THE FLEXIBLE NATURE OF A CRITICAL PEPTIDE REGION COMMON TO ALL ELAPIDAE 'SHORT' NEUROTOXINS

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1. Introduction

In a study undertaken by two of the authors [1] with modified Chou-Fasman protein structure prediction rules [2], the three major categories of homologous toxic snake venom proteins were compared. It was found that the short neurotoxins, long neurotoxins and cytotoxins all shared a common distribution of secondary structure, suggesting a common shape and related mode of action. Shortly after this work was completed, the X-ray crystallographic structures for erabutoxin b [3] and Philippines sea snake toxin b [4] were published. Comparison of our predictions with these short neurotoxin structures showed good agreement, especially in detecting the extensive areas of β-sheet. An important disagreement, however, concerned the positioning of the β-turn at the end of the central loop of erabutoxin b as depicted at 31–34 by Low et al. [3]. Our predictions failed to indicate a β-turn at this site, but nevertheless showed that most turn potential lay in the overlapping tetrapeptide 32–35. In addition, examination of the peptide backbone maps produced by the two groups of X-ray workers shows apparently different stereochemistries in the exposed chain reversals, including the 31–35 region. This is surprising, since there is thought to be little, if any, difference between the two toxins investigated [5]. Excepting the erabutoxins, all other short neurotoxins possess histidine instead of phenylalanine-32, giving an extremely high β-turn potential for the segment 32–35.

Possible reasons for the statistically unusual turn at 31–34 include intermolecular interactions and sensitivity to media in the crystallographic analysis. With regard to the latter, both groups of workers used high salt media to obtain their toxin crystals – Tsernoglou and Petsko crystallised Philippines sea snake toxin b from ammonium sulphate while Low et al. used sodium sulphate for erabutoxin b. In order to establish whether the structure of the central loop belonging to this class of molecule is altered at high salt concentration, another homologous short neurotoxin, 4.11.3 from *Dendroaspis viridis*, was examined by circular dichroism in the presence and absence of both salts. Change in structure with increasing temperature was investigated as a further measure of the lability of this region.

2. Methods

Isotropic absorption and circular dichroism measurements were undertaken with a Cary 17 and Jasco J4OCS respectively. Variable temperature spectra were obtained using a heated cell holder and the thermistor probe of a United Systems Corp. Digital Thermometer inserted directly into the solution

Purified *Dendroaspis viridis* toxin 4.11.3 was the

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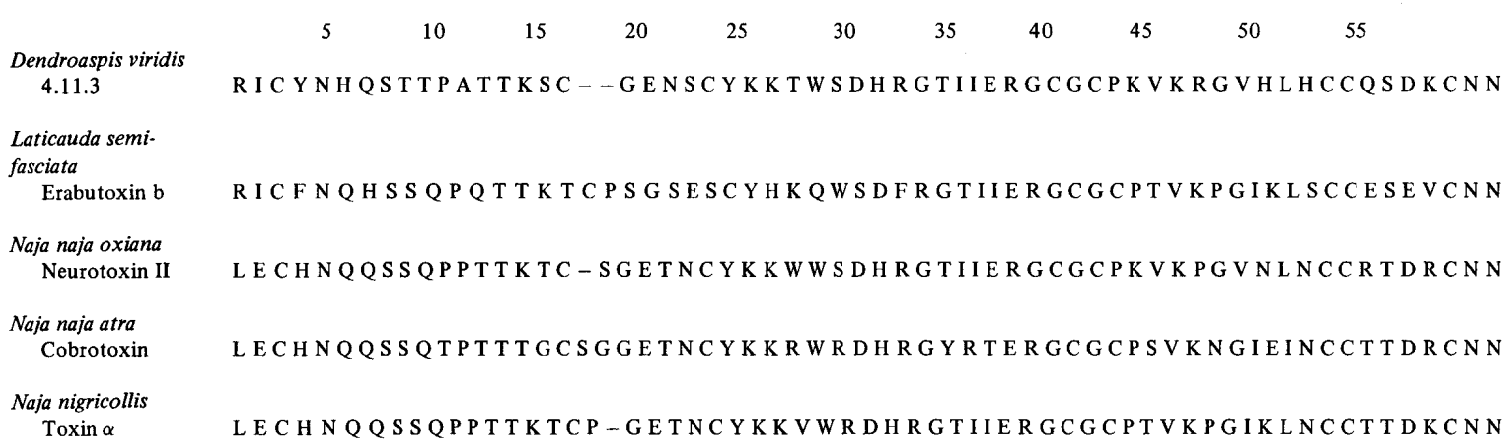

