	
	Physical properties	
S value	9*S* 13.7*S* as dimer (*4*)	
Stokes radius	72 Å (*4*)	
Isoelectric point	4.9 (*4*)	
Specific activity	1 α-bungarotoxin per 110 ± 15 × 10^3 daltons*	
	Composition (residues) (7)	
	$D_{37}T_{25}S_{27}E_{35}P_{21}G_{19}A_{16}C_3V_{26}M_{10}I_{28}L_{30}Y_{13}F_{16}W_4K_{21}H_{11}R_{14}$	40K
	$D_{49}T_{27}S_{34}E_{50}P_{31}G_{28}A_{27}C_4V_{31}M_7I_{26}L_{40}Y_{15}F_{18}W_4K_{23}H_{10}R_{19}$	50K
	$D_{61}T_{28}S_{38}E_{58}P_{33}G_{34}A_{29}C_6V_{32}M_7I_{35}L_{50}Y_{19}F_{23}W_8K_{29}H_{11}R_{21}$	60K
	$D_{68}T_{30}S_{42}E_{60}P_{36}G_{35}A_{30}C_6V_{34}M_{10}I_{38}L_{53}Y_{20}F_{25}W_{12}K_{29}H_{12}R_{23}$	65K
	Summary	
Carbohydrate	~ 75 residues per molecule	of AcChR (*7*)
o-Substituted serine	~ 22 residues per molecule	
o-Substituted threonine	~ 23 residues per molecule	
Phosphoserine	~ 7 residues per molecule	

*The AcChR specifically binds α-neurotoxins with a specific activity, measured by ^{125}I-labeled α-bungarotoxin binding, as given.

The AcChR from *Torpedo californica* occurs as monomeric (9*S*) or dimeric (13.7*S*) form (*4*) and the 13.7*S* dimers are the predominant form in postsynaptic membranes (*5*) as a result of the formation of one or more specific disulfide bridges between one type of polypeptide (65 × 10^3 daltons in the 9*S* form). The 9*S* form (250 × 10^3 to 270 × 10^3 daltons) (*6*) is a glycoprotein containing small amounts of galactose, mannose, glucose, and *N*-acetylglucosamine (*7*).

The subunit structure of the AcChR has been widely debated. Affinity labeling studies initially suggested that a 40 × 10^3 dalton species was involved in AcChR function (*8*). The earliest reports of the polypeptide composition of purified preparations showed such a component in addition to other peptides of lower and higher molecular mass (*9*).

In more recent studies, several lines of evidence indicate that four peptides—40 × 10^3, 50 × 10^3, 60 × 10^3, and 65 × 10^3 daltons—are components of the native receptor. These polypeptides were first observed in highly purified membrane fragments (*10*) and in preparations purified by affinity chromatography after detergent solubilization of such membranes. Prevention of Ca^{2+}-activated proteolysis also allowed purification of AcChR from crude unfractionated membranes (*11–13*), and these preparations contained the same four subunits. In several species of electric ray the same four peptides were found as components of purified AcChR, and each peptide is immunologically cross-reactive to its counterpart among different species. However, the four peptides were not cross-reactive among themselves, an indication that they were not derived from each other by degradation (*14*). In addition, a variety of photolabeling methods (*15*) have implicated the four polypeptides as part of the receptor protein.

On the basis of this evidence we considered that the AcChR was a complex composed of four distinct polypeptides (*15*). However, the simplicity of a model in which a single 40 × 10^3 dalton subunit (*16*) was suggested to form a hexamer of 240 × 10^3 daltons (*17*) was appealing. The evidence that we present here, based on the amino acid sequence analysis of the four subunits, demonstrates that they are four distinct but homologous polypeptides (Table 1).

The AcChR was purified by sucrose gradient centrifugation of *T. californica* electroplax membranes and subsequent treatment at *p*H 11 to remove peripheral proteins (*3*). Subsequently, four AcChR subunits (40 × 10^3, 50 × 10^3, 60 × 10^3, and 65 × 10^3 daltons) were isolated by

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preparative electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels (*14*). The purified subunits were subjected to amino terminal sequence analysis by automated Edman degradation on a spinning cup sequenator as described (*18*). The sequence of each subunit was determined for 54 residues (Fig. 1). These analyses indicate the following.

