The N-terminal extracellular region (amino acids 1–209) of the α-subunit of the nicotinic acetylcholine receptor (nAChR) from Torpedo marmorata electric tissue was expressed as inclusion bodies in Escherichia coli using the pET 3a vector. Employing a novel protocol of unfolding and refolding, in the absence of detergent, a water-soluble globular protein of 25 kDa was obtained displaying approximately 15% α-helical and 45% β-structure. The fragment bound α-[3H]bungarotoxin in 1:1 stoichiometry with a KD value of 0.5 nM as determined from kinetic measurements (4 nM from equilibrium binding). The kinetics of association of toxin and fragment were of second order, with a similar rate constant (8.2 × 10^3 M^-1 s^-1) as observed previously for the membrane-bound heteropentameric nAChR. Binding of small ligands was demonstrated by competition with α-[3H]bungarotoxin yielding the following KD values: acetylcholine, 69 μM; nicotine, 0.42 μM; anatoxin-a, 3 μM; tubocurarine, 400 μM; and methyllycaconitine, 0.12 μM. The results demonstrate that the N-terminal extracellular region of the nAChR α-subunit forms a self-assembling domain that functionally expresses major elements of the ligand binding sites of the receptor.

So far, most of our knowledge on the three-dimensional structure of neuroreceptors is based on a combination of electronmicroscopic, biochemical and immunological data obtained for the nicotinic acetylcholine receptor (nAChR) from the electric ray Torpedo (1, 2). These studies have elucidated the overall dimensions of the receptor protein (3), its position with respect to the surrounding lipid bilayer, the locations of functional domains and amino acid residues belonging to the integral ion channel (4, 5), its gating structures (6, 7), and the binding sites for several classes of ligands (1, 2, 7–12). However, a high resolution three-dimensional structure of the nAChR or any other neuroreceptor is still missing. If available, it could provide a molecular correlate for the recognition function of the receptor and thereby also for rational drug design.

Promoted by the fact that all attempts to crystallize the detergent-solubilized whole nAChR protein have been unsuccessful for the past more than 20 years, we have begun to overexpress selected domains of the receptor in bacterial expression systems. If successfully renatured, the domains that are not transmembranous should be water-soluble and thus should provide a better material for protein crystallization than the detergent-solubilized whole receptor protein. Moreover, if small enough in size, such fragments should be suited for multidimensional NMR analysis (13). As presented here for the N-terminal extracellular region of the nAChR α-subunit, we attempted and achieved expression as a water-soluble globular protein that displays ligand binding properties comparable to or better than those of the SDS gel-isolated α-subunit. To achieve appropriate renaturation, we developed a protocol for the complete unfolding (by means of chaotropic agents and disulﬁde-reducing agents) and refolding (by means of an oxidizing shuffling system and 1-arginine as structure-stabilizing agent) of the expressed polypeptide (14). The experimental conditions were such that self-organization of the unfolded protein was favored at the expense of (unwanted) aggregation and denaturation. In this way, we are able to prepare large quantities of the functional ligand binding domain from inclusion bodies of transformed Escherichia coli bacteria. Our results confirm that the N-terminal extracellular domain indeed harbors major elements of the ligand recognition function of the nAChR, as has long been suggested on the basis of affinity labeling and immunological studies (for recent reviews, see Refs. 1, 2, 8, 9, 15, and 16).

EXPERIMENTAL PROCEDURES

Materials—nAChR-rich Torpedo membrane fragments (3 mg/ml; 5 nmol of ACh sites/mg) were obtained by homogenization of Torpedo marmorata electric organ, followed by several centrifugation steps and alkaline treatment as described (11). N-(propionyl-3H)bungarotoxin (54–68 Ci/mmol) was obtained from Amersham Pharmacia Biotech. All other chemicals were of analytical grade and were obtained from major commercial sources.

