

Analysis of Early Events in Acetylcholine Receptor Assembly

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Abstract. Mammalian cell lines expressing nicotinic acetylcholine receptor (AChR) subunit cDNAs from *Torpedo californica* were used to study early events in AChR assembly. To test the hypothesis that individual subunits form homooligomeric intermediates before assembling into $\alpha_2\beta\gamma\delta$ pentamers, we analyzed the sedimentation on sucrose density gradients of each subunit expressed separately in cell lines. We have shown previously that the acute temperature sensitivity of *Torpedo* AChR subunit assembly is due, in part, to misfolding of the polypeptide chains (Paulson, H. L., and T. Claudio. 1990. *J. Cell Biol.* 110:1705–1717). We use this phenomenon to further analyze putative assembly-competent intermediates. In nonionic detergent at an assembly-permissive temperature, the majority of α , β , γ , and δ subunits sediment neither as 3–4S monomers nor as 9S complexes, but rather as 6S species whether synthesized in fibroblasts, myoblasts, or differentiated myosyncytia. Several results indicate that

the 6S species are complexes comprised predominantly of incorrectly folded subunit polypeptides. The complexes represent homoaggregates which form rapidly within the cell, are stable to mild SDS treatment and, in the case of α , contain some disulfide-linked subunits. The coprecipitation of α subunit with BiP or GRP78, a resident protein of the ER, further indicates that at least some of these internally sequestered subunits also associate with an endogenous protein implicated in protein folding. The majority of subunits expressed in these cell lines appear to be aggregates of subunits which are not assembly intermediates and are not assembly-competent. The portion which migrates as monomer, in contrast, appears to be the fraction which is assembly competent. This fraction increases at temperatures more permissive for assembly, further indicating the importance of the monomer as the precursor to assembly of $\alpha_2\beta\gamma\delta$ pentamers.

A problem currently of interest to cell biologists is the assembly of multimeric membrane proteins (Rose and Doms, 1988; Hurlley and Helenius, 1989). The molecular and cellular phenomena involved in the assembly process have been most extensively studied with viral membrane proteins composed of identical subunits, such as the hemagglutinin trimer of influenza virus (Gething et al., 1986; Copeland et al., 1986) and the G protein of vesicular stomatitis virus (Kreis and Lodish, 1986; Doms et al., 1987). Among eukaryotic proteins, the nicotinic acetylcholine receptor (AChR)¹ provides a model membrane protein complex with which to examine the assembly of heterologous subunits. The AChR from skeletal muscle and *Torpedo* electric organ is the most extensively studied member of a gene superfamily of ligand-gated ion channels, which also includes neuronal AChRs, GABA_A, and glycine receptors (reviewed in Claudio, 1989). The AChR is an intrinsic membrane glycoprotein composed of four different (although homologous) subunits with apparent molecular masses of ~40 (α), 50 (β),

60 (γ), and 65 (δ) kD, which together form an $\alpha_2\beta\gamma\delta$ pentamer of ~250 kD (for reviews see Popot and Changeux, 1984; Karlin et al., 1986; McCarthy et al., 1986; Maelicke, 1988; Claudio, 1989).

Most known ligand-gated ion channels are heterooligomers. Expression studies in *Xenopus* oocytes or transfected somatic cells have shown that a single subunit type usually is not sufficient to generate a surface-expressed, functional channel complex (Mishina et al., 1984; Mixter-Mayne et al., 1987; Sumikawa and Miledi, 1989; Pritchett et al., 1989) although partially functional homooligomeric channels have been observed (Boulter et al., 1987; Sontheimer et al., 1989). The muscle or electroplax AChR is an example of an obligate heterooligomer, as it appears to require at least three of its four subunit types in order to form functional complexes (Mishina et al., 1984; Mixter-Mayne et al., 1987; Sumikawa and Miledi, 1989). It is nevertheless unclear whether early in receptor biogenesis, AChR subunits form homooligomeric complexes before assembling into proper $\alpha_2\beta\gamma\delta$ pentamers. What is known about the assembly of the AChR largely concerns the biosynthesis and posttranslational maturation of the α subunit, the subunit which binds both acetylcholine and the high affinity antagonist α -bungarotoxin (BuTx). In the murine nonfusing muscle cell line BC₃H-1, it has been shown

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; BiP, binding protein; BuTx, α -bungarotoxin; DSP, dithiobis(succinimidylpropionate); LB, lysis buffer (1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% NaN₃, 10 μ g/ml gelatin).

that nascent α polypeptide sediments as a 5S species, does not associate with heterologous subunits and does not bind BuTx (Merlie and Lindstrom, 1983). Approximately 15 min after synthesis, unassembled α subunits acquire the ability to bind BuTx, and ~ 75 min later, assemble into 9S pentamers. The 5S sedimentation of unassembled α suggests that newly synthesized subunits do not first associate as large homooligomers, although 5S is larger than expected for a monomer. In a series of cell-free translation studies, Anderson and Blobel (1983) found that each of the four *Torpedo* subunits formed large, apparently homooligomeric, complexes sedimenting broadly at ~ 7 -13S. Their data led them to propose that newly synthesized AChR subunits form homooligomers before associating with heterologous subunits. It has not been possible to test this proposal rigorously, but a recent study showed that a single subunit of the glycine receptor also forms homooligomers, or homo-aggregates, when expressed by itself (Sontheimer et al., 1989).

In vivo, it is difficult to establish whether individual AChR subunits form homooligomers before assembling because interactions with heterologous subunits cannot be prevented. Moreover, the in vitro translation experiments described above are limited by the fact that functional channel expression cannot be achieved in such a system. Using transfection and/or viral infection, we have established mammalian fibroblast and muscle cell lines which express one or multiple *Torpedo* AChR subunits cDNAs (Claudio, 1987; Claudio et al., 1987, 1989a,b; Green et al., 1991; Ross et al., 1991). Fibroblasts containing the four different subunit cDNAs express fully functional AChRs on the cell surface (Claudio et al., 1987; Sine et al., 1990), demonstrating that the mouse fibroblast can execute proper AChR subunit biosynthesis and assembly, as well as transport and insertion of the complex into the plasma membrane. In the experiments described here, we use several of these cell lines to investigate the events preceding heterologous subunit assembly. Our expression system affords us the advantages of being able to analyze a given subunit's behavior in the absence of heterologous subunits and to analyze interactions between subunits and cellular proteins that may participate in the assembly process.

Another advantage of this system is the temperature sensitivity of *Torpedo* AChR assembly. At 37°C no assembly of subunits occurs, however, by lowering the temperature to 20°C efficiencies of ~ 20 -35% can be achieved (Ross et al., 1991). Mouse AChR in muscle cell lines also assembles with an efficiency of ~ 30 % but its assembly is not temperature sensitive and it cannot be regulated by this mechanism. Using our temperature-sensitive assembly system, by analyzing several properties of a single subunit expressed at different permissive and nonpermissive temperatures, we are better able to distinguish between functional and nonfunctional assembly intermediates. The similarities among *Torpedo* AChRs, mouse AChRs, and the rest of the ligand-gated ion channels suggests that the assembly scheme established for *Torpedo* AChRs will have general implications for at least this superfamily of channels.

Materials and Methods

Cell Lines

The *Torpedo* AChR subunit cDNAs used in establishing the various cell

lines have already been described (Claudio, 1987). Cell lines stably expressing individual subunits were established either by transfection using calcium phosphate coprecipitation or by viral infection using packaged retroviral recombinants (Claudio et al., 1987, 1988, 1989a,b). For viral infections, we used the direct orientation retrovirus vectors pDOL (Korman et al., 1987), pDOI (generously provided by J. Morgenstern and H. Land, Imperial Cancer Research Fund, London, United Kingdom, and also referred to as pDORneo, Penn et al., 1990), and pDOP (generously provided by R. C. Mulligan, The Whitehead Institute, Cambridge, MA), which permit the simultaneous expression of the neomycin resistance (*neo*^r) selectable marker gene and an inserted sequence. In all three vectors, the long terminal repeat from Moloney murine leukemia virus controls expression of the AChR subunit cDNA and an internal SV-40 promoter drives expression of the *neo* gene. pDOL- α , pDOL- β , pDOI- α , or pDOP plasmid DNA was transfected onto the packaging cell line φ_2 (Mann et al., 1983), virus was harvested, and used to infect NIH3T3 or rat L6 muscle cells. Several hundred colonies were pooled to establish the 3T3-DOL- α_{mass} and 3T3-DOL- β_{mass} cell lines whereas pDOL- α_1 , pDOL- α_2 , pDOL- β_4 , 3T3-DOI- α_3 (Claudio et al., 1989a), and L6-DOP (L6 cells containing pDOP vector sequences without cDNA insert; Claudio et al., 1988) are each clonal isolates. For transfection experiments, we used a pSV2 expression vector in which the SV-40 early promoter is located 5' to a subunit cDNA or the *neo*^r gene (constructions are described in Claudio et al., 1987). 3T3-pSV2- $\gamma_{1,2}$ was established by cotransfection of pSV2- γ and pSV2-*neo* onto NIH3T3 cells. L-pSV2- γ_5 and L-pSV2- δ_2 were established by cotransfection of pSV2- γ or pSV2- δ with the thymidine kinase gene (*tk*) into cells deficient in *tk* and adenine phosphoribosyltransferase (Ltk⁻aprt⁻ cells; Claudio et al., 1989a).

Cell Culture

Cell lines were grown at 37°C in the presence of 5% CO₂ in DMEM supplemented with 10% calf serum (3T3 and L cells) or 10% fetal calf serum (L6 and BC₃H-1 cells). The L-pSV2- δ_2 and L-pSV2- γ_5 cell lines, originally selected in HAT (15 μ g/ml hypoxanthine, 1 μ g/ml aminopterin, and 5 μ g/ml thymidine), were grown in the continued presence of HAT. L6 myoblasts were induced to differentiate by removing the normal growth medium from 70% confluent cells and replacing it with DMEM containing 2% horse serum. Cells typically were well fused within 4-5 d. Only plates on which 70% of the cells had fused into large myosyncytia (containing 8-30 nuclei per syncytium) were used in experiments requiring differentiated L6 cells. Confluent dishes of BC₃H-1 cells were induced to differentiate by replacing normal growth medium with DMEM containing 1% fetal calf serum. For experiments in which the temperature sensitivity of subunit assembly was analyzed, cells were grown at 20 or 26°C in the presence of 5% CO₂. 10 mM sodium butyrate (Sigma Chemical Co., St. Louis, MO) was added to the medium of cell lines containing subunit cDNAs under the control of an SV-40 promoter (3T3-pSV2- $\gamma_{1,2}$, L-pSV2- γ_5 and L-pSV2- δ_2) in order to increase subunit mRNA levels and polypeptide expression (Claudio et al., 1987).

