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# COMPARATIVE STUDY OF THE CYTOTOXINS OF THE VENOM OF THE CENTRAL ASIAN COBRA *Naja naja oxiana* BY THE INTRINSIC-FLUORESCENCE METHOD

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The intrinsic fluorescence of cytotoxin $V_c1$ and $V_c5$ from the venom of the Central Asian cobra due to the tyrosine residues and differing with respect to quantum yield for these two polypeptides because of the nonidentity of the microenvironments of the tyrosyl residues has been measured and characterized. The dependence of the quantum yield on the pH and the temperature also indicate this nonidentity.

Membrane-active substances – cytotoxins $V_c1$ and $V_c5$, which are polypeptides consisting of 60 amino acid residues with molecular weights of about 7000 D – have been isolated from the venom of the Central Asian cobra [1]. Cytotoxin $V_c1$ contains three tyrosine residues, and $V_c5$ contains two [2]. The interaction of the cytotoxins with membranes takes place in two stages: the adsorption of the cytotoxin molecules on the membrane surface takes place through electrostatic forces, and then the free hydrophobic parts of the cytotoxin molecules penetrate within the hydrophobic region of the membranes causing structural rearrangements of them [3, 4]. This shows that the spatial organization of the cytotoxin molecules has a definite significance in their functioning. The method of intrinsic fluorescence is an effective one for studying the structure of proteins in solution [5, 6], and we have used it for studying these cytotoxins.

The position of the maximum and the half-width of the fluorescence spectrum of each cytotoxin, on excitation at 280.4 nm, are 305 and 33 nm, respectively. The quantum yields at 25°C and pH 6.0 are 1.67 ±0.04% and 1.27 ±0.04% for cytotoxins $V_c1$ and $V_c5$, respectively. The spectral parameters show that the intrinsic fluorescence of the cytotoxins is determined completely by tyrosine residues which is in agreement with the fact that there are no tryptophan residues in the cytotoxin molecules [2]. They therefore belong to fluorescent class A [6]. Since the quantum yields of the two cytotoxins, like those of other proteins of class A, are an order of magnitude lower than the fluorescence yield of free tyrosine, it may be considered that the tyrosine in the cytotoxins is subjected to quenching by the side chains of amino acids and (or) by peptide groups (having hydrogen bonds with the hydroxyl of tyrosine [7]) forming their microenvironment [6]. Apparently, the difference in the quantum yields of $V_c1$ and $V_c5$ may be a consequence of the fact that the two tyrosine residues in $V_c5$ and the three in $V_c1$ have different microenvironments. Bearing in mind the primary structures of analogous cytotoxins from other courses and their homology with cytotoxins from the venom of the Central Asian cobra, it may be expected that two of the three tyrosyls in $V_c1$ will have similar quantum yields to the two tyrosyls in $V_c5$ and their microenvironment may lead to the quenching of the fluorescence to a greater degree than for the third tyrosyl of $V_c1$. This tyrosyl is $Tyr^{11}$, as follows from an analysis of the primary sequence of the polypeptide chains of the cytotoxin molecules [2].

Among the factors causing the quenching of fluorescence in proteins, an important role is played by charged residues, and the charge of a cytoxin molecule has functional significance. It therefore appeared of interest to determine the influence of the pH of the

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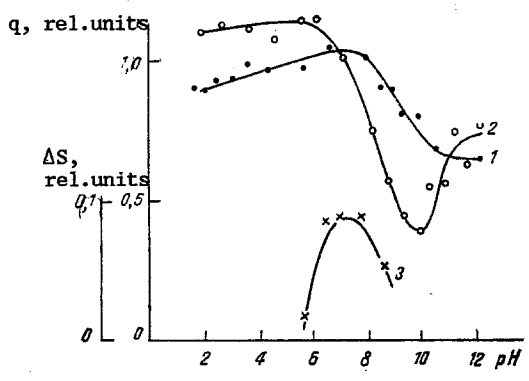


Fig. 1

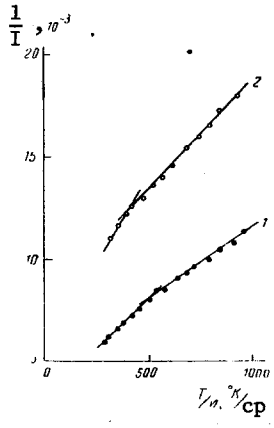


Fig. 2

Fig. 1. Influence of the pH on the quantum yield (q) of the fluorescence of aqueous solutions of cytotoxins $V_c1$ (1) and $V_c5$ (2) and on the order parameter S of the ESR spectrum of a spin probe (5-doxylstearic acid) on the interaction of cytotoxin $V_c5$ with model membranes at room temperature. Information on S taken from [12].

