Formation of a Ligand-binding Site for the Acetylcholine Receptor in Vitro

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Investigation of the mechanisms by which the subunits of ligand-gated ion channels fold and associate to form oligomers has been hampered by the lack of an in vitro system in which these reactions occur. We have established conditions in a rabbit reticulocyte translation system supplemented with canine pancreatic microsomes under which the α and δ subunits of the nicotinic acetylcholine receptor (AChR) fold and assemble to form a heterodimer with a cholinergic binding site comparable with that found in the intact AChR. Folding of the α subunit was followed by its ability to bind α-bungarotoxin. Folding efficiency was highly sensitive to changes in the redox potential of the translation medium and was favored by an oxidizing environment. Acquisition of the toxin binding conformation required N-linked core glycosylation but not oligosaccharide trimming, suggesting that oligosaccharide-dependent interaction of chaperones with the α subunit is not essential for correct subunit folding. The conformationally mature α subunit specifically associated with the δ subunit but not the β subunit to form a heterodimer with a high affinity ligand-binding site. These data demonstrate, for the first time, correct folding and assembly of the AChR subunits in an in vitro system.

The muscle acetylcholine receptor (AChR) is the best understood member of a family of ligand-gated ion channels that mediate fast synaptic transmission in the nervous system. All members of the family share a common structure in which five subunits form a pseudosymmetric array around a central aqueous pore. When the channel is activated by binding neurotransmitter, the pore opens, allowing ions to flow into and out of the extracellular region of the receptor (1). The two ligand-binding sites that each receptor possesses are associated with the N-terminal domains of the two α subunits (4, 5). The sites, which have distinctive pharmacological properties, are thought to lie at or near the interface between each α subunit and either the γ or δ subunit, respectively (6, 7).

Each of the subunits of the muscle AChR is synthesized as a separate polypeptide chain (8) that is translocated into the ER, where the signal sequence is cleaved (8), the core glycosyl residues are added (8, 9), and the N-terminal domain is folded (10, 11). The folded, glycosylated polypeptide chains then associate with each other to form the pentameric AChR (11, 12). The assembled receptor is transported to the Golgi, where further modifications such as complex carbohydrate addition and fatty acid acylation (13, 14) take place. The mature AChR is then transported to the surface. The entire process takes about 90 min (11).

Assembly of the AChR subunits occurs by a defined pathway in which the first step is the formation of the αδ and αγ heterodimers (15, 16) (for an alternative view of the assembly pathway, see Ref. 17). The heterodimers associate with the β subunit to form the pentameric receptor. Prior to association with the δ and γ subunits, the α subunit undergoes a conformational change in which it acquires the ability to bind α-bungarotoxin (BTX) (10, 11). Correct folding of the α subunit is a relatively inefficient process. In muscle cells, about 30% of the α subunit acquires toxin binding activity and is subsequently incorporated into the AChR (11), while the remainder does not acquire BTX binding activity and is degraded (12). The rate-limiting step in the folding reaction is postulated to be disulfide bond formation (11) between two pairs of cysteine residues in the extracellular region of the α subunit (18). The other subunits of the muscle AChR contain a single disulfide bridge in the extracellular region (19–21); however, it is not known whether they undergo a similar intramolecular folding reaction or whether conformational maturation is induced following association with the folded α subunit.

The factors that influence AChR subunit maturation and association are unknown. Studies of other glycoprotein complexes processed in the ER have demonstrated a critical role for molecular chaperones and folding enzymes in conformational maturation, oligomeric assembly, and retention of misfolded and unassembled monomers inside the ER lumen (22, 23). These factors facilitate protein folding and oligomerization by preventing irreversible aggregation and by catalyzing disulfide bond formation and prolyl isomerization (23–26). Molecules such as BiP, Grp94, and calnexin associate transiently with polypeptides that are in the process of folding (23, 27–29) and may play a role in subunit assembly (27, 30, 31). Both BiP and calnexin have been reported to be associated with subunits of the AChR in muscle cells (32–34). Although the kinetics of the observed association and dissociation of the subunits with BiP...
are incompatible with a role in folding or assembly (33), calnexitin and the a subunit form a transient complex whose kinetics of formation are consistent with that of an intermediate in the folding or assembly pathways (34).

Elucidation of the factors that influence AChR subunit folding and oligomerization has been limited by the lack of a system in which these processes could be easily studied. Subunit folding and assembly have been previously observed only in intact cells (5, 10, 11), in which it is difficult to manipulate conditions. Although subunit synthesis and processing have been reported in an in vitro translation system, correct folding of the a subunit and heterooligomerization have not been previously observed (8, 9, 35). Using an in vitro translation system, we have established conditions under which the a subunit of the AChR folds to acquire BTX binding activity through a process that shows sensitivity to the redox potential and requires core glycosylation but does not require oligosaccharide trimming. Further, we demonstrate specific association of the a subunit with the b subunit as evidenced by formation of a high affinity cholinergic ligand-binding site.