Labeling and Solubilization

70-80% confluent 10-cm dishes of 3T3 cells or undifferentiated L6 myoblasts, or 100% confluent dishes of L cells, BC₃H-1 cells, or differentiated L6 myotubes, were incubated 15 min in leucine-free medium, then 20 min with 2 ml of leucine-free medium containing 400 μ Ci of [³H]leucine (Amersham Corp., Arlington Heights, IL). In other experiments, cells were labeled with 330 μ Ci of [³⁵S]methionine (³⁵S-TRANS; ICN Biochemicals, Irvine, CA) in methionine-free medium. Unless specified otherwise, labeling was performed at 37°C. After labeling, cells were rinsed twice with 4°C PBS and lysed with 740 μ l of lysis buffer (LB: 1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% NaN₃, 10 μ g/ml gelatin). 2 mM phenylmethylsulfonyl fluoride (Calbiochem-Behring Corp., San Diego, CA), 2 mM *N*-ethylmaleimide, and 10 μ g/ml each of chymostatin, leupeptin, pepstatin, and tosyl-lysine chloromethyl ketone (Sigma Chemical Co.) were added to the lysis buffer immediately before use. In experiments where subunits were analyzed under nonreducing conditions, 10 mM iodoacetamide was included in the initial lysis buffer. In experiments testing the effect of low levels of SDS on subunit sedimentation, cells were lysed with LB containing 0.1% SDS. At 4°C, cell lysates were vortexed several times over a 10-30-min period, spun 10 min in an Eppendorf centrifuge (model No. 5414; Brinkmann Instruments Co., Westbury, NY), and the supernatants collected. This lysis procedure completely solubilized all four subunit types from the insoluble pellet, whether cells had been grown and labeled at 20, 26, or 37°C. Samples were next precleared by incubation for 30 min at 4°C

with 40 μ l of a 1:1 solution of protein A-Sepharose (Sigma Chemical Co.) or goat anti-rat IgG (Cappel Laboratories, Malvern PA) coupled to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.), and the recentrifuged for 60 s. In our initial experiments, the resultant supernatants were also centrifuged at 100,000 g for 30 min. Regardless of whether this step was included, labeled subunit sedimented identically on sucrose gradients, hence the 100,000 g spin was eliminated from later experiments. Lysates typically had a protein concentration of 9–11 mg/ml as determined by the method of Lowry et al. (1951).

Gradient Centrifugation

500 μ l of cell lysate (total lysate volume from a 10-cm plate was usually \sim 900 μ l) was layered onto 12.5-ml 5–20% continuous sucrose density gradients. Gradients were generated in LB containing 100 mM NaCl instead of 150 mM NaCl. In experiments testing the effect of SDS on subunit sedimentation, 0.1% SDS was included throughout the gradient as well as in the lysate. Gradients were spun at 5°C in a rotor (SW41; Beckman Instruments, Inc. Palo Alto, CA) for a total $\omega^2 t$ of 8.66×10^{11} to 1.20×10^{12} . Fractions collected from the top of the gradient were then immunoprecipitated with the appropriate antiserum. $S_{20,w}$ values were calculated for the midpoint of each fraction according to the method of McEwen (1967). Calculations were corroborated by comparing the sedimentation of markers of known S value 4S human hemoglobin and 11S bovine liver catalase (Boehringer Mannheim Diagnostics, Inc., Houston, TX), and in most cases by running a parallel sucrose gradient containing either 125 I-BuTx-labeled native *Torpedo* AChR (kindly provided by Arthur Karlin, Columbia University, New York, NY), which sediments as two peaks of 9S (250 kD “monomer”) and 13S (500-kD disulfide-lined “dimer”), or *Torpedo* AChR expressed in fibroblasts, which sediments only as the 9S species (Claudio et al., 1987). Pellets at the bottom of the tube were occasionally resuspended in SDS and immunoprecipitated to assay for the presence of subunit. Variable but small (usually \sim 2–5% but always $<$ 20%) amounts of each subunit could be found in the pellet. In some experiments, cells were solubilized with the detergent octaethylene glycol dodecyl ether ($C_{12}E_8$; Calbiochem-Behring Corp.), and centrifuged on gradients containing $C_{12}E_8$. The lysis buffer contained 10 mM $C_{12}E_8$, 100 mM NaCl, 20 mM Hepes, 5 mM EDTA, 0.02% NaN_3 , with protease inhibitors added immediately before use. $C_{12}E_8$ gradients were generated in a solution containing 3 mM $C_{12}E_8$ and 50 mM NaCl, but otherwise identical to the lysis buffer.

Immunoprecipitation and SDS–Gel Electrophoresis

Portions of lysates or fractions from sucrose gradients were immunoprecipitated with rabbit polyclonal antisera directed against denatured α , β , γ , or δ subunits of *Torpedo* AChR (Claudio and Raftery, 1977), or with the rat mAb 147 and 64 (generously provided by Jon Lindstrom, The Salk Institute, La Jolla, CA), which are specific for *Torpedo* and mammalian α subunits, respectively (Tzartos et al., 1982; Kordossi and Tzartos, 1987) or mAb 168 (generously provided by Socrates Tzartos, The Hellenic Pasteur Institute, Athens, Greece) which is specific for *Torpedo* β subunit. Immunoprecipitations of heavy chain binding protein (BiP) were performed using an anti-BiP mAb (Bole et al., 1986) kindly provided by David Bole (Yale University, New Haven, CT). After incubation with antisera at 4°C for 6 h, lysates were incubated 3–5 h with 50 μ l of twofold diluted protein A-Sepharose or, for mAbs 147 and 64, goat anti-rat Sepharose. For immunoprecipitations of gradient fractions, Sepharose pellets were washed four times with 800 μ l of LB. For other immunoprecipitations (unless specified otherwise), the first two washes were with LB containing 0.5 M NaCl and 0.1% SDS, the last two with LB containing 0.1% SDS. Protein was eluted from the Sepharose pellet in SDS–gel electrophoresis sample buffer and run on 7.5 or 10% discontinuous SDS–polyacrylamide gels as previously described (Claudio, 1987), except that samples were not heated to 100°C before electrophoresis (except where indicated). Gels were fixed, incubated with Fluoro-Hance (Research Products International, Mount Prospect, IL), as specified by the supplier, dried, and exposed to Kodak XAR film at $-70^\circ C$. In some experiments, lysates were denatured in 1% SDS at room temperature for 45 min then renatured in 5% Triton X-100 before immunoprecipitation at 4°C (stringent conditions). In experiments where subunits were analyzed under nonreducing conditions, 10 mM iodoacetamide was included in the initial lysis buffer and added again both before the denaturation step prior to immunoprecipitation and before gel electrophoresis.

Toxin Binding

Lysates of 3T3-DOL- α_{mass} and 3T3 cells were incubated with 50 nM

125 I-BuTx (specific activity of 140–170 cpm/fmol; ICN Biochemicals Inc., Irvine, CA) for 6 h, centrifuged on gradients 21 h for a total $\omega^2 t$ of 1.2×10^{12} , and fractions immunoprecipitated with α antiserum. The immunoprecipitates were then counted in a gamma counter and analyzed by gel electrophoresis and immunoblotting. Preliminary binding experiments with 3T3-DOL- α_{mass} membranes indicated that association of BuTx (4.7 nM) is $>$ 95% complete within 8–10 h at 4°C (data not shown).

Cross-linking

Dithiobis(succinimidyl propionate) (DSP) (Pierce Chemical Co., Rockford, IL) was used to cross-link cell lysates or gradient fractions. Aliquots of metabolically labeled cell extracts or gradient fractions were diluted threefold with LB and cooled on ice. DSP, freshly made as 10 or 100 mM stocks in dimethylsulfoxide, was added to the desired final concentration, samples were immediately vortexed, then continuously rotated for 30 min at 4°C. Samples were next denatured in 1% SDS (in the presence of iodoacetamide), immunoprecipitated under stringent conditions, and analyzed on 7.5% SDS–polyacrylamide gels under nonreducing and reducing conditions. Bovine liver catalase (Boehringer Mannheim Diagnostics, Inc.) and purified *Torpedo* AChR were cross-linked as positive controls, and yielded discreet cross-linked products at $<$ 100 μ M DSP in the same buffers, while bovine serum albumin (Sigma Chemical Co.) did not cross-link at these concentrations.

Immunoblots

Samples were electrophoresed on 7.5 or 10% SDS–polyacrylamide gels then transferred to ZetaBind filters (American Laboratory Supply, Natick, MA) in a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA) for 6 h at 4°C according to the manufacturer's instructions. ZetaBind filters were quenched overnight at 45°C with 5% Carnation nonfat dry milk in PBS with 0.05% NaN_3 . All subsequent steps were performed at room temperature. Filters were incubated with α antiserum (diluted 1:500 in 2% milk in PBS) in heat-sealed bags for 3–6 h. After a 1 \times 15-min wash with PBS containing 0.1% Triton X-100 and 3 \times 15-min washes with PBS/milk, filters were incubated with 125 I-protein A (Amersham Corp.) for 150 min. 1.0×10^6 cpm of 125 I-protein A (specific activity of 30 mCi/mg) were used per lane. Filters were washed in PBS 1 \times 15 min, 1 \times 10 h, 2 \times 15 min, and then exposed to Kodak XAR film at $-70^\circ C$.

Membrane Vesicle Preparation

3T3-DOL- α_{mass} or 3T3 cells incubated at 26°C were scraped from a 10-cm dish into 500 μ l of 0°C 10 mM phosphate buffer containing 10 mM EDTA and 10 mM iodoacetamide. On ice, cells were homogenized with 12 strokes in a 1 ml Dounce homogenizer (Wheaton Industries, Inc., Millville, NJ) using a tight fitting pestle. The homogenate was pelleted in an airfuge (Beckman Instruments, Inc.) at 30 p.s.i. for 10–15 min at 4°C, and resuspended in LB supplemented to 3% with Triton X-100 and containing protease inhibitors and 10 mM iodoacetamide. Aliquots of the lysate were made 1 \times in SDS–gel electrophoresis sample buffer and electrophoresed on 7.5% SDS–polyacrylamide gels under nonreducing and reducing conditions.

