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Yeast Expression and NMR Analysis of the Extracellular Domain of Muscle Nicotinic Acetylcholine Receptor α Subunit*

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The α subunit of the nicotinic acetylcholine receptor (AChR) from *Torpedo* electric organ and mammalian muscle contains high affinity binding sites for α-bungarotoxin and for autoimmune antibodies in sera of patients with myasthenia gravis. To obtain sufficient materials for structural studies of the receptor-ligand complexes, we have expressed part of the mouse muscle α subunit as a soluble, secretory protein using the yeast *Pichia pastoris*. By testing a series of truncated fragments of the receptor protein, we show that α211, the entire amino-terminal extracellular domain of AChR α subunit (amino acids 1–211), is the minimal segment that could fold properly in yeast. The α211 protein was secreted into the culture medium at a concentration of >3 mg/liter. It migrated as a 31-kDa polypeptide with *N*-linked glycosylation on SDS-polyacrylamide gel. The protein was purified to homogeneity by isoelectric focusing electrophoresis (pI 5.8), and it appeared as a 4.5 S monomer on sucrose gradient at concentrations up to 1 mM (~30 mg/ml). The receptor domain bound monoclonal antibody mAb35, a conformation-specific antibody against the main immunogenic region of the AChR. In addition, it formed a high affinity complex with α-bungarotoxin (k_D 0.2 nM) but showed relatively low affinity to the small cholinergic ligand acetylcholine. Circular dichroism spectroscopy of α211 revealed a composition of secondary structure corresponding to a folded protein. Furthermore, the receptor fragment was efficiently ^{15}N-labeled in *P. pastoris*, and proton cross-peaks were well dispersed in nuclear Overhauser effect and heteronuclear single quantum coherence spectra as measured by NMR spectroscopy. We conclude that the soluble AChR protein is useful for high resolution structural studies.

The nicotinic AChRs[1] are members of the superfamily of ligand-gated ion channels that mediate fast ion flow across postsynaptic membranes in neural and muscle cells. This family of proteins includes the receptors for glycine, γ-aminobutyric acid, glutamate, and serotonin (1, 2). The AChR from *Torpedo* electric organ and skeletal muscle is a large (~290 kDa) heteropentameric complex consisting of four homologous subunits in the stoichiometry of $\alpha_2\beta\delta\gamma$ (*Torpedo* and embryonic muscle) or $\alpha_2\beta\delta\epsilon$ (adult muscle (3, 4)). The subunits are arranged in the order α-γ-α-δ-β to create a cylindrical complex around the ion channel (5–7). Each subunit is a single polypeptide chain that has a large extracellular amino-terminal domain followed by three transmembrane domains, a long intracellular loop, a fourth transmembrane domain, and a short extracellular carboxyl-terminal tail (8–10). The large extracellular domain of the AChR confers high affinity binding activity for major agonists and competitive antagonists. The neurotransmitter ACh interacts with two nonequivalent sites near the interface of the αδ and αγ subunits (2, 11, 12). The binding site for α-bungarotoxin (α-BuTx) is located largely on the amino-terminal extracellular domain of the α subunit (13–16). In addition, this part of the subunit also possesses the main immunogenic region, which stimulates the production and forms the binding site for autoimmune antibodies in sera of patients with myasthenia gravis (17).

In early binding studies using synthetic peptides corresponding to sequences of *Torpedo* α subunit, a major determinant of the α-BuTx binding site, was mapped to residues 185–196 and the main immunogenic region to residues 67–76, respectively (15, 18–22). Recent NMR studies have delineated further the structural details of the toxin-peptide complexes (23, 24). In comparison with the full-length α subunit, however, the peptide ligands bound α-BuTx with significantly lower affinities, suggesting that other residues outside of the peptide sequence may be part of the high affinity binding sites found on the native receptor protein (15, 25, 26). Electron microscopic studies have imaged the intact *Torpedo* AChR proteins at a resolution of 4.6 Å but were unable to reveal great details of the ligand binding sites (27). Recently, an ACh-binding protein (AChBP) from glial cells of the snail *Lymnaea stagnalis* has been crystallized (28). The molluscan AChBP is a soluble protein with 210 amino acids, which shares structural similarities with extracellular domains of the nicotinic AChR. Notably, 23.9% of residues in the protein were found to be identical to the α7 subtype neuronal AChR (29). However, the AChR-binding protein has much lower sequence identity with the α subunits of *Torpedo* and muscle AChR. In addition, the snail protein does not possess the main immunogenic region epitope

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[1] The abbreviations used are: AChR(s), acetylcholine receptor(s); α-BuTx, α-bungarotoxin; AChBP, acetylcholine-binding protein; α211, recombinant fragment of the amino-terminal extracellular region (amino acid 1–211) of the α subunit of the AChR; Endo H, endoglycosidase H; HSQC, heteronuclear single quantum coherence; IEF, isoelectric focusing; mAb, monoclonal antibody; Ni-NTA, nickel-nitrilotriacetic acid; NOESY, nuclear Overhauser effect spectroscopy; TROSY, transverse relaxation-optimized spectroscopy.

for interaction with autoimmune antibodies, and structural attributes of the α-BuTx binding site on the AChR-binding protein remain unknown.

Structural analysis of AChR at atomic resolution has been hampered by an insufficient supply of native receptor proteins and the difficulty in crystallizing membrane proteins. The full-length and truncated fragments of AChR subunits have been expressed as folded proteins in *Xenopus* oocytes, and mammalian and baculovirus-infected insect cells (30–34). These eukaryotic expression systems, however, were incapable of producing milligram quantities of receptor material needed for structural determination. Bacterial expression of AChR proteins has proven to be problematic because of the denaturing conditions required to solubilize the protein and aggregation of receptor subunits (35–38). In previous studies, we have shown that the entire amino-terminal extracellular domain of mouse muscle AChR α subunit (α211) can fold properly in the absence of other parts of the receptor subunit, and the protein is secreted to the culture medium when expressed in transfected COS cells (39, 40). Here we have made use of the yeast *Pichia pastoris* to generate large quantities of α211 as a soluble protein. The yeast-produced receptor domain includes the appropriate post-translational modifications and forms high affinity complexes with α-BuTx and the monoclonal antibody mAb35. Circular dichroism (CD) and NMR measurement further suggest that the protein was properly folded and hence amenable to structural determination by x-ray diffraction and multidimensional NMR techniques.

EXPERIMENTAL PROCEDURES

cDNAs, Expression Vectors, Strains, and Antisera—The full-length cDNA coding for the mouse muscle AChR α subunit was kindly provided by the late Dr. John Merlie (Washington University, St. Louis (41)). The yeast expression vector *pPICZαA* and *P. pastoris* strain *KM71 mutS* were purchased from Invitrogen. mAb210 and mAb35, two monoclonal antibodies against the NH_2-terminal extracellular domain of the AChR α subunit, were purchased from CRP Inc. (Richmond, CA (42,43)).

Chemicals and Reagents—Restriction and modification enzymes for DNA cloning were purchased from Invitrogen. Endoglycosidase H (Endo H) was obtained from New England Biolabs, Inc. (Beverly, MA). Synthetic oligonucleotide primers were made by Integrated DNA Technology (Coralville, IA). Nickel-nitrilotriacetic acid (Ni-NTA) metal affinity resin was the product of Qiagen, Inc. (Valencia, CA). [^{15}N]Ammonium sulfate was obtained from Cambridge Isotope Laboratories (Andover, MA). α-^{125}I-BuTx was purchased from Amersham Biosciences. Unlabeled α-BuTx and all other chemicals were obtained from Sigma.

