# PHOTOAFFINITY DERIVATIVES OF α-BUNGAROTOXIN AND α-*NAJA NAJA SIAMENSIS* TOXIN

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## 1. Introduction

The nicotinic acetylcholine receptor from the electric organ of *Torpedo californica* appears to be a protein complex composed of several different polypeptide chains (reviewed in [1]). Four predominant chains have been identified by SDS–polyacrylamide gel electrophoresis. Their apparent molecular weights are (± 5%): 40 000 (α); 48 000 (β); 62 000 (γ); 68 000 (δ) [2–4]. The stoichiometry of these chains in the receptor complex is still unclear, but from cross-linking experiments a total mol. wt 270 000 and a pentameric quaternary structure was deduced [5]. A chain composition of $\alpha_2\beta\gamma\delta$ was proposed [6]. A mol. wt ~270 000 was also obtained by various other methods, [6,7]. However, the participation of β, γ and δ in the formation of the receptor complex has been questioned [8]. There is a general agreement that the α-chain contains the binding site for cholinergic agonists and antagonists, but the functional role of the other polypeptide chains in the regulation of the ion permeability of the postsynaptic membrane is still unclear. After incubation with reducing agents, the α-chain reacts with an SH-group directed affinity reagent [2]. In the native state α- and β-chains react preferentially with a photoaffinity antagonist but α-neurotoxin from cobra venom inhibited the labeling of only the α-chain [4]. The conclusion from these results is that the α-chain is the receptor in the true sense of the word, the other chains possibly being involved in ion translocation or other regulatory or structural functions.

The snake venom polypeptide toxins as α-bungarotoxin and α-*Naja naja* toxin [9] have been essential tools in the biochemical characterization of acetylcholine receptors. However, their binding sites in the receptor complex have been deduced only indirectly, since the resolution of the complex into its subunits under non-denaturating conditions has not been achieved. Here we describe covalently reacting photoaffinity derivatives of these toxins. With these compounds we find that the toxins bind to the α-chains as expected but surprisingly the δ-chain and another chain hitherto unknown also incorporated the label. These findings as well as the general applicability of radioactive photoaffinity toxins for the detection of acetylcholine receptors in biological tissues are discussed.

A preliminary account of these experiments has been presented [10].

## 2. Materials and methods

### 2.1. *Chemicals*

α-*Naja naja siamensis* toxin was prepared according to [11]. α-Bungarotoxin was purchased from Boehringer, Mannheim. 4-Fluoro-3-nitrophenylazide was a product from Pierce, Rockford. Buffer substances, materials used for SDS–polyacrylamide gel electrophoresis and for column chromatography were of the highest purity commercially available.

### 2.2. *Electric fish*

All experiments were performed with *Torpedo californica* obtained live from Biomarine Supply, Venice, CA. The animals were killed immediately before the preparation. Excess electric tissue was

frozen with liquid nitrogen, stored at −60°C and used in < 4 weeks.

2.3. *Receptor rich membranes*

Membrane fragments enriched in acetylcholine receptor were prepared as in [12] with the modifications in [13]. Phenylmethylsulfonylfluoride (0.1 mM) and 0.1 mM EDTA were present during homogenization of the electric organ to inhibit proteolysis. The specific activity of the membrane fragments used in the photoaffinity experiments was 1.2–2.0 nmol $^{125}$I-labeled *Naja naja siamensis* toxin bound/g protein.

2.4. *Receptor assay*

Two different assay procedures were used to determine the binding of cobra toxin to acetylcholine receptor. For the assay of the specific organs and for the determination of the binding capacity of the photoaffinity derivative of *Naja naja siamensis* toxin the Millipore filtration assay according to [14] was used. With the photoaffinity derivative of α-bungarotoxin the Millipore assay yielded very high backgrounds. Therefore the binding of this compound to receptor-rich membranes was determined by ultracentrifugation: membranes containing 8 μg protein were incubated in 2 ml Ringer's solution with increasing amounts of a solution containing 1.5 μg/ml $^{125}$I-labeled NAP-α-bungarotoxin. Incubation was performed in the dark at room temperature for 1 h. As a control the same incubation took place, but before adding the $^{125}$I-labeled NAP-α-bungarotoxin the receptor-rich membranes had been saturated by 1 h preincubation with 60 μg toxin from other fractions of the ion-exchange chromatography shown in fig.1 or with native bungarotoxin to show competition with the radioactive photoaffinity toxin. To determine binding the incubation mixtures were centrifuged 40 min at 140 000 × *g* at 10°C and the unbound toxin was determined by measuring the radioactivity in the supernatant.

