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Purification of the nicotinic acetylcholine receptor protein by affinity chromatography using a regioselectively modified and reversibly immobilized α-toxin from *Naja nigricollis*

Philippe Ringler [a,1], Pascal Kessler [b], André Ménez [b], Alain Brisson [a,c,*]

[a] *Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 rue Laurent Fries, 67404 Illkirch, France*
[b] *Département d'Ingéniérie et d'Etude des Proteines, C.E.A. / Saclay, 91191 Gif-sur-Yvette, France*
[c] *Biophysical Chemistry, Department of Chemistry, GBB, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*

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Abstract

A new method of affinity chromatography purification of the detergent-solubilized nicotinic acetylcholine receptor protein (nAChR) is presented, based on the reversible coupling of a chemically monomodified α-toxin from *Naja nigricollis* to a resin. The α-toxin was monothiolated on the ϵ-amino group of its lysine-15 by reaction with *N*-succinimidyl-3-(2-pyridyldithio)propionate and was covalently linked in a reversible manner to a thiopropyl-activated agarose resin by thiol-disulfide exchange. We found that 50% of the immobilized toxin molecules were effective for purifying nAChR, indicating a high accessibility of resin-bound toxins to their binding sites on the receptor protein. Purified α-toxin/nAChR complexes were eluted with nearly 100% recovery by reduction of disulfide bridges with dithiothreitol. nAChR solutions of high purity were obtained, as shown by polyacrylamide gel electrophoresis. A comparison was made with two other procedures of affinity chromatography using: (1) α-bungarotoxin from *Bungarus multicinctus* polymodified on several amines and covalently linked to a resin in a reversible manner, and (2) a commercial agarose resin bearing irreversibly immobilized α-cobrotoxin from *Naja naja kaouthia*. We conclude that: (1) the use of a selected regioselective linking of a peptidic ligand to a chromatography resin results in an increased efficiency of protein binding, and (2) a high yield of protein recovery is obtained via reversible covalent linking.

Keywords: Affinity purification; Nicotinic acetylcholine receptor; α-Neurotoxin; Chemical modification; SPDP

1. Introduction

The nicotinic acetylcholine receptor (nAChR) is a glycoprotein acting as a ligand-gated cation channel involved in signal transduction between nerve and muscle cells (reviews in Refs. [1–3]). At the neuromuscular junction, binding of acetylcholine to its receptor protein located in the post-synaptic membrane triggers the transient opening of an ion channel

Abbreviations: nAChR, nicotinic acetylcholine receptor; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; NEM, *N*-ethylmaleimide; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate

* Corresponding author, at address c. Fax: +31 (50) 3634800.
[1] Present address: M.E. Müller-Institut, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

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through which cations diffuse passively, leading to membrane depolarization and ultimately to muscle contraction [4]. The nAChR is a pentameric membrane protein of about 290 kDa composed of four subunits with stoichiometry $\alpha_2\beta\gamma\delta$, arranged symmetrically around a central channel [5–8]. This eukaryotic membrane protein has been extensively studied and provides a paradigm for the family of receptors for neurotransmitters. The two main factors which have contributed to such a popularity is the possibility of obtaining large quantities of protein from fish electric organs and the availability of high-affinity ligands made of snake venom α-toxins which have facilitated the isolation of this receptor protein.

Our understanding of the function of biological macromolecules requires a detailed description of their structure, if possible at atomic resolution. In the case of membrane proteins, both structural approaches of X-ray crystallography [9,10] and electron crystallography [11,12] can be used for providing this information. In both cases, one major requirement is the availability of large amounts of membrane protein solutions of high purity and monodispersity.The current three-dimensional structure of the nAChR is limited at a resolution of 9 Å [8], by electron image analysis of crystals of tubular nature which form spontaneously in native membrane suspensions [13]. Our aim is to obtain large amounts of detergent-solubilized pure nAChR for crystallization experiments.