1) Each of the four subunits represents a single polypeptide chain, since yields at the first step for each of the subunits ranged from 80 to 90 percent (*19*). No contaminating sequences were present at detectable levels (> 3 percent) for each subunit (*20*). We observed no sequences other than these four by amino terminal sequence analysis of both intact membrane-bound AcChR and intact AcChR purified by affinity chromatography. The high initial yields of the major sequences eliminate the possibility that another polypeptide (even with a blocked amino terminus) could be present in amounts equimolar with the four subunits.

2) The four subunits have distinct but homologous amino acid sequences (Fig. 1). At 11 of 54 positions, all four subunits have the same amino acid. The sequence identities present in the amino terminal regions of the four polypeptides range between 35 and 50 percent (Table 2). Because of the extent of sequence differences among the four subunits, it is not surprising that serological (*14, 21-23*) or peptide mapping analysis (*23*) failed to detect subunit homology. The homology alignment of the subunits in the region investigated requires the introduction of just a single (two residue) insertion in the 65×10^3 dalton subunit (Fig. 1). These data suggest that the genes encoding each of the four subunits descended from a single ancestral coding sequence.

3) The four subunits (40×10^3, 50×10^3, 60×10^3, and 65×10^3 daltons) exist in the AcChR as a 2:1:1:1 stoichiometric complex (Table 3). This subunit stoichiometry was determined by amino terminal analyses of a mixture of the four subunits in both base-extracted membrane-bound AcChR and in Triton-solubilized AcChR purified by affinity chromatography (*24*). The stoichiometry agrees with other results based on recovery of protein from SDS gel electrophoresis (*21*). It clearly demonstrates, along with the amino acid sequence homology, that the 9*S* (255×10^3 dalton) form of *T. californica* AcChR is a pentamer composed of two equivalent plus three pseudoequivalent subunits. Accordingly, these four evolutionarily related subunits appear to be fundamental components of the AcChR. Clearly, the AcChR has four distinct components and is not a single subunit type as has been suggested (*16*).

Table 2. Subunit amino terminal sequence homology (percentage).

	40K	50K	60K	65K
40K		35	39	41
50K	35		39	35
60K	39	39		50
65K	41	35	50	

The pentameric structure is consistent with independently determined values for the molecular mass of *T. californica* AcChR (*6*). These ranged from 250×10^3 to 270×10^3 compared with that of 255×10^3 calculated from the subunit molecular mass and the stoichiometry and agree with estimates made from the dimensions of the receptor particles observed in AcChR-enriched membranes (*25*). These particles have been demonstrated in electron microscopy studies of negatively stained membranes (*26*), with dimensions of the particles being ~ 85 Å in diameter for cylindrical objects containing a central depression or hole ~ 25 Å in diameter (*27*). The particles are considered to be composed of from four to six substructures, presumably subunits. Optical diffraction patterns of these membranes are consistent with a cylindrically averaged structure containing a central hole (*28*). Since all subunits are part of the functional AcChR, the particle cannot have true internal symmetry; in correspondence with this, a lattice in which they occur cannot have any true internal symmetry because one 9*S* receptor monomer represents the protein content of one unit cell.

A genealogical tree based on the evolutionary pathway by which the four contemporary subunits can be generated from a single ancestral sequence via minimum nucleotide substitution (*29*) is shown in Fig. 2. The genes encoding the four AcChR subunits appear to have been generated by fourfold gene duplication early in the evolution of the receptor and subsequently have diverged from one another. If a single early gene duplication gave rise to the genes encoding two AcChR subunits and these genes were in turn duplicated at a much later time, then the genealogical tree should show two major branches, each with minor divergences for the four subunit species. Instead, the almost total divergence initially of the four branches from one another suggests that the four subunit genes were all generated nearly simultaneously. We assume that the subunits evolved to perform discrete functions in

Table 3. Acetylcholine receptor subunit stoichiometry.