Constructs of AChR α Subunit—All amino acids were numbered according to their position in the mature protein sequence. The cDNA construct α211 encodes the entire NH_2-terminal extracellular portion of mouse muscle AChR α subunit (from amino acid serine at position 1 up to proline at position 211; see Fig. 1*A*). It was made by amplification of the corresponding sequence in the full-length α subunit cDNA using PCRs. cDNA constructs α216 and αM1 express proteins containing the entire extracellular domain plus the first 6 amino acids of the first transmembrane domain (M1) and the complete M1 domain (up to amino acid 241) of the α subunit, respectively.

cDNAs encoding shorter fragments of the extracellular domain of the α subunit were created in a similar way by PCR amplification. The carboxyl-terminal deletion constructs α207, α208, α209, and α210 encode the α extracellular domain starting at amino acid number 1 (serine) and terminating immediately after amino acid number 207, 208, 209, and 210, respectively. The amino-terminal deletion constructs α5–211, α10–211, and α15–211 code for α subunit proteins whose sequences start at amino acid 5, 10, and 15 of the mature mouse AChR, respectively, and they all terminate immediately after amino acid 211 (Fig. 1*A*). For the convenience of cloning, an *Eco*RI site (GAATTC) was added to the 5′-end of all forward PCR primers, thereby introducing two additional amino acids (Glu and Phe) to the amino terminus of each α subunit protein. These two residues did not affect protein folding and the level of expression (data not shown). Besides, an *Xba*I site (TCTAGA) was added to the 3′-end of all reverse primers after the stop codon. After amplification by PCR, the cDNA products were digested with *Eco*RI/*Xba*I. They were then cloned into *Eco*RI/*Xba*I sites of the *Pichia* expression vector *pPICZαA*, downstream of the sequence for the α-mating factor signal peptide from *Saccharomyces cerevisiae* and the Glu-Ala-Glu-Ala repeat sequence (44). The sequence of all constructs was confirmed by automated DNA sequencing (performed at the University of Pittsburgh Biotech Center).

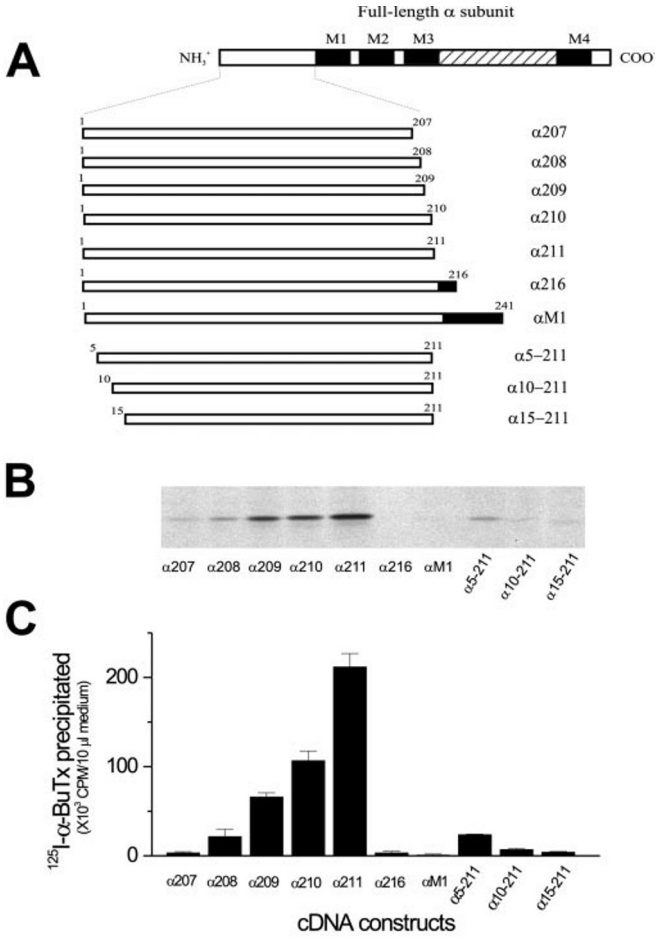

FIG. 1. **Expression of amino-terminal extracellular domains of mouse muscle AChR α subunit in *P. pastoris*.** *Panel A*, schematic representation of α subunit fragments expressed in yeast. *Top*, protein encoded by full-length α subunit cDNA. *Bottom*, proteins encoded by a set of truncated α subunit cDNAs containing deletions in the carboxyl- or amino-terminal portion of the coding region. The *numbers* indicate the first and the last amino acids encoded by each of the truncated subunit cDNAs. Sequences are shown in one-letter amino acid notation. Hydrophobic transmembrane domains of the constructs are shown in *black*. *Panel B*, expression levels of α subunit fragments vary with the length of the constructs. 48 h after methanol induction, the culture supernatant was collected by centrifugation. Total α subunit proteins secreted were separated by SDS-PAGE and immunoblotted with mAb210. *Panel C*, the amount of folded receptor proteins in culture supernatant was determined by α-BuTx binding assays. 10 μl of yeast culture medium was incubated with 5 nM α-^{125}I-BuTx in a buffer containing 0.1 M sodium phosphate (pH 7.4), 0.25 M NaCl, and 0.5% bovine serum albumin for 1.5 h at 4 °C. Toxin binding activities were determined by immunoprecipitation using the α subunit-specific monoclonal antibody, mAb210. Each data point represents results obtained from four separate experiments.

Yeast Transformation and Screening of Positive Clones—Plasmids carrying receptor cDNA constructs were linearized with the restriction enzyme *Pme*I and transformed by electroporation into the *KM71 mutS* strain of *P. pastoris* (45). The promoter regulating the production of alcohol oxidase (*aox1)* was used to drive the expression of the receptor protein (46, 47). Positive transformants with receptor cDNA integrated into the *aox1* locus on yeast chromosome were selected by growth on plates with Zeocin (Invitrogen). Single colonies were picked up randomly from the plates, and each was grown in 2 ml of the induction medium BMMY (1% yeast extract, 2% peptone, 0.1 M potassium phos-

