SUBFRACTIONATION AND RECOMBINATION OF A NEUROTOXIC COMPLEX FROM THE VENOM OF THE BULGARIAN VIPER *(VIPERA AMMODYTES AMMODYTES)*

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1. Introduction

In our previous studies [1] we isolated and purified the strongest toxic protein (NVC) of the venom from Bulgarian viper. It was shown that NVC is an electrophoretically homogeneous protein complex consisting of two components: one TC with an isoelectric point in the alkaline region and the other NC with an isoelectric point in the acidic region [2]. Systematic studies on the isolated NVC components have shown that TC is a phospholipase A_2 while NC has not got an enzymatic activity but partially inhibits the phospholipase A_2 activity of the TC when bound (1:1 ratio) in NVC [3,4]. The properties of NVC have been compared with those of the analogous crotoxin complex [3,4].

Toxic phospholipases A_2 have been isolated from *Vipera ammodytes* venom [5] and *Vipera berus* venom [6] but up to now their binding in any kind of protein complex has not been established.

Abbreviations: NVC neurotoxic viper complex, TC toxic component, NC nontoxic component

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2. Materials and methods

The isolation of NVC from the Bulgarian viper crude-venom was carried out according to [1] and its components were obtained by decomposition of NVC in acidic water/urea solutions (pH 4.0) [2].

The differential NVC ultraviolet spectra were obtained with a Specord (DDR, Jena) at a concentration of 2.4×10^{-6} M. Fluorescence spectra of NVC and its components (at a concentration of 3.8×10^{-5} M) were obtained with a specially constructed spectrofluorimeter [7]. The spectral values were corrected for the readsorption and spectral sensitivity of the apparatus. The concentrations of NVC, TC and NC were determined by their ϵ_m = 51, 26 and 28 $mM.cm^{-1}$ respectively [8]. pH-Values were measured with a precise pH-meter OP-302/1 (Reanal, Hungary). The studies were conducted in salt-free solutions of 0.005 M Tris/HCl, pH 7.4, at 25°C.

Alkaline NVC subfractionation was carried out on QAE-Sephadex A-25 (Pharmacia, Uppsala): approximately 14 mg NVC dissolved in 0.5 ml 7 M urea, pH 10 (pH adjusted with 1,2-diaminoethane, BDH) was loaded on an 8 × 140 mm column equilibrated with the same buffer. When 30 ml of the buffer

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(25 ml/h) had passed through the column, a linear-gradient of 0–0.3 M NaCl was started. After dialysis and liophilization of the fractions, about 5 mg of both TC and NC were obtained.

Isoelectric focusing was carried out in a 5% polyacrylamide gel (0.1–0.2 mg protein/sample) using a standard apparatus (Reanal, Budapest). The ampholine solutions (pH 3–10) were supplied by LKB (Stockholm).

3. Results and discussion

NVC contains many tyrosine and tryptophan residues, 20 and 5 respectively [8]. We aimed to clarify the effect of pH (pH 2–12) on the state and stability of NVC by analysis of the absorption and fluorescence spectra of the toxin (fig.1). From the experimental data the pH-region observed can be divided into five parts. One of these (III, pH 5.5–7.5) is the region of complex stability in which no marked changes occurred in the NVC state of NVC chromophores. The fluorescence maximum was at a short-wave length (λ_{max} = 335 nm) corresponding to the tryptophan residues being in a hydrophobic environment [9]. In the neighbouring two regions (II and IV) changes occurred in the state of the tyrosine residues indicated by the change in absorption. The whole ultraviolet spectrum showed fixed isosbestic points at 258 nm on acidic titration and at 230 nm and 275 nm on alkaline titration. In region IV (pH 7.5–10.5) a titration of about three tyrosine residues occurred based on ϵ_{244} = 9500 M.cm^{-1}/tyrosine residue [10]. In region II (pH 2.8–5.5) titration of the aspartic and glutamic acid residues occurred and also of some of the tyrosine residues ($\Delta\epsilon_{244} \neq 0$). Both the fluorescence maxima in regions II and IV were at short-wave lengths (λ_{max} 334–336 nm) which proved that the local environment of the tryptophan residues was approximately the same as in region III. At pH < 2.8 (region I) and at pH > 10.5 (region V) the isosbesticity titration was disturbed, an indication of the participation of more than one structural form in the H^+-equilibrium. Regions I and V were probably regions of acidic and alkaline denaturation. Removal of the fluorescence maxima to long-wavelengths is consistent with regions I and V being regions of denaturation, an indication that on denaturation the accessibility of the indole chromophores to the solution molecules increased.