Subunit	Triton-solubilized AcChR		Membrane-bound AcChR	
	Preparation 1	Preparation 2	Preparation 3	Preparation 4
40,000	1.93 ± 0.13	1.92 ± 0.14	1.96 ± 0.04	2.05 ± 0.16
50,000	1.02 ± 0.08	1.07 ± 0.09	1.03 ± 0.04	1.02 ± 0.01
60,000	1.00 ± 0.10	1.02 ± 0.21	1.01 ± 0.03	1.00 ± 0.07
65,000	1.04 ± 0.07	1.00 ± 0.13	1.01 ± 0.08	0.93 ± 0.08

Average	
40,000	1.97 ± 0.12
50,000	1.03 ± 0.06
60,000	1.01 ± 0.10
65,000	0.99 ± 0.09

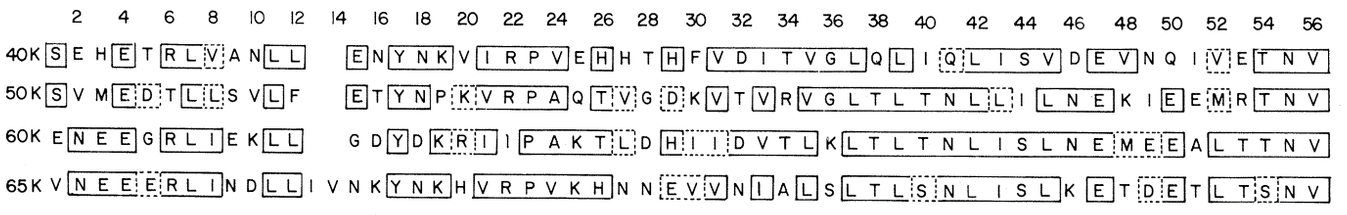

Fig. 1. Sequence homology at the amino termini of AcChR subunits. A solid box around the letters denotes identity between at least two subunits. A dotted box denotes the presence of structurally similar amino acids in the subunits.

the receptor complex. In this regard, it is important to keep in mind the distinct size differences among the subunits, for size differences as well as sequence differences characterize the subunits and presumably are related to their distinct functions.

Whether AcChR subunits also exhibit internal homology units and, if so, whether these are related to the molecular weight differences remains to be determined. That the receptor subunits may be composed of homology units invites comparison with the immunoglobulin molecules, which are composed of two distinct subunits, light and heavy polypeptides, that are evolutionarily related (*30*). The immunoglobulin polypeptides are made up of homology units about 100 amino acid residues in length (two units for light and four or five for heavy) that suggest all immunoglobulin genes descended from a common ancestral gene coding for a primordial homology unit.

The sequences from the four AcChR subunits were subjected to a homology search with sequences for all polypeptides present in the data bank of the National Biomedical Research Foundation by Dr. M. O. Dayhoff. No significant homology was detected between any of the four subunits and these other sequences.

Reports on the purification of detergent-solubilized AcChR from various sources including *Electrophorus electricus*, rat, and cat denervated muscle, and a nonfusing muscle cell line have appeared (*23, 31*). Although there is variability in the recorded values of subunit mass, the general trend in these studies appears to be that a major subunit of 40×10^3 to 45×10^3 daltons is observed in addition to lesser amounts of two to three polypeptides of greater mass. In many instances these range from 48×10^3 to 56×10^3 and from 60×10^3 to 72×10^3 daltons. Even with these limited data, it appears reasonable to suggest that AcChR from most, if not all, species will have the structure seen in *Torpedo*. This hypothesis appears particularly attractive because of the apparently very early duplication and divergence of the genes encoding *Torpedo* AcChR subunits.

Affinity labeling studies with maleimido derivatives (antagonists of AcCh) were conducted on whole cells of *E. electricus* (*8*) and were later extended to purified preparations from the same source and from *T. californica* (*11*). In all cases a single component of 40×10^3 daltons was labeled. More recently it has been shown that the agonist bromoacetylcholine also reacts specifically with SH groups on the same subunit of *Narcine braziliensis* (*13*) or *T. californica* AcChR (*32*). In view of the homologous structures of the subunits we must consider the possibility that putative binding sites may exist on the subunits other than those of 40×10^3 daltons. Such sites might bind agonists, antagonists, local anesthetics, or histrionicotoxin since all these classes of compounds are now known to associate with the AcChR protein (*2, 3, 13, 32–34*).