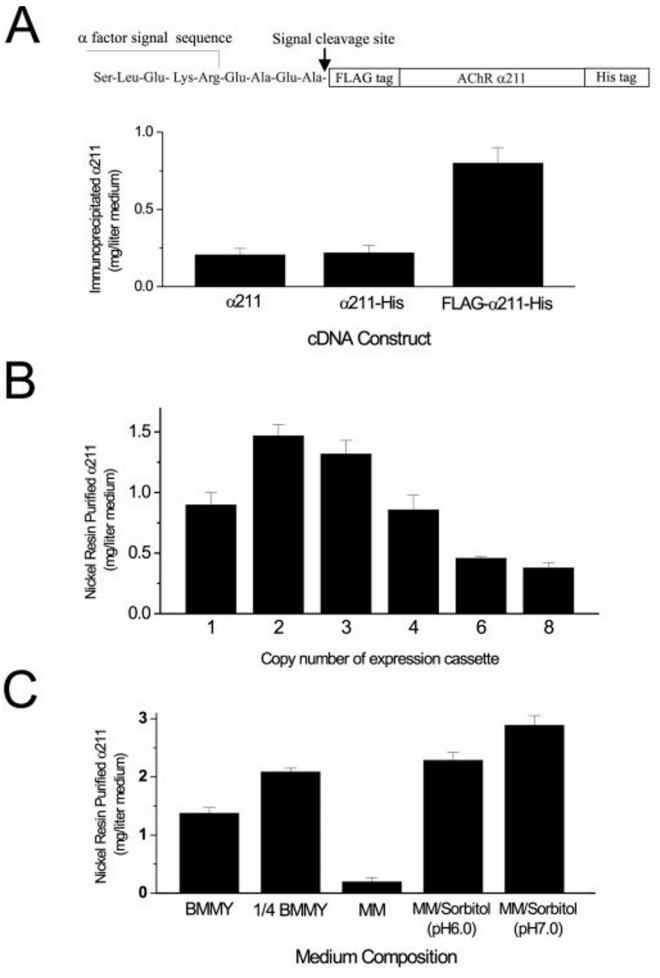

FIG. 2. **Optimization of conditions for α211 expression in *Pichia*.** *Panel A*, diagram of the expression cassette, which includes the signal sequence from the α-mating factor of *S. cerevisiae*, a Glu-Ala-Glu-Ala repeat, the FLAG/hexahistidine tags, and the α211 protein. The *arrow* indicates the site where signal peptide cleavage is predicted to occur in *Pichia*. 20 μl of yeast culture medium was incubated with 5 nM α-^{125}I-BuTx in a buffer containing 0.1 M sodium phosphate (pH 7.4), 0.25 M NaCl, and 0.5% bovine serum albumin for 1.5 h at 4 °C. Bound α-^{125}I-BuTx was pulled down by immunoprecipitation using mAb210 and protein G-Sepharose beads and counted with a gamma counter. The concentration of α211 protein was calculated based on the level of precipitated radioactivity and the specific activity of 220 Ci/mmol α-^{125}I-BuTx. *Panel B*, the copy number of the expression cassette affects secretion of α211 protein in *Pichia*. α211 protein was purified from the yeast culture medium using a Ni-NTA column. Protein concentration was determined by the BCA method. *Panel C*, *Pichia* was grown in culture media with different compositions. 48 h after methanol induction, the α211 protein was purified from the culture supernatant using a Ni-NTA column. Protein concentration was determined by the BCA method. Each data point represents results obtained from four separate experiments.

phate (pH 6.0), and 1% methanol) at 30 °C. After 48 h in culture, the supernatant was collected by centrifugation, and α subunit proteins secreted were determined by immunoblotting with mAb210 and by measuring α-^{125}I-BuTx binding activity (39). Clones that secreted the highest level of the receptor protein were chosen for large scale protein expression experiments.

Large Scale Expression and Purification of α211 Protein—The cDNA construct employed for large scale expression of α211 protein was tagged by an 18-nucleotide sequence encoding hexahistidine residues at the 3′-end of α211 coding region before the stop codon. In addition, a sequence for the FLAG tag (DYKDDDDK) was fused in-frame to the 5′-end of α211 cDNA (Fig. 2*A*). The dual epitope tags facilitated protein purification and enhanced secretion of α211 in yeast (Fig. 2*A*). The expression cassette was subcloned into the vector *pPICZaA* and stably transformed into a *KM71 mutS* yeast strain (*His3*$^+$). A single colony harboring the α211 cDNA was grown in 50 ml of BMGY medium (1% yeast extract, 2% peptone, 0.1 M potassium phosphate (pH 6.0), and 1% glycerol) at 30 °C in an incubator shaker overnight. The starting culture was used to inoculate 1 liter of a minimal medium containing 0.1 M potassium phosphate (pH 7.0), 3.4 g of yeast nitrogen base without amino acid, 0.5 g of ammonium sulfate, 0.0004 g of biotin, and 0.5 g of sorbitol. For ^{15}N labeling of the receptor protein, unlabeled ammonium sulfate was replaced by [^{15}N]ammonium sulfate. The culture was grown in batch mode in a 1.25-liter vessel on a New Brunswick BioFlo 3000 Fermentor at 30 °C. Agitation speed was set at 350 rpm, and the dissolved oxygen concentration was maintained above 25% through the entire fermentation process. 0.5% methanol and 0.05% sorbitol were added each day to induce protein expression and to increase the cell density. At 48 h after growth in the minimal medium, yeast supernatant was harvested by centrifugation, loaded on to a metal affinity column packed with Ni-NTA Superflow resin, washed with 250 mM NaCl plus 10 mM imidazole in 50 mM phosphate-buffered saline (pH 7.4), and eluted with 200 mM imidazole in 50 mM phosphate buffer (pH 7.4). The protein was dialyzed in 5 mM phosphate buffer (pH 7.4), and concentrated using Aquacide II (Calbiochem-Novabiochem Corp., San Diego). The sample was then purified using the Rotofor IEF system (Bio-Rad). A small aliquot was taken from each of the fractions, separated by SDS-PAGE, and detected with SimplyBlue SafeStain (Invitrogen). Fractions containing pure α211 protein were pooled, concentrated, and loaded onto a HiPrep26/10 desalting column (Amersham Biosciences) to remove the ampholytes.

Protein Analysis—The recombinant proteins were characterized with several biochemical and biophysical techniques. SDS-PAGE using 12.5% gels was carried out based on the method of Laemmli (48). Western immunoblotting experiments were performed as described by Wang *et al*. (39). Protein concentrations were routinely measured using a BCA colorimetric assay (Pierce) with bovine serum albumin as standard. Protein deglycosylation was carried out by incubating a purified protein sample with Endo H in 50 mM sodium phosphate buffer (pH 7.4), at 37 °C for 2 h. Sucrose gradient ultracentrifugation was performed as described by Wang *et al*. (39). Circular dichroism spectra were measured in a CD spectrometer (Aviv model 202) with the purified protein in 20 mM Tris (pH 7.4) at 22 °C.

Ligand Binding Studies—Radioligand binding at equilibrium was determined using a pull-down assay with the Ni-NTA Superflow resin. Briefly, protein samples were incubated with 220 Ci/mmol α-^{125}I-BuTx (Amersham Biosciences) in a buffer containing 0.1 M sodium phosphate, 0.25 M NaCl, and 0.5% bovine serum albumin at 4 °C. 40 μl of Ni-NTA Superflow resin was added to each reaction tube and incubated for an additional 30 min in a rotary mixer. The resins were precipitated by centrifugation, washed with phosphate-buffered saline, and counted for bound α-^{125}I-BuTx with a gamma counter.

The kinetics of association was measured by incubating α211 protein with α-^{125}I-BuTx at room temperature. To quantify the amount of toxin-receptor complex formed at various times after initiation of the reaction, the mixture was filtered through a Ni-NTA minicolumn (Qiagen), washed briefly, and counted for bound radioligand with a gamma counter. The association constant, k_{on}, was calculated from the time course of association using the integrated second order rate equation (49).

$$\ln\left(\frac{(LR)_e((L)_T - (LR)(LR)_e/(R)_T)}{(L)_T((LR)_e - (LR))}\right) = k_{on} \cdot t\left(\frac{(L)_T(R)_T}{(LR)_e} - (LR)_e\right) \quad \text{(Eq. 1)}$$

In Equation 1 $(L)_T$ is the total concentration of α-^{125}I-BuTx, $(R)_T$ the total concentration of α211 protein, $(LR)_e$ the concentration of the toxin-α211 complex at equilibrium, and (LR) the concentration of toxin-α211 complex at time t.