MATERIALS AND METHODS

Constructs and Antibodies—Full-length cDNAs coding for the a, b, and d subunits of the mouse muscle acetylcholine receptor were obtained from Drs. J. P. Merlie and N. Davidson (a, Ref. 36; b, Ref. 19; d, Ref. 20). Each cDNA was cloned into the pSP65 vector (Promega). Site-directed mutagenesis was carried out using standard techniques (37). Rat monoclonal antibodies against the a subunit, mAb 61 and mAb 210 (38), and against the b subunit, mAb 124 (39), were a kind gift of Dr. J. Lindstrom. Mouse monoclonal antibody against the d subunit, mAb 8SB (40), was a generous gift of Dr. S. Frohner.

RNA Synthesis—RNA was prepared according to a method previously described (41), except that capped transcripts were synthesized. Transcriptions were carried out with SP6 RNA polymerase (Promega) in the presence of 40 mCi Tris acetate, pH 7.6, 6 mM magnesium acetate, 2 mM spermidine, 10 mM DTP, 100 lM bovine serum albumin, 500 units/ml ribonuclease inhibitor (RNasin, Promega), 5 mM RNA cap analog (GpppG; New England Biolabs), 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.1 mM GTP, 80 lM linearized template DNA, and 800 units/ml SP6 RNA polymerase (Promega). Reactions were incubated at 37 °C for 90 min. After 60 min, an additional 400 units/ml of SP6 polymerase was added, and the reactions were incubated for various amounts of time. At the end of the incubation period the 125I-BTX bound to the a subunit was collected by immunoprecipitation with mAb 61. Nonspecific binding was assessed at each time point by omission of mAb 61 in the immunoprecipitation. Duplicate samples for each time point were processed. To control for possible protein degradation during the long incubations, all samples were solubilized and harvested at the same time. Radioactivity was measured in a y counter. Nonspecific binding was usually <10% of total binding.

To calculate the rate of association of 125I-BTX with the a subunit, the solubilized a subunit was incubated with 125I-BTX (200 Ci/mmol; Amersham) for 2 h at 4 °C. A 2500-fold excess of unlabeled BTX was added, and the reactions were incubated for various amounts of time. At the end of the incubation period the 125I-BTX bound to the a subunit was collected by immunoprecipitation with mAb 61. Nonspecific binding was assessed at each time point by omission of mAb 61 in the immunoprecipitation. Duplicate samples for each time point were processed. To control for possible protein degradation during the long incubations, all samples were solubilized and harvested at the same time. Radioactivity was measured in a y counter. Nonspecific binding was usually <10% of total binding.

To calculate the rate of association of 125I-BTX with the b subunit, the protein was solubilized and incubated with 125I-BTX for various amounts of time. At the end of the incubation period the 2500-fold excess of unlabeled BTX was added, and samples were harvested by immunoprecipitation. For these experiments, mAb 210 was used instead of mAb 61 due to the limited amount of the latter antibody; both antibodies precipitated BTX-bound a subunit with similar efficiency. Nonspecific binding was determined at each time point by omitting the primary antibody. All samples were solubilized and immunoprecipitated at the same time and in duplicate.

To measure the association efficiency of the d and b subunits with the folded a subunit, the a subunit was cotranslated with either the d or the b subunit. Following solubilization and incubation with 125I-BTX, the toxin-bound complexes were collected via immunoprecipitation with antibodies against each subunit. Nonspecific binding was determined by omission of the primary antibody. Total specific binding was defined as the amount of BTX-bound a subunit precipitated with mAb 210 minus nonspecific binding.

To assess inhibition of BTX binding by cholinergic ligands to the a subunit synthesized alone or in the presence of the b subunit, the protein was solubilized and preincubated with various concentrations of ligand for 15 min on ice. The ligand was then incubated with 125I-BTX for 10 min (to maintain initial rate conditions), at which time a 2500-fold excess of unlabeled BTX was added and the samples were harvested by immunoprecipitation with mAb 210. Nonspecific binding was determined as above.

To measure inhibition of BTX binding by cholinergic ligands to the a/b heterotetramer, the subunit complex was solubilized and preincubated with various concentrations of ligand for 15 min on ice. The v-lysate was then incubated with 125I-BTX for 90 min (at which time less than 50% of sites are occupied by BTX; data not shown). Incubations were terminated with the addition of an excess of unlabeled BTX, and the toxin-bound complexes were immunoprecipitated with mAb 88B.