Results

Based on an analysis of *Torpedo* subunits synthesized in vitro, Anderson and Blobel (1983) proposed that early in receptor biosynthesis, each AChR subunit self-associates as large (\sim 9S) homooligomers. Presumably these multimers would disassemble as appropriate contacts with heterologous subunits were established. If homooligomer formation does occur before AChR assembly, then cells synthesizing a single subunit should express a stable pool of homooligomers which could be detected by velocity sedimentation analysis. We thus performed a series of experiments designed to answer whether any of the *Torpedo* AChR subunits sediment on sucrose density gradients as complexes of identical subunits.

The cell lines used in this study each contain and express one of the four AChR subunit cDNAs from the electric ray *Torpedo*. When examined by SDS–PAGE, the expressed subunits either comigrate precisely with subunits isolated from

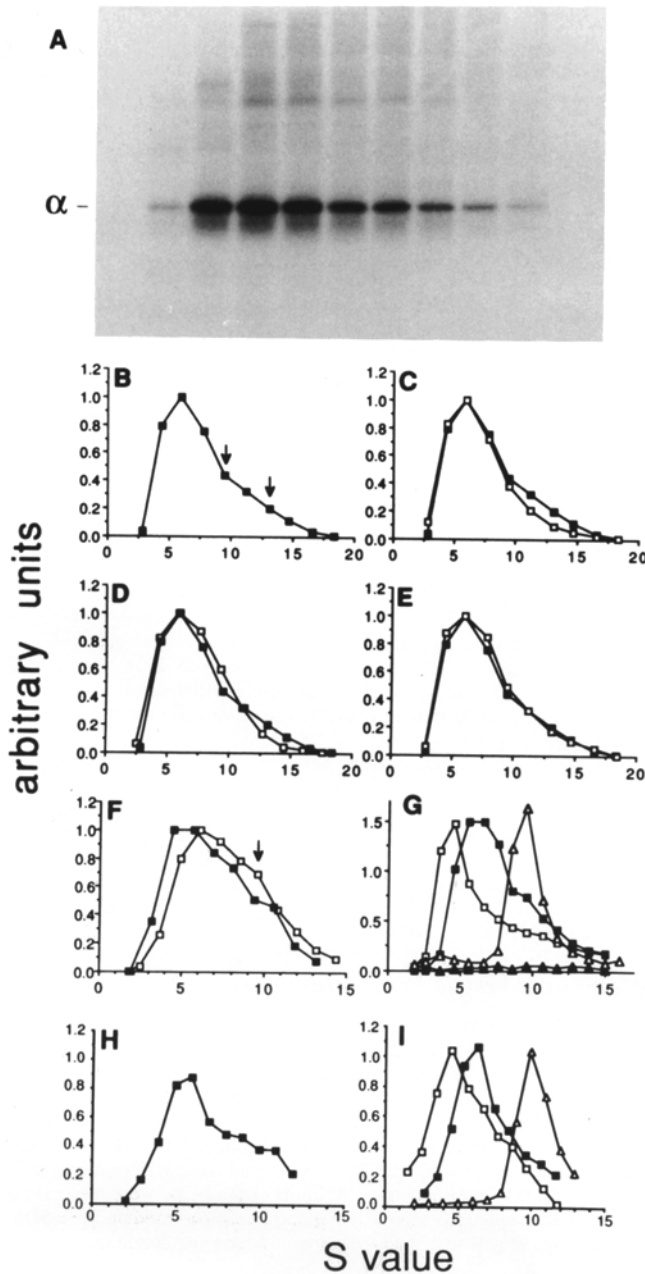


Figure 1. Velocity sedimentation analyses of *Torpedo* AChR α subunit expressed in mammalian cells and of mouse myocyte BC₃H-1 α subunit. (A) Fluorograph and (B) sedimentation profile illustrating the 6S sedimentation of α synthesized in 3T3-DOL- α_{mass} fibroblasts. Cells were labeled 20 min with [³H]leucine at 37°C, solubilized in LB, and centrifuged on 5–20% sucrose density gradients. α subunit was immunoprecipitated from gradient fractions and analyzed by SDS-PAGE and fluorography. Out of a total of 13 fractions, fractions 2–12 (top to bottom of gradient) are shown. Fractions 1 and 13 contained negligible amounts of α subunit and are not shown. The sedimentation profile in B was obtained by densitometrically scanning the α bands in A, and plotting the relative density of each band as a function of S value, calculated for the midpoint of each fraction. In this and subsequent gradients, the calculated S values corresponded accurately to known sedimentation markers including ¹²⁵I-BuTx-labeled *Torpedo* AChR (9S and 13S monomer and dimer, respectively, indicated by arrows in B). (C–E) Sedimentation profiles showing that α subunit sediments as a 6S species when the lysate is diluted fivefold with LB for 1 h before centrifugation (C, open squares), when the lysate and gradient are supple-

Torpedo electroplaque (α , β , δ) or migrate slightly faster (γ) (Claudio et al., 1987). Experiments with tunicamycin and endoglycosidase H indicate that in these cells, all four subunit types are translocated into the membrane of the ER and acquire the correct number of N-linked oligosaccharides (Claudio et al., 1989a). For the purpose of this study, it is important to note that *Torpedo* subunits can assemble into fully functional AChR in mouse fibroblasts (Claudio et al., 1987; Sine et al., 1990). Given this fact, if homooligomers do play a functional role in the assembly process, we should be able to detect them in fibroblasts expressing individual subunits.

Torpedo α Sediments As a 6S Complex

α -expressing 3T3 cells were metabolically labeled, solubilized in a Triton X-100 lysis buffer (LB), centrifuged on a 5–20% sucrose density gradient, and subunits in each gradient fraction were immunoprecipitated and analyzed by SDS-PAGE and fluorography (Fig. 1 A). Densitometric scanning of the α bands revealed that α sedimented predominantly at 6S, although the profile was polydisperse and extended broadly toward higher S values (Fig. 1 B). Under the same solubilization and gradient conditions, purified *Torpedo* AChR sedimented appropriately in two peaks at 9S and 13S, representing receptor monomer (~250 kD pentameric complex) and disulfide-linked dimer (~500 kD) (arrows in Fig. 1 B).

A sedimentation peak at 6S is consistent with small oligo-

mented with 0.1% SDS (D, open squares), or when *Torpedo* α is expressed in L6 myoblasts instead of fibroblasts (E, open squares). In C–E, the standard sedimentation profile from B (filled squares) is shown for comparison. (F) Sedimentation profile showing that fibroblast-expressed *Torpedo* α (filled squares) sediments similarly to endogenous α in BC₃H-1 myocytes (open squares). Cells were labeled 6 min with [³H]leucine at 37°C, solubilized in LB, and centrifuged in parallel. The arrow indicates the sedimentation on a parallel gradient of ¹²⁵I-BuTx-labeled AChR from BC₃H-1 cells. (G) Sedimentation experiment showing that the subset of α subunit displaying high affinity binding of BuTx sediments as a smaller species than most α subunit. 3T3-DOL- α_{mass} and 3T3 fibroblasts at 26°C were solubilized in LB and incubated with 50 nM ¹²⁵I-BuTx before centrifugation. Gradient fractions were then immunoprecipitated with α antiserum. After the immunoprecipitated ¹²⁵I-BuTx counts in each fraction were determined, total α subunit was quantitated by immunoblot. The percentage of α subunit possessing high affinity BuTx binding was calculated to be <3% of total α subunit. Total α (filled squares) subunit (arbitrary units); (open squares) ¹²⁵I-BuTx cpm ($\times 10^{-3}$) precipitated from 3T3-DOL- α_{mass} gradients in which each point represents the mean of two parallel gradients differing by <5% in all cases; (filled triangles) background cpm ($\times 10^{-3}$) precipitated from 3T3 gradient; (open triangles) ¹²⁵I-BuTx-labeled *Torpedo* AChR from all 11 fibroblasts sedimented on a parallel gradient (cpm $\times 10^{-4}$). (H) Sedimentation profile showing that γ subunits at 20°C migrate as a 6S species. L-pSV2- γ_5 cells were labeled with [³⁵S]methionine for 48 h at 20°C and γ subunits were immunoprecipitated with γ -specific mAb 168 after sedimentation. (I) Same as G with the following modifications. 3T3-DOL- α_{mass} cells were grown to confluence, shifted to 20°C for 3 d, and labeled with 100 μ Ci/ml [³⁵S]methionine for the last 24 h. Cells were lysed and labeled with 50 nM ¹²⁵I-BuTx before centrifugation. Fractions were immunoprecipitated as in G and analyzed by SDS-PAGE. The α subunit band was quantified by densitometry.