The single protein complex of five homologous subunits is responsible for binding of agonists (*13, 32*), cholinergic antagonists (*15*), α-toxins (*2*), histrionicotoxin (*33*), local anesthetics (*34*), and also for cation translocation (*35*). The specific elements within the complex responsible for these functions have, however, not yet been delineated. It is known that all four subunits are present on the outer (synaptic) face of the membrane (*36*) and that the complex is a transmembrane protein (*37*). It remains to be determined which polypeptides are transmembrane and what exact elements of those constitute the putative ion channel or ion discrimination site or sites, that is, whether homologous structures are part of a channel or whether unique protein structures have evolved for this function.

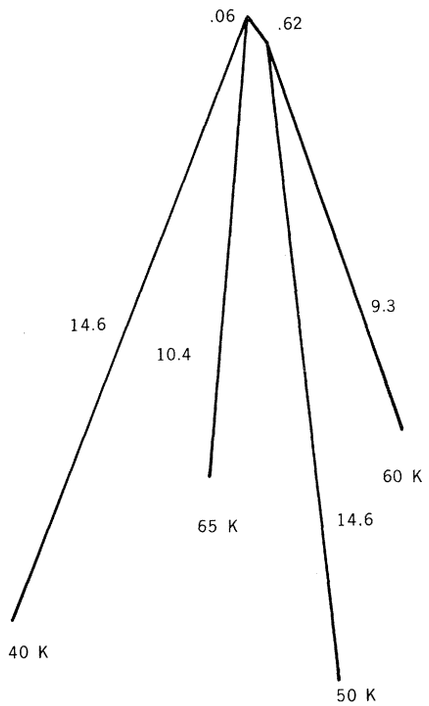

Fig. 2. A phylogenetic tree generated from the amino terminal regions of the four AcChR subunits. Each branch point represents a nodal or ancestral sequence. The four subunits are represented as the terminal twigs of the tree. The minimum number of single base substitutions separating two nodal sequences is indicated by numbers in the center of the line joining two corresponding branch points on the tree.

In conclusion we can unequivocally state, based on the work reported here, that the AcChR from *Torpedo* is a pentameric protein complex comprised of four distinct and homologous subunits that appear to be responsible for the functionality of the receptor.

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19. We calculated the percentage of protein with the major sequence for each subunit relative to the total protein applied to the sequenator. This was done by assuming 95 percent recovery of the *P*th aliphatic amino acids (*18*) and extrapolating the observed yields of these *P*th amino acids in the sequenator runs back to cycle 0. By comparing these quantities from the sequenator analysis to those for the total protein obtained by amino acid analyses of separate portions of each subunit, we obtained initial yields of 80 percent (for 9.6 nmole of 40 × 10^3 dalton subunit), 80 percent (for 3.1 nmole of 50 × 10^3 dalton subunit), 80 percent (for 2.9 nmole of 60 × 10^3 dalton subunit), and 91 percent (for 11.0 nmole of 65 × 10^3 dalton subunit).
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24. Subunit stoichiometry was calculated by comparing the yields of the *P*th amino acids assigned to positions 9, 10, and 14 of the four subunits where each of the four subunits has a different amino acid residue. The yields of the *P*th amino acids were calculated by subtracting the background *P*th amino acid levels and then correcting for the differing recoveries of the *P*th amino acids (alanine, 95 percent; serine, 35 percent; glutamic acid, 80 percent; asparagine, 65 percent; valine, 95 percent; lysine, 50 percent; aspartic acid, 60 percent; and threonine, 40 percent) observed in sequenator analysis of the isolated subunits. These values represent the efficiency of conversion of the amino acid thiazolinones to the corresponding *P*th amino acids in the sequenator.
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Probing the Component Structure of a Maize Gene with Transposable Elements