Several methods of purification of the nAChR have been developed, the most successful being based on affinity chromatography with various types of ligands [14–16]. The curarimimetic α-neurotoxins from snake venoms, polypeptides of 60–75 amino acids, have been extensively used because they bind to the nAChR with high affinity ($K_d = 10^{-8}$ to 10^{-12} M) and high specificity [17,18]. However, recovery of nAChR has been limited by the quasi-irreversible binding between α-neurotoxins and AChR [19]. Affinity purification using cholinergic ligands of lower affinity has provided higher yield of recovery, however, purification was often incomplete and possible contamination with acetylcholine esterases rendered this approach inappropriate for crystallization studies [20].

We present here a method of affinity purification of nAChR–α-toxin complexes, using a chemically monomodified α-toxin covalently bound to a resin in a reversible manner, which fulfils both requirements of high purification and recovery.

2. Materials and methods

2.1. Chemicals

Dithiothreitol (DTT), carbamoylcholine, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), *N*-ethylmaleimide (NEM), α-bungarotoxin from *Bungarus multicinctus*, thiopropyl-activated agarose and α-cobrotoxin from *Naja naja kaouthia* immobilized on 4% beaded agarose were purchased from Sigma (St. Louis, MO, USA). *N*-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was from Pierce Europe (Amsterdam, The Netherlands). α-Toxin from *Naja nigricollis* was obtained from Pasteur Institute (Paris, France). [α-^{3}H]Bungarotoxin (78 Ci/mmol) was from Amersham (Poole, UK). Other chemicals were of best commercial grade.

2.2. Thiolation of α-toxins

The derivatization of *Naja nigricollis* α-toxin with SPDP and the purification of monomodified toxins were performed as previously described [21–23]. Briefly, after incubation of equimolar amounts (1 μmol) of SPDP and α-toxin, to which a trace amount of tritiated toxin was added [24], the resulting derivatives were separated according to their isoelectric point by cationic exchange chromatography (Fig. 1A). The α-toxin derivative harboring an additional disulfide on lysine-15 was used in this study (15 μM stock solution in 160 mM ammonium acetate, containing about 600 dpm/pmol). Its purity was checked by reverse phase HPLC (Fig. 1B). The presence of a monomodification was also checked by isoelectrofocusing gel electrophoresis and the position of the modification on lysine-15 was verified by N-terminal peptide micro-sequencing, as previously described [21].

Lyophilized α-bungarotoxin from *Bungarus multicinctus* was reconstituted at 1 mg/ml in a buffer made of 1 mM EDTA, 100 mM sodium phosphate (pH 7.5) and mixed with a small amount of [α-^{3}H]bungarotoxin (78 Ci/mmol) to give a final spe-