2.5. *Synthesis of 4-azido-2-nitro-α-bungarotoxin (NAP-α-bungarotoxin)*

The method originally proposed for insulin [15] was modified: 1 ml dimethyl sulfoxide containing 26 mg (3.25 μmol) α-bungarotoxin and 15 mg (15 μmol) triethylamine was mixed with 1 ml dimethylsulfoxide containing 1.8 mg (10 μmol) 4-fluoro-3-nitrophenylazide. The mixture was shaken for 24 h at room temperature in the dark. Then it was lyophilized and the residue was resuspended in 2 ml 0.01 M ammonium acetate buffer (pH 7.6) and the NAP-α-bungarotoxin was separated from other reaction products by column chromatography with Sephadex G-10 (bed vol. 1 × 25 cm). The eluant was 0.01 M ammonium acetate buffer (pH 7.6). The chromatography was performed at 7°C in the dark. The fractions containing the protein were collected and lyophilized. The lyophilized material was dissolved in 2 ml 3.3 mM sodium phosphate buffer (pH 7.4) and transferred to a Whatman CM 52 ion-exchange column (0.8 × 5 cm). Elution was performed with 3.3 mM phosphate (pH 7.4) and a linear gradient of 0–8 mM NaCl. After fraction 75 was collected (2 ml each) a steeper gradient (8–80 mM NaCl) was applied. The elution profile is shown in fig.1.

2.6. *Synthesis of 4-azido-2-nitro-α-*Naja naja*-toxin (NAP-*Naja naja siamensis *toxin)*

Dimethyl sulfoxide, 1 ml containing 5 mg (0.6 μmol) *Naja naja siamensis* toxin and 15 mg (15 μmol) triethylamine was mixed with 1 ml dimethyl sulfoxide containing 1.8 μg (10 μmol) 4-fluoro-3-nitrophenylazide. The mixture was shaken for 24 h at room temperature in the dark. The reaction products were lyophilized and purified by chromatography with Sephadex G-10 as described for NAP-α-bungarotoxin. The NAP-*Naja naja siamensis* toxin was not purified further by ion-exchange chromatography because most of the compound could not be eluted from the resin even at high salt concentrations. The concentration of 4-fluoro-3-nitrophenylazide used in this experiment, although in excess, was found to be optimal. With larger excess of reagent to toxin water-insoluble derivatives were obtained which probably represent protein with several NAP groups attached to it.

2.7. *Radioactive labeling of the NAP-toxins*

NAP-α-bungarotoxin and NAP-*Naja naja siamensis* toxin were iodinated with $^{125}$I by the lactoperoxidase method [16]. The specific radioactivity was $6.4 \times 10^9$ cpm/μmol $^{125}$I-labeled NAP-α-bungarotoxin and $5.4 \times 10^9$ cmp/μmol $^{125}$I-labeled NAP-*Naja naja siamensis* toxin, counted in omnifluor/dioxane (8 g/l) with the tritium channel of a Beckman LS 100 scintillation counter.