Expression of a nAChR 1–209 in E. coli—A cDNA fragment coding for amino acids 1–209 of the nAChR α-subunit from T. marmorata was obtained by polymerase chain reaction amplification from a full-length cDNA clone (provided by Dr. Kretschmer, Leverkusen, Germany) using primers complementary to the nucleotide sequences 1–18 and 610–627. The primers were supplemented with restriction sites for the T7 expression system following IPTG induction of transformed bacteria. The α nAChR 1–209 polypeptide was found almost exclusively in E. coli inclusion bodies. Bacteria were disrupted by ultrasonic treatment in extraction buffer containing Tris-HCl (10 mM),

The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; α nAChR1–209, recombinant fragment of the N-terminal extracellular region (amino acids 1–209) of the α-subunit of the nAChR; CD, circular dichroism; IPTG, isopropyl-β-thiogalactoside; PAGE, polyacrylamide chain reaction; NMR, nuclear magnetic resonance.

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† To whom correspondence should be addressed. Tel.: 49-6131-395997; Fax: 49-6136-45094; E-mail: schratter@mail.uni-mainz.de.

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NaCl (10 mM), MgCl₂ (10 mM), pH 8.0, and inclusion bodies were harvested by centrifugation at 12,000 × g for 50 min and subsequently washed with guanidinium chloride (3 M), NaCl (0.1 M), Tris (0.01 M), pH 8.4.

Renauration of the α-nAChR₁–209 Fragment—The pellet containing the α-nAChR₁–209 fragment was solubilized in guanidinium chloride (6 M), Tris-HCl (0.1 M), dithioerythritol (0.1 M) (pH 8.5) by ultrasonic treatment and incubation for 2 h at 20 °C. In order to remove dithioerythritol, the mixture was dialyzed in a hollow-fiber membrane module (Variperm L, BITOP, Witten, Germany) (18) for 24 h against a 50-fold volume of Tris-HCl (0.1 M), EDTA (0.01 M), guanidinium chloride (6 M), pH 8.4. For a typical renaturation batch of 100 μl of this dialysate, were diluted in 20 ml of renaturation buffer, consisting of Tris-HCl (100 m M), glutathione (reduced form) (5 M), glutathione (oxidized form) (0.5 mM), EDTA (0.1 M), arginine (1000 mM), pH 9.5, and incubated for 24 h at 10 °C. Subsequently the renaturation solution was dialyzed three times for 24 h against storage buffer, consisting of Tris-HCl (10 mM), NaCl (100 mM), sucrose (10 mM), pH 7.4, and the resulting solution of α-nAChR₁–209 fragment was concentrated by ultrafiltration using Centricon-10 concentrators (Amicon) at 3000 × g and 4 °C to 2–10 mg/ml (80–400 μl). The final concentration was determined by the BCA method (19) following the protocol of the supplier (Pierce). Fragment solutions were stored at 10 °C.

SDS Gel Electrophoresis and Isoelectric Focusing—SDS-PAGE (12.5%) of the purified α-nAChR₁–209 (12 kDa) was performed according to Laemmli (20). Isoelectric focusing was performed using nonlinear pH 3–10 Immobiline® DryStrips in a MultiphorII electrophoresis system from Amersham Pharmacia Biotech according to a protocol of the supplier. Theoretical pI calculations were performed using Expasy facilities at the Swiss Institute of Bioinformatics (21).

Sequence Analysis—After SDS-PAGE, the α-nAChR₁–209 Protein was electroblotted onto polyvinylidene difluoride membranes as described previously (22), and the membrane was directly applied to an Applied Biosystems pulsed liquid-phase HPLC system (Applied Biosystems). The first order rate constant of dissociation was calculated from Equation 2 to the slope of the plot,

\[ \ln \left( \frac{C_{b}^{eq}}{C_{b}^{eq} - C_{b}^{eq}(R_{0} - L_{0})} \right) = \frac{1}{k_{eq}} \left( \ln \left( \frac{C_{b}^{eq}}{C_{b}^{eq} - C_{b}^{eq}(R_{0} - L_{0})} \right) + \ln \left( \frac{R_{0}}{L_{0}} \right) \right) \]  

(Eq. 1)

against the incubation time in s (26) \((C_{b}^{eq}, \text{concentration of bound ligand at equilibrium}; C_{b}^{eq}, \text{concentration of bound ligand at time } t; R_{0}, \text{total concentration of binding protein}; L_{0}, \text{total concentration of radioligand}).

The first order rate constant of dissociation was calculated from Equation 3 (calculations according to Ref. 26). Division of \(k_{eq}\) by \(k_{eq}\) gave the \(K_{d}\) value.