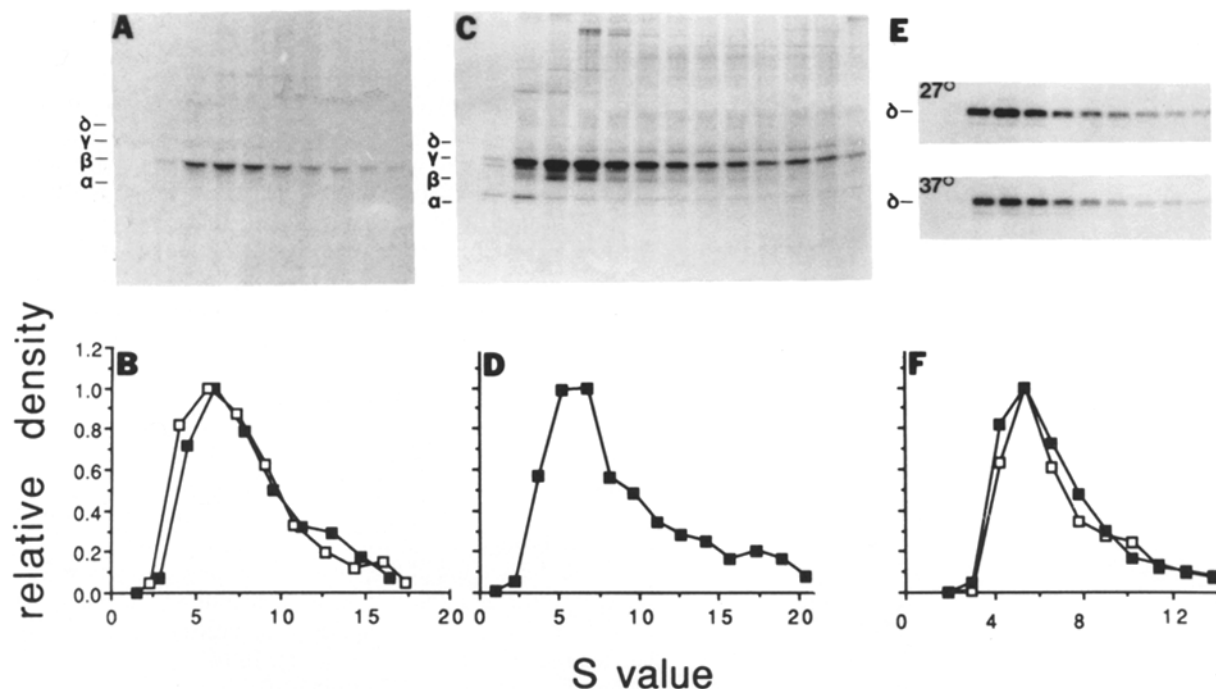


Figure 2. Sedimentation of *Torpedo* β , γ , and δ subunits individually expressed in mammalian fibroblasts. (A) Fluorograph illustrating the sedimentation of β subunit in 3T3-DOL- β_{mass} cells. Cells were labeled 20 min with [^3H]leucine at 37°C, solubilized in LB, and analyzed on a sucrose density gradient as described in the legend to Fig. 1 A. (B) Sedimentation profile of β subunit in the preceding fluorograph (filled squares) is compared to that of β subunit expressed in L6-DOL- β myoblasts (open squares). Myoblasts were labeled, extracted, and analyzed on a gradient as in A. (C and D) Fluorograph and profile illustrating the sedimentation of γ subunit expressed in 3T3-pSV2- $\gamma_{1,2}$ cells. Sedimentation analysis was performed as in A, except cells were labeled with [^{35}S]methionine. (E) The relevant portions of fluorographs illustrating the sedimentation of δ subunit synthesized at 37 and 27°C. Parallel dishes of L-pSV2- δ_2 cells were incubated overnight at 37 or 27°C, labeled 20 min with [^3H]leucine, solubilized in LB, and centrifuged on parallel gradients. (F) Sedimentation profile of δ in E: (filled squares) 37°C; (open squares) 27°C. In A, C, and E, the positions of *Torpedo* AChR standards are indicated; in C, the band migrating close to the β position is a variant γ band which is an artifact of the electrophoresis procedure.

mers of α , or with limited associations between α subunit and endogenous cellular proteins. To ensure that the 6S species represented fully solubilized subunit, we altered the amount and type of detergent used. If the cell lysate was diluted fivefold in LB (Fig. 1 C) or centrifuged on gradients containing 0.1% SDS in addition to 1% Triton X-100 (Fig. 1 D), a 6S sedimentation profile was still obtained. Our 6S profile differs from those obtained by Anderson and Blobel (1983) who found that after synthesis in a cell-free translation system, *Torpedo* subunits sedimented broadly from ~ 7 to 13S with a peak at $\sim 9.5\text{S}$. Using conditions similar to those of the cell-free study (same detergent, buffers, and gradients), we typically observed a 6S sedimentation peak for *Torpedo* subunits and only obtained peaks $>7\text{S}$ if the detergent to protein ratio was reduced (not shown); presumably this is due to insufficient solubilization. A study of the sedimentation profiles of newly synthesized (6 min pulse; Fig. 1 F) and steady-state (immunoblot of the entire cellular pool; Fig. 1 G) α showed nearly identical sedimentation patterns for *Torpedo* α . These results suggest that the individually expressed subunits rapidly and stably form a 6S species. Furthermore, using our solubilization and gradient conditions, newly synthesized mouse α in BC₃H-1 myocytes also sediments as a 6S species (Fig. 1 F), demonstrating that the sedimentation of *Torpedo* α expressed in a fibroblast closely parallels that of mammalian α expressed in intact muscle cells. Since neither *Torpedo* α expressed in mammalian cells nor BC₃H-1

α form large 9S complexes, these results suggest that large complexes such as homopentamers do not play a role in assembly.

Assembly-competent α Sediments at 4S

Do the 6S complexes represent productive intermediates in the assembly process? To address this question, we selectively examined the sedimentation of a subset of *Torpedo* α subunits that are likely to be assembly competent: those that bind the AChR antagonist BuTx with high affinity. Our assumption that this high affinity BuTx-binding subset of α subunits is assembly competent is based on observations in BC₃H-1 myocytes (Merlie and Lindstrom, 1983). Newly synthesized α cannot bind BuTx but it later acquires high affinity BuTx-binding before assembling with β , γ , and δ subunits. We previously determined the BuTx-binding capabilities of fibroblast-expressed *Torpedo* α subunits (Paulson and Claudio, 1990). A broad binding profile was observed with specific binding detected from ~ 0.1 nM to 1 μM and half-maximal binding at $\sim 4 \times 10^{-7}$ M. Approximately 2–3% of α subunits existed in a high affinity (defined as ~ 1 nM) BuTx-binding conformation. In the present experiments, we selectively examined the high affinity BuTx-binding subset by incubating 3T3-DOL- α_{mass} cell lysate with 50 nM [^{125}I]BuTx, sedimenting the lysate on gradients, then immunoprecipitating gradient fractions. Using this procedure, the

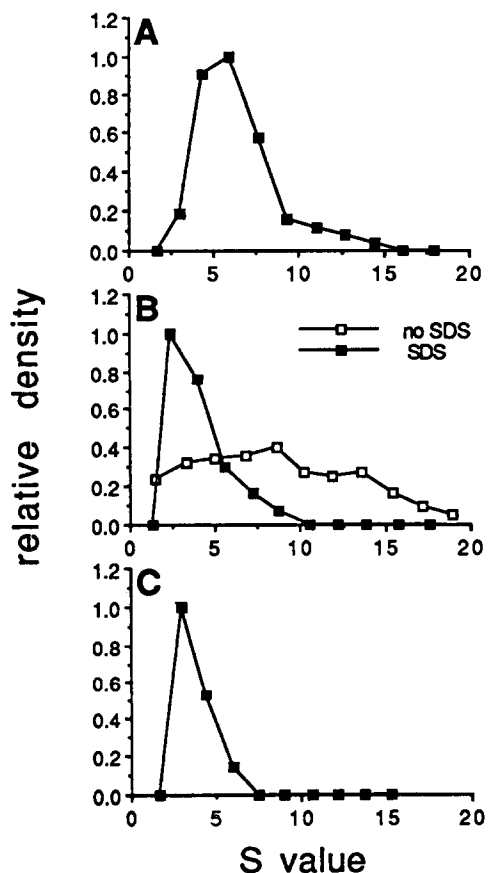


Figure 3. Sedimentation of endogenous α versus *Torpedo* α in L6 myotubes: differential SDS sensitivity. (A) Sedimentation profile, in the presence of 0.1% SDS, of total (endogenous plus *Torpedo*) α subunit in L6-DOL- α myosyncytia. Fused myosyncytia were labeled 20 min with [3 H]leucine at 37°C, solubilized with LB containing 0.1% SDS, and sedimented on a gradient supplemented with 0.1% SDS. α subunit in each fraction was immunoprecipitated with α antiserum that recognizes both species of α . In separate experiments, species-specific immunoprecipitations demonstrated that 80–95% of the α subunit synthesized in L6-DOL- α myosyncytia is *Torpedo* (not shown). (B) Endogenous α in L6-DOL- β myosyncytia sedimented on gradients with (filled squares) and without (open squares) 0.1% SDS. A single plate of cells was labeled 20 min with [3 H]leucine at 37°C, solubilized with LB, and the lysate divided into two equal aliquots which were centrifuged on parallel gradients. (C) Sedimentation profile, in the presence of 0.1% SDS, of endogenous α produced in L6-DOP myosyncytia (L6 cell line containing vector sequences without cDNA insert). Cells were labeled, solubilized, and analyzed as in A.

125 I-BuTx-binding α subunits which are ultimately assayed probably have a binding affinity for BuTx of <1 nM for two reasons: (a) toxin molecules that are unbound or dissociate from α subunits during centrifugation become physically separated from the faster sedimenting α subunit and therefore cannot rebind, and (b) the BuTx concentration becomes even further diluted during the subsequent immunoprecipitations and washings making it unlikely during incubations and impossible after precipitations for dissociated BuTx to rebind. As shown in Fig. 1 G, subunits binding BuTx with high affinity sedimented distinctly from the bulk of α subunits. The high affinity subset (in this experiment, constituting $<3\%$ of total subunit) sedimented at 4.5S while the

usual 6S profile was observed for the majority of α subunit polypeptides. A similar 4.5S profile was observed for the high affinity subset even when subunits were solubilized in $C_{12}E_8$ (data not shown), a detergent less disruptive than Triton X-100 to some membrane protein complexes (Wagner, 1989; Paulson, H., unpublished observations). The sedimentation at 4.5S establishes that the 6S species observed with both *Torpedo* and BC $_3$ H-1 α subunits cannot be monomers. The results also indicate that a putative assembly-competent pool of subunits, the high affinity BuTx-binding subset, tends not to reside in the 6S or larger complexes. This does not rule out the involvement of the 6S or larger complexes in assembly, but suggests they do not represent immediate precursors to heterologous subunit assembly.

To further investigate the high affinity BuTx-binding material, we incubated α -expressing 3T3 cells at 20°C. At this temperature, 23–36% of each subunit assembles as opposed to only 1.7–2.9% at 26°C (Ross et al., 1991). As shown in Fig. 1 I, the high affinity BuTx-binding material still migrates at 4S and the amount of this material increased about twofold relative to the amount obtained when cells are incubated at 26°C (compare the BuTx-binding peak with the metabolically labeled material in Fig. 1, G and I). In two separate experiments, the amount of high affinity BuTx-binding material was quantitated (using 50 nM 125 I-BuTx) and expressed as a percentage of total α subunit synthesized (determined by quantitative immunoblot) at 20 and 26°C. Increases of 1.4- and 3.4-fold at 20 compared with 26°C were obtained. We have established a series of mammalian-*Torpedo* hybrid AChR cell lines and know that *Torpedo* α is not the only temperature-sensitive subunit and that the other three subunits probably all contribute to the temperature-sensitive assembly process (Loutrari, H., S. M. Sine, A. F. Ross, unpublished observations). If each subunit contributes equally to the assembly process, then we would only expect ~ 1.7 -fold increase in the assembly efficiency of each subunit in order to account for the observed 23–36% assembly efficiency observed at 20°C (see Discussion). Thus the results that at 20°C the amount of high affinity BuTx-binding increases 1.4- to 3.4-fold and the 4S α peak similarly increases, are all supportive of the idea that the 4S monomer peak is the precursor to heterologous subunit assembly.