Fig.1. Amino acid sequences of short neurotoxins referred to in text. The standard one-letter code is used.

kind gift of Dr R. A. Shipolini. Throughout, concentrations of 1 mg/ml of this toxin were used.

3. Results and discussion

The circular dichroism spectrum of 4.11.3 in Tris buffer (10 mM, pH 7.4) shows bands at 216 nm, 228 nm and in the vicinity of 280 nm (fig.2). These are very similar to those reported for the short neurotoxins erabutoxin b and *Naja nigricollis* toxin α [6], with the 216 nm peak being due to the $n-\pi^*$ transition of the protein backbone and indicative of the high content of antiparallel β-sheet suggested by X-ray crystallography [3].

The actual assignments of the 228 nm and 280 nm band systems in proteins has been the subject of much research [6–8]. The reader is referred to these papers for a more detailed discussion, but suffice it to say that the vibronic fine structure of the 280 nm band indicates that the major absorbing species being observed is tryptophan. The presence of the isosbestic point in the variable temperature study (fig.2) demonstrates that if the 228 nm band is not a tryptophan absorption, it is certainly associated with it.

The invariance of the 216 nm peak in the variable temperature study shows that the bulk of the molecule is not conformationally labile, although the variation of the 228 nm and 280 nm bands points to a local conformational change associated with a tryptophan moiety. These results are complementary to those of Menez et al. [6] who demonstrated that both the latter absorptions in erabutoxin b and *Naja nigricollis* toxin α are similarly sensitive to trifluoroethanol. They too concluded that changes at these wavelengths probably indicate a local conformational change involving tryptophan. The Θ_{288}^{max} versus temperature profile (fig.3) shows that at room temperature in Tris buffer (10 mM, pH 7.4) almost all the tryptophan is in the low temperature conformation, and its degree of exposure increases steadily with temperature. Above 93°C there was evidence of large conformational changes associated with both the tryptophan residue and the overall protein backbone. Upon cooling, the CD spectrum was never completely reversible, indicating a small degree of irreversible denaturation. Similar findings have been reported by Yee-Hsiung Chen et al. [9] for cobrotoxin. That the protein backbone conformational change occurs at a lower temperature indicates the presence of a less