To study the kinetics of dissociation (k_{off}), toxin binding was allowed to reach equilibrium (90 min). Excess cold 20 μM toxin was then added to initiate dissociation, and the amount of bound α-^{125}I-BuTx was determined at the indicated times. The first order rate constant of dissociation was determined using Equation 2 (49). In this equation, $(LR)_0$ is the concentration of the toxin-α211 complex just prior to addition of excess cold toxin, and (LR) is the concentration of complex at time t after the initial dissociation. Division of k_{off} by k_{on} gave the K_D value.

$$\ln((LR)/(LR)_0) = -k_{off} \cdot t \quad \text{(Eq. 2)}$$

In competition studies with small cholinergic ligands, the concentrations of α211 and α-^{125}I-BuTx were kept constant at 1 and 5 nM,

respectively. Toxin binding activity in the absence of small ligands was normalized to 100%, and the amount of bound ligands was calculated from the reduction in α-^{125}I-BuTx binding. K_I values were calculated from the IC_{50} concentration of competing ligands by the equation of Cheng and Prusoff (50).

NMR Measurement of α211 Protein—Samples of unlabeled and ^{15}N-labeled α211 protein were prepared and concentrated to 10 mg/ml (0.32 mM) in 50 mM sodium phosphate (pH 7.4) and 50 mM sodium chloride. A two-dimensional sensitivity-enhanced TROSY-^{15}N-^{1}H HSQC (51) and a TROSY-three-dimensional ^{15}N-separated NOESY spectra (52) were measured at 25 °C on a Bruker DRX-800 MHz NMR spectrometer equipped with a z-gradient triple resonance probe (Bruker). Acquisition times and number of complex points in each dimension in the HSQC spectrum were 25.8 ms, 700* (f_2, ^{1}HN) and 41.9 ms, 110* (f_1, ^{15}N). 12 scans were recorded for each hypercomplex point for a total measurement time of 75 min. Acquisition times and number of complex points in each dimension in the NOESY spectrum were 20.0 ms, 512* (f_3, ^{1}HN), 4.5 ms, 40* (f_2, ^{1}H), and 11.4 ms, 24* (f_1, ^{15}N), and the mixing time was 70 ms. 32 scans were recorded for each hypercomplex point for a total measurement time of 57 h.

Selective flipback pulses on the water resonance were applied to compensate for the faster exchange rate of the amide protons observed at neutral and basic pH (53, 54). The WATERGATE (WATER suppression by GrAdient Tailored Excitation) pulse sequence was applied for additional water suppression (55). Data were processed and analyzed on an Octane work station (Silicon Graphics) using XWINNMR and NMRPipe (56).

RESULTS

In previous studies we have shown that α211, the entire amino-terminal extracellular domain of mouse AChR α subunit, is secreted as a soluble protein in transfected COS cells (39, 40). When the receptor domain with its native signal peptide sequence was expressed in *P. pastoris*, α211 protein was not detected in yeast culture medium as measured by α-^{125}I-BuTx binding activity. Immunoblotting of cell lysates using mAb210 indicated that the native receptor signal sequence was not cleaved off, and the protein was retained intracellularly. When the native signal peptide of the receptor protein was replaced by a signal sequence from α-mating factor of *S. cerevisiae*, however, low levels of α211 were secreted. A further increase in the secretion of α211 protein was detected when a Glu-Ala-Glu-Ala repeat was placed between the yeast signal peptide and α211 protein (data not shown and Ref. 44). Based on these results, the signal peptide of yeast α-mating factor and a Glu-Ala-Glu-Ala repeat were employed for the expression of α211 in all experiments described below.

Minimal Extracellular Domain of AChR α Subunit—In a previous study using transiently transfected COS cells, we noted that the levels of secretion of truncated extracellular fragments of AChR α subunit were affected markedly by the length of subunits expressed (39). As the first step toward setting up the yeast expression system, we sought to define a minimal domain of α subunit which is able to fold efficiently and give maximal secretion in *P. pastoris*. In one set of the experiments, we tested a series of α subunit fragments whose carboxyl-terminal sequences were truncated. Thus, the constructs α210, α209, α208, and α207 encode amino-terminal extracellular domains terminated immediately after amino acid 210, 209, 208, and 207, respectively (Fig. 1*A*). *Pichia* transformed with each of the mutant cDNAs was grown in BMMY and induced to express the proteins with methanol. 48 h after induction, the culture medium was collected by centrifugation, and total α subunit proteins secreted were detected by immunoblotting with mAb210 and protein G-Sepharose beads (39). As illustrated in Fig. 1*B*, α211 protein was produced at the highest level, whereas other shorter fragments (α210, α209 and α208, and α207) were secreted at significantly lower levels. In contrast, α fragments with part (α216) or the entire first transmembrane domain (αM1) were hardly detectable in the culture supernatant. These longer proteins are presumably retained in the endoplasmic reticulum as integral membrane proteins. In fact, we have shown in a previous study that they are resistant to extraction by alkaline buffers from transfected COS cell membrane (39). To examine whether the yeast-secreted proteins folded properly to assume a native receptor-like conformation, we measured their binding activities to α-^{125}I-BuTx. Among all of the fragments detected in yeast medium, α211 folded most efficiently as shown by its high toxin binding activity (Fig. 1*C*). Our data thus suggest that α subunit sequences up to proline at position 211 are indispensable for high level expression in *Pichia*.

In another set of experiments, we examined the expression of α211 protein with truncation of the NH_2-terminal sequence. The cDNA constructs α5–211, α10–211, and α15–211 encode proteins whose N terminus starts at amino acid 5, 10, and 15, respectively. The carboxyl termini of these constructs were terminated immediately after residue 211. As shown in Fig. 1*B*, truncation of the first five amino-terminal residues (construct α5–211) reduced the concentration of α211 protein in the culture medium dramatically. When more residues at the amino terminus were truncated as in the case of α10–211 and α15–211, little proteins were secreted, and α-^{125}I-BuTx binding activity was virtually undetectable in the yeast supernatant. Thus, we conclude that amino acids 1–211 in the primary sequence of α subunit constitute the minimal ligand binding domain for efficient folding and secretion in *Pichia*. Accordingly, the α211 construct was employed for all experiments described below.

Factors That Affect the Secretion of α211 Protein—Because a major goal of our research is to obtain protein materials sufficient for NMR measurement and for crystallization, we examined conditions that are key to high level expression of the receptor domain in *Pichia*. Epitope tagging by adding hexahistidine residues at the carboxyl-terminal end of α211 protein had little effect on protein yield but facilitated its purification by metal-chelating chromatography. Virtually all receptor domains in the culture supernatant bound the Ni-NTA resin, and ~95% of proteins eluted from the column appeared to be α211 (Fig. 3*A*, *lane 3*). In contrast, adding a FLAG tag (DYKDDDDK) to the amino terminus of α211 resulted in a remarkable increase in protein expression (Fig. 2*A*). Because the FLAG sequence is rich in charged residues, it may possibly enhance the overall solubility of the extracellular domain of the α subunit and thus account for the increased secretion of α211 in *Pichia*. For these reasons, we have employed the α211 construct with both FLAG and hexahistidine tags (see Fig. 2*A*) in all of the experiments described below (Figs. 2*B* through Fig. 9).

A major advantage of the *Pichia* expression system is more than one copy of foreign genes can be integrated into the *aox1* loci on chromosome DNA by homologous recombination (57). We have therefore introduced tandem expression cassettes containing several copies of the FLAG/hexahistidine-tagged α211 cDNA to *Pichia* genome by electroporation. The effect of cDNA copy number on the level of expression is depicted in Fig. 2*B*. Yeast with two copies of receptor cDNA secreted highest level of α211 protein. Adding more copies of the cDNA failed to enhance the expression of α211 further. In fact, protein yields started to decrease in cells carrying more than four copies of the expression cassette.