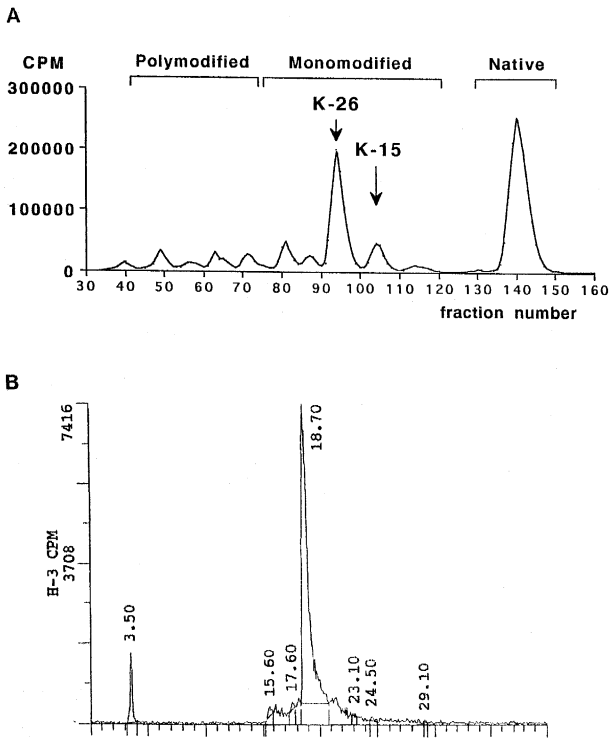

Fig. 1. Biochemical characterization of lysine-15 monomodified α-toxin from *Naja nigricollis*. (A) Purification of the products resulting from the derivatization of α-toxin from *Naja nigricollis* with SPDP by cation exchange chromatography on Biorex 70. The α-toxin contains seven amino groups: the NH_2-terminal group and six lysines, at positions 15, 25, 26, 46, 50 and 58 along the sequence. In a preparative-scale experiment using 60 mg of α-toxin, about 40% of the protein was not modified, 40% was monomodified on the various NH_2 groups and 20% was polymodified, as reported previously by Kessler et al. [21]. The monothiolated derivatives are resolved into five fractions. Major monoderivatization is on lysine-26 (56% of the monomodified toxins) which belongs to the toxic site. The derivative on lysine-15 is the second major product (16% of the monomodified product). (B) Elution profile of α-toxin from *Naja nigricollis* monomodified on lysine-15 by reverse phase HPLC on C4-Vydak column, using a 10–30% acetonitrile gradient. The radioactivity of each fraction was measured. The major peak contains 88% of the total radioactivity in the sample. The small peak eluted at low acetonitrile concentration is unexpectedly due to sodium azide.

cific radioactivity of 0.013 Ci/mmol. Thiolation of α-bungarotoxin free amines was performed by mixing the toxin with a 10-fold molar excess of the SPDP reagent: 1 ml of protein solution was mixed with 93 μl of a freshly prepared 15 mM SPDP solution in ethanol for 1 h at room temperature. The reaction medium was used as such for further incubation with the activated resin.

2.3. Preparation of affinity resins

In a first step, free sulfhydryls on the thiopropyl-activated 4% beaded agarose were deprotected by reduction with DTT. Briefly, 1 volume of thiopropyl–agarose was loaded in a 0.8-cm diameter column, washed with 10 volumes of a solution containing 100 mM NaCl, 1 mM EDTA, 3 mM NaN_3, 20 mM sodium phosphate (pH 6.5) (buffer A), and the hydroxypropyl 2-pyridyl disulfide linked to the resin (extent of activation: 20–35 μmol/ml) was reduced by elution with 5 volumes of buffer A supplemented with 100 mM DTT. The excess of DTT and the released thiopyridine were eliminated by extensive washing with at least 20 volumes of buffer A.

In a second step, the activated resin was incubated in batch with the solutions of modified α-toxins. 1.4 ml of lysine-15 monomodified α-toxin from *Naja nigricollis* at 15 μM were gently mixed during 1 h at room temperature with 4.7 ml of freshly reduced resin. Similarly, 1 ml of polymodified α-bungarotoxin was mixed with 2.2 ml of resin. Unbound toxins were eliminated by washing with buffer A.

In the last step, unreacted free sulfhydryl groups on the resins were quenched by reaction with an excess of NEM to avoid nonspecific binding of proteins during purification. A 5-volume solution of 100 mM NEM was deposited onto 1 volume of resin in a column. A final washing was made using 10 volumes of buffer A supplemented with 0.4% CHAPS.

2.4. Preparation of solubilized nAChR

AChR-rich membrane fragments were prepared from either fresh or frozen *Torpedo marmorata* electric organ (Institut de Biologie Marine, Arcachon, France) according to Sobel et al. [25] except that the purification step by centrifugation on a sucrose gradient was omitted. Membrane suspensions contained about 10% of nAChR-rich membranes, as estimated by their content in α-bungarotoxin binding sites, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and by transmission electron microscopy. Protein concentration was determined by the method of Bradford [26] using bovine serum albumin as a standard.

For membrane solubilization, one volume of nAChR-rich membranes at 2.5 mg protein/ml in 0.1 M Tris (pH 6.8) was centrifuged for 10 min at 30 krpm in a Beckman-R50 rotor. The supernatant was discarded and the pellet was mixed by vortexing with 2.5 volumes of buffer A supplemented with 1% CHAPS [27]. Non-solubilized material was discarded by centrifugation for 30 min at 30 krpm.

2.5. Purification of nAChR by affinity chromatography using reversibly immobilized α-toxins

Solubilized nAChR (1 mg protein/ml) was loaded on the two different types of resin and cycling of the samples at natural flow rate through the column was allowed for 30 min at 4°C. Unbound material was kept for evaluating the amount of nAChR bound to the resin or for further purification. The columns were then washed with 10 volumes of the following series of 0.4% CHAPS-supplemented elution buffers: buffer A, buffer A supplemented with 1 M NaCl and again buffer A.