Sucrose Gradient Sedimentation Analysis—Microsomes from a 50-µl
Subunits of the AChR Fold and Assemble in Vitro

**RESULTS**

**Folding of the α Subunit in an in Vitro Translation System**—In devising an in vitro translation system that would allow a subunit folding, we focused initially on finding a redox potential that is compatible with both translation and disulfide bond formation. Standard preparations of rabbit reticulocyte lysates and dog pancreas microsomes are customarily made and stored in a reducing environment for maximum efficiency of translation and polypeptide chain translocation. To provide an oxidizing environment, we used reticulocyte lysate prepared without DTT and further raised the redox potential of the reaction by adding GSSG. To determine if the in vitro translation system prepared under these conditions processed the α subunit in a normal manner, we incubated α subunit mRNA with rabbit reticulocyte lysate in the presence and absence of dog pancreas microsomes and in the presence and absence of an inhibitor of glycosylation, the acetylated tripeptide AcNYT (42). The products were analyzed by SDS gel electrophoresis. In the absence of microsomes, a single 38 S-labeled polypeptide of apparent molecular mass 43 kDa was observed (Fig. 1A, lane 2), which represents the newly synthesized uncleaved and unglycosylated α subunit. When microsomes were added and N-linked glycosylation was inhibited with the tripeptide, a faster migrating band (42 kDa) of processed, unglycosylated peptide was observed (Fig. 1A, lane 3). When the tripeptide was omitted, a 44-kDa band corresponding to the glycosylated, processed α subunit was formed (Fig. 1A, lane 4). These results are consistent with those previously reported in conventional in vitro translation systems (8, 43). Although all forms of the α subunit were immunoprecipitated with the monoclonal antibody specific for the AChR α subunit (mAb 61) (38), the antibody bound with highest affinity to the fully mature (44-kDa) form (Fig. 1B, lane 2).

To test whether the α subunit synthesized under these conditions acquired BTX binding activity, we lysed the microsomes, incubated the lysate with α-bungarotoxin Sepharose (BTX-Sepharose), and then eluted the bound protein with SDS sample buffer and analyzed it by SDS gel electrophoresis (Fig. 1B, lane 3). Approximately 15% of the α subunit synthesized during the 60-min incubation bound BTX-Sepharose. The efficiency of folding of the α subunit synthesized in vitro is somewhat lower than that observed in muscle cells (30%) (11) but is comparable with that seen when the α subunit is expressed in heterologous cells (5).

**Properties of the Folded α Subunit**—To determine if the toxin-binding form of the α subunit synthesized in vitro corresponds to the correctly folded, mature α subunit, we examined a number of its properties. Previous studies of the α subunit expressed in heterologous cells established that the hallmark of the mature α subunit is its high affinity for BTX (5). Since the rate of association of BTX with the α subunit is slow, the high affinity of the binding reaction is achieved by the extreme stability of the toxin-subunit complex (5). These same two characteristics were found for the interaction of BTX with the α subunit synthesized in vitro. To determine the association rate constant for the BTX-binding reaction, the newly synthesized α subunit was incubated with 1.9 nM [125I]BTX, and the amount of toxin-subunit complex formed at various times was measured by immunoprecipitation. The reaction showed bimolecular kinetics with a calculated rate constant of 1.5 × 10^6 M^-1 s^-1, which is similar to the value of 4.6 × 10^6 M^-1 s^-1 calculated from the data of Blount and Merlie (5), who expressed the α subunit in fibroblast cells. Dissociation of the toxin-subunit complex was measured by incubating the solubilized α subunit with [125I]BTX for 2 h, followed by the addition of a 2500-fold excess of unlabeled BTX. Immunoprecipitation at various times thereafter showed that the half-time of dissociation of the complex was >36 h (data not shown). The calculated dissociation constant for the binding reaction (K_D < 3.5 × 10^-11 M) is similar to that observed for the α subunit expressed in vitro (5).

The affinity for cholinergic ligands of the α subunit synthesized in vitro was measured by determining their ability to decrease the initial rate of toxin binding to the solubilized α subunit. After preincubation with ligand, we incubated the α subunit in the presence of [125I]BTX for 10 min to maintain initial rate conditions. Incubations were terminated by the addition of an excess of unlabeled BTX followed by immunoprecipitation. The cholinergic agonist carbamylcholine had little effect on the rate of toxin binding, even at very high con-
centrations (IC$_{50}$ > 10$^{-2}$ M), while the antagonists d-tubocurarine (IC$_{50}$ = 1.6 × 10$^{-3}$ M) and gallamine (IC$_{50}$ = 4.7 × 10$^{-3}$ M) inhibited initial rates of BTX binding (Fig. 2). The calculated affinities were similar to, but somewhat lower than, those observed for the subunit expressed in fibroblast cells (5). This discrepancy is likely due to differences in the binding assays employed. We used solubilized subunit in our binding assay and observed a faster time course of association of BTX to the subunit, which may have reduced the apparent affinity of competitive ligands for the subunit.