Pichia containing two copies of α211 cDNA was then fermented in batch mode (see "Experimental Procedures"), and optimal conditions for the culture were determined. In BMMY, a standard rich medium used widely for protein expression in yeast, the cells secreted ~1.4 mg/liter α211 protein. Surprisingly, the yield of receptor protein increased to 2.1 mg/liter when the cells were grown in diluted BMMY medium (1/4

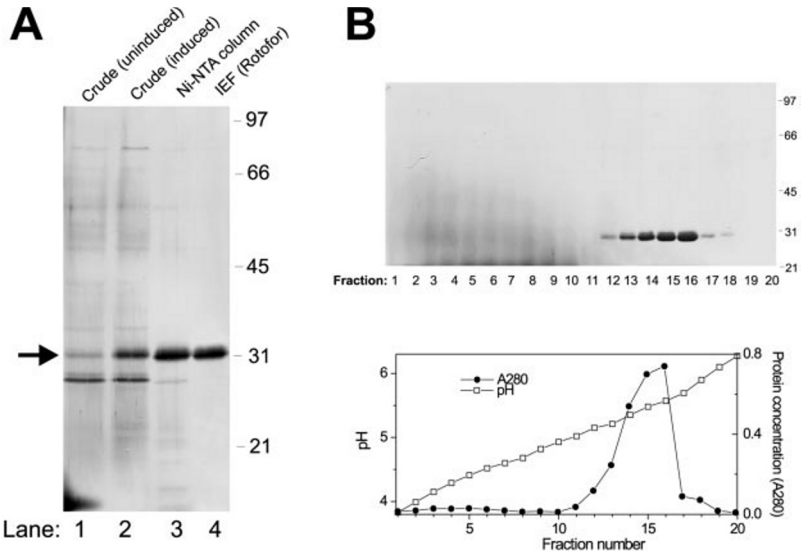

FIG. 3. **Purification of α211 from the yeast culture medium.** *Panel A*, SDS-PAGE separation and Coomassie Blue staining of of crude and purified protein samples. *Lane 1*, crude culture medium before induction; *lane 2*, crude medium from 0.5% methanol-induced culture; *lane 3*, α211 protein purified using a Ni-NTA column followed by IEF (*lane 4*). The *arrow* indicates the location of α211 protein on a 12.5% gel. *Panel B*, purification of protein by IEF. *Top*, protein samples were run in the Rotofor apparatus, fractionated, and separated by SDS-PAGE (12.5% gel). The gel was stained with Coomassie Blue. *Bottom*, α211 protein displays a pI value of ~5.8.

strength). Presumably the diluted medium may help to prevent overproduction and aggregation of α211 in the ER of yeast cells and hence facilitate the secretion of folded proteins. To optimize conditions for ^{15}N and ^{13}C labeling of α211 protein, we have grown *Pichia* in a protein-free minimal medium (pH 6.0). As shown in Fig. 2*C*, little receptor protein was generated (0.2 mg/liter) because of the sluggish growth of the cells (OD ~2.0 at 48 h). Addition of sorbitol (0.5 g/liter) to the minimal medium stimulated the growth of *Pichia* (OD ~14) and boosted α211 secretion (2.4 mg/liter). For many other recombinant proteins that have been successfully expressed before, the optimal pH for culture of *Pichia* was found to be 6.0 (57, 58). This condition, however, was not favorable for the expression of α211 presumably because it is close to the pI value (5.8) of the protein. In fact, when the culture was carried out at pH 7.0, the amount of α211 secreted into the medium increased to >3 mg/liter (Fig. 2*C*).

Biochemical and Pharmacological Characterization of α211 Protein—Because *P. pastoris* secreted very low levels of yeast proteins, α211 thus comprised the vast majority of the secretory proteins in the minimal medium, thereby simplifying the purification procedures (Fig. 3*A*). It was readily purified to >95% purity using metal-chelating chromatography with a Ni-NTA column (Fig. 3*A*). IEF electrophoresis in the Rotofor apparatus further purified the protein to homogeneity (Fig. 3, *A* and *B*). At the end of purification, ~75% of total receptor protein in the crude medium was recovered, and the final yield of α211 was 2.3 mg/liter culture. Amino-terminal sequencing of an aliquot of the sample confirmed that complete signal peptide cleavage had occurred in the yeast (data not shown). The purified α211 showed a pI value of 5.8 on IEF (Fig. 3*B*) and migrated as a 31 kDa single band on SDS-PAGE (Figs. 3*A* and 4*A*). Treatment with Endo H reduced the size of the protein to ~28 kDa, indicating that it was homogeneously glycosylated (Fig. 4*A*). When a glycosylation-defective mutant ($Asn^{141} \rightarrow$ Ala) of α211 was expressed in *Pichia*, we could not detect any receptor proteins in the yeast culture medium, suggesting that the *N*-linked glycosylation is essential for folding and secretion of the receptor domain (data not shown).

The yeast-secreted receptor protein folded correctly as determined in the following two experiments. First, purified α211 bound to α-BuTx-conjugated Sepharose 4B resin in a pull-down assay (Fig. 4*B*). Inclusion of excess free 0.5 μM α-BuTx prevented protein binding to toxin beads, suggesting that the interaction is specific. Second, the α211 protein could be immunoprecipitated by mAb35, a mononclonal antibody against a conformation-specific epitope in the amino-terminal extracellular domain of AChR α subunit (Fig. 4*B* and Refs. 42 and 43). The antagonist binding affinity of α211 was determined quantitatively in an equilibrium binding assay using α-^{125}I-BuTx as radioligand (Fig. 5*A*). The binding reaction was saturable with a K_D value of 1.2 ± 0.2 nM. A Scatchard plot of the data showed that the purified receptor fragment contains a single class of equivalent and independent binding site. Based on the B_{max} value of the binding reaction and the binding capacity curve (Fig. 5, *A* and *B*), we estimated that more than 95% of α211 protein employed in the assays bound ^{125}I-BuTx, suggesting a stoichiometry of 1:1 for the toxin-receptor complex.

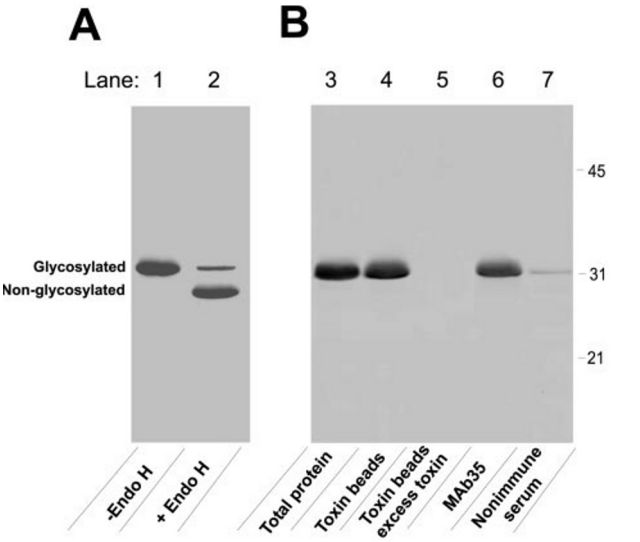

FIG. 4. **Folding of the purified α211 protein.** *Panel A*, 2 μg of purified α211 protein was digested with Endo H, separated by SDS-PAGE, and revealed by Coomassie Blue staining. *Panel B*, the α211 protein interacts with α-BuTx and mAb35. *Lane 3*, 2 μg of purified α211 protein on SDS-PAGE. *Lane 4*, the protein was precipitated with α-BuTx-Sepharose 4B resin and then separated by SDS-PAGE. *Lane 6*, α211 was immunoprecipitated by mAb35 and protein G-Sepharose. In *lane 5*, excess free 0.5 μM α-BuTx was included in the pull-down assay by α-BuTx-Sepharose 4B resin. *Lane 7*, α211 protein was not immunoprecipitated with nonimmune serum.