The kinetic data obtained determined the appropriate conditions for competition studies with small nicotinic ligands. In these experiments, the concentrations of Torpedo α-nAChR₁–209 fragment and tritiated α-bungarotoxin were kept constant at 1 and 6 nM, respectively. \(K_{i}\) values were calculated from the IC₅₀ concentrations of competing ligands according to Cheng and Prusoff (27). Data were fitted using appropriate equations of the program Origin 5.0.

RESULTS

Expression, Unfolding, and Refolding of the α-nAChR₁–209 Fragment—After induction by IPTG, the α-nAChR₁–209-pET 3a-transformed competent E. coli bacteria expressed large quantities (100–200 mg from 1 liter of bacterial culture) of Torpedo α-nAChR₁–209 largely in aggregated, denatured states as inclusion bodies. Overexpression was demonstrated in SDS gels of total bacterial extracts and of washed and stabilized inclusion bodies (Fig. 1). In particular, as shown in Fig. 1, lane 4, the washed inclusion bodies contained a very high percentage of α-nAChR₁–209.

To obtain the α-nAChR₁–209 fragment in water-soluble and purified form, we developed an unfolding/refolding procedure that made use of strategies recently developed for other pro-
teins overexpressed in *E. coli* as inclusion bodies (14, 28, 29). After extensive washing, in order to remove bacterial impurities, the inclusion bodies were incubated under moderate alkaline conditions with a large excess of chaotropic and disulfide-reducing agents (6 M guanidinium chloride, 0.1 M dithioerythritol, pH 8.5). The completely unfolded polypeptide was then dialyzed against 6 M guanidinium chloride, 10 mM EDTA, 100 mM Tris, in order to remove the disulfide-reducing agent and to prevent metal ion-catalyzed reoxidation. The solution was then diluted 200-fold, so as to provide optimal conditions for intramolecular interactions (self-organization) as compared with intermolecular interactions (aggregation). The renaturation buffer also contained an oxido shuffling system (5 mM reduced glutathione and 0.5 mM oxidized glutathione) and L-arginine (1 mM) as a protein-stabilizing agent. Incubation was performed at 10 °C for 24 h. Finally, the renatured α nAChR<sub>1–209</sub> fragment was dialyzed against storage buffer consisting of Tris-HCl (10 mM), NaCl (100 mM), sucrose (10 mM), pH 7.4, and concentrated by ultrafiltration to the wanted level (up to 10 mg/ml, i.e. 400 μM). No free sulfhydryl groups were detectable in the concentrated renatured solution by Ellman’s reaction.

**Biochemical and Biophysical Characterization of the Renatured α nAChR<sub>1–209</sub> Fragment**—As a result of the refolding procedure described above, a single polypeptide of apparent molecular mass of approximately 26 kDa was obtained, as demonstrated by SDS-PAGE (Fig. 1, lane 6). The band was electroblotted onto a polyvinylidene fluoride membrane and subsequently submitted to automated Edman degradation. A single sequence was obtained (SEHETRLVANLLENY) with an initial yield of 8 pmol. It corresponded to the first 15 amino acids of the N-terminal region of the *Torpedo* nAChR α-subunit (57). The molecular mass of the fragment protein, as determined by matrix-assisted laser desorption/ionization mass spectroscopy, was 24,896.2 Da, which conforms quite well with the calculated mass of 24,796.3. The difference of 100 is not unusual, as it may be due to random acrylamide adducts on cysteines (+70) and/or ionization involving K⁺ ions (+39); the accuracy of the method in this range of masses is approximately ±50 (58). The mass spectogram (not shown) did not indicate the presence of any significant impurities.

Additional purification schemes involving size exclusion gel permeation materials (Sephadex G50, G75, and G100, and Superdex 75) and subsequent SDS-PAGE analysis of fractions indicated a monomeric state of the *Torpedo*-on-α nAChR<sub>1–209</sub> fragment (data not shown). UV absorption measurements at 280 nm of a 10 μM solution of *Torpedo*-on-α nAChR<sub>1–209</sub> fragment yielded an extinction coefficient of 61,000 ± 500 cm<sup>2</sup> mM<sup>−1</sup>, which conforms satisfactorily well to the value calculated from the known content of aromatic amino acid residues (59,840 cm<sup>2</sup> mM<sup>−1</sup>) (21).