Our experiments show that the subunit synthesized in vitro resembles that made in vivo both in its binding of BTX and in its affinity for cholinergic ligands. To determine whether the folded polypeptide made in vitro is oligomeric or associated with other proteins, we used velocity sedimentation in a sucrose gradient. A lysate of a translation reaction was subjected to sedimentation in a 5–20% sucrose gradient, and fractions from the gradient were analyzed for total protein and for toxin binding activity. Whereas the total subunit formed a broad peak centered at 5.6 S but skewed toward higher molecular weights (Fig. 3A, filled squares), the toxin-binding form migrated in a narrow, symmetrical peak centered at 4.5 S (Fig. 3A, open squares), the position found for monomeric subunit expressed in vitro (5, 11). Thus, the subunit folded in vitro is in monomeric form.

Kinetics of Acquisition of BTX Binding—To establish the time course of acquisition of BTX-binding by the subunit, we pulse-labeled the polypeptide in the presence of $^{35}$S-methionine for 10 min and incubated the reaction in the presence of an excess of unlabeled methionine (chase). Aliquots of the translation reaction were removed at various times and placed on ice. Further disulfide bond formation was blocked by alkylation of free cysteines with 20 mM N-ethylmaleimide (44). Samples from each time point were then analyzed for total protein and BTX binding activity. Both the total polypeptide and the folded subunit increased gradually during the chase period (Fig. 4A). The acquisition of BTX binding by the subunit, however, was significantly slower than its synthesis (Fig. 4A). Quantitation of this and other experiments showed that folding of the subunit into its BTX binding conformation occurred with a delay of 15–20 min (Fig. 4B). A similar lag in folding was observed in vivo using pulse-labeled muscle cells (10, 11) or fibroblasts expressing the polypeptide in the absence of other AChR subunits (5). These data demonstrate that the folding reaction in vitro exhibits similar kinetics to that seen in vivo.

Folding Efficiency Depends on the Redox Potential—The rate-limiting step in the folding pathway of the subunit is thought to involve the formation of correct disulfide bonds that stabilize the mature conformation of the polypeptide (11). To determine the effect of changing the redox potential on the folding efficiency of the subunit, we added increasing amounts of GSSG to the translation reaction and measured the amount of the subunit that bound BTX (Fig. 5A). In the
leading to degradation of misfolded proteins (45). When cosylated proteins does not induce the rapid stress response the cleaved, nonglycosylated form of the linked glycosylation was inhibited by the AcNYT tripeptide, onset of the folding (data not shown). Thus, the redox environment at the N-linked glycosylation (12, 46). Since deoxymannojirimycin, as specific inhibitor of the ER glucosidases I and II, and deoxymannojirimycin, a specific inhibitor of the α-mannosidase was produced (Fig. 6A, compare lanes 2 and 4). This was increased and assayed via immunoprecipitation and BTX binding. Not that increasing concentrations of GSSG slightly inhibited the translation efficiency. B, a reducing environment lowers the efficiency of folding. The fluorograms showing mAb 61 immunoprecipitation and BTX binding were exposed for different lengths of time. B, quantitation of the fluorograms shown in A. Results were quantitated using a PhosphorImager.

![Subunits of the AChR Fold and Assemble in Vitro](Image)

**Fig. 4.** The newly synthesized α subunit folds with a delay. A, kinetics of α subunit synthesis and BTX binding. The α subunit was synthesized in the presence of [35S]methionine for 10 min (pulse) and in the presence of excess unlabeled methionine for various times thereafter (CHASE). Some of the polypeptide chains labeled during the pulse period were elongated during the chase interval, allowing detection of subunit synthesis. At the times indicated during the chase period, an aliquot from the translation reaction was removed and alkylated with 20 mM N-ethylmaleimide to block further disulfide bond formation. Each aliquot was divided into two portions and analyzed via immunoprecipitation and BTX binding. The upper band precipitated with mAb 61 corresponds to the mature, glycosylated subunit, and the lower band corresponds to the unglycosylated subunit from which the signal sequence has been removed. The fluorograms showing mAb 61 immunoprecipitation and BTX binding were exposed for different lengths of time.

Absence of additional GSSG, little or no folding occurred. With increasing concentrations of GSSG, as the redox potential became more oxidizing, the amount of α subunit that bound toxin increased. Although the addition of GSSG slightly inhibited the efficiency of translation, quantitation of the data in Fig. 5A showed that extent of folding relative to the total amount of the α subunit synthesized was maximal at 2 mM GSSG. In the presence of 2 mM GSSG, increasing concentrations of DTT dramatically lowered the efficiency of folding (Fig. 5B). Since shifting the redox potential toward a more oxidizing state appeared to be essential for correct folding, we determined whether the timing of this oxidative shift was also important by adding GSSG at different times after translation was initiated. In all cases delayed addition of GSSG lowered the efficiency of folding (data not shown). Thus, the redox environment at the onset of the α polypeptide synthesis is critical for proper folding of the α subunit. These data suggest that in a reducing environment, the α subunit folds incorrectly to assume conformations that are not easily reversed.