β , γ , and δ Subunits Sediment As 6S Complexes

We also determined the sedimentation behavior of β , γ , and δ subunits expressed individually in mouse fibroblasts. With our standard solubilization and gradient conditions, each subunit had a broad sedimentation profile very similar to that of α , with a peak at ~ 6 S (Fig. 2). As with α subunit, the sedimentation profiles extended into higher S values. More extensive sedimentation analyses of β subunit included diluting the sample fivefold with LB, adding 0.1% SDS to the sample and gradient, and altering the length of the labeling period (6, 20, or 90 min), all of which resulted in essentially identical sedimentation profiles (data not shown). γ and δ subunits were also examined under the dilution and SDS conditions and again sedimentation profiles were unchanged. By analogy with the results obtained with α subunit, the 6S species of β , γ , and δ subunits represent protein complexes larger than subunit monomers.

We next compared the sedimentation profiles of β , γ , and δ subunits expressed at permissive (20 or 26°C) and nonper-

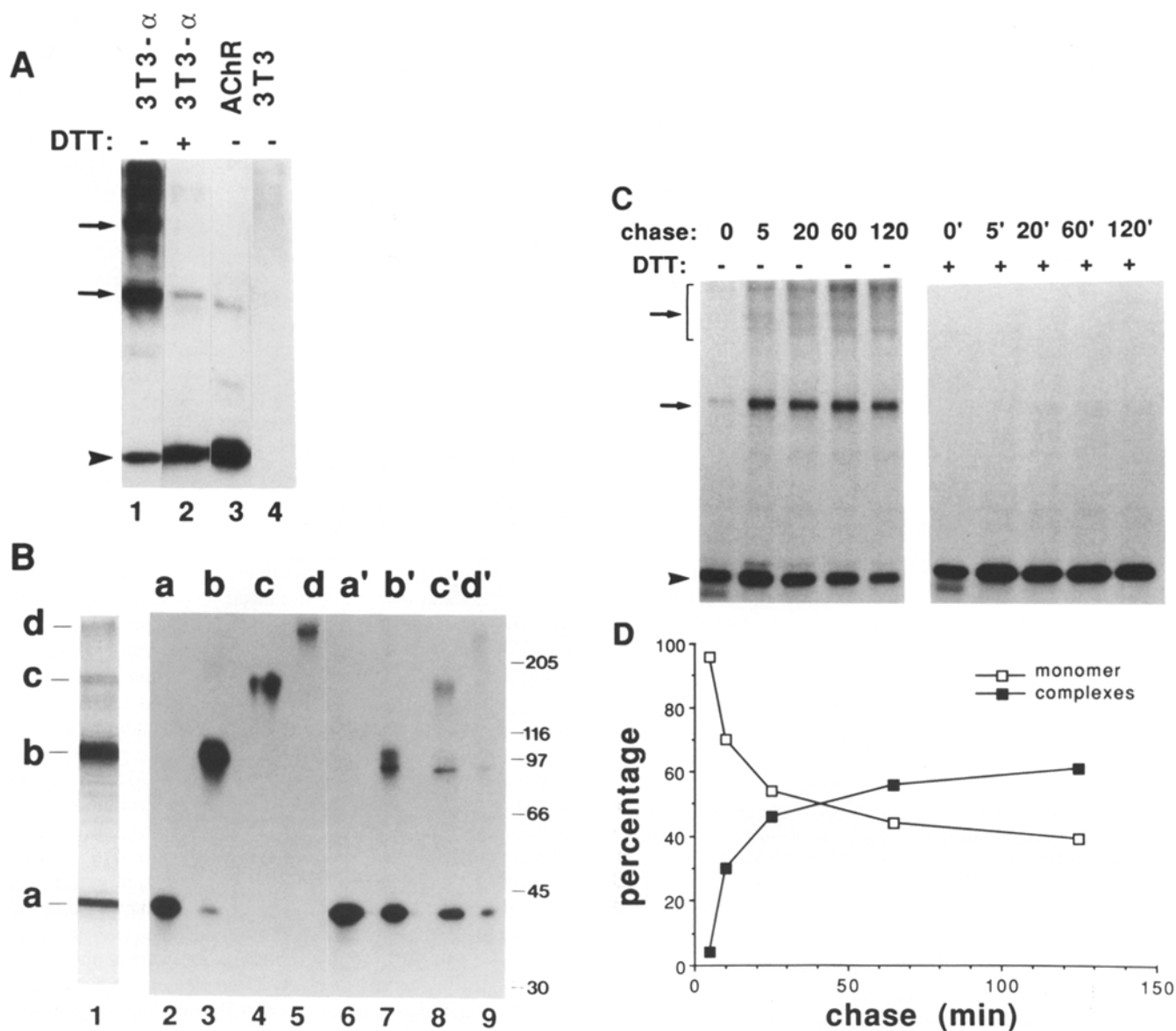


Figure 4. Disulfide-linked complexes of α subunit in fibroblasts. (A) Immunoblot analysis of α subunit under nonreducing and reducing conditions. Solubilized membranes from 3T3-DOL- α_{mass} or 3T3 cells (incubated at 26°C) were electrophoresed in the presence or absence of DTT, and immunoblotted with α antiserum. Lane 1, 3T3-DOL- α_{mass} , no DTT; lane 2, 3T3-DOL- α_{mass} , DTT; lane 3, purified AChR from *Torpedo* electroplax, no DTT; lane 4, 3T3, no DTT. The arrowhead marks the position of the 40-kD α monomer and the arrows mark two disulfide-linked complexes at \sim 100 and 170 kD. (B) Reelectrophoresis of disulfide-linked complexes under nonreducing and reducing conditions. 3T3-DOL- α_{mass} cells at 26°C were labeled 60 min with [35 S]methionine, solubilized, immunoprecipitated under stringent conditions, and subjected to electrophoresis using nonreducing conditions. After autoradiography of the unfixed gel (lane 1), bands representing monomeric α (a), the 100- (b), and 170-kD (c) disulfide-linked complexes, and a high molecular mass aggregate at the top of the separating gel (d) were excised and equal aliquots of the bands were reelectrophoresed in the absence (lanes 2–5) or presence (lanes 6–9) of DTT. The letters above the gel indicate the excised band; a'–d' indicate the presence of DTT. Molecular mass standards are shown on the right. (C) Time course of disulfide linkage. 3T3-DOL- α_{mass} cells at 26°C were pulse-labeled 5 min with [35 S]methionine, chased for the indicated times, solubilized in LB, immunoprecipitated under stringent conditions, and equal aliquots of the immunoprecipitate electrophoresed in the absence (left) or presence (right) of DTT. The arrowhead indicates α monomer and the arrow indicate 100-kD α dimer and higher molecular mass complexes. (D) The relative levels of α monomer and disulfide-linked complexes in C were determined by scanning densitometry and the percentages of monomeric and complexed α plotted versus time. The 5-min pulse-label is included in the indicated chase times.

missive (37°C) temperatures to test whether temperature would alter a subunit's ability to interact with itself or with endogenous cellular proteins. Fig. 2, E and F show that δ synthesized at 27 or 37°C sedimented identically. Similar findings were obtained for the other subunits (α subunit at 26°C is shown in Fig. 1 G; results with β and γ are not shown).

The sedimentation profile of the γ subunit synthesized at 20°C is shown in Fig. 1 H and those of α are shown in Fig. 1 I. In both cases, the majority of each subunit still migrates as a broad polydisperse peak centering at \sim 6S. These results show that all four subunits behave similarly on sucrose density gradients and imply that the degree to which subunits

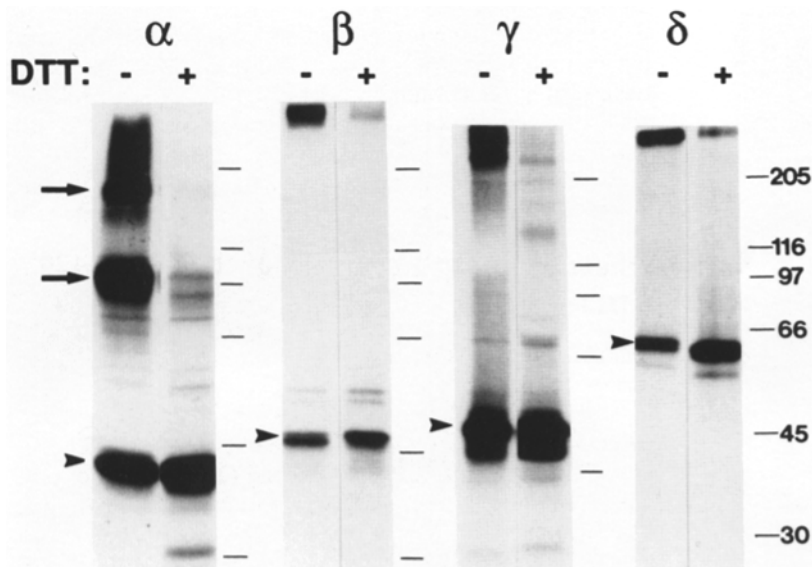


Figure 5. Electrophoretic analysis of α , β , γ , and δ subunits under nonreducing conditions. 3T3-DOL- α_{mass} , 3T3-DOL- β_{mass} , L-pSV2- γ_5 , and L-pSV2- δ_2 cells at 26°C were labeled 20 min with [^{35}S]methionine, solubilized in LB, immunoprecipitated under stringent conditions, and equal aliquots of the immunoprecipitate electrophoresed in the presence and absence of DTT. Arrowheads indicate (from left to right) monomeric α , β , γ , and δ subunit, and arrows indicate disulfide-linked α dimers and trimers. Under nonreducing conditions, a significant fraction of β , γ , and δ subunits electrophorese as aggregates at the top of the separating gel; the stacking portion of each gel did not contain subunit (not shown). Molecular weight standards are shown at right.

self-associate or interact with cellular proteins does not differ greatly at permissive and nonpermissive temperatures. As we will show later, however, the quality of these subunit interactions does differ at the nonpermissive temperature, with increased formation of disulfide-linked α subunits being expressed at nonpermissive temperatures.