Purified nAChR–α-toxin complexes were detached from the resin by reduction with 100 mM DTT in buffer A containing 0.4% CHAPS [28]. The amount of released α-toxins in the collected fractions was measured by counting the radioactivity of aliquots and the concentration of nAChR was determined as described below.

2.6. Purification of nAChR by affinity chromatography using irreversibly-bound α-cobrotoxin

The amount of insolubilized α-cobrotoxin in the commercial batch used for the present experiments was 120 nmol/ml resin. Loading of solubilized nAChR and washing were identical as described above. Purified nAChR was displaced from the α-cobrotoxin-agarose (1 ml gel) by incubation in batch for 3 h at 4°C with 3 ml buffer A supplemented with 1 M carbamoylcholine and 0.4% CHAPS [29].

2.7. Estimation of the concentration of α-toxin binding sites in nAChR solutions

The specific activity of nAChR solutions was determined by ultra-filtration with Centricon 100 concentration devices (Amicon, Beverly, MA, USA), using a method developed by Sutisna et al. [30]. Briefly, detergent-solubilized nAChR solutions were incubated with a known excess of [α-^{3}H]bungarotoxin, diluted to 1 ml if necessary with detergent-containing buffer and concentrated to 40 μl in a Centricon 100 device by centrifugation at 3000 rpm for 1 h. Due to the molecular cut-off of 100 kDa of the ultra-filtration membrane, the nAChR–α-toxin complexes are concentrated whereas unbound α-toxin molecules pass through the ultra-filtration membrane. Aliquots were counted before and after concentration and these values were compared to control experiments performed in the absence of nAChR. As α-neurotoxins were incubated in slight excess and at a minimal concentration of 100 nM, the low and high affinity binding sites of nAChR were assumed to be occupied by α-toxin molecules [31].

In the case of AChR solutions purified by reversible covalent chromatography, as an excess of tritiated α-toxin was already present in the sample, no further incubation with [α-^{3}H]bungarotoxin was needed.

With samples of nAChR purified on α-cobrotoxin-agarose, the high concentration of unbound agonist (1 M carbamoylcholine) was reduced by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) before incubation with [α-^{3}H]bungarotoxin, as described below.

2.8. DEAE anion exchange chromatography

In order to eliminate unbound ligands, such as α-neurotoxin or carbamoylcholine, or DTT, in the affinity purified samples of nAChR, anion exchange chromatography on DEAE Sepharose CL-6B was performed [32]. Briefly, a volume of resin was equilibrated with a buffer made of 50 mM NaCl, 1 mM EDTA, 3 mM NaN_3, 5 mM sodium phosphate (pH 6.5), 0.4% CHAPS (buffer B). Pooled fractions of purified α-toxin–nAChR complexes were directly loaded on the resin whereas carbamoylcholine-containing fractions were diluted 10-times with buffer B before loading. Washing was performed with buffer B and purified nAChR or α-toxin–nAChR complexes were displaced from the resin by elution with buffer B supplemented with 1 M NaCl.

2.9. Gel electrophoresis

SDS-PAGE was performed in 0.8-mm-thick slab gels. Separating gels contained 10% acrylamide and 0.26% bis-acrylamide, stacking gels contained 5% acrylamide and 0.13% bis-acrylamide. SDS was added at 0.02%. Gels were stained according to standard procedures. Molecular mass markers (LMW standard kit, Biorad) were boiled with β-mercaptoethanol prior to deposition on the gels whereas samples containing nAChR were not boiled because this treatment is known to induce aggregation of this protein.

2.10. Electron microscopy

A 5 μl drop of 0.1 to 0.2 mg protein/ml samples was adsorbed onto carbon-coated grids rendered hydrophilic by glow discharge in air under reduced pressure [33]. Grids were washed with two drops of water in order to remove most of the detergent and were stained with 1% uranyl acetate for 1 min. Electron microscopy was performed with a Philips CM12 operating at 100 kV under low electron dose conditions. Electron micrographs were recorded on Kodak SO163 films and developed under standard conditions.