**Core Glycosylation, but Not Oligosaccharide Trimming, Is Essential for the Acquisition of BTX Binding—Experiments in vivo that have examined the role of glycosylation in α subunit folding have been complicated by the rapid degradation of the unglycosylated polypeptide. We therefore examined α subunit folding in the in vitro system, in which accumulation of unglycosylated proteins does not induce the rapid stress response leading to degradation of misfolded proteins (45). When N-linked glycosylation was inhibited by the AcNYT tripeptide, the cleaved, nonglycosylated form of the α subunit (42 kDa) was produced (Fig. 6A, compare lanes 1 and 2); however, this nonglycosylated peptide bound BTX poorly (Fig. 6A, compare lanes 5 and 6). We also generated a mutated α subunit in which the consensus core glycosylation signal was disrupted by replacing the Asn residue at position 141 with Asp (N141D). The N141D α polypeptide also does not bind BTX efficiently (Fig. 6B, compare lanes 4 and 6). Thus, little or no folding of the α subunit occurs in the absence of glycosylation. These data are in agreement with in vivo studies using tunicamycin and site-directed mutagenesis to block N-linked glycosylation (12, 46).

We also examined the role of oligosaccharide trimming in the maturation process. Trimming of the core oligosaccharide has recently been implicated in protein folding because it promotes a specific interaction between glycoproteins and a lectin-like chaperone molecule, calnexin (31, 47). We used castanospermine, a specific inhibitor of ER glucosidases I and II, and deoxymannojirimycin, a specific inhibitor of the α-mannosidase
Subunits of the AChR Fold and Assemble in Vitro

Core glycosylation, but not oligosaccharide trimming, is essential for BTX binding. A, inhibition of N-linked core glycosylation and oligosaccharide trimming. α subunit was translated either in the absence of any additional treatment (NT) or in the presence of AcNYT, an inhibitor of core glycosylation; castanospermine (CST; 1 mM final), an inhibitor of glucosidases I and II; and deoxymannojirimycin (DMJ; 1 mM final concentration), an inhibitor of ER mannosidase. Aliquots from each translation reaction were analyzed via immunoprecipitation and BTX binding. A single fluorogram depicting both mAb 61 immunoprecipitation and BTX-binding is shown. Quantitation of these data revealed that 18% of the glycosylated subunit acquired the BTX binding conformation, while less than 2% of the unglycosylated subunit folded to bind toxin. B, inhibition of N-linked core glycosylation with AcNYT and by site-directed mutagenesis. The glycosylation consensus sequence was mutated by replacing the asparagine residue at position 141 with an aspartic acid residue. The mutated subunit and the wild type subunit in the presence and the absence of AcNYT were translated. Aliquots from each translation were solubilized and assayed via immunoprecipitation and BTX binding. The fluorograms showing mAb 61 immunoprecipitation and BTX binding were exposed for different lengths of time.

(48) to block the trimming of the α subunit synthesized in vitro. Incubation of microsomes with castanospermine and deoxymannojirimycin did not affect translation or translocation; however, the glycosylated α subunit that was produced had a lower mobility, consistent with it being linked to an untrimmed oligosaccharide (Fig. 6A, lanes 3 and 4, compared with lane 1). Blockade of the trimming reactions did not lower the ability of the α subunit to bind BTX (Fig. 6A, compare lanes 7 and 8 with lane 5). In fact, inhibition of the trimming reactions appeared to facilitate folding of the α subunit. Thus, oligosaccharide trimming is not essential for correct folding of the α subunit into its BTX binding conformation.

The α and δ Subunits Assemble into a Heterodimer—Studies of cells transfected with various combinations of AChR subunits revealed that the first step in the assembly pathway is formation of heterodimers between the α subunit and either the δ or the γ subunit (15, 16). Once the heterodimers are formed, the β subunit associates with the subunit complexes to form the pentameric receptor (16). Thus, the correctly folded α subunit can distinguish between the δ subunit, with which it forms a heterodimer, and the β subunit, with which it does not. To determine if the α subunit folded in vitro can specifically assemble with the δ subunit, we coexpressed the α subunit with either the δ subunit or the β subunit, incubated the complex with [125I]BTX, and measured the ability of subunit-specific antibodies to immunoprecipitate the BTX-bound complex. Since neither the δ nor the β subunit binds BTX in the absence of the α subunit (Refs. 15 and 16 and data not shown), precipitation of BTX with antibodies against these subunits indicates association with the BTX-bound α subunit. Approximately 40% of the δ subunit associated with the folded α subunit, while only approximately 5% of the β subunit formed a complex with the α subunit (Fig. 7). These data demonstrate that the folded α subunit specifically assembles with the δ subunit but not with the β subunit. The efficiency of α-δ assembly was unaffected by treatment with castanospermine (data not shown), suggesting that glucose residue trimming is not essential for subunit assembly.