After the renatured polypeptide was identified as the α<sub>1–209</sub> fragment of *Torpedo* nAChR, it was further characterized by biochemical and biophysical means. Isoelectric focusing (not shown) yielded an isoelectric point of 5.5, which is close to the value of 5.4 calculated on the basis of the amino acid composition (21).

In order to obtain a rough estimate of the secondary structure content of the renatured fragment, CD spectra were taken in Tris-HCl (10 mM), NaCl (50 mM), pH 7.4 (Fig. 2). Using the CONTIN and JFIT programs (24, 25), we calculated 15% α-helical and 45% β-sheet structure. These values may be compared with those obtained for the membrane-bound and solubilized whole *Torpedo* nAChR by CD measurements (30–32), results from Fourier transformed infrared and Raman spectroscopy (33, 34), theoretical modeling (35, 36), and cryoelectron microscopy (37), which suggested between 20 and 40% α-helical and 20 and 45% β-sheet structure (Table I).

Taken together, the biophysical data suggest that the renaturation procedure resulted in a refolded α nAChR<sub>1–209</sub> fragment that appeared to be structurally homogenous and to display a secondary structure composition that is in contradiction to previous predictions.

**Interaction of α nAChR<sub>1–209</sub> Fragment with α-Bungarotoxin—α-Bungarotoxins**—α-Bungarotoxin is an established high-affinity competitive inhibitor of the binding of acetylcholine to *Torpedo* nAChR. We employed the toxin to test whether renaturation of the α nAChR<sub>1–209</sub> fragment restored the ligand binding function of the fragment. Binding studies were performed with a filter disc assay (26) employing *N*-α-[propionyl-<sup>3</sup>H]bungarotoxin as radioligand. The assay makes use of the acidic nature of the fragment and the fragment-toxin complex in that these are collected by binding to a DEAE-cellulose filter matrix, whereas the free toxin is washed through. The assay is applicable only to complexes that do not dissociate to any significant extent during the time of filtration and washing (1–3 min). Fig. 3 shows a typical binding curve, the related Scatchard plot, and a binding capacity determination for the α nAChR<sub>1–209</sub> fragment. As is demonstrated by the linearity of the Scatchard plot, the fragment preparation exhibited a single class of binding sites for the toxin, with an equilibrium dissociation constant *Kₐ* = 4 ± 0.8 nM. The *B₎ₐₜ₅ₐₓ* value derived from the Scatchard plot (2.7 nM) is very close to the concentration of fragment employed in the assays as determined by the bicinchoninic acid method (3 nM), demonstrating a stoichiometry of 1:1 for the toxin-fragment complex. Clearly, the α nAChR<sub>1–209</sub> fragment binds the neurotoxin with high affinity, suggesting that the water-soluble renatured ligand binding domain assumes a conformation that in regard to toxin binding is close to the conformation in the native α-subunit. Furthermore, the fragment preparation appeared to consist of a single homogenous population of toxin binding sites.

Using the same DEAE filter disk assay as for the equilibrium binding experiments described above, the kinetics of association and dissociation of toxin and fragment were assayed. As shown in Fig. 4, the association kinetics were of second order, yielding an association rate constant *kₐ* = 8.2 × 10⁵ M⁻¹ s⁻¹.

![CD spectrum of the α nAChR<sub>1–209</sub> fragment of *Torpedo* nicotinic receptor. A 40 μM solution of α nAChR<sub>1–209</sub> in Tris-HCl (10 mM), NaCl (50 mM), pH 7.4, was prepared, and the CD spectrum was obtained as described under “Experimental Procedures.” Using the CONTIN and JFIT programs (24, 25), we calculated 15% α-helical and 45% β-sheet structure. These values imply a relatively low content of α-helix as compared with those obtained by a variety of methods (see Table I) for the membrane-bound and solubilized whole *Torpedo* nAChR, suggesting 20–35% α-helical and 30–55% β-sheet sheet.](http://www.jbc.org/Downloaded from)
TABLE I
Secondary structure predictions for the Torpedo nicotinic acetylcholine receptor and its extracellular ligand-binding domain

| Author           | Protein                          | α-Helical | β-Sheet |
|------------------|----------------------------------|-----------|---------|
| Moore et al.     | Whole nAChR                      | 34%       | 29%     |
| Yager et al.     | Whole nAChR                      | 35%       | 33%     |
| Mielke and Wallace | Whole nAChR                  | 23%       | 43%     |
| Wu et al.        | Whole nAChR                      | 40%       | 20%     |
| Méthot et al.    | Whole nAChR                      | 39%       | 35%     |
| Unwin (37)       | nAChR-extracellular domain       | 30%       | ND      |
| Tsigelny et al.  | nAChR-extracellular domain°     | 20%       | 25%     |
| Oettle (38)      | nAChR-nonmembrane regions°      | 29%       | 24.9%   |

° Theoretical model.