The first successful subfractionation of NVC was carried out by ion-exchange chromatography on CM-cellulose in acidic 7 M urea solution [2]. Data from this acidic decomposition and the appeareance of fig.1 lead to the expectation of an analogous decomposition in region IV, which was confirmed by experimental alkaline subfractionation of NVC (See Materials and methods). The elution-curve (fig.2) was the same as that obtained in acidic decomposition of NVC [2] and the components were eluted in the reverse order due to the use of QAE-Sephadex A-25 instead of CM-cellulose. In both cases, before the gradient was started a nontoxic fraction was eluted

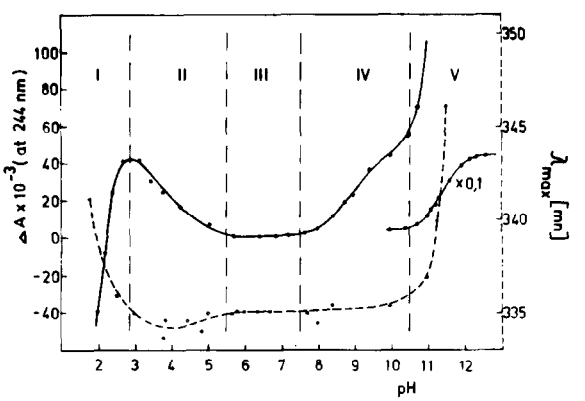

Fig.1. pH-Dependence of NVC absorption at 244 nm as shown by difference spectrum (——) and of the position of the fluorescence maxima of NVC (– – –).

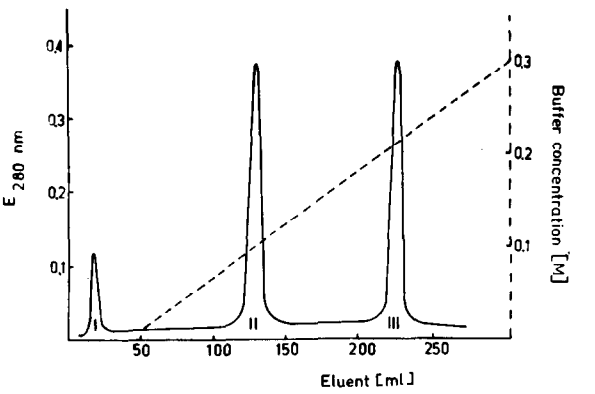

Fig.2. Subfractionation of NVC at alkaline pH on QAE-Sephadex A-25. The conditions are described in Materials and methods. Fraction I - nontoxic fraction, fraction II - toxic component (TC), fraction III - nontoxic component (NC).

in negligible amounts. The appearance of this fraction could be explained by toxin denaturation. The basic phospholipase A_2 (TC) obtained by alkaline subfractionation showed a lower toxicity and lower enzymatic activity than the TC isolated in the acidic region. Besides, the TC activity varied over a broad range, probably due to partial denaturation in the isolation conditions. Consequently acidic subfractionation of NVC [2] remains the better method for the isolation of TC and NC. However, alkaline NVC decomposition confirmed our assumptions that:

(i) NVC consists of two components only.

(ii) The two components bind by strong electrostatic bonds.

(iii) Separation of the two components is accompanied by changes in the ionization state of the tyrosine residues.

(iv) Because subfractionation of NVC did not occur in the absence of urea either at pH 4 or pH 10, we may suppose that hydrophobic bonds are formed when NVC is formed.

The toxicity [2] and enzymatic activity [3] of NVC were restored when the two freshly isolated components were mixed, showing the reversibility of NVC formation and decomposition, in appropriate conditions. To confirm this phenomenon isoelectric focusing of NVC and its components were carried out. The two components (TC and NC) differ strongly in their isoelectric points [2]. When the two components were mixed in 1:1 ratio and isoelectrically focused, only one protein band was observed – in the same neutral zone as with intact NVC.

The changes that occur in the environment of the tryptophan residues on subfractionation and recombination of the NVC components are shown in fig.3. The fluorescence spectra of the tow mixed components (1:1 ratio) observed at λ_{ex} = 296 nm (fig.3, curve 1) differed considerably from the sum of their individual spectra (fig.3, curve 2) and practically coincided with the spectrum of intact NVC. If no essential changes occured in the conformations of TC and NC after they are bound in NVC, it can be concluded that there are tryptophan residues in the binding faces.

The complex formed by TC and NC is very stable while TC isolated from NVC gradually lost its biological activity over several days [2–4]. Therefore the conclusions regarding NVC recombination are only valid for freshly isolated components.

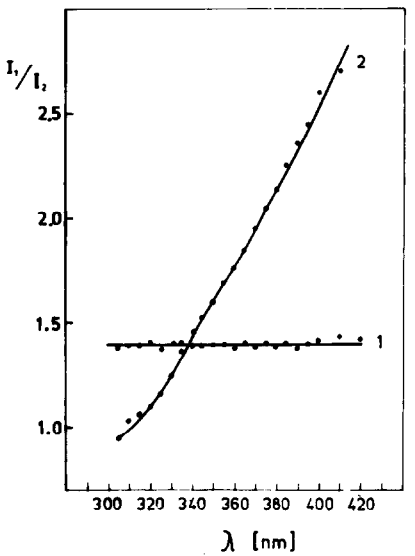

Fig.3. Dependence of the ratio I_1/I_2 on the fluorescence wavelength: (1) I_1 – Intensity of fluorescence of the two components (mixed in 1:1 ratio), I_2 – intensity of fluorescence of intact NVC. (2) I_1 - The sum of the fluorescence intensities of the two isolated components TC and NC, I_2 – as for curve 1.

More detailed studies on the reversibility of NVC subfractionation and recombination have been carried out based on analysis of absorption and CD spectra [8]. They will be published elsewhere.

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