The kinetics of association between toxin and α211 was shown to be of second order with an association rate constant $k_{on} = 1.06 \times 10^6\ M^{-1}\ s^{-1}$ (Fig. 6). To determine the kinetics of dissociation, the binding reaction was allowed to reach equilibrium (90 min), and an excess of nonlabeled 20 μM α-BuTx was then added to initiate dissociation. The dissociation kinetics

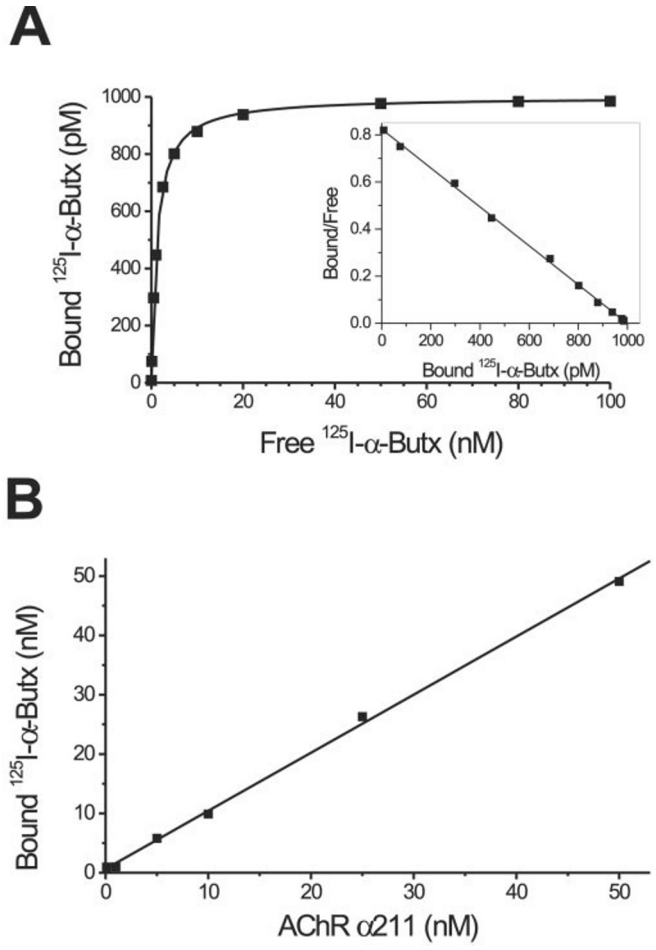

FIG. 5. **Saturation binding of ^{125}I-α-BuTx to purified α211 protein.** *Panel A*, 1 nM purified α211 protein was incubated with various concentrations of α-^{125}I-BuTx for 20 h. Bound toxin was separated by precipitation with Ni-NTA beads, and the radioactivity was measured in a gamma counter. Nonspecific binding was determined by competition with a 1,000-fold excess of nonlabeled α-BuTx and was subtracted from the total binding. Each data point is the mean of triplicate determinations. A Scatchard plot of the data is shown in the *inset*. *Panel B*, linear relationship of α211 protein concentration and toxin binding at saturation concentration (500 nM) of α-^{125}I-BuTx. The binding reaction was carried out in a total volume of 0.25 ml for each data point. Nonspecific binding was determined by competition with a 500-fold excess of nonlabeled α-BuTx and was subtracted from the total binding.

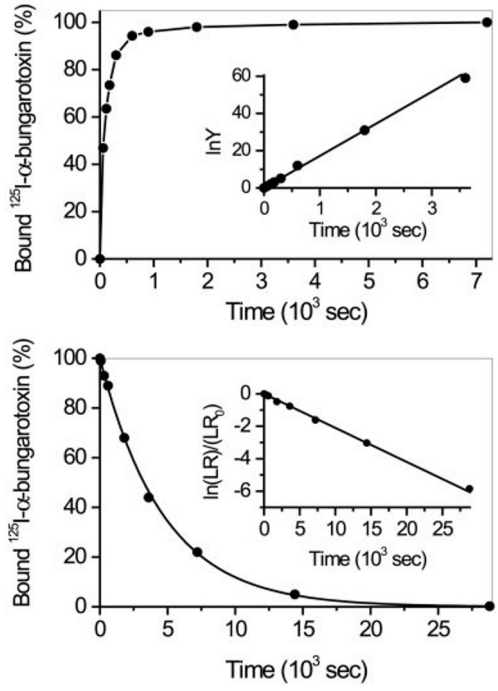

FIG. 6. **Kinetics of association and dissociation of ^{125}I-α-BuTx and α211 protein.** *Top*, 10 nM purified α211 protein was incubated with 25 nM α-^{125}I-BuTx at room temperature. Aliquots were removed at the times indicated and assayed for receptor-toxin complex using Ni-NTA minicolumns. *Panel B*, to study the kinetics of dissociation (k_{off}), toxin binding was allowed to reach equilibrium (90 min). Excess cold 20 μM toxins were then added to initiate dissociation, and the amount of bound α-^{125}I-BuTx was determined at the indicated times by filtering the sample through the Ni-NTA columns.

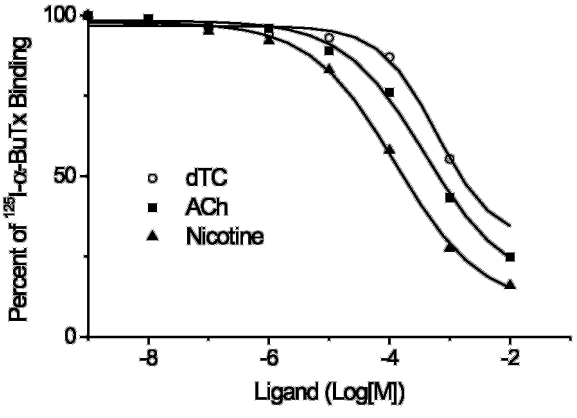

FIG. 7. **Competition binding assays with small cholinergic ligands.** The concentrations of α211 protein and α-^{125}I-BuTx were kept constant at 1 and 5 nM, respectively. Toxin binding activity in the absence of small ligands was normalized to 100%, and the amount of bound ligands was calculated from the reduction in α-^{125}I-BuTx binding.

was of first order, yielding the rate constant $k_{off} = 0.21 \times 10^{-3}$ s^{-1}. The dissociation constant K_D calculated from the association and dissociation rates (k_{off}/k_{on}) is 0.2 nM. This value is close to those obtained previously with the native AChRs (59–61) but is considerably lower than those with bacteria-expressed α extracellular domains (K_D = 130 nM) (37) or with synthetic peptides corresponding to α subunit sequence (K_D = 10 μM) (18–20). Thus, we conclude that the yeast-expressed α211 protein appears to assume a conformation very close to that in the native AChR α subunit.

The affinity of α211 to small cholinergic ligands was determined by equilibrium competition assays using 6 nM α-^{125}I-BuTx as radioligand and acetylcholine, *d*-tubocurarine, and nicotine as competing reagents. Based on the IC_{50} concentrations of competing ligands, K_I values were calculated using the Cheng and Prusoff equation (50) as 1.3×10^{-4} M, 3.3×10^{-4} M, and 4.2×10^{-5} M, respectively, for acetylcholine, *d*-tubocurarine, and nicotine (Fig. 7). These results are consistent with previous studies demonstrating that the α subunit alone does not bind cholinergic ligands well, and formation of high affinity ACh binding sites involves protein domains from adjacent δ and γ subunits in the receptor pentamer (2, 11, 12).