This rate constant is very similar to that obtained for the association of neurotoxin and the native membrane-bound receptor protein (26). Dissociation of toxin-fragment complexes was studied following addition of a 300-fold excess of unlabeled toxin to the toxin-fragment reaction mixture. The resulting dissociation kinetics were of first order, yielding a rate constant $k_{off} = 0.4 	imes 10^{-3}$ min. The quotient of $k_{off}$ and $k_{on}$, 0.5 nM, is lower than the $K_D$ value of 4 nM obtained from equilibrium binding experiments. The difference can be explained taking into account the second order kinetics of association: in the range of lower concentrations, $\tau_{on}$ is slower than in the case of near-saturating concentrations (38, 59), which results in apparently lower concentrations of bound radioligand. As summarized in Table II the $K_D$ value of 0.5 nM may be compared with those obtained previously with the same toxin and the whole solubilized receptor protein ($K_D = 0.01$ nM) (39), the detergent-solubilized isolated α-subunit ($K_D = 10$ nM) (40, 41), or synthetic peptides matching in sequence the region around cysteines 192 and 193 ($K_D = 10 \mu$M) (42). From the dissociation rate constant, a half-life of the toxin-fragment complexes $\tau_{off}$ is 30 min was obtained.

Equilibrium competition studies were performed using N-[propionyl-3H]bungarotoxin (6 nM) as radioligand and methyllycaconitine, anatoxin-a, nicotine, D-tubocurarine, and acetylcholine as competing nicotinic ligands. The concentration of Torpedo α nAChR1–209 fragment was 1 nM. Fig. 5 shows: $K_I$ values were calculated from the IC$_{50}$ concentrations of competing ligands (Fig. 4) according to Cheng and Prusoff (27) as $1.2 \times 10^{-3}$, $3.0 \times 10^{-6}$, $4.2 \times 10^{-7}$, $4.0 \times 10^{-4}$, and $6.9 \times 10^{-5}$ M, for methyllycaconitine, anatoxin-a, nicotine, tubocurarine, and acetylcholine, respectively (see Table II). As controls, atropin (a ligand of muscarinic acetylcholine receptors) and procain (a local anesthetic with binding sites within the open channel pore of the receptor) were included. Neither substance was able to displace tritiated α-bungarotoxin binding to the α nAChR1–209 fragment.

DISCUSSION

The Torpedo nAChR has been established as the prototypic model for fast ligand-gated ion channels in the central and peripheral nervous system due to its abundance in electric organs of electric fish (Torpedo and Electrophorus), which made it accessible to detailed biochemical and biophysical studies. To date, it has been impossible to obtain high resolution structural information at the atomic level due to resistance of the receptor protein to crystallization attempts (for recent reviews, see Refs. 1, 2, 8, 9, 15, and 16).

Therefore, attention has focused on the expression of relevant functional domains of the nAChR (such as the large extracellular loops of nicotinic α-subunits, which contain most of the determinants for binding of agonists and competitive antagonists) in heterologous systems, always with the hope of finding a general method to obtain substantial amounts of material, suitable for structural analysis at the atomic level by NMR or x-ray crystallography.

Large scale expression of recombinant proteins in E. coli often results in confinement of the desired protein in so-called inclusion bodies, which concentrate the heterologous protein in a denatured and aggregated conformation. Recently, methods have been developed to restore the functional native conformation of recombinant proteins by ensuring conditions that allow correct refolding and disulfide bond formation starting from