2.8. *Photoaffinity labeling of acetylcholine receptor-rich membranes*

In a typical photoaffinity labeling experiment, 100 μl of a suspension of receptor-rich membranes in Ringer's solution (1 mg/ml) were incubated with 20 μl $^{125}$I-labeled NAP-*Naja naja siamensis* toxin (0.15 mg/ml) for 1 h at room temperature in the dark. The incubation mixture was then irradiated with white light with a 250 W medium pressure mercury lamp (Applied Photophysics, London) mounted ~6 cm from a 0.2 × 1 cm glass cuvette. An irradiation time of 30 s was found to be sufficient. In control experiments designed to show specificity of the reaction of the photolabels, the membranes had been preincubated with 20 μl unmodified *Naja naja siamensis* toxin (1 mg/ml)/100 μl membrane suspension. Previous control experiments had shown that irradiation in the absence of the label did not affect the receptor protein [4].

2.9. *Identification of membrane components reacting with the photoaffinity labels*

Before and after irradiation of the NAP-toxin/membrane complexes the membranes were washed 3 times by centrifugation (40 min at 20 000 × *g*) and resuspension to remove excess label and water-soluble reaction products. The purified membranes were then analysed by SDS–polyacrylamide gel electrophoresis on 10% acrylamide slab gels according to [17]. The gels were stained with 0.1% Coomassie-blue in isopropanol/acetic acid/$H_2O$ (25/10/65), destained with methanol/acetic acid/water (30/10/60), dried and subjected to autoradiography.

## 3. Results and discussion

Nitroazidophenyl groups can be easily incorporated into proteins converting them into molecules which can be activated by light to react with other molecules in their neighbourhood. If the protein is a radioactive ligand of a receptor, this NAP-derivative can be used as a photoaffinity label for the receptor. We show that labels of this type are useful for the characterization of neurotoxin binding sites in excitable membranes. We used an analogous approach with NAP-derivatives of anemone toxin from *Anemonia sulcata* for the identification of components of the voltage-dependent axonal sodium channel [18].

3.1. *Synthesis and purification of NAP-α-neurotoxins*

The original method for the synthesis of NAP-derivatives of proteins as described for insulin [15] proposes a 300-fold excess of 4-fluoro-3-nitrophenylazide over the protein. Under this condition a mixture of mostly water-insoluble reaction products probably poly-substituted peptides is obtained. To solubilize these reaction products the authors [15] used high concentrations of urea which was not feasible for our purpose. We found that a 3-fold excess of the reagent is sufficient to obtain NAP-α-bungarotoxin with a yield of > 80% (fig.1). Purification of the products by ion exchange chromatography with a linear NaCl gradient gave 5 protein peaks only 4 of which absorbed light at 480 nm, the characteristic absorbance of the nitroazidophenyl group. The fifth peak therefore was unreacted toxin. The 4 peaks showing light absorption at 480 nm most likely represent protein with the nitroazidophenyl group in different positions of the amino acid sequence.

Alternatively the polypeptide toxin may have incorporated 1, 2 or even higher amounts of the label. The exact stoichiometry of the reaction cannot be determined from the $A_{480}$ because the absorption of the nitroazidophenyl group may be influenced by its local environment in the polypeptide. However, the ratio $A_{480}/A_{280}$ which may be correlated with the amount of label incorporated per molecule protein varies from 0.18–0.26.

Increasing the excess of the reagent over the protein from 3–10 fold caused the disappearance of the peak of the elution profile containing no NAP. Therefore for the synthesis of NAP-*Naja naja siamensis* toxin a 17-fold excess was chosen. After removal of low molecular weight reaction product and water insoluble toxin derivatives by chromatography with Sephadex G-10 a NAP-*Naja naja siamensis* toxin was obtained which was not further separated into isomers.

3.2. *Light spectroscopy*

Figure 1 shows the absorption spectrum of NAP-α-bungarotoxin with its characteristic $A_{262}$ and $A_{480}$ maxima. Irradiation with white light in a glass cuvette