Fig. 2. Dependence of the reciprocal intensity of the fluorescence of cytotoxins $V_c1$ (1) and $V_c5$ (2) on the reduced temperature of the solution $T/\eta$ (pH 6.0).

solution on the quantum yields of the two cytotoxins. In the acid region, the quantum yield did not change (Fig. 1). A fall in the yield was observed in the range of pH values above 7.5 with a half-transition value of 9.3 for $V_c1$, repeating the fall in the functional activity of the cytotoxin with a change in the pH. For $V_c5$ a fall in the yield took place in the pH 7-10 region with the half-transition at pH 8.2, while in the pH-10-12 region was an increase in yield with a half-transition at pH 10.7. The change in the quantum yield with a variation in the pH of the solutions was determined with allowance for the screening and reabsorption of the tyrosyls with the deprotonated residues appearing in the alkaline region. Apparently, the tyrosine residues are located in those regions of the cytotoxin macromolecules that are responsible for the performance of their functions, as is indicated by the coincidence of the pH range in which the decrease in the quantum yield is accompanied by a fall in membrane activity (the change in membrane activity is characterized by the order parameter S determined from the ESR spectrum of a spin probe [4]).

The fall in the fluorescence of the cytotoxins in the weakly alkaline region may take place as a consequence of an increase in quenching by the peptide carbonyl group due to a conformational transition The deprotonation of ammonium groups cannot lower the quantum yield, since the quenching agent is in fact the ammonium, and not the amine, group. In the pH range above 10, the molecules of cytotoxins $V_c1$ and $V_c5$ exhibit different sensitivities to alkaline denaturation. The increase in the yield of tyrosine fluorescence of cytotoxin $V_c5$ at a pH above 10 corresponds completely to alkaline denaturation (the denaturation increase in the yield of $V_c5$ is apparently connected with the cleavage of a hydrogen bond).

Another approach to the study of proteins and their conformational transitions in the investigation of temperature dependences. The influence of the temperature on the tyrosine fluorescence of cytotoxin is shown in a trivial temperature quenching. In the coordination $1/I$ versus $T/\eta$, where T is the absoluted temperature and $\eta$ is the viscosity of the solution, temperature quenching in the absence of a conformational transition should be described by a linear function [8]. In our case (Fig. 2,), for both cytotoxins breaks are observed in the straight lines where the linear sections on the graphs obviously correspond to temperature-diffusional quenching. The deviation from linearity at 40-45°C for $V_c1$ and at 30-35°C for $V_c5$ means the existence of temperature-dependent conformational transitions [8]. The slight difference in the temperatures of these transitions shows a difference in the noncovalent interactions forming the microenvironments of the tyrosine residues. The relatively low temperatures of the conformational transitions obviously show that these interactions are comparatively weak, and that the transitions are not denaturation processes. However, they affect the region of the tyrosine residues and may reflect a physiologically significant

transition that is known for many proteins [9]. It must be mentioned that the relationships that we obtained (Fig. 2) do not fully correspond to information in the literature [6], and this requires further investigation.

## EXPERIMENTAL

The cytotoxins $V_c1$ and $V_c5$ were obtained as described by Grishin et al. [2]. Absorption spectra were measured on a EPS-3T recording spectrophotometer (Hitachi). Optical densities were determined with allowance for light-scattering [10]. Fluorescence spectra with excitation at 280.4 nm were measured on a spectrofluorimeter of the institute of Biochemistry of the Academy of Sciences of the Uzbek SSR (medium-pressure mercury lamp; MDR-2 excitation microchromators; observation at an angle of 90°; thermostated cell-holder; OP-204 amplifier (Hungary); recording on a KSP-4 potentiometer; measurement of the temperature of the solution directly in the cell with copper-constantan thermocouple, the thermo-emf being recorded on a F-30 digital voltameter with an accuracy of 2 μV (0.05°C) at a comparison temperature of 37°C). The fluorescence spectra were corrected for the spectral sensitivity of the fluorimeter. The quantum yields were determined relative to the fluorescence of an aqueous solution of tyrosine, which was taken as 0.21 [5]. The pH values of the solutions were measured on an OP-211/1 pH-meter (Hungary).

## SUMMARY

The intrinsic fluorescence of cytotoxins $V_c1$ and $V_c5$ from the venom of the Central Asian cobra has been measured and characterized , it is determined by the tyrosine residues and differs with respect to quantum yield for these two polypeptides because of the nonidentity of the microenvironments of the tyrosin, which results in a difference between the dependences of the quantum yield on the pH and on the temperature.

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