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SRTX-d, a new native peptide of the endothelin/sarafotoxin family

A. Bdolah, Z. Wollberg, G. Fleminger* and E. Kochva

Departments of Zoology and **Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

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The primary structure of a new sarafotoxin, SRTX-d, from the venom of *Atractaspis engaddensis* is described. SRTX-d differs from SRTX-b in two substitutions: Ile^{19} instead of Val and Thr^{2} instead of Ser. The toxicity of SRTX-d and its vasoconstriction potency are very low in comparison to SRTX-a and SRTX-b, whereas its IC_{50} for ^{125}I-SRTX-b binding is similar to that of SRTX-b. It is suggested that the Thr to Ser substitution, which is shared by two additional weak members of the endothelin/sarafotoxin family, SRTX-c and ET-3, affects the biological activity of SRTX-d as well.

Sarafotoxin; Endothelin; Snake venom; Aorta

1. INTRODUCTION

Since the discovery of the mammalian endothelins and the sarafotoxins from the venom of the snake *Atractaspis engaddensis* [1–3], attempts were made to define the domains which are essential for specific binding to receptors, immunoreactivity and vasoconstriction activity [4–7]. These attempts were approached by (i) using modified versions of the peptides and (ii) comparison of the structure/function relationships of the various natural peptides [6,7]. The latter approach was quite fruitful as it pointed to the importance for activity of the N-terminal half of the molecule in addition to the C-terminal tail. We have stressed previously the importance of the Thr to Ser substitution in position 2 in both SRTX-c and ET-3, the weakest members of the endothelin/sarafotoxin family [8,9]. The difference between the two amino acids, however, appeared too small in order to explain such differences in activity, and the reduction in activity was therefore ascribed to the other substitutions found in these two molecules.

We have now established the amino acid sequence of SRTX-d, an additional sarafotoxin; this isotoxin differs from SRTX-b in two substitutions, again threonine instead of serine in position 2 and isoleucine instead of valine in position 19. The latter substitution is found in all ETs described thus far, including the vasoactive intestinal contractor peptide (VIC) reported recently [10], but in none of the SRTXs. SRTX-d shows a very low potency in vasoconstriction, a weak toxic activity, but a high binding affinity to aortal preparations.

Correspondence address: A. Bdolah, Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Abbreviations: SRTX, sarafotoxin; ET, endothelin

2. EXPERIMENTAL

SRTX-b was isolated and purified from the venom of *Atractaspis engaddensis* as described previously [2,3]. SRTX-d was purified from fraction S6a of the DEAE-cellulose step [3] by reverse phase chromatography on a Lichrosorb RP-18 column (250 × 4 mm, Merck, Darmstadt, FRG); the column was developed at 1 ml/min with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid (TFA) using a Gilson model 303 HPLC system. Two protein peaks with retention times of 26.8 min and 29.7 min were obtained; the first, larger peak corresponded to SRTX-a; the second peak is here

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designated as sarafotoxin-d (SRTX-d). The latter peak was rechromatographed on the same system before further analysis.

Amino acid analysis was carried out by AminoLab (Rehovot, Israel) on samples hydrolyzed in 6 N HCl in evacuated sealed tubes at 110°C for 22 h. Cysteine was estimated as cysteic acid after performic acid oxidation.

Sequence analysis of the pyridylethylated reduced peptide [11] or the untreated peptide was performed at the Department of Virology, Weizmann Institute of Science (Rehovot, Israel) using a model 470A liquid phase protein sequencer with an online model 120A PTH analyzer (Applied Biosystems, Inc.). C-terminal sequence analysis was performed with carboxypeptidase-Y. An amount of 150 μg of SRTX-d was incubated with 50 μg of the enzyme in 0.1 M pyridine-acetate buffer, pH 5.5, 5 μg/ml pepstatin, 1 mM EDTA, at 37°C. Samples containing 25 μg SRTX-d were withdrawn at 0, 10 and 60 min and at 24 h. The samples were then analyzed for amino acid composition.