To determine if the α and δ subunits assemble into a heterodimer or a larger aggregate, we analyzed the sedimentation properties of the α-δ complex in a sucrose gradient. A lysate containing the α subunit coexpressed with the δ subunit was incubated with [125I]BTX and sedimented through a 5–20% linear sucrose gradient. Fractions were immunoprecipitated with an anti-δ subunit antibody to detect the BTX-bound α-δ complex and with an anti-α subunit antibody to reveal the sedimentation profile of the folded α subunit. The α-δ complex migrated in a narrow peak with a sedimentation coefficient of 6.3 S (Fig. 3B, filled circles), a value demonstrated for the α-δ heterodimer in vivo (49). Although some of the folded α subunit remained unassembled and migrated as a monomer with a sedimentation coefficient of 4.5 S, a major portion of the α subunit associated with the δ subunit to form heterodimers (Fig. 3B, open squares).

In vivo, assembly of the α and δ subunits into a heterodimer produces a high affinity cholinergic ligand-binding site whose properties are indistinguishable from the α-δ site of the native AChR (15). To ascertain whether a cholinergic ligand-binding site is formed in vitro, we measured the ability of cholinergic ligands to inhibit initial rates of BTX binding to the α subunit expressed either with the δ subunit or with the β subunit. After preincubating the lysate containing either the α-δ or the α-β subunit combination with ligand, we incubated the subunits in the presence of [125I]BTX under conditions that maintain initial rate binding kinetics (see “Materials and Methods”). Incubations were terminated by the addition of unlabeled BTX, and the complexes were immunoprecipitated with either the anti-α or the anti-δ subunit antibody. d-Tubocurarine and carbamylcholine at concentrations that did not inhibit BTX binding to the α subunit expressed with the β subunit (Fig. 6A), efficiently
units. We have established conditions that allow efficient signal sequence cleavage, core glycosylation, and maturation of the subunits as demonstrated by the ability of the α polypeptide to assume its BTX binding conformation. The α subunit expressed in this system folded into a conformation that was indistinguishable from the α subunit made in vivo as evidenced by the kinetics of BTX binding and the affinity of the subunit for cholinergic ligands (5). The correctly folded α subunit preferentially associated with the δ subunit to form a heterodimer with a high affinity cholinergic ligand-binding site.

Sucrose gradient sedimentation analysis demonstrated that the folded α subunit migrated as a monomer, while coexpression of the α and the δ subunits resulted in formation of heterodimers with sedimentation coefficients similar to those reported for the subunit monomers and dimers expressed in vivo (5, 11, 49). In contrast to the folded subunit, the total population of α protein exhibited a shift toward a larger molecular weight, suggesting an association with other proteins in the microsomes. Studies of muscle and transfected cells have previously reported an association between the α polypeptide and the molecular chaperone BiP (32, 33), and recent experiments have shown that unfolded α subunit is transiently bound to calnexin (34). Since only a portion of the α subunit attains the folded conformation, the rest of the polypeptide chains are probably in an unfolded or misfolded state and are likely to be complexed with chaperones or other folding enzymes in the ER-derived microsomes (50, 51). Furthermore, the α subunit associated in the absence of other subunits may be preferentially associated with chaperones that play a role in oligomeric assembly (27, 52).

Folding of the α polypeptide into its native conformation is a relatively inefficient process. Folding efficiency is highest in muscle cells, where approximately 30% of the α subunit attains BTX binding activity and becomes incorporated into the mature receptor (11). The remainder of the α subunit is presumed to be misfolded, aggregated, and targeted for degradation (12). The folding efficiency of 15% that we have observed in the in vitro translation system is lower than the efficiency reported in muscle cells, but is comparable with the percentage of the α subunit that acquired the BTX binding conformation in fibroblast cells (5). Likewise, the efficiency of αδ heterodimer formation, which varied from 40 to 70%, depending on the ratio of RNA used in the cotranslation experiment (Figs. 3B and 7), is lower than that reported in muscle cells, in which all of the folded α subunit becomes assembled into the pentameric receptor (11). The lower efficiency in vitro is likely due to the requirement that both the α and the δ polypeptide chains be translocated into the same microsome, where subunit association takes place.

In the in vitro translation system, we have observed that folding of the α subunit is highly sensitive to the redox potential in the reaction medium, requiring the addition of an oxidizing agent (GSSG) for maximum efficiency. Elevation of the redox potential is presumably necessary to overcome exogenous reducing agents such as DTT and β-mercaptoethanol present in the microsomes and the [35S]methionine, respectively, and to restore the redox state to a level that is favorable for disulfide bond formation. In the in vitro translation system, the endogenous transport of glutathione across the membrane (53) and the buffering capacity of proteins in the reticulocyte lysate and the microsomes are likely to contribute to a redox state that closely approximates the potential maintained in vivo. Several studies comparing folding (51, 54) and assembly (55, 56) of glycoprotein complexes in vitro with that in vivo used oxidized glutathione in the same range of concentrations as required for optimal folding of the α subunit.