Biophysical Properties of α211 Protein—Unlike receptor fragments made with bacterial expression system (38), the yeast-generated α211 protein remained soluble at concentrations up to 1 mM (~30 mg/ml) in 50 mM phosphate buffer (pH 8.0). Ultracentrifugation on a sucrose gradient displayed a single peak of α-^{125}I-BuTx binding activity at ~4.5 S, suggesting that the receptor domain is a monomer (Fig. 8*A*). Secondary structure analysis of CD spectra indicated that the protein contained considerable β-pleated sheets with only a small amount of α-helical structure (14% α-helix, 46% β-sheet, 21%

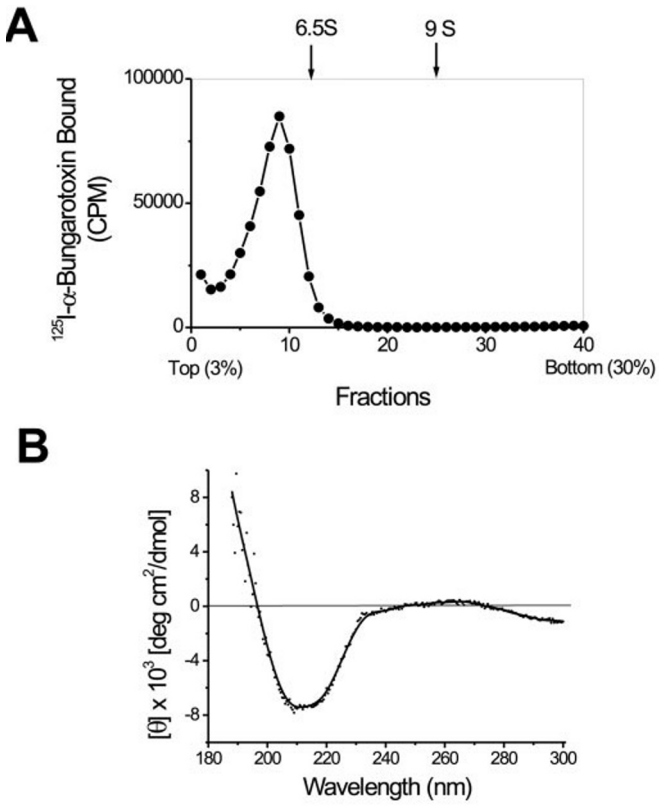

FIG. 8. **Biophysical characterization of the α211 protein.** *Panel A*, analysis by sucrose gradient sedimentation. Purified α211 protein was concentrated to 1 mM, and an aliquot of the sample was separated on 3–30% sucrose gradients. The fractions were assayed for α-^{125}I-BuTx binding activity as described under "Experimental Procedures." *Panel B*, analysis of 100 μM α211 in 20 mM Tris (pH 7.4) at 22 °C by CD spectrometry. The CD spectra show that the protein fragment is rich in β-sheet structure.

β-turn, and 19% random coils based on the CDPro program, Fig. 8*B*). This composition of α211 is very close to that of a glycosylphosphatidylinositol-linked mouse α210 protein expressed on surface of CHO cells (33).

NMR Analysis of α211 Protein in Solution—The high solubility of α211 protein facilitates protein structural studies using NMR spectroscopy. Moreover, the optimal growth of *Pichia* in minimal medium has allowed us to label α211 using [^{15}N]ammonium sulfate. The labeled receptor protein was concentrated to 10 mg/ml (0.32 mM) in the absence of detergent and used for multidimensional NMR analysis. By measuring the ^{1}H spectra and ^{1}H-^{15}N HSQC spectra (Fig. 9), we have optimized the sample conditions (salt concentration, pH, and temperature) to narrow the resonance line widths. At lower pH values (pH 4–7) more suitable for protein NMR, α211 precipitated at 35 °C, and T2 measurements indicated protein aggregation. At pH 7.4, α211 precipitation was minimal, but amide protons were subject to a faster exchange rate, which could result in the disappearance of amide protons in flexible regions of the protein. The incorporation of water flip-back pulses and WATERGATE water suppression into the pulse sequences, as well as measurement at lower temperatures (25 °C) partially alleviated the difficulties caused by the faster exchange rate. Of 215 expected cross-peaks (227 amino acids of which 12 are prolines) 195 cross-peaks were observed, a remarkable achievement considering the low concentration of α211 (0.32 mM), the short measurement time (75 min), and the expected partial overlap in a two-dimensional spectrum of a protein of this size. Both the HSQC and the NOESY spectra showed that the proton resonances are well dispersed, highly indicative of a folded protein. Our initial NMR studies thus suggest that the yeast-expressed receptor domain is amenable to further high resolution multidimensional NMR analysis.

DISCUSSION

In the present study, we have reported expression of the entire amino-terminal extracellular domain of mouse muscle AChR α subunit in a native-like form using *P. pastoris*. Several lines of evidence suggest that the α211 protein we produced is properly folded. First, the receptor fragment was expressed as a secreted protein in *Pichia*, and it remained soluble at high concentrations. Second, the protein bound the competitive antagonist α-BuTx with affinities approaching those reported for the native AChR pentamers in muscle and *Torpedo* electric organ (59–61). In addition, CD spectra showed that the receptor domain displays a composition of secondary structure similar to a membrane-anchored α210 protein expressed in Chinese hamster ovary cells (33). Furthermore, the proton resonances of α211 are well dispersed in NMR spectra, suggesting that the recombinant yeast protein assumes a folded conformation suitable for structural determination at high resolution.

Extracellular domains of the AChR have been expressed before as soluble proteins using transfected mammalian cell lines and baculovirus-infected insect cells (33, 34, 39). The receptor fragments were able to fold properly in these systems, but the quantities of protein produced were insufficient for crystallization or NMR determination. The full-length subunits of *Torpedo* AChR have also been expressed using *S. cerevisiae*. The yeast system, however, failed to yield functional proteins presumably because of insufficient cleavage of receptor signal sequences. Moreover, polypeptides of AChR subunits assumed wrong orientations in the endoplasmic reticular membrane of *S. cerevisiae* (62–64). Recently, the extracellular fragments of *Torpedo* AChR α subunit have been produced in inclusion bodies of bacteria in quantities sufficient for structural studies (36–38). This approach, however, has proven to be problematic because of the denaturing conditions required to solubilize the protein as well as the difficulty in refolding the receptor domain. Furthermore, bacteria-generated receptor fragments are not suitable for structural studies because they aggregate at high concentrations presumably resulting from the absence of post-translational modifications (38).

In the present study, we show that the length of extracellular domain is critical to the soluble expression of the receptor protein in *Pichia*. Protein fragments with part of (α216) or the entire first transmembrane domain (αM1) were not secreted by *Pichia* and were probably retained in yeast endoplasmic reticulum as integral membrane proteins. In our previous study, they were shown to be resistant to extraction by alkaline buffers from membranes of transfected COS cells (39). Other laboratories have employed α210, the entire amino-terminal extracellular domain of the α subunit, as well as fragments α209 and α208 for protein expression in Chinese hamster ovary cells, baculovirus-infected insect cells, and bacteria (33, 34, 36, 37). In the present study, however, we found that these shorter fragments were secreted in low levels by *Pichia*. Maximal protein expression was obtained with α211, which contains a proline residue in the first transmembrane domain of the α subunit sequence. Because the synthetic peptide derived from amino acid sequence 173–204 of *Torpedo* α subunit can bind α-BuTx (19), it seems unlikely that residue 211 participates directly in interacting with the toxin molecule. Instead, the proline residue in the first transmembrane domain may be critical for peptide folding to acquire a proper tertiary structure with an accessible high affinity toxin binding site. Alternatively, it may help to stabilize the receptor protein in yeast (39).