The Differentiated State Does Not Alter *Torpedo* Subunit Sedimentation

The preceding results were obtained with subunits synthesized in fibroblasts. We also analyzed *Torpedo* subunits expressed in differentiating muscle cell lines, as they might better reflect the biosynthetic environment typically experienced by the subunits during assembly. For these analyses, we used rat L6 cell lines in which *Torpedo* α or β subunit cDNAs or vector sequences alone (L6-DOP) had been stably integrated.

In unfused L6-DOL- α and L6-DOL- β myoblasts, α (Fig. 1 E) and β (Fig. 2 B) *Torpedo* subunits displayed sedimentation profiles similar to those seen in fibroblasts. Myoblast-expressed subunits still sedimented at 6S when lysates were diluted fivefold before centrifugation, when 0.1% SDS was included in the sample and gradient, and when subunits were synthesized at 27 or 37°C (data not shown). In fully differentiated myotubes, α subunit sedimented at 6S in the presence (Fig. 3 A) or absence (not shown) of 0.1% SDS. Although the α signal in L6-DOL- α myotubes (Fig. 3 A) included both *Torpedo* and endogenous α , the sedimentation profile largely reflects *Torpedo* α since these cells produce 10- to 20-fold more *Torpedo* than endogenous α (determined by immunoprecipitations with species-specific monoclonal antibodies; data not shown). Immunoprecipitations of gradient fractions with a *Torpedo*-specific mAb confirmed that *Torpedo* α expressed in these myotubes sedimented as an SDS-resistant 6S species. Similarly, *Torpedo* β subunit expressed in myotubes, also sedimented as an SDS-resistant 6S complex (data not shown).

In contrast, endogenous α in L6 myotubes has a strikingly altered sedimentation profile when the gradient is run in the presence of 0.1% SDS. In nonionic detergent, endogenous α

in L6-DOL- β myotubes sedimented broadly, but the addition of 0.1% SDS to the gradient caused it to sediment sharply at \sim 3–4S (Fig. 3 B). This 3S peak was also observed in L6-DOP myotubes (Fig. 3 C). Immunoprecipitations of gradient fractions with monoclonal antibodies specific for mammalian or *Torpedo* α subunit confirmed that, even in L6-DOL- α myotubes, endogenous α sedimented as a 3–4S species in the presence of 0.1% SDS while *Torpedo* α sedimented as a \sim 6S species (not shown). This SDS sensitivity pertains only to newly synthesized, unassembled endogenous α ; α subunit that has been incorporated into surface AChR is resistant to 0.1% SDS (not shown). In five separate experiments with low levels of SDS, we consistently observed this shift in the sedimentation of rat α , yet never observed it with any of the four *Torpedo* subunits (Figs. 1 D, and 3 A, and data not shown). *Torpedo* subunits could be made to sediment at 3S but only if they were first denatured by heating at 60°C in 1% SDS and renatured in Triton X-100 before running sucrose gradients (data not shown). These results further illustrate that true AChR subunit monomers migrate at \sim 3–4S. Subunits sedimenting at 6S therefore, must represent either small homooligomeric complexes or individual subunits associated with endogenous cellular proteins.

Disulfide-linked Aggregates of Subunits

We performed several experiments to determine whether the 6S (and larger) complexes represented subunits interacting with homologous subunits or with endogenous cellular proteins. In one set of experiments, subunits were analyzed by SDS-PAGE under nonreducing conditions. When membrane fragments from 3T3-DOL- α_{mass} fibroblasts were immunoblotted under nonreducing conditions, α subunit electrophoresed not only as 40-kD monomer, but also as bands of M_r \sim 100, 170, and $>$ 200 kD (Fig. 4 A, lane 1). In the presence of reducing agent, a 40-kD monomer was the predominant band (Fig. 4 A, lane 2). Similar results were obtained with lysates of whole cells and with a second α -expressing cell line, 3T3-DOJ- α_3 . In four such experiments, the percentage of α subunit electrophoresing as 40-kD monomer

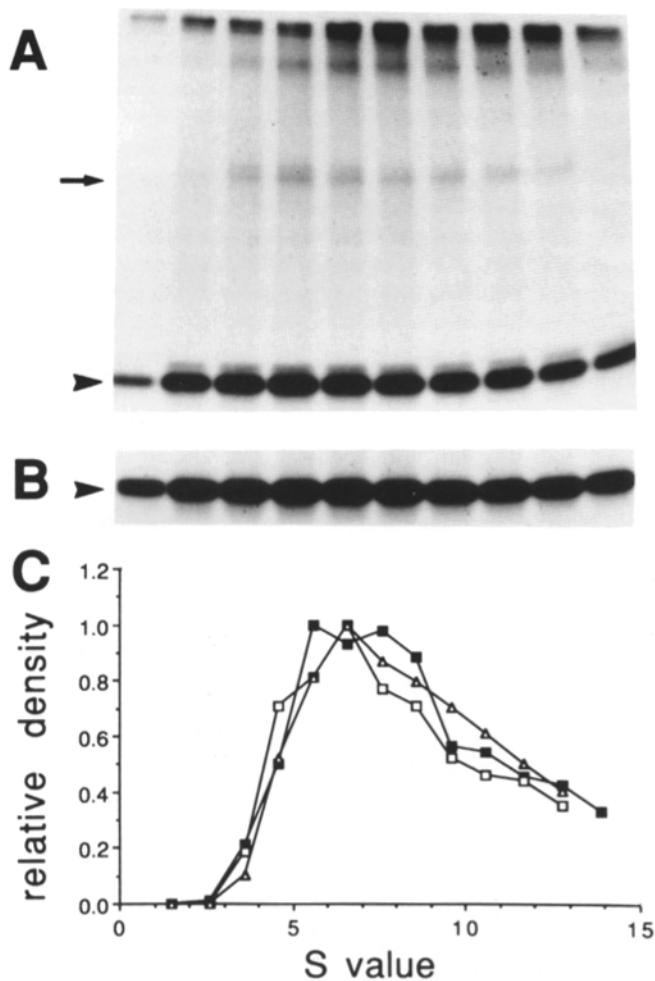


Figure 6. Analysis of α subunit sedimentation under nonreducing and reducing conditions. 3T3-DOL- α_{mass} cells at 26°C were labeled 60 min with [^{35}S]methionine, solubilized in LB, centrifuged on gradients containing either DTT or iodoacetamide, and gradient fractions immunoprecipitated with α antiserum. The α immunoprecipitates from the nonreduced gradient were divided into two equal aliquots and electrophoresed under nonreducing (A) and reducing (B) conditions. In A, α monomer (arrowhead), dimer (arrow), and higher molecular aggregates can be seen; in B, under reducing conditions, all α signal electrophoresed as monomer, hence only the relevant portion of the gel is shown. In both A and B the lanes represent, from left to right, fractions 4–13 of a total of 15.5 fractions. (C) Sedimentation profiles showing monomeric α in A (open triangles), total α in B (open squares), and total α centrifuged in parallel under reducing conditions (filled squares).

under nonreducing conditions ranged from 10 to 40% (mean of 23%), and the percentage electrophoresing as the 100-kD species ranged from 15 to 50% (mean of 32%).

Similar experiments were performed with metabolically labeled cells (Figs. 4 B and C, and 5). 3T3-DOL- α_{mass} cells were incubated with [^{35}S]methionine, solubilized in LB, immunoprecipitated with α antiserum, then electrophoresed in the presence or absence of reducing agent. For these experiments, *N*-ethylmaleimide and iodoacetamide were included in the lysis buffer to prevent disulfide-bond formation, and the cell lysates were denatured with 1% SDS before immunoprecipitation in order to remove noncovalently associated proteins. Consistent with the preceding immunoblot results,

nonreduced α subunit migrated at ~ 40 , 100, 170 kD, and higher molecular mass species. Although these complexes were seen in each of nine similar experiments, their relative abundance varied considerably. The average ratio of 40-kD monomer to the 100-kD complex was 2:1, ranging from 0.6:1 to 5:1. We believe this variability may arise, in part, from events occurring after cell lysis since lysates which are subsequently centrifuged on gradients appear relatively devoid of 100- and 170-kD complexes (see, for example, Figs. 6 and 7).

Nonsubunit proteins, if also present in these disulfide-linked complexes, should be detectable as discreet bands after reduction, provided they have incorporated radiolabel. Except for a faint band of ~ 30 kD (seen in Fig. 5, α panel), we do not observe associated nonsubunit bands whether cells are labeled for short (20 min) or long (2 h) periods of time with [^{35}S]methionine or [^3H]leucine (data not shown). This result implies that most, if not all, of the intermolecular disulfides in the higher molecular mass complexes are between α polypeptides. To verify this conclusion, complexes were excised from a gel and reelectrophoresed with and without DTT (Fig. 4 B). In the absence of DTT, each band reelectrophoresed appropriately as the 40-, 100-, 170-, or >250-kD species (Fig. 4 B, lanes 2–5). In the presence of DTT, each of the higher molecular mass complexes was largely reduced to α monomer (Fig. 4 B, lanes 6–9). The small amounts of protein that did not reduce to monomer, electrophoresed either identically to the starting material or in a band of $M_r \sim 90$ kD (visible in Fig. 4 B, lanes 7–9). We believe this 90-kD band represents fully reduced α dimer that is resistant to SDS because it comigrates with an α dimer band formed in very small amounts when excised monomer is reelectrophoresed under either reducing or non-reducing conditions (not shown). These results lead us to conclude that the 100- and 170-kD species are α dimers and trimers, respectively, and that the >250-kD species are large complexes of disulfide-linked α aggregates. Pulse-chase experiments showed that all of the covalently linked complexes form quickly: at 26°C, α could be detected in higher molecular weight aggregates within 5 min of synthesis (Fig. 4, C and D).