**FIG. 8. Inhibition of BTX binding by cholinergic ligands.** A, low concentrations of cholinergic ligands do not inhibit BTX binding to the α subunit expressed with the β subunit. The α subunit was coexpressed with the β subunit to simulate cotranslation conditions used to synthesize αδ heterodimers. The subunits were lysed and incubated in the presence of the indicated concentrations of ligand (in μM) for 15 min prior to the addition of 1.7 nM [125I]BTX. Incubations were terminated after 10 min (to maintain initial rate binding conditions) with the addition of an excess of unlabeled BTX. [125I]BTX-bound complexes were immunoprecipitated with an antibody against the α subunit (mAb 210). Non-specific binding was measured by omission of the primary antibody. Radioactivity was measured in a γ counter. The results for each ligand concentration were plotted as percentage of the total specific [125I]BTX-binding in the absence of any ligand. Each value is the mean ± S.E. of three determinations. B, cholinergic ligands inhibit BTX-binding to the αδ heterodimer. The α subunit was coexpressed with the δ subunit and treated as described above, except that preincubation with ligand was carried out for 45 min and incubation with [125I]BTX was carried out for 90 min. Under these conditions less than 50% of the αδ sites are occupied with BTX (data not shown). BTX-bound complexes were immunoprecipitated with an anti-δ subunit antibody and plotted as described above. NL, no ligand; d-Tc, d-tubocurarine; carb, carbamylcholine.

inhibited BTX binding to the αδ heterodimer (Fig. 8B). These data demonstrate formation of the cholinergic ligand-binding site in vitro.

**DISCUSSION**

We have used an in vitro expression system consisting of the rabbit reticulocyte lysate supplemented with canine pancreatic microsomes to study folding and assembly of the AChR subunits. We have established conditions that allow efficient signal sequence cleavage, core glycosylation, and maturation of the subunits as demonstrated by the ability of the α polypeptide to assume its BTX binding conformation. The α subunit expressed in this system folded into a conformation that was indistinguishable from the α subunit made in vivo as evidenced by the kinetics of BTX binding and the affinity of the subunit for cholinergic ligands (5). The correctly folded α subunit preferentially associated with the δ subunit to form a heterodimer with a high affinity cholinergic ligand-binding site.
Maximal folding efficiency was dependent not only on the concentration of oxidized glutathione but also on the timing of its addition to the translation reaction. Delayed addition of oxidized glutathione failed to produce efficient folding of the α subunit. These data suggest that the folding process may begin co-translationally. Consequently, in the absence of an oxidizing environment at the onset of subunit synthesis irreversible misfolding and aggregation may occur. A similar requirement for oxidizing conditions at the time of translational initiation in vitro was also reported for intramolecular folding of prolactin (50) and pancreaticzymogens (54, 57) as well as for heterooligomeric assembly of the major histocompatibility complex class I (55) and class II (56) immunoglobulins. In contrast, influenza hemagglutinin folding could be initiated with oxidized glutathione both co- and post-translationally (51).

Previous studies on AChR subunit expression in vitro reported signal sequence cleavage and core glycosylation but did not observe subunit folding or heterooligomeric assembly (8, 9, 35). Our data demonstrating the stringent requirement for optimal redox conditions at the initiation of subunit synthesis may explain the failure of earlier studies to achieve folding and assembly of AChR subunits in vitro. The reducing environment maintained in the translation reaction presumably resulted in subunit misfolding and aggregation. Consequently, Anderson and Blobel (8) observed homooligomerization of AChR subunits, which has not been reported in vivo (11, 49) and was not observed in our in vitro system.

Studies of α subunit maturation in vivo (5, 10, 11) and our data in vitro revealed a delay in the ability of the newly synthesized α polypeptide to bind BTX. Merlie and Lindstrom (11) originally suggested that correct disulfide bond formation was the rate-limiting step in the folding pathway. Our data demonstrating that folding of the α subunit is dependent on the redox potential in the translation reaction are consistent with the hypothesis that disulfide bond formation is important for correct folding (11, 18). In preliminary experiments, we further tested this idea by eliminating one of the two disulfide bonds by mutating cysteines 192 and 193 to serines and measuring the folding efficiency. We observed that the extent of correct folding is approximately 2-fold greater for the mutated subunit than for the wild type subunit. Our data suggest that the efficiency of folding for the wild type α subunit is in part limited by the formation of incorrect disulfide bonds and provides additional support to the notion that disulfide bond formation is critical for proper folding of the α subunit.

One of the earliest steps in glycoprotein processing in the ER is N-linked core glycosylation. N-Glycans have been suggested to play a role in protein stability and maturation by facilitating interactions with various chaperone molecules that prevent aggregation and aid in the folding and assembly pathways (31, 47, 58). Using tunicamycin to block glycosylation, studies with muscle and transfected cells demonstrated a reduced number of cell surface AChRs and an increased rate of subunit degradation (12, 46). We have shown that blockade of N-linked glycosylation with a competitor tripeptide or via site-directed mutagenesis abolishes the capability of the α subunit to acquire high affinity BTX binding. Thus, core glycosylation is critical for conformational maturation of the α subunit.