Abstract. *Three instances of* R *gene instability were found in maize stocks carrying the controlling elements Dissociation (*Ds*) and Modulator (*Mp*). In each,* Ds *or a* Ds*-like element had transposed to* R*, inhibiting kernel pigmentation irregularly. When* Mp *was removed from the genome,* R *expression stabilized at low to intermediate levels. Strong pigmenting action was restored through recombination in heterozygotes of the three new forms with an* R *allele that specifies only plant pigmentation. The sites of* Ds *insertion mapped distal to the region that specifies seed versus plant expression. The evidence suggests that an* R *functional unit consists of one component that both governs tissue-specific expression and another that is common to alleles of different tissue-specific activities.*

Controlling elements are mobile units in the maize (*Zea mays*) genome that alter the expression of standard genes. The behavior of a number of different controlling elements, some acting singly and others as interacting pairs, has been worked out in detail (*1*). In the present study, controlling elements were used to probe the organization of a maize gene. The approach is analogous to the use made of transposable insertion sequences in *Escherichia coli* for determining the arrangement of ribosomal protein genes in operons (*2*).

The target locus *R* is one of a series of genes required for anthocyanin (flavonoid pigment) formation. The *R* alleles collected from diverse sources differ in distribution, intensity, and time of pigment formation (*3*). A given allele may comprise one or more units of independent function. Stadler (*4*) designated these units as genic elements since the individual elements of compound *R* alleles behave as separate genes. It has been shown that a number of alleles composed of multiple elements carry duplicated chromosome segments (*5*). An allele consisting of a single genic element was chosen for the present study in order to avoid the complexities attending chromosome rearrangement and multiple *R* elements. This allele, *R-sc:124*, specifies intense pigmentation both in the outer layer of the seed's endosperm (the aleurone) and the scutellum of the embryo (*6*). (Vegetative parts of the plant are not colored.) The element is designated (Sc) for self-colored, denoting the solid appearance of kernels. It contrasts with elements that effect pigmentation either in plant tissues exclusively or in some combination of kernel and plant tissues (*7*).

Are there, in addition to components that distinguish *R* elements, ones that are not tissue-specific in their effect? The latter might code, for example, for a product affecting a given step in pigment synthesis in any plant or seed part. Such a component would have gone undetected if it were invariant in the alleles studied so far.

Mutations in *R* were induced in a search for components whose effects are not tissue-specific. The general plan was to establish variants of a given *R* element that are of null or low-level activity and then to map the site of mutation relative to the site of the tissue-specific com-

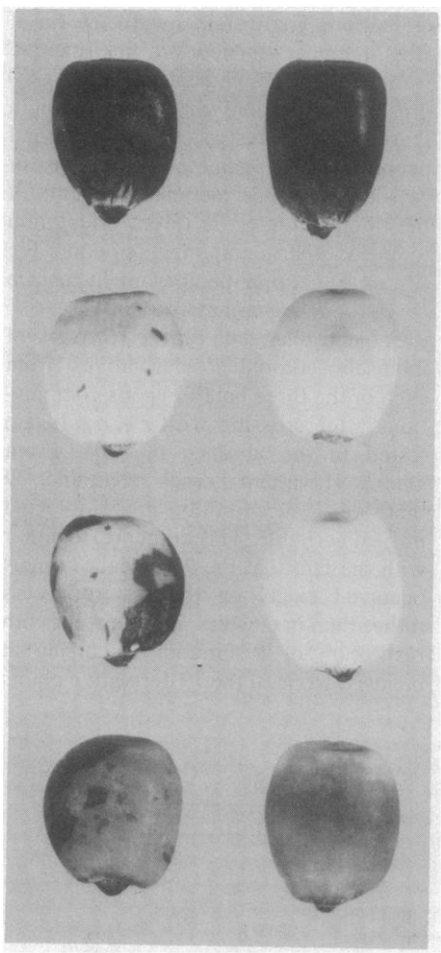

Fig. 1. Variations in aleurone pigmentation of maize endosperm due to controlling element action at the *R* locus. The *R* alleles are parental *R-sc* (top row) through *r*-mutable phenotypes m1, m3, and m9 (bottom row). In the left column, *Mp* is present in the genome; in the right column it is absent. (Pericarps have been removed.)

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