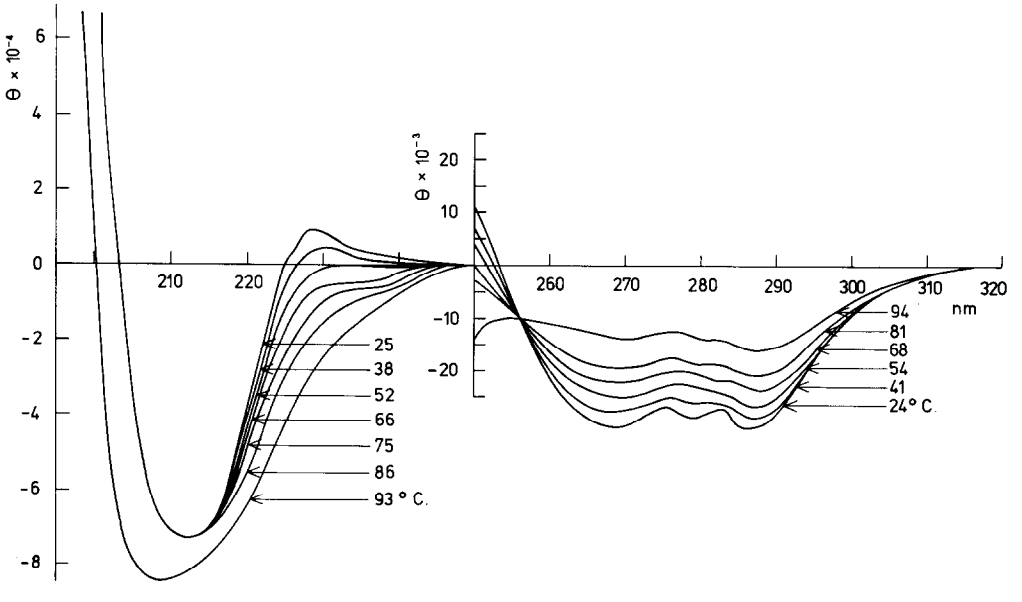

Fig.2. Variation of the CD of 4.11.3 in Tris buffer (10 mM, pH 7.4) with temperature. Θ is expressed in terms of cm^2 degree $dmol^{-1}$.

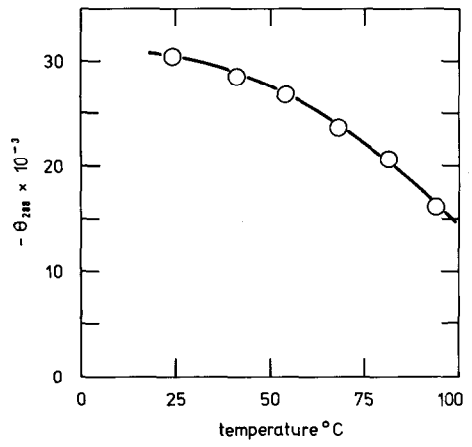

Fig.3. Variation of Θ_{288}^{max} with temperature for 4.11.3 as obtained from fig.2.

stable β-sheet in this toxin. This suggestion correlates well with the β-structure predictions for cobrotoxin and 4.11.3 [1].

It has also been demonstrated that the effect of added salt can be similar to these temperature effects. Thus, whilst the addition of sodium sulphate (1 M) to a solution of 4.11.3 in Tris buffer (10 mM, pH 7.4) has little effect on the CD spectrum, both ammonium sulphate (2.5 M (fig.4) and tetraethylammonium chloride (6 M) (fig.5) reduce the CD spectrum in the 228 nm and 280 nm regions. A possible explanation of this effect on 4.11.3 is that the latter two salts cause appreciable structuring of the aqueous environment, thereby reducing unfavourable interactions between hydrophobic residues and the solvent. Any conformational change that involves exposure of aromatic residues like tryptophan to the external medium would, therefore, be facilitated. Such an effect is quantitatively described as a change in the dielectric constant, Σ, of the medium. Extrapolation between salts shows that for ammonium sulphate (2.5 M) $\Sigma \simeq 50$ at 20°C, whereas for water $\Sigma = 80$ at the same temperature [10,11]. The tetraethylammonium ion would be expected to be an overall solvent structure breaker, but in fact clathrate-like structures associated with the ethyl residues are formed [12], again resulting in $\Sigma \simeq 50$ at 20°C. Sodium sulphate (1 M), however, is less effective at structuring the solvent, possessing an estimated $\Sigma \simeq 65$ at 20°C.

Thus it is clear that high salt concentrations and heating similarly perturb the conformation of 4.11.3 compared to that in physiological media. The isosbestic point obtained near 256 nm during the heat treatment suggests two major conformations of the toxin are involved, the difference between them being independent of the overall β-sheet structure. It is possible, therefore, that the X-ray crystallography shows the central loops of the two short neurotoxins studied to be more exposed to the solvent than is to be expected in the tissues of the snakes victim. If the β-bend in erabutoxin b is placed at 32–35 according to our predictions [1], then the tryptophan