Recently, the role of N-glycans in protein folding and assembly has become more defined as some of the mechanistic details by which core oligosaccharides facilitate maturation of proteins in the ER have become clarified (31, 47, 58). Almost immediately after the addition of the core oligosaccharide, the outermost glucose and mannose residues are trimmed by two types of glucosidases (I and II) and an α-mannosidase, respectively (59). The monoglycosylated form of the oligosaccharide has been demonstrated to be the preferential substrate for a lectin-like ER-resident molecular chaperone, calnexin (29, 47). Calnexin may also play a role in glycoprotein oligomerization, since it was found associated with subunit monomers in the process of assembly (52, 58, 60) and remained associated until their export out of the ER (60). Association and dissociation with calnexin is controlled through a cycle of deglycosylation by glucosidases and reglucosylation by a lumenal UDP-glucose: glycoprotein glucosyltransferase, which ensures that unfolded and unassembled proteins are retained in the ER, while the mature proteins that are not reglucosylated are then exported out of the ER (31).

The involvement of oligosaccharide trimming, and specifically of calnexin, in glycoprotein folding and assembly makes this process an attractive candidate for playing a role in the maturation pathway of AChR subunits. Expression of the α polypeptide in the presence of glucosidase I and II and α-mannosidase inhibitors, however, did not reduce the efficiency with which the α subunit acquires the ability to bind BTX. In fact, we consistently observed an enhanced efficiency of BTX binding in the presence of these inhibitors. Blockade of oligosaccharide trimming also had no effect on subunit assembly, demonstrating that oligosaccharide trimming is not essential for correct folding or oligomerization of AChR subunits. These data are in agreement with a previous study that reported no effect on subunit folding and receptor assembly in cultured muscle cells treated with an inhibitor of glucose residue trimming (61). If oligosaccharide trimming is critical for the association between glycoproteins and calnexin (47), then our data suggest that calnexin does not play an essential role in the maturation process of AChR subunits. Alternatively, calnexin may act as a chaperone via a different mechanism that is independent of oligosaccharide trimming (62) but involves a direct interaction with transmembrane domains on target proteins (63). Although a recent study of cultured muscle cells showed that calnexin associates with the α subunit prior to completion of the folding reaction (34), further experiments will be necessary to demonstrate a direct role for calnexin in the maturation pathway of AChR subunits.

In vivo, folding and assembly of AChR subunits occurs in a stepwise fashion. Studies with cells transfected with various combinations of subunits revealed that the first step in maturation of the AChR is formation of heterodimers between the α subunit and either the δ or the γ subunit (15, 16). Only after the heterodimers are assembled does the β subunit become incorporated into the complex to form trimers (16). Thus, in vivo, the mature form of the α subunit preferentially interacts with the δ or the γ subunit but not with the β subunit to form high affinity ligand-binding sites that are indistinguishable from the sites found in the native AChR (15). Our experiments in vitro showed that when the α subunit was expressed with either the δ or the β subunit, the BTX-binding form of the α subunit associated specifically with the δ subunit and not with the β subunit. Moreover, assembly of the β and the δ subunits led to the formation of heterodimers that bind cholinergic ligands with affinities much higher than those exhibited by the unassembled α subunit. Thus, formation of the high affinity cholinergic ligand-binding site demonstrates that maturation of the AChR subunits in vitro follows a pathway similar to that observed in vivo.

The in vitro translation system supplemented with dog pancreas microsomes is a powerful tool with which to pursue studies of membrane glycoprotein folding and assembly. The iso-
lated microsomes contain enzymes and factors necessary for efficient translocation, signal sequence cleavage, core glycosylation, and intramolecular folding. The external environment and the internal milieu of the microsomes can be easily manipulated, allowing a detailed dissection of the protein folding and assembly pathways. We have used this system to establish the importance of the redox potential and N-linked core glycosylation in the maturation pathway of the AChR α subunit. We have also demonstrated that the α subunit folds into a native-like conformation and specifically assembles with the δ subunit. Since the mechanism of subunit folding and assembly of the muscle AChR serves as a model system for assembly of other ligand-gated ion channels, the conditions that we have established using the in vitro translation system can be employed to elucidate the precise mechanism of subunit recognition and association that controls which combinations of subunits are assembled into functional receptors in vivo.

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Note Added in Proof—After submission of this paper, Gelman and Prives (Gelman, M. S., and Prives, J. M. (1996) J. Biol. Chem. 271, 10709-10714) reported experiments on the effects of DTT on the folding and assembly of AChR subunits that also show the importance of redox potential for maturation of the α subunit.

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