Disulfide-linked homoaggregates often form from misfolded membrane proteins (Machamer and Rose, 1988; Hurtley et al., 1989). To determine whether the disulfide-linked α complexes represent misfolded aggregates or potential assembly intermediates, we compared their production at temperatures permissive (26°C) and nonpermissive (37°C) for *Torpedo* AChR assembly. We have previously determined that there is greater misfolding of fibroblast-expressed α subunits at 37 than at 26°C (Paulson and Claudio, 1990). A corresponding increase in the production of disulfide-linked subunits at 37°C would be consistent with dimers representing misfolded, assembly-incompetent, aggregates. We obtained similar results from experiments in which the entire cellular pool (immunoblots) or just the newly synthesized (radiolabeled) α monomers and dimers were visualized. An immunoblot of 3T3-DOL- α_{mass} cells maintained at 26°C contained 15% α monomer whereas only 5% remained as monomer after a 3 h shift to 37°C. Similarly, 3T3-DOL- α_{mass} cells labeled for 30 min with [^{35}S]methionine at 26 or 37°C, showed 49% α monomer at 26°C and only 22% at 37°C. Because the amount of disulfide cross-linking increases at the nonper-

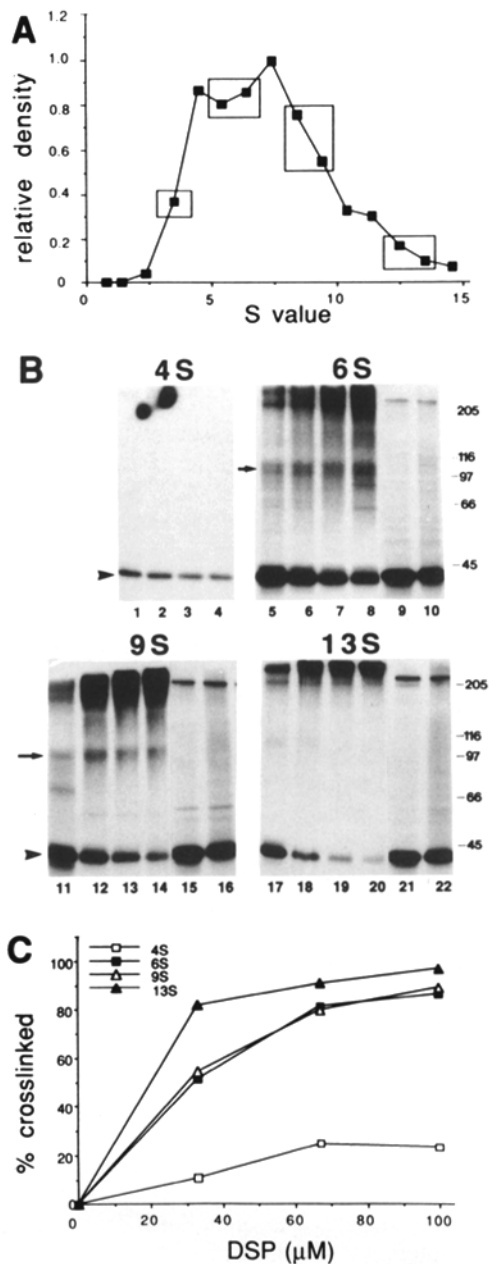


Figure 7. Cross-linking analysis of α subunit gradient fractions. (A) 3T3-DOL- α_{mass} cells at 26°C were labeled 60 min with [^{35}S]methionine, solubilized, and centrifuged on sucrose gradients under standard conditions. Shown is the sedimentation profile of α subunit, determined by immunoprecipitating small aliquots of each fraction. The remainder of the fractions in each box were pooled separately and crosslinked with DSP. (B) Aliquots of the boxed fractions in A (corresponding to sedimentation coefficients of $\sim 4\text{S}$, 6S, 9S, and 13S) were cross-linked with increasing concentrations of DSP, immunoprecipitated under stringent conditions with α antiserum, and electrophoresed in the presence or absence of DTT. Lanes 1, 5, 11, and 17, no DSP, no DTT; lanes 2, 6, 12, and 18, 33 μM DSP, no DTT; lanes 3, 7, 13, and 19, 67 μM DSP, no DTT; lanes 4, 8, 14, and 20, 100 μM DSP, no DTT; lanes 9, 15, and 21, no DSP, 10 mM DTT; lanes 10, 16, and 22, 100 μM DSP, 10 mM DTT. Arrowheads mark α monomer and arrows mark α dimer. Molecular weight standards are shown at right. (C) For each of the crosslinked fractions, the percent α subunit cross-linked was determined by scanning densitometry and is plotted versus the concentration of DSP.

missive temperature, these results are consistent with the interpretation that the disulfide-linked complexes are nonproductive, misfolded aggregates. They further indicate that although the sedimentation profiles of subunits are not altered at the nonpermissive temperature (see Fig. 2 and text), the nature of subunit interactions does differ at permissive and nonpermissive temperatures.

Under nonreducing conditions, *Torpedo* β , γ , and δ subunits also form large aggregates which electrophorese near the top of 7.5% SDS-polyacrylamide gels (Fig. 5). Unlike α , however, discrete β , γ , or δ disulfide-linked dimers and trimers are not readily apparent. The percentage of β , γ , or δ subunits present in high molecular weight aggregates does not greatly increase after a chase with unlabeled methionine, or when cells are incubated at 37°C (not shown). After reduction however, the amount of these aggregates decreases with a corresponding increase in monomer. We have not determined whether these high molecular weight aggregates also contain nonsubunit proteins.

Cross-linking and Immunoprecipitation Experiments

Noncovalent interactions appear to contribute to complex formation since only a subpopulation of α subunits sedimenting at 6S were found to be disulfide-linked to one another. To determine the extent to which noncovalent interactions are involved in causing the 6S profile, 3T3-DOL- α_{mass} cell lysates were centrifuged on gradients in the presence or absence of reducing agent (Fig. 6). Monomeric α isolated from a nonreduced density gradient and electrophoresed under nonreducing conditions (Fig. 6, A and C) had the same 6S profile as total α isolated from a parallel, reduced gradient (Fig. 6 C). These results demonstrate that noncovalent associations are sufficient to account for the 6S migration of subunits on density gradients.

To further characterize these noncovalent associations, we performed a series of cross-linking and immunoprecipitation experiments. In cross-linking experiments (Fig. 7), metabolically labeled α subunit was centrifuged, and fractions corresponding to $\sim 4\text{S}$, 6S, 9S, and 13S (boxed fractions in Fig. 7 A) were incubated with increasing amounts of DSP, a thiol-cleavable bifunctional cross-linking reagent. After cross-linking, subunit was immunoprecipitated under stringent conditions (see Materials and Methods) and analyzed on nonreducing SDS-gels. α subunit sedimenting at 4S was not efficiently cross-linked even at the highest concentration of DSP (100 μM ; Fig. 7 B, lane 4), consistent with it being monomeric. In contrast, subunit sedimenting at 6S, 9S, and 13S was efficiently cross-linked into large complexes. At lower DSP concentrations (33 and 67 μM), α subunit in the 6S and 9S fractions was partially cross-linked into complexes which coelectrophoresed with α dimer (Fig. 7 B, lanes 6, 7, 12, and 13). Analysis of the cross-linked material under reducing conditions showed that it contained no prominent nonsubunit proteins (Fig. 7 B, lanes 10, 16, and 22), implying that most of the cross-linking occurred between homologous subunits. The results suggest that α sedimenting from 6S to 13S resides primarily in variably sized homoaggregates.

In an effort to identify any cellular proteins that may be associating with these homoaggregates, we immunoprecipitated each subunit under varying conditions of stringency (Fig. 8). 3T3-DOL- α_{mass} , 3T3-DOL- β_{mass} , L-pSV2- γ_5 , and

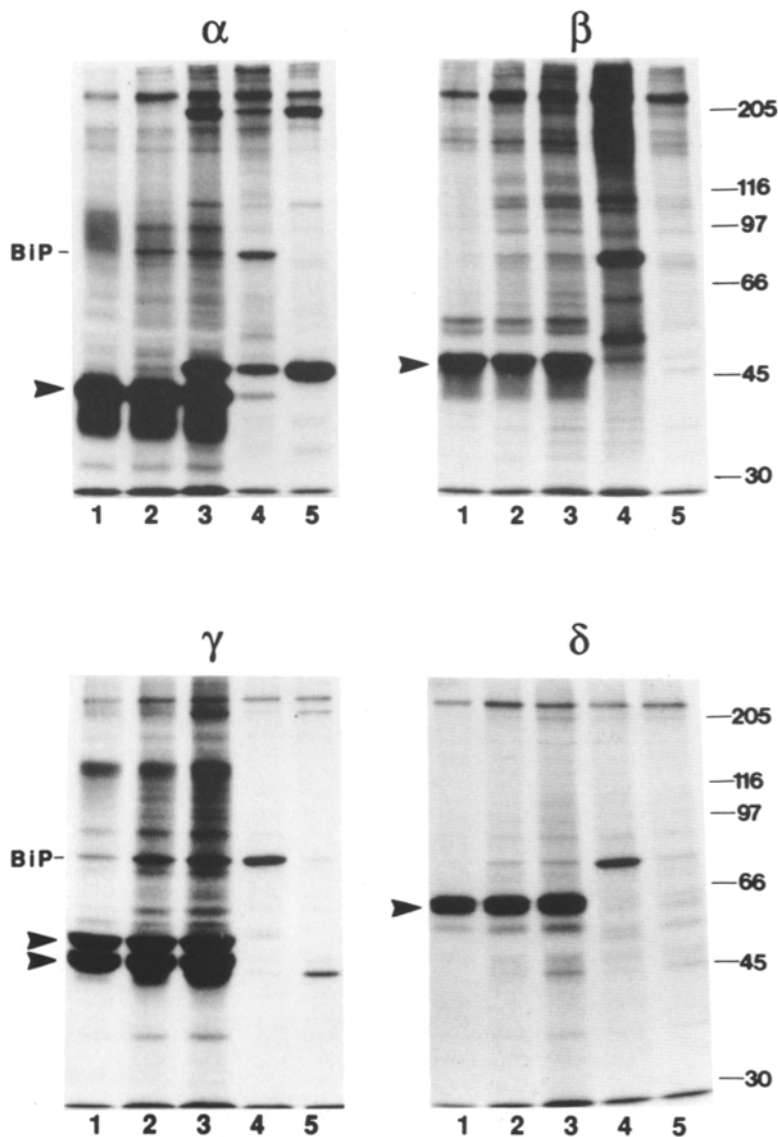


Figure 8. Immunoprecipitation analysis of individually expressed α , β , γ , and δ subunits. 3T3-DOL- α_{mass} , 3T3-DOL- β_{mass} , L-pSV2- γ_5 , and L-pSV2- δ_2 cells at 26°C were labeled 60 min with [^{35}S]methionine, solubilized in LB, and immunoprecipitated with the appropriate subunit antiserum under the following conditions; lane 1, fully denatured in 1% SDS; lane 2, high salt (0.5 M NaCl) and low SDS (0.1%); lane 3, low salt (0.15 M NaCl) and low SDS. Equal aliquots of the lysates were also immunoprecipitated with preimmune antisera (lane 5) and anti-BiP antisera (lane 4) under low salt and low SDS conditions. After heating to 100°C for 3 min, samples were electrophoresed on 7.5% SDS-gels in the presence of DTT. The arrows indicate α , β , γ , and δ subunit. Molecular weight standards are shown on the right. γ subunit's migration as a widely spaced doublet is an artifact of the heating step; the ~ 130 -kD band in lanes 1-3 of the γ panel is consistently observed under these electrophoresis conditions and probably represents SDS-resistant γ dimer based on its migration relative to molecular weight standards.