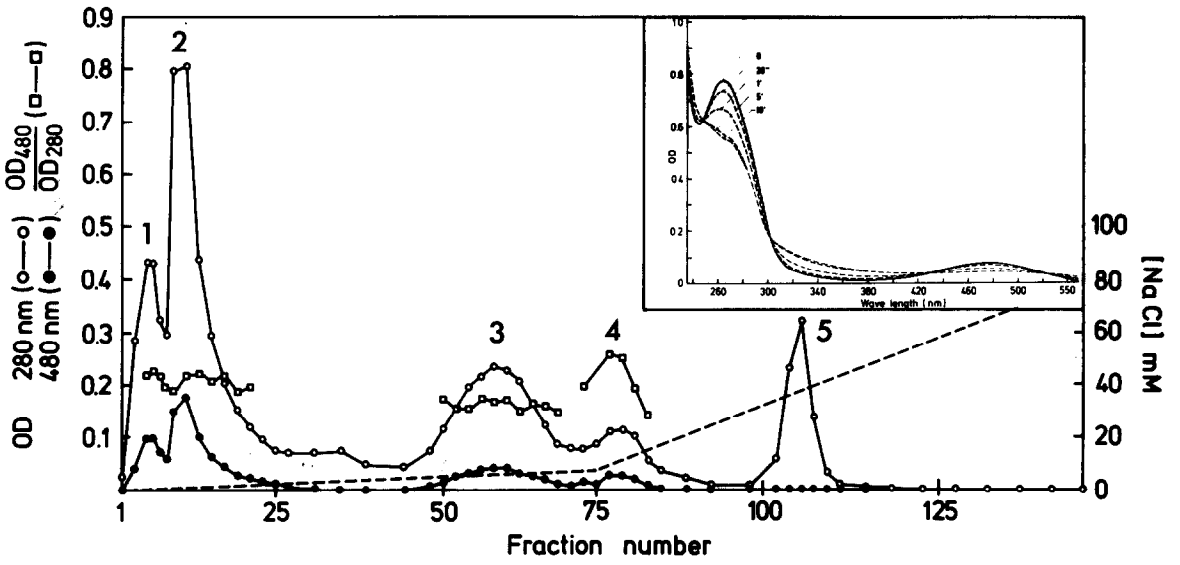



Fig.1. Purification of NAP-α-bungarotoxin. Ion exchange chromatography with Whatman CM 52 and two linear NaCl gradients (dotted line). Peaks 1–4 represent NAP-labeled protein. Insert: Absorption spectrum of NAP-α-bungarotoxin. The dotted lines represent absorption after 20 s, 1, 5 and 10 min of irradiation, respectively.

caused a decrease of both absorption maxima probably due to photoreactions of the azido group. After 5 min irradiation no significant further change in the absorption was observed.

### 3.3. *Radioactive labeling and binding studies*

NAP-α-bungarotoxin and NAP-*Naja naja siamensis* toxin can be labeled radioactively with $^{125}I$ by the lactoperoxidase method. The resulting radioactive NAP toxins still bind to the nicotinic acetylcholine receptor of the *Torpedo* electric organ (fig.2). Binding can be prevented by preincubation of the receptor membranes with native α-bungarotoxin. Furthermore binding of the radioactive NAP toxins is saturable. Both these observations can be taken as indications of the specificity of the toxin derivatives for acetylcholine receptor binding sites.

Binding of $^{125}I$-labeled NAP-α-bungarotoxin prepared by iodination of protein from peak 3 (fig.1) is also inhibited by non-radioactive NAP-proteins from peak 1 and 2. This shows that these isomers also retained their binding capacity for the acetylcholine receptor. 4-Fluoro-3-nitrophenylazide reacts with amino groups of the protein and none of the groups involved appear to be essential for the binding of the protein to the receptor.