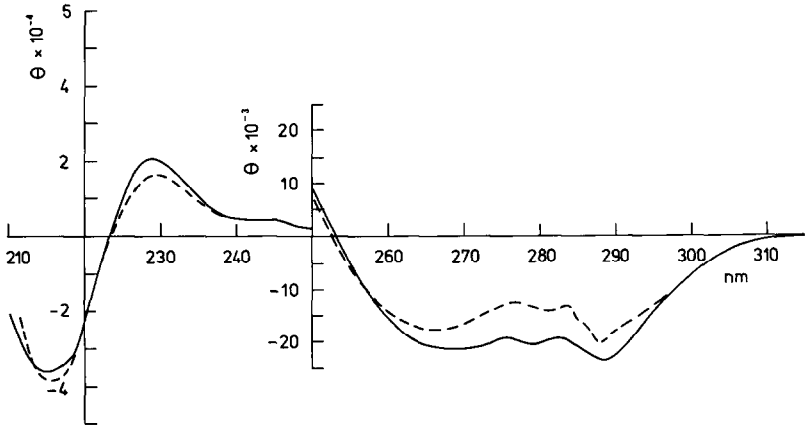

Fig.4. Effect of added ammonium sulphate (2.5 M) on the CD spectrum of 4.11.3. Below 220 nm, the salt obscures the true absorption. (—) 4.11.3 in Tris buffer (10 mM, pH 7.4). (- -) 4.11.3 in $(NH_4)_2SO_4$ (2.5 M)/Tris.

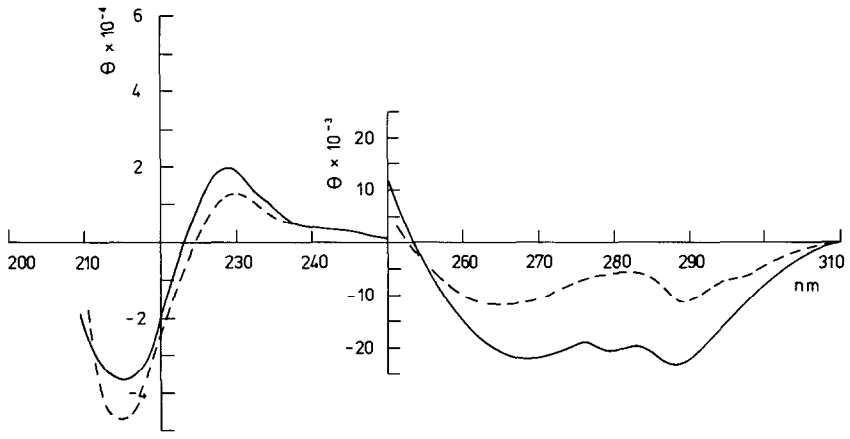

Fig.5. Effect of added tetraethylammonium chloride (6 M) on the CD spectrum of 4.11.3. Below 220 nm, the salt obscures the true absorption. (—) 4.11.3 in Tris buffer (10 mM, pH 7.4). (- -) 4.11.3 in TFE (6 M)/Tris.

assumes a more 'buried' position, becoming aligned with phenylalanine-32 and more proximate to aspartate-31. Such a change would give agreement with the NMR data of Arseniev et al. [13] who concluded that the tryptophan-29 in neurotoxin II from *Naja naja oxiana* (which like most short neurotoxins possesses histidine instead of phenylalanine-32) has histidine-32 and a carboxylate in its microenvironment. The changes in CD observed in this study are consistent with the tryptophan-29 of 4.11.3 moving away from such a buried position involving a neighbouring aromatic residue and/or ion pair interaction [8]. Interestingly, when this change in bend position is made, the residues lysine-47, arginine-33, tryptophan-29 and phenylalanine/histidine-32 common to all short neurotoxins form a close stereochemical mimic to curare.

McCammon et al. [14] have postulated that the small peptide loop 25–28 which emerges from the extensive secondary structure of bovine pancreatic trypsin inhibitor undergoes considerable fluctuations in shape, reflecting an intrinsic softness in this part of the molecule. The short neurotoxin loop 30–35 therefore presents an analogous situation and might be expected to behave in a similar fashion. The possible significance to neurotoxicity of having an extremely stable molecular structure with many of the 'essential' residues being located in a small flexible region is currently under investigation in our laboratory.

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