L-pSV2- δ_2 cells were labeled 60 min with [^{35}S]methionine at 26°C, solubilized with LB containing 0.1% SDS, and aliquots of the lysate were immunoprecipitated in the presence of low salt, in the presence of high salt, or after denaturation of the sample in 1% SDS (Fig. 8, lanes 3, 2, and 1, respectively). An equal aliquot (Fig. 8, lane 4) was also immunoprecipitated with an antibody directed against BiP. Because protein interactions with BiP are disrupted by ATP (Hurtley et al., 1989; Flynn et al., 1989), cell lysates were depleted of ATP by adding hexokinase and glucose to the lysis buffer. The results demonstrate that only a few nonsubunit proteins coprecipitated with each subunit. One of these proteins may be BiP since proteins are seen on the gels with the same electrophoretic mobility (78 kD) as BiP (Fig. 8, lane 1, α and γ panels). BiP antiserum coprecipitated some α subunit (lane 4, α panel), thus indicating some interaction between at least α and BiP. Because the anti-BiP immunoprecipitations were not quantitative, we cannot estimate how much α subunit is associated with BiP. However, sedimentation experiments have shown that BiP is associated with α subunits across the gradient from 6S to >15S (data not shown). These

data thus imply that part of the 6S profile of individually expressed *Torpedo* subunits is due to interactions with endogenous cellular proteins.

Discussion

We have used mammalian fibroblast and muscle cell lines which stably express individual or all four *Torpedo* AChR subunits (Claudio et al., 1987, 1989a,b; Green et al., 1991; Ross et al., 1991) in order to address specific questions regarding early events in AChR biogenesis. Some subunit members of the superfamily of ligand-gated ion channels can self-associate to form homooligomeric complexes which are expressed on the cell surface. It has been our aim with this expression system to approximate the conditions encountered by a newly synthesized channel subunit before it associates with heterologous subunits and to determine the fate of an individually expressed ion channel subunit. Do the individual subunits associate as homooligomeric complexes before establishing contacts with heterologous subunits or do they assemble from monomers? Do subunits interact with specific

cellular proteins and do any such interactions represent productive assembly intermediates?

We found that individually expressed *Torpedo* α , β , γ , δ , and mouse BC₃H-1 α subunits sedimented predominantly as 6S complexes on sucrose density gradients run in the presence of nonionic detergents. Increasing the detergent to protein ratio or adding 0.1% SDS to the gradient did not alter the sedimentation profile. The *Torpedo* subunit sedimentation profiles were similarly unaffected by expressing the subunits at permissive or nonpermissive temperatures, or expressing them in fibroblasts, myoblasts, or differentiated muscle syncytia. We know that AChR pentamers migrate as 9S complexes and show here that subunit monomers migrate at \sim 3–4S. An investigation of the composition of the 6S and larger complexes showed that they are composed of disulfide-linked homoaggregates, noncovalently associated homoaggregates, and noncovalently associated complexes of subunits and endogenous cellular proteins. The homoaggregates form quickly (within 6 min) and are the predominant species of the entire cellular pool of α subunits.

Some of the homoaggregates formed in our expression system contain intermolecular disulfide linkages. These in particular, we believe to represent an off-pathway of misfolded polypeptides that have formed nonproductive aggregates. One reason for this belief is that the formation of disulfide-linked α dimers and trimers increases when cells are shifted from permissive to nonpermissive temperatures. This correlates with an increase in the misfolding of α subunits when cells are shifted to the nonpermissive temperature (Paulson and Claudio, 1990). Another reason is that no such covalently linked oligomers have been found which serve as transient intermediates in the assembly of multimeric membrane proteins. In contrast, there are many examples of mutated or misfolded membrane proteins which lead to the formation of disulfide-linked aggregates (Machamer and Rose, 1988; Hurtley et al., 1989).

We further believe that the complexes of noncovalently associated subunits do not participate in the assembly process. This is based on our finding that almost all of the α subunits in these complexes have not acquired the ability to bind BuTx with high affinity. It has been reported that α polypeptides first fold into a BuTx-binding conformation before assembling with heterologous subunits. Subunits that display high affinity BuTx-binding therefore can be thought of as assembly competent. In our expression system, an analysis of this subset of high affinity BuTx-binding α subunits showed that at 26°C, it comprised <3% of the total α pool and sedimented largely as 4S monomers (Fig. 1 G). As only \sim 2–3% of α polypeptides assemble into AChR pentamers at 26°C (Paulson and Claudio, 1990; Ross et al., 1991), the number of α monomers expressed agrees well with the number of α peptides that end up in assembled AChR complexes. The numbers are consistent with the view that the high affinity BuTx-binding subset of α subunits represents the pool of assembly-competent precursors. This conclusion was further corroborated by analyzing the behavior of α polypeptides at 20°C (Fig 1 I), a temperature at which subunit assembly efficiencies of 23–36% can be achieved (Ross et al., 1991). High affinity BuTx-binding to α subunits increases \sim 1.4- to 3.4-fold as the temperature is lowered from 26 to 20°C. If each *Torpedo* subunit is temperature sensitive (as indicated by experiments employing different combinations of mouse-

Torpedo subunit hybrid AChR complexes), then each subunit might be expected to contribute equally to the assembly process. A 1.7-fold increase in the number of assembly competent α subunits would result in \sim 14-fold (1.7⁵) increase in the assembly efficiency of the AChR complex. Thus an assembly efficiency of 1.7–2.9% at 26°C would be expected to be \sim 24–40% at 20°C (we achieved 23–36%). We also observed at 20°C that there was an increase in the size of the 4S high affinity BuTx-binding peak. Both sets of results are consistent with the high affinity BuTx-binding subset of α subunits being the assembly-competent precursor.

We investigated whether subunits interact with specific cellular proteins and whether any such interactions comprise productive assembly intermediates. A limited interaction could be detected between α polypeptides and BiP. BiP is one of a growing number of recognized chaperone proteins involved in the folding, unfolding, assembly, or translocation of proteins across membranes (Pelham, 1986, 1988; Ellis, 1987; Flynn et al., 1989). BiP may perform one of these roles or it may serve to recognize misfolded proteins or to retain unassembled subunits within the ER (Hurtley and Helenius, 1989). Our results indicate that one or more of these functions may operate in BiP's association with AChR subunits. First, unassembled AChR subunits remain sequestered within the cell (Claudio et al., 1989a), probably in the ER. Second, many subunits expressed in fibroblasts are misfolded (Paulson and Claudio, 1990), and hence are probable candidates for either refolding or degradation. Third, studies of AChR assembly in mouse cells have shown that α subunit maturation occurs slowly, suggesting that its correct folding may require, or be facilitated by, interactions with chaperone proteins.

The observation that the majority of each *Torpedo* AChR subunit expressed in mammalian cells resides in assembly-incompetent polypeptide aggregates is not unique either to *Torpedo* or to AChR subunits. In mouse muscle BC₃H-1 cells, most α subunit migrates not as a 4S monomer, but as a 6S complex (Fig. 1 F). In cells transfected with a single subunit of another ligand-gated channel, the glycine receptor, subunits form similar (\sim 7S) complexes of incompletely or incorrectly folded polypeptides (Sontheimer et al., 1989). We suspect that these glycine receptor subunit aggregates are analogous to those formed by individually expressed AChR subunits. They remain sequestered within the cell (as do AChR subunits) and lack high affinity strychnine binding, unlike the small percentage of subunits that successfully assemble into surface-expressed, glycine-gated chloride channels. In light of these similarities, it will be important to determine whether subunits from related ligand-gated ion channels suffer a similar fate when expressed in the absence of the remaining heterologous subunits normally comprising the receptor.

Aggregate formation is not a general feature of assembly of eukaryotic membrane proteins. The observation that individually expressed (or overexpressed) channel subunits form aggregates may be a reflection of the unique structural and functional features of ion channels. Specifically, channel subunits face the biosynthetic dilemma of both residing within the hydrophobic membrane and forming the hydrophilic pore which traverses this membrane. In the fully assembled receptor, this problem is solved by having each subunit contribute a relatively polar transmembrane domain (such as the

M2 domain in AChRs) to the centrally located ion pore. Before assembly, as yet unresolved and possibly channel-specific mechanisms may be at play which protect the hydrophobic domains. In vitro studies of the folding and association of cytosolic proteins have suggested that, in general, subunits assemble into functional oligomers only after first folding into "structured" monomers, and that aggregates are generated by a competing off-pathway in which incompletely folded monomers associate (Jaenicke, 1987; Goldenberg, 1988). There are nevertheless many proteins whose intramolecular folding steps are not completed until after subunits associate. The results here indicate that the correct or mature folding of a given channel subunit may be facilitated by the presence of its heterologous partner subunits. Viewed this way, homoaggregates formed by individually expressed subunits represent polypeptides either trapped in an early intermediate step in channel biosynthesis, or misfolded via an off-pathway. Our results indicate that the heterologous 5-13S population of subunits represents an off-pathway of assembly and that the 4S monomers are the assembly-competent form.

We thank David Bole, Jon Lindstrom, and Socrates Tzartos for monoclonal antibodies, Arthur Karlin for purified *Torpedo californica* AChR, and Richard Mulligan, Jay Morgenstern, and Hartmut Land for retrovirus vectors.

This work was supported by National Institutes of Health grants NS21714 and HL38156.

Received for publication 25 January 1991.

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