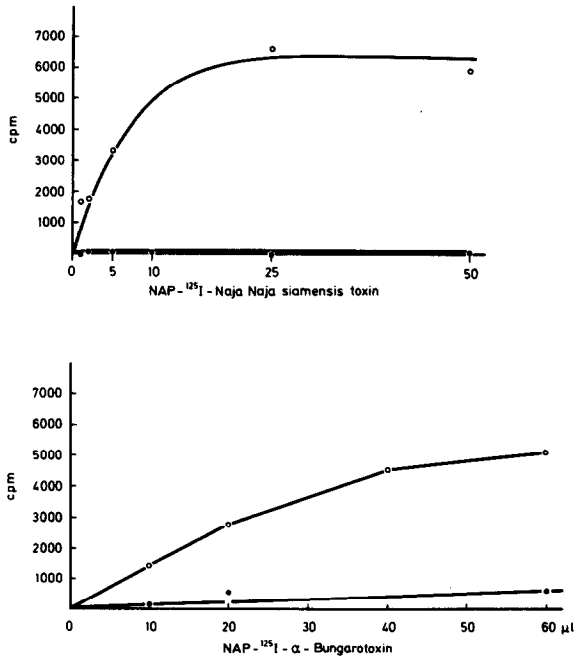
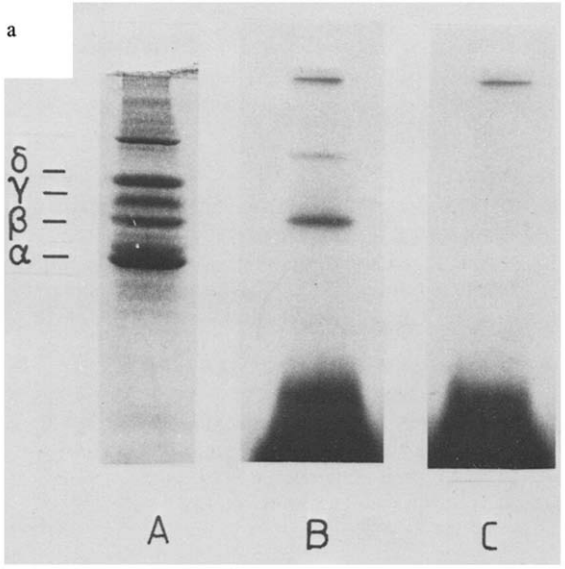



Fig.2. Binding of NAP-labeled α-neurotoxins to acetylcholine receptor-rich membrane fragments from *Torpedo californica*. (o—o—o) binding in the absence; (●—●—●) in the presence of an excess of unlabeled α-bungarotoxin. (for detailed conditions see section 2).

### 3.4. *Photoaffinity labeling of acetylcholine receptor-rich membranes*

Reversibly bound NAP toxins can be attached covalently to its binding site by irradiation of the receptor–toxin complex. We incubated receptor-rich membrane fragments from the electric organ of *Torpedo californica* with $^{125}$I-labeled NAP-α-bungarotoxin, removed the unbound excess of the toxin by centrifugation of the membranes and activated the photoaffinity label by irradiation. The experiment was performed in a glass cuvette to protect the protein from light in the 280 nm range. The reaction products were analysed by SDS–polyacrylamide gel electrophoresis and subsequent autoradiography (fig.3a). The autoradiographs showed that only a few of the membrane proteins had incorporated radioactivity. Mainly two bands could be detected on the film (besides radioactivity migrating with the tracking dye). They migrated with an app. mol. wt 48 000 and 78 000 (± 5%). Assuming that the reacting membrane proteins each incorporated one molecule of NAP-toxin of mol. wt 8000, the resulting mol. wt 40 000 and 70 000 are similar to the molecular weights of the α-chain (40 000) and δ-chain (68 000) of the acetylcholine receptor complex. NAP-toxin from peak 2 (fig.1) gave the same result, peak 1 and 4 were not investigated.

$^{125}$I-labeled NAP-*Naja naja siamensis* toxin on the other hand reacted differently. Only very little radioactivity was found migrating with an app. mol. wt 48 000. Instead a band corresponding to $M_r$ 62 000 was detected (fig.3b) besides the $M_r$ 78 000 band which had been obtained with the NAP-bungarotoxin. This photoaffinity label apparently reacted with the δ polypeptide chain and with a protein of $M_r$ 54 000, again assuming that only one molecule of the label was incorporated per protein molecule. Several control experiments were performed to prove the specificity of the photoaffinity labeling. First of all, none of the radioactive bands could be detected if the receptor-rich membranes had been saturated with α-bungarotoxin before applying the photolabel (fig.3ab, trace C). Furthermore incubation of the