3. Results

3.1. Purification of nAChR by affinity chromatography with reversibly bound α-neurotoxins

A scheme describing the principle of the method of purification is presented in Fig. 2. A high-affinity peptidic ligand of the nAChR is monomodified with the introduction of a disulfide structure and covalently coupled to a resin bearing sulfhydryl groups (1) by thiol–disulfide exchange (2). Solubilized nAChR binds to its ligand by molecular recognition (3) and the whole protein–ligand complex is released by reduction of disulfide bridges by DTT (4).

3.1.1. Synthesis of an affinity chromatography resin bearing α-toxin from Naja nigricollis monothiolated on lysine-15

The α-toxin from *Naja nigricollis* is a 6.8 kDa single polypeptide chain of 61 residues which belongs to the class of short post-synaptic α-neurotoxins [34]. A number of studies have identified the residues involved in the molecular interaction between α-neurotoxins and the nAChR, showing that they are highly invariant and localized in one loop of the three-looped structure [35–37]. Based on this knowledge, lysine-15 was chosen as the amino-acid

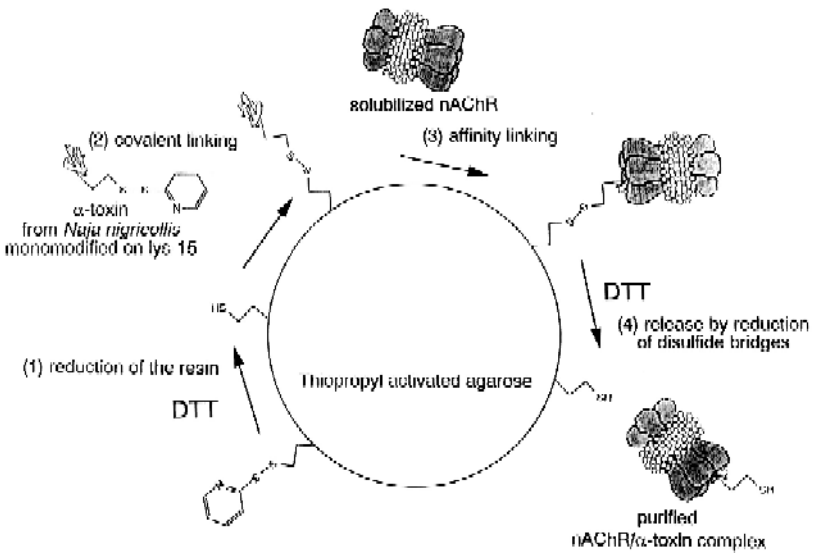

Fig. 2. Scheme describing the principle of the method of affinity purification of nAChR by reversible covalent linking of regioselectively monomodified α-toxin from *Naja nigricollis* to a resin.

to derivatize for subsequent coupling to the affinity resin because it is the most abundant lysine monomodified by SPDP which does not belong to the toxic site (see Fig. 1).

Several experiments were performed to optimize the efficiency of the purification procedure. Due to the high cost of the monomodified toxin, our rationale was to minimize the ratio between the number of toxin molecules immobilized on a resin and the amount of purified nAChR. In one typical experiment carried out in such optimal conditions, 21 nmoles of α-toxin monothiolated on lysine-15 were incubated with 4.7 ml propyl-agarose resin after deprotection of the sulfhydryls by DTT. By measuring the amount of non-bound toxins by radioactivity, we found that nearly 7% of toxin molecules – 1.5 nmol – bound to the resin, giving a concentration of 0.3 μM α-toxin in the gel. This value was verified by direct counting of the radioactivity associated with an aliquot of the resin. These conditions were considered as optimal as the same amount of nAChR could be purified with this resin (containing 0.3 μM α-toxin) and with resins containing up to 8 μM α-toxin (results from five experiments).

