TRITIUM LABELLING OF THE α-NEUROTOXIN OF *NAJA NIGRICOLLIS*

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1. Introduction

The venom of *Naja nigricollis* is a complex mixture of a variety of enzymes and polypeptide toxins. One of its most active principles is the α-neurotoxin [1, 2], a polypeptide of 61 amino acids (M.W. = 6787) with 4 disulfide bridges, which has been shown to act as a potent neuromuscular blocking agent [3]. This toxin, like α-bungarotoxin [4, 5] binds with a high selectivity, both *in vivo* and *in vitro*, to the cholinergic receptor protein integrated in an excitable membrane or dispersed in solution [6]. Because of these exceptional properties, it was of primary interest to label the toxin with a radioactive isotope.

Several neurotoxins with similar properties have been recently labeled with ^{131}I [7, 8]. In this letter we describe the tritiation of the α-neurotoxin from *N. nigricollis*. The principle of the method is first to iodinate the toxin and, subsequently, to replace iodine by tritium. Interestingly, in our experimental conditions, iodine does not react with tyrosyl residues, as it does with other polypeptides [9, 10], but attacks a histidine side chain. The tritiated toxin has a specific radioactivity of 14,000 Ci per mole and exhibits all the properties of the native toxin.

2. Results and discussion

2.1. *Iodination*

0.88 μmole of α-neurotoxin prepared according to [1] were dissolved in 0.8 ml of 0.1 M ammonium chloride ammonia buffer, pH 8.5. To the chilled solution 6 μmole of ^{125}ICl in 150 μl methanol were added; after 30 sec an excess of Na thiosulfate was added to destroy the unreacted iodine. The resulting solution was filtered on a Biogel P2 column (50–100 mesh, 1 × 20 cm) prepared in 0.1 M acetic acid and the column was subsequently washed with 0.1 M acetic acid. The toxin passed unretained through the column, and thus was separated from the salts. 0.85 μmole of peptide containing 0.65 μatom iodine was recovered in acetic acid solution. Spectroscopic measurements did not show any shift of absorption in the tyrosine absorbing region at 280 nm as compared to the native peptide, indicating no modification of the tyrosyl residue.

2.2. *Tritiation*

The acetic acid solution of toxin was evaporated and the dry iodopeptide dissolved in 0.5 ml 0.004 M Na phosphate buffer pH 7.4 and placed in the special flask previously described [10]. The solution was frozen, and 10 mg of palladium-rhodium-alumina

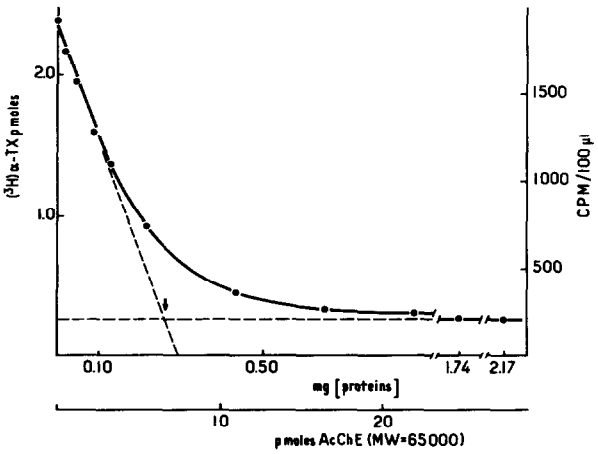

Fig. 1. Binding of tritiated α-neurotoxin to excitable membrane fragments purified from homogenate of electric organ. Membrane fragments were purified by the method previously described [15] and added as a suspension in 0.7 M sucrose at the indicated concentration in the toxin solution. The toxin concentration was kept equal to 4.6 pmole per ml in a total volume of 0.5 ml containing: 1.6×10^{-1} M NaCl, 5×10^{-3} M KCl, 2×10^{-3} M $MgCl_2$, 2×10^{-3} M $CaCl_2$, 3×10^{-4} M sodium phosphate pH 7.0 (physiological Ringer's solution) and 0.35 M sucrose. The mixture of membranes and toxin was incubated for 45 min at room temperature and subsequently centrifuged in a Beckman Ultracentrifuge Model L with a rotor no. 30 at 30,000 rpm (approximately 100,000 *g*) for 45 min at 4°. Aliquots of the supernatant were counted in a Packard Scintillation counter in Bray's solution. The amount of radioactive toxin remaining in the supernatant was plotted as a function of the total amount of membrane added to the solution.