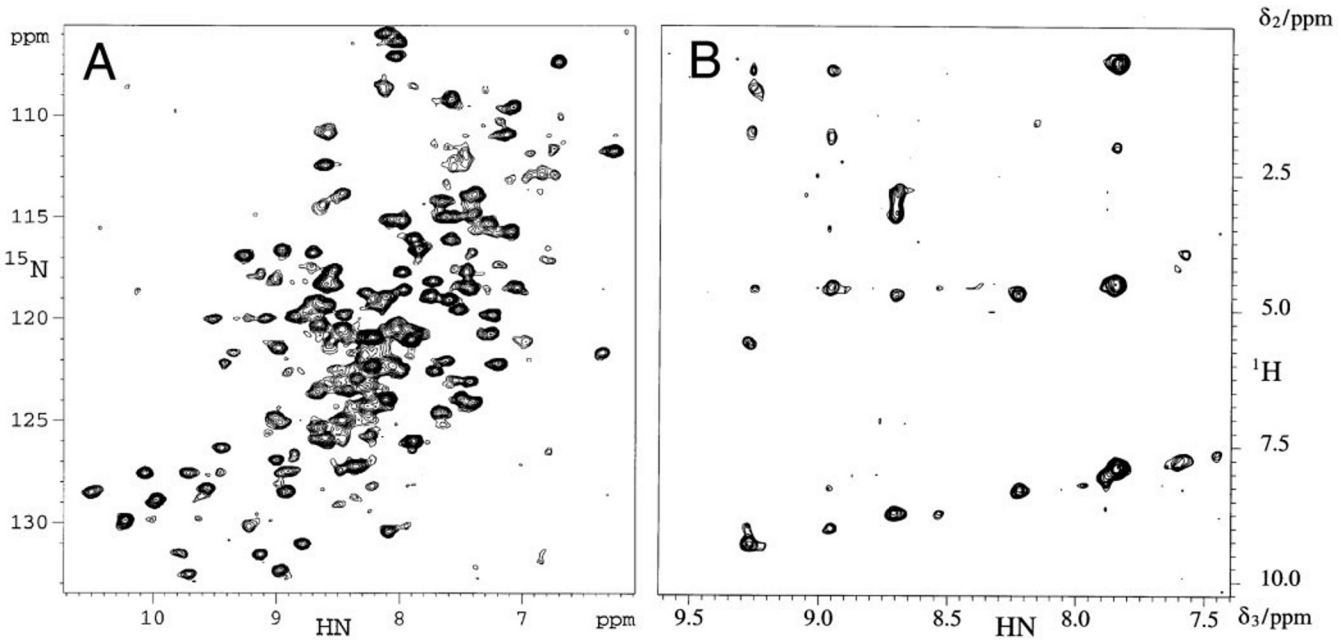

FIG. 9. **NMR study of the α211 protein in solution.** *Panel A*, a two-dimensional sensitivity-enhanced TROSY-^{15}N-^{1}H HSQC. *Panel B*, a representative plane of a TROSY three-dimensional ^{15}N-separated NOESY spectrum of α211 protein measured at 25 °C. The spectra were measured on a Bruker 800 MHz spectrometer at a protein concentration of 10 mg/ml (0.32 mM) in 50 mM sodium phosphate (pH 7.4) and 50 mM sodium chloride.

In agreement with the x-ray structure of the snail AChBP (28), the CD spectra of the yeast-expressed α211 indicate that the protein contains considerable β-pleated sheets with only a small amount of α-helical structure. The 14% of α-helical conformation measured by CD represents ~30 amino acids in α211 protein. In contrast, only 12 residues in the amino terminus of the snail AChBP were found to be in the α-helix (28). This discrepancy is not surprising in view of the low sequence identity between the AChBP and muscle AChR (29). In fact, the composition of α211 revealed by CD is consistent with early amphipathic analysis of *Torpedo* AChR sequences (65). It is also very close to that of a glycosylphosphatidylinositol-linked mouse α210 protein expressed on the surface of Chinese hamster ovary cells (33).

The yeast *P. pastoris* reported here offers several major advantages over other expression systems adopted previously for AChR expression. First, it is faster and less expensive to use than mammalian or insect cells and gives higher expression levels. Although the concentration of α211 in the culture medium is relatively lower compared with other secretory proteins that have been produced with *Pichia* before (57, 58), the addition of a FLAG tag to the amino terminus of α211 could increase the yield considerably. This effect is likely the result an enhanced solubility of the receptor protein because the tag sequence is rich in charged residues. Alternatively, the presence of FLAG tag at the amino terminus may help to facilitate signal peptide cleavage.

Although it is as easy to manipulate as *Escherichia coli*, the expression system we report here is superior to bacteria with regard to the efficiency of protein processing and folding. In *Pichia*, the yeast signal peptide is cleaved completely from α211 sequence, a key step that allows the receptor protein to enter the secretory pathway where it is folded properly to assume ligand binding activity (44, 64). In addition, the α211 protein was modified by *N*-linked glycosylation in *Pichia*, and the oligosaccharide chain was cleavable by Endo H. Many secretory proteins have been found to be hyperglycosylated in *S. cerevisiae* (50–150 mannose residues/side chain). In *P. pastoris*, however, the length of sugars added post-translationally to proteins usually averages 8–14 mannose residues, thus resembling the glycoprotein structure of higher eukaryotes (66–68). Glycosylation of α211 appeared to be homogeneous on SDS-PAGE, and it increased the size of the protein by ~3 kDa. The extracellular domain of AChR α subunit is known to possess a putative consensus sequence for glycosylation on residue Asn^{141}. Previous studies have detected *N*-linked oligosaccharides of similar size and composition on α subunit purified from skeletal muscle or expressed in heterologous systems (10, 31–33, 39, 69, 70). Accumulating evidence suggests that the *N*-linked glycosylation is required for efficient protein folding and secretion (69–71). A further advantage of the post-translational modification in yeast is the enhanced solubility of the recombinant protein. Biophysical studies using sucrose gradient ultracentrifugation, CD, and NMR spectroscopy all suggest that the α211 protein remains in a nonaggregated state even at high concentrations. Although glycosylation may sometimes render it difficult to crystallize proteins for x-ray diffraction, it generally does not interfere with protein structural determination by NMR. Indeed, the dispersed spectra of α211 in our HSQC and NOESY experiments support this notion. Finally, yeast cells are capable of secreting the receptor domain when they are grown in minimal medium supplemented with sorbitol, thereby enabling labeling of protein backbone by ^{15}N and C^{13} for multidimensional NMR studies.

In summary, we have described a yeast expression system for production of a soluble extracellular domain of AChR α subunit. Pharmacological and biophysical studies suggest that the α211 protein appears to be suitable for structural determination by multidimensional NMR and for crystallization. Because the AChR is highly homologous to other members of the ligand-gated ion channel family including γ-aminobutyric acid, glycine, and the serotonin receptors (1, 2), the approach we introduce here may open a new avenue for large scale production of soluble domains of these proteins. Because the receptors are known to be targets of drugs for pain management, treatment of mental illness, and other pathological events such as seizure and stroke, their high resolution structure information

is essential to the rational design of more specific and effective therapeutic agents.

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