3.1.2. Synthesis of an affinity chromatography resin bearing polythiolated α-bungarotoxin from Bungarus multicinctus

The general scheme presented in Fig. 2 is also valid for polymodified peptidic ligands. This approach was applied to α-bungarotoxin from *Bungarus multicinctus*. This toxin, as well as α-cobratoxin from *Naja naja kaouthia*, belongs to the class of long α-neurotoxins (70–74 amino acids) which have a general topology with three loops similar to that of the short α-neurotoxins [37]. For polymodification of the NH_2-groups, lysines were randomly modified by incubating α-bungarotoxin with a 10-fold molar excess of SPDP. When 140 nmol of polymodified α-bungarotoxin were incubated with 2 ml reduced thiopropyl-agarose resin, 12% – 17 nmol – of the protein was reversibly bound to the resin through disulfide bridges, giving a concentration of 9 μM in the gel.

3.1.3. Purification of nAChR by affinity chromatography with reversibly bound α-neurotoxins

The main results of the affinity purification experiments are given in Table 1. The maximal binding capacity of the resins towards nAChR was first determined. We found that a 2-fold excess of nAChR, with respect to the amount of resin-bound α-toxin, was sufficient to reach saturation in the case of the lysine-15-coupled resin. As mentioned above, the amount of purified nAChR did not increase when resins with concentrations of α-toxin larger than 0.3 μM were used. We also found that the capacity of nAChR binding, determined at saturation, was almost the same with the resin bearing polymodified toxin, although the relative concentrations of toxin bound to the resins differ by more than one order of magnitude (0.3 μM and 9 μM).

Reduction of disulfide bridges with DTT resulted in an almost quantitative ($>$ 90%) release of α-toxin

Table 1
Comparative results of affinity chromatography purification experiments

Origin of the α-neurotoxins	*Naja nigricollis*	*Bungarus multicinctus*	*Naja naja kaouthia*
Mode of binding of α-neurotoxins to resins	Reversible binding, unique via lysine-15	Reversible binding, multiple	Irreversible binding, multiple
Amount of α-neurotoxin/ml resin	300 pmol	9000 pmol	120 000 pmol
Amount of bound nAChR/ml resin	85 pmol	90 pmol	4000 pmol
Amount of purified nAChR/ml resin	80 pmol	85 pmol	500 pmol
Bound α-toxin/purified nAChR	4	100	240

molecules bound to the resins, as measured by radioactivity. Similarly, the release of nAChR–α-toxin complexes by DTT was complete, as determined by ultrafiltration on Centricion 100. The amount of purified nAChR–α-toxin complexes was very similar for both resins: 80–85 pmol/ml resin. The nAChR–α-toxin complex was stable at 100 mM DTT and pH 6.5, as demonstrated by the Centricon-based assay. The specific activity of purified nAChR–α-toxin solutions was around 4 μmol bound α-toxin per g protein.

The purity of the nAChR solutions was evaluated by SDS-PAGE (Fig. 3A, B). Four major bands corresponding to the four subunits α, β, γ and δ of nAChR were resolved. The staining intensity of the band corresponding to the α subunit was higher, in agreement with the known stoichiometry and with common results [6]. The presence of a band corresponding to the associated protein of 43 kDa was also observed [38]. No other contaminants were visible even with silver-staining, indicating that the samples are of high purity with both procedures of purification. Estimated molecular masses, taking the standards as reference for calculation, gave mean values for the two gels of 41, 47, 52 and 61k Da for the α, β, γ and δ subunits, respectively. These values are in good agreement with values from the literature [6]. This also indicates that the α-toxin–AChR complexes dissociate during electrophoresis. We verified by fluorography that α-toxins indeed migrated with the tracking dye in these 10% gels (data not shown).

The purity of nAChR solutions was confirmed by electron microscopy (data not shown).

3.2. Purification of nAChR by affinity chromatography with irreversibly bound α-cobrotoxin

One type of commercially available affinity resin, extensively used for AChR purification, is prepared by irreversible binding of α-cobrotoxin from *Naja naja kaouthia* to agarose with bromocyanogen [29]. The amount of insolubilized ligand in the batch used for the present experiments was 120 nmol/ml resin. The capacity of binding of the resin towards nAChR was 4 nmol/ml (Table 1). Displacement of nAChR from the α-cobrotoxin–agarose with 1 M carbamoylcholine was only partial (25%) and provided 500 pmol nAChR per ml of resin.