catalyst (Engelhardt and Co) added. 10 Ci of pure tritium gas were then introduced, at a pressure of 500 mm Hg. The mixture was brought to room temperature and shaken with the catalyst for 30 min at 25°. After removal of the tritium gas the suspension was poured on a Biorex 70 column (1 × 15 cm, 4°) prepared in 0.05 M Na phosphate buffer, pH 7.2. Under these conditions the toxin adsorbs on the column. Labile tritium atoms were first removed by extensive washing (500 ml) of the column with phosphate buffer and the ^3H toxin finally eluted by increasing the concentration of phosphate buffer up to 0.2 M. The column effluents were monitored with an LKB densitometer at 280 nm. Only one peak of UV absorbing material was eluted. This peak contained exclusively peptide-bound tritium and no iodine at all; the iodinated neurotoxin remained firmly bound to the Biorex resin. The specific radioactivity of the tritiated material in each fraction of the peak was identical and equal to 14 Ci per mmole. A total of 0.36 µmole of ^3H labeled peptide was recovered in this experiment. The labeled toxin gave only one band at pH 4.5 in polyacrylamide gel electrophoresis performed according to Davis [11].

The iodinated peptidic material submitted to the dehalogenation step contained 0.61 µmole iodotoxin and 0.21 µmole of unreacted toxin. Since the Biorex column separated the iodotoxin from the nonhalogenated peptide, the material recovered after dehalogenation should be made up of the unreacted toxin (0.21 µmole) and of the tritiated peptide: by difference, 0.14 µmole. Since the specific radioactivity of tritium gas is 30 Ci per milliatom tritium, then the calculated radioactivity of the mixture of cold and labeled toxin should be approximately 12 Ci per mmole. This number is, indeed, very close to the 14 Ci per mmole found experimentally. This suggests that the sequence of events described is correct, and that the tritiation was carried out without dilution of the tritium gas by the hydrogen of the solvent. As a consequence, the rate of histidine dehalogenation has to be more rapid than that of tritium-proton exchange.

2.3. *Identification of the labeled amino acid*

An aliquot of the ^3H-labeled peptide was diluted with 1 mg of native α-neurotoxin and hydrolysed with 6 N HCl at 105° for 16 hr in a sealed vial. The amino acids obtained were separated in a Technicon analyser according to the procedure of Spackman et al. [12]. About 10% of the calculated tritium atoms were recovered bound to histidine and only to this amino acid. This indicates that histidine was the amino acid labeled. The low yield of radioactivity recovered in this experiment is the consequence of an exchange between aromatic hydrogen and protons of the solvent promoted by the acid hydrolysis of the peptide.

In the course of the iodination procedure no spectral shift at 280 nm occurred which means that in these conditions the tyrosyl residue did not react with iodine. If, however, the pH was raised to 11, then the spectrum changed: iodination of the tyrosyl

residue took place. Titration of the tyrosyl residue was completed only after several hours exposure to high pH. This suggests that in the native form of this toxin, like in the *erabutoxin* [8] or the *cobrotoxin* [7], the tyrosyl residue is buried inside the polypeptide chain [13].

2.4. *Biological tests*

The lethal activity of the tritiated toxin was assayed on mice as previously described [1]. The LD_{50} of the tritiated toxin was 2.4 μg and the LD_{100} 3.35 μg, as compared to an LD_{50} of 1.7 μg and an LD_{100} of 2.0 μg for the native toxin. The immunoreactivity of the labeled toxin was checked by immuno-diffusion according to Ouchterlony [14] and was found to be identical to that of the native material: in addition, the precipitates of native α-toxin and of labeled toxin on the same preparation were coalescent.

The curare-like action of the tritiated toxin was then assayed on the electroplax preparation from *Electrophorus electricus*. 1 μg/ml of either tritiated or native toxin gave approximately 80% blocking of the electrical response to 2.5×10^{-5} M carbamylcholine [6]. As shown in the figure the tritiated toxin binds *in vitro* to excitable membrane fragments purified from homogenates of electric organ. The number of ^{3}H-toxin binding sites was found to be 8.4 nmole/g protein. Assuming for AcChE a turnover number of 750 mole of acetylcholine hydrolysed per hr and per g of protein and a M.W. per site of 65,000, then we find that, in the membrane fragments, there are approximately 2.7 times more catalytic sites of AcChE than toxin binding sites. This result is in perfect agreement with previous findings with the native α-neurotoxin [6]. The same figure indicates that in the presence of an excess of membrane fragments, approximately 90% of the tritiated toxin adsorbed. The contamination of the toxin preparation by non-binding tritiated material was thus less than 10%. Binding of the radioactive toxin to the membrane fragments was prevented by reversible and irreversible reagents specific for the cholinergic receptor site. The tritiated toxin had thus the same pharmacological and biochemical properties as the native toxin.

Acknowledgements

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