Fig. 3. Binding of α-[3H]bungarotoxin to renatured α nAChR1–209 fragment. Non-specific binding was determined by competition with a 1000-fold excess of non-labeled α-bungarotoxin and amount to 10%. A, the binding curve of N-α-[propionyl-3H]bungarotoxin to a 5 nM solution of renatured α nAChR1–209 fragment was fitted by a one site binding equation of the program Origin 5.0. The dissociation constant ($K_D$) for N-α-[propionyl-3H]bungarotoxin is 4.0 ± 0.8 nM. B, the Scatchard plot of the binding data shown in A is linear, suggesting a single binding site for α-bungarotoxin and confirming the concentration of the α nAChR1–209 fragment (3 nM) with a similar value for maximal binding sites ($B_{max} = 2.7$ nM). C shows the linear relationship of protein concentration of α nAChR1–209 fragment solutions (see under "Experimental Procedures") and radioligand binding at saturating concentrations of radioligand (500 nM). The slope of the plot is 0.7, again indicating a highly homogeneous solution of the protein in a native conformation and a 1:1 stoichiometry of α-bungarotoxin-binding.
solubilized denatured material (14, 28, 29, 43).

Using an oxido shuffling system with reduced and oxidized glutathione and l-arginine as a stabilizing agent, we have been able to obtain large amounts of an α-nAChR fragment that is soluble in aqueous buffers without the use of detergents and contains the first 209 N-terminal amino acids, including relevant binding sites for agonists and competitive antagonists, as demonstrated by radioligand binding experiments. The affinity for α-bungarotoxin (Kᵣ value, 0.5 nM) is lower than for whole membrane-bound receptor (0.01 nM) (26, 39), but higher than for detergent-solubilized isolated α-subunit (100 nM) (40, 41). These differences in affinity probably arise from the fact that in native nAChR, ligand binding sites are composed of discontinuous subunits, which contribute to the actual ligand binding domain. Most of these determinants reside within α-subunits, although there is also some contribution from neighboring (mainly β) subunits. Moreover, evidence from photoaffinity labeling experiments seemed to indicate location of toxin binding sites at the interface of subunits (for recent reviews, see Refs. 1, 2, and 16). The missing interaction of ligands with these additional subunits could be the explanation for the 40-fold lower affinity of α-bungarotoxin to α nAChR₁₋₂₀₉ fragment compared with the native pentameric receptor. On the other hand, the affinity of α-bungarotoxin to isolated and solubilized nAChR α-subunit is even 200-fold weaker when compared with α nAChR₁₋₂₀₉ fragment, probably because the use of detergents is disturbing crucial secondary structure elements (see also Table II).

In native nAChR, there are different agonist-binding states, namely low and high affinity states. Events leading to opening of the receptor intrinsic ion channel involve sequential binding of two molecules of agonist to the two α-subunits at relatively low affinity (EC₅₀ of 0.1–10 μM, depending upon agonist used), followed by conformational changes that are associated with an approximately 100–1000-fold increase in affinity for nicotine, for example, and a desensitized state of the receptor, which can no longer be activated by agonist (for recent reviews, see Refs. 1, 2, 15, 16, and 55).

Table II

| Ligand         | Native nAChR | Solubilized α-nAChR | α-nAChR₁₋₂₀₉ | Authors                  |
|----------------|--------------|---------------------|--------------|--------------------------|
| α-Bungarotoxin | 0.01         | 120–400             | 0.5          | Maelicke et al. (28)     |
|                |              | 1–7                 | 420          | Haggerty and Froehner (40) |
|                |              | 930                 |              | Tzartos and Changeux (60) |
|                |              | 120                 |              | Blanchard et al. (59)    |
|                |              |                     |              | Maelicke et al. (26)     |
|                |              |                     |              | Haggerty and Froehner (40) |
|                |              |                     |              | Weber and Changeux (38)  |
| Nicotine       | 8            | ~1000               | 69,000       | Haggerty and Froehner (40) |
| Acetylcholine  | ~800         |                     |              | Cooper et al. (45)       |
| Anatoxin-a     | 50–100       |                     | 3,000        | Pereira et al. (46)      |
| Methyllycaconitine | ~1000   |                     | 120          | Blanchard et al. (59)    |
| d-Tubocurarine | 200          |                     | 400,000      | Haggerty and Froehner (40) |
|                |              |                     | 4,000        | Gershoni et al. (41)     |

*Pereira et al. (46).
that high affinity binding states for ACh might result from conformational changes recruiting additional subsites from other subunits, possibly at interfaces between subunits (see Table II for summary and comparison of $K_I$ and $K_D$ values).