The four subunits α, β, γ and δ of nAChR were also resolved by SDS-PAGE analysis of the purified nAChR sample (Fig. 3C, lane 3). When this sample was deposited without preincubation with β-mercaptoethanol, the band corresponding to the δ subunit was extremely weak and one additional band was observed with an apparent molecular mass of 126 kDa (Fig. 3C, lane 4), indicating that the purified nAChR molecules are mainly present as dimers linked by disulfide bridges between the δ subunits [39,40].

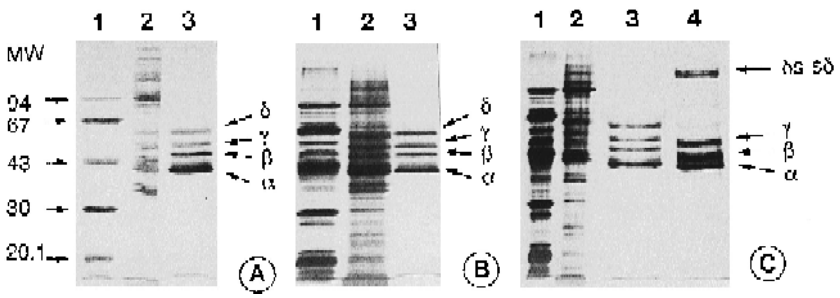

Fig. 3. SDS-PAGE analysis of purified nAChR solutions. (A) Purification with reversibly immobilized lysine-15 α-toxin from *Naja nigricollis*. (B) Purification with randomly modified and reversibly immobilized α-bungarotoxin. (C) Purification with irreversibly immobilized α-cobrotoxin. Gels were silver-stained. The four bands corresponding to nAChR subunits are labeled α, β, γ and δ. (1) A, B, C: molecular mass markers; molecular masses are indicated in the first left column. (2) A, B, C: crude solubilized membranes (1 μg protein in A, 5 μg protein in B, C) prior to purification. The four bands corresponding to nAChR subunits are visible, together with other proteins. (3): A, B: $\approx$ 4 μg purified nAChR after concentration on Centricon 100; C: 20 μl of purified nAChR ($\approx$ 1 μg) in the presence of β-mercaptoethanol. (4) C: same as in 3C without β-mercaptoethanol. The band corresponding to the disulfide linked δ–δ dimer is strong while the δ band is extremely weak.

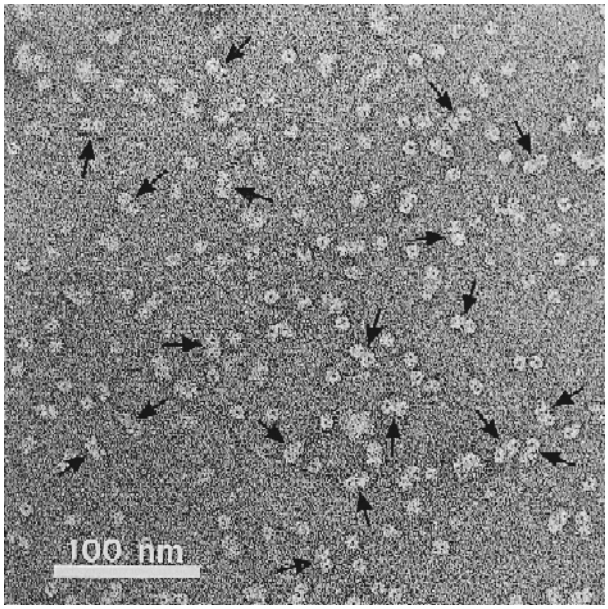

Fig. 4. Transmission electron micrograph of negatively stained nAChR molecules purified with the α-cobrotoxin resin. nAChR molecules appear as annular-shaped particules of nearly 8 nm diameter. Most of the particles present a stain deposit at their centre which outlines the central ion-channel. Numerous dimeric associations of nAChR molecules are visible (see arrows). Other particles are also present, mainly ill-defined aggregates which are likely to have resulted from the negative staining process. Scale bar = 100 nm.