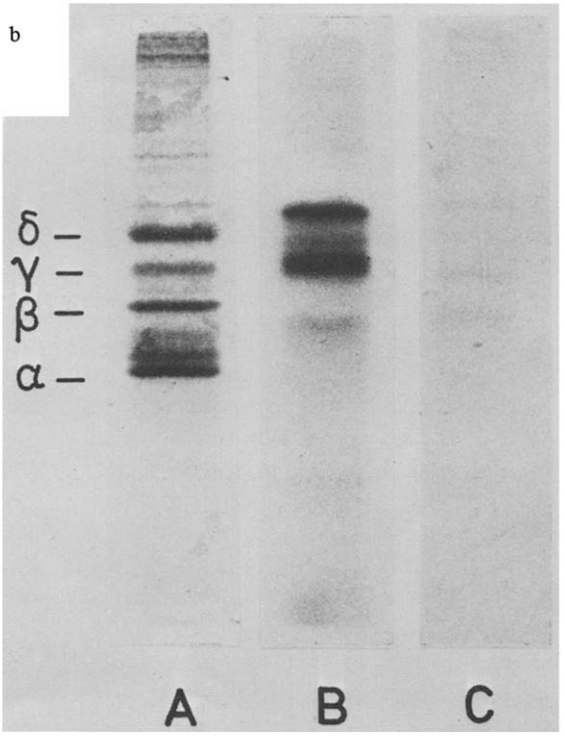




Fig.3. SDS–polyacrylamide gel electrophoresis and autoradiography of acetylcholine receptor-rich membranes labeled with $^{125}$I-labeled NAP-α-neurotoxin. (a) $^{125}$I-labeled NAP-α-bungarotoxin-labeled membranes. (b) $^{125}$I-labeled NAP-*Naja naja siamensis* toxin-labeled membranes. (A) Coomassie blue-stained gel of unlabeled membranes; (B) autoradiogram of gel with labeled membranes; (C) control: $^{125}$I-labeled NAP-neurotoxin labeling with membranes which were preincubated with unlabeled α-bungarotoxin. The autoradiogram shows no significant incorporation of label.

receptor–photolabel complex in the dark or irradiation of the photolabel in the absence of membranes gave no radioactivity with $R_F$ values similar to the ones described above (experiments not shown here).

## 4. Conclusions

Although the binding sites for cholinergic agonists and antagonists are assumed to be located on the α-polypeptide chains of the acetylcholine receptor protein complex the photoaffinity derivatives of α-bungarotoxin and *Naja naja siamensis* toxin do not react exclusively with these sites. NAP-*Naja naja siamensis* toxin hardly reacts with the α-chain at all. Instead it labels a protein which appears to be different from the main polypeptide chains α–δ, shown by crosslinking experiments to be part of the receptor complex [10]. From this we conclude that the photoaffinity labels do not only react with the toxin binding site itself but with other proteins located close to it in the receptor-rich membrane. α-Bungarotoxin and *Naja naja siamensis* toxin are polypeptides of $M_r$ 8000 which may extend to areas outside the binding pocket of the acetylcholine receptor, and the nitroazidophenyl group attached to it may be located on such an extending part of the toxin molecule. This assumption is supported by the observation that the nitro-azidophenyl group does not interfere with the binding of the toxin to the receptor to a significant extent.

Therefore we may conclude that the δ polypeptide chain is located in close proximity to the α chain in the *Torpedo* receptor. The experiment with the NAP-*Naja naja siamensis* toxin suggests that there is a further polypeptide chain with an app. mol. wt 54 000 also nearby.

A protein of mol. wt 55 000 has been shown by photoaffinity labeling to contain a binding site for ATP [19] and therefore may be part of the endogenous kinase/phosphatase system discovered by Gordon et al. [20]. At present it is not clear if this protein is identical with the mol. wt 54 000 polypeptide chain labeled in our experiment by the $^{125}$I-labeled NAP-*Naja naja siamensis* toxin.

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