Secondary structure determination of the renatured α nAChR $\alpha_{1-209}$ fragment by CD spectroscopy indicated 15% α-helical and 45% β-strand structure. Whereas the value for β-strand structure is consistent with data obtained for the membrane-bound and solubilized whole Torpedo nAChR by CD measurements (30–32), results from Fourier transformed infrared and Raman spectroscopy (33, 34) and theoretical modeling (35, 36) (Table I) show that 15% α-helical structure is lower than the values of approximately 30 (37) and 20% (35) that have been obtained in two studies published so far explicitly concerning the extracellular N-terminal domain of nicotinic α-subunits. The first study used cryoelectron microscopy (36), and the differences observed could be explained in terms of subtle conformational changes due to interactions at interfaces to other subunits; the theoretical model makes a prediction (20% α-helix) that is nearer to the value we obtained (15%) than to the 30% α-helix derived from cryoelectron microscopy. Taken together, the biophysical data suggest that the renaturation procedure resulted in a α nAChR $\alpha_{1-209}$ fragment that appeared to be structurally homogenous and to display a secondary structure composition that is consistent with previous predictions.

Expression of recombinant proteins in E. coli results in the absence of posttranslational modifications, like N-glycosylation, which are not part of the synthetic repertoire of bacteria. Native α-subunits of Torpedo nAChR are glycosylated on Asn 141, glycosylation does not affect ligand binding properties but rather seems to play an important role in transport of the different types of subunits to the plasma membrane and in the assembly of the receptor pentamer (see, e.g. Ref. 56 and, for reviews, Refs. 1, 2, 15, and 16).

Thus, the expression of the extracellular ligand binding domain of the α-subunit of Torpedo nAChR in E. coli and subsequent renaturation to a native conformation resulted in a water-soluble protein with ligand-binding properties very similar to the native receptor. The biochemical parameters confirm the homogeneity and functionality of the protein, which presents an example for the production and characterization of ligand binding domains of other nAChR, namely neuronal subtypes, which are not available from natural sources in high amounts, or from other members of the multigene superfamily of five-subunit ionotropic receptors, like γ-aminobutyric acid$_A$, glycine, 5-hydroxytryptamine$_\text{g}$ or glutamate receptors.

To date, only the expression of short nicotinic receptor-fragment complexes, comprising mainly the assumed acetylcholine-binding site around the two adjacent cysteines 192 and 193, has been achieved in E. coli (41), and a NMR solution structure of such peptides in complex with α-bungarotoxin has been published (44). Recently, there were entries in the Protein Data Bank at Brookhaven National Laboratory of the NMR solution structures of the M2 membrane-spanning domains (which are assumed to contribute to the ion channel pore) of rat nAChR and human glutamate receptor of N-methyl-D-aspartate-subtype NR1, which have been expressed in E. coli and reconstituted in artificial micelles (Protein Data Bank accession numbers 1A11 and 2NR1). In summary, expression in E. coli and reconstitution of functional domains of neurotransmitter receptors appears to be an attractive possibility to obtain detailed structural information in the absence of successful crystallization of the whole receptor protein. Alternative strategies include recent successful attempts to express the N-terminal domain of mouse muscle α-nAChR in the membranes of Xeno- pus oocytes or Chinese hamster ovary cells (53) and rat α7 nAChR in Xenopus oocytes (54). Solubility and yield remained problematic, although these systems have advantages with respect to posttranslational modifications.

Also, for drug screening, large scale expression of neuroreceptor ligand binding domains offers attractive perspectives: pathological alterations of ligand-gated neuroreceptor ion channels are implicated in diseases or pathological conditions such as endogenous depression (γ-aminobutyric acid$_A$ receptors and 5-hydroxytryptamine$_\text{g}$ receptors) (47), stroke and amyotrophic lateral sclerosis (excitotoxicity and glutamate receptors) (48), schizophrenia (nAChR and γ-aminobutyric acid$_A$ receptors) (49 and 50), myasthenia gravis, morbus Alzheimer, morbus Parkinson, and Chagas’s disease (nAChR) (51, 52). Altogether, this work demonstrates the feasibility of large scale production of functional ligand binding domains of neuroreceptors in a prokaryotic expression system for structural investigations applying NMR or x-ray crystallography, at the same time offering possibilities with regard to pharmacological screening systems.

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