The high proportion of nAChR dimers in this sample was confirmed by electron microscopy (Fig. 4).

4. Discussion

We present here a new method of affinity purification of the nAChR based on the regioselective coupling of a high-affinity ligand to a resin in a reversible manner. The heterobifunctional reagent SPDP was chosen for protein modification as it introduces a disulfide structure (2-pyridyl disulfide) allowing binding of the ligand to a thiol-bearing resin by a disulfide link which is ultimately easily cleavable with reducing agents. The α-toxin from *Naja nigricollis* monoderivatized at lysine-15 was selected as modifications at this position are known not to hinder its binding to the nAChR [21,37]. This strategy requires the engineering of the ligand but achieves both high efficiency of binding (50%) and high recovery of the protein (100%) because the whole protein–ligand complex can be displaced chemically from the resin. The affinity chromatography resin is of single use, but as only four immobilized ligands are needed for purifying one protein–ligand complex, it is reasonable to prepare fresh resin extemporaneously. The current limiting factor in the final amount of purified receptor is directly the amount of α-toxin bound to the resin.

The use of regioselectively monomodified ligands presents several advantages with respect to random polymodification. When α-bungarotoxin, a similar high affinity ligand of nAChR, was randomly modified with an excess of SPDP and bound via disulfide bonds to the same resin, a significantly larger amount (30-times) of immobilized ligands was needed to bind the same amount of nAChR and then to yield the same amount of purified receptor–ligand complexes. This result indicates that the majority ($> 95\%$) of the immobilized α-bungarotoxin molecules are not able to bind nAChR, either because the α-toxin binding sites are no longer accessible to the receptor or because their affinity for the nAChR has significantly decreased. Similarly, with the commercial resin obtained by bromocyanogen coupling of α-cobrotoxin, the number of bound toxins is 400-times larger than obtained with lysine-15 α-toxin, but the capacity of AChR binding is only 6-times larger, indicating that the major proportion of bound α-cobrotoxin molecules are non-effective in binding solubilized nAChR. The limited binding of both lysine-15 α-toxin and polymodified α-toxin to the resin, as compared to α-cobrotoxin, is certainly due to the fact that covalent reaction between disulfides on toxin molecules and SH groups on the resin is inefficient, due to steric effects. The presence of trace amounts of DTT remaining after resin activation might also explain this relatively weak binding. To improve the yield of this step which constitutes the limiting step in the whole purification procedure, an alternative strategy would be to bind the mono-modified toxin to the resin by affinity, by means for example of the biotin–streptavidin system [41]. Affinity binding reactions present the advantage to impose less stringent requirements in the relative positioning of the participating atoms as compared to covalent reactions.

Another potential advantage of the regioselective modification is that purified nAChR–α-toxin com-

plexes are homogeneous, which is of utmost importance in view of crystallization experiments. In the case of the classical α-cobrotoxin affinity resin tested here, 75% of bound nAChR molecules could not be displaced from the solid support by elution with high concentration of carbamoylcholine, a competitive ligand of lower affinity. It is highly likely that the elution procedure provides a non-homogeneous purified material, releasing the most weakly bound AChR molecules, while the most firmly bound, which are likely to be the most native ones, are not displaced. This is supported by the fact that δ–δ dimers predominate in purified nAChR solutions, as seen by SDS-PAGE and electron microscopy, while these dimers constitute only a minor population in non-purified solutions. One possible explanation is that dimers of nAChR have higher dissociation rates from the resin than monomers and are displaced first.

We anticipate that this approach using cleavable heterobifunctional reagents together with high-affinity ligands, such as offered by the biotin–streptavidin system [41], could be useful in the purification of macromolecular complexes in large quantity. Purification of monodisperse solutions of membrane protein–ligand or more generally of protein–protein complexes is of potential interest for crystallization studies. Not only proteins are stabilized in a given conformational state, but also the addition of an hydrophilic domain can help growing 3-D crystals of membrane proteins, as it has been elegantly demonstrated in the case of the cytochrome *c* oxidase–Fv complexes [10]. Furthermore, co-crystallizing a protein of interest with another protein of known structure can help in solving the structure of that protein by molecular replacement techniques [42].

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