Binding competition experiments were performed with 5 nM ^{125}I-SRTX-b as radioligand. The particulate fraction of rabbit aorta was prepared according to Gurwitz et al. [12] with 25 mM Tris-HCl buffer, pH 7.4, containing EDTA, EGTA and protease inhibitors. Aliquots of 50 μl containing about 200 μg protein were incubated for 45 min at 30°C with various concentrations of SRTX-b or SRTX-d in Tris buffer (total volume 200 μl). The reaction was terminated by the addition of 3 ml ice-cold buffer followed by centrifugation at 4000 × g for 15 min at 4°C. The precipitates were then washed twice with 3 ml cold buffer. For nonspecific binding, incubation was carried out in the presence of 2 μM unlabeled SRTX-b. Assays were performed in triplicate. Data are expressed as specific binding (total, minus nonspecific).

Vasoconstriction was examined on isolated rabbit aorta strips (opened rings) as detailed previously [7].

3. RESULTS AND DISCUSSION

The primary structure of SRTX-d was determined by automatic sequencing and verified by amino acid analysis; in addition, carboxypeptidase digestion of the C-terminal tail was carried out up to the amino acid residue at position 19 (Ile). The results show that this isotoxin differs from SRTX-a in three positions, Ile^{19} instead of Val, Tyr^{13} instead of Asn and Thr^{2} instead of Ser and from SRTX-b in two – Ile^{19} instead of Val and Thr^{2} instead of Ser (fig.1). The Thr to Ser substitution is probably very important as it is shared by two additional 'weak' members of the endothelin/sarafotoxin family, SRTX-c and ET-3, with apparently considerable functional implications (see below). In order to ascertain this finding we have performed several sequence analyses using unmodified and reduced and alkylated peptide preparations; in all cases Thr clearly appeared in the second (as well as in 7th) position and the amino acid composition of SRTX-d showed two threonine residues and no significant amount of serine, as expected from the amino acid sequence.

The toxicity of SRTX-d, as determined by i.v. injections, showed an LD_{50} of about 350 ng/g mouse; this toxicity is very low in comparison with SRTX-a and b and ET-1 (LD_{50} of all three is about 10 ng/g) and is within the toxicity range of SRTX-c [9].

Radioligand binding experiments in brain (not shown) and aorta showed that SRTX-d competes with ^{125}I-SRTX-b with an IC_{50} similar to that of SRTX-b (fig.2).

In the vasoconstriction experiments, cumulative doses as high as 40 nM of SRTX-d did not elicit any noticeable response. Higher doses induced a

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Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp   SRTX-a
Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Val-Ile-Trp   SRTX-b
Cys-Thr-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Ile-Ile-Trp   SRTX-d
Cys-Thr-Cys-Asn-Asp-Met-Thr-Asp-Glu-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp   SRTX-c
Cys-Thr-Cys-Phe-Thr-Tyr-Lys-Asp-Lys-Glu-Cys-Val-Tyr-Tyr-Cys-His-Leu-Asp-Ile-Ile-Trp   ET-3
Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp   ET-1
Cys-Ser-Cys-Ser-Ser-Trp-Leu-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp   ET-2
Cys-Ser-Cys-Asn-Ser-Trp-Leu-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp   VIC
 1                  5                  10                  15                  20
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Fig.1. Amino acid sequence of SRTX-d in comparison with the other sarafotoxins and endothelins.

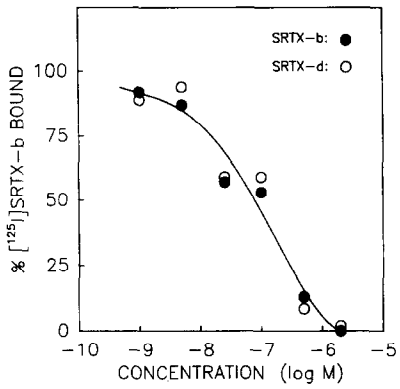


Fig.2. Inhibition of ^{125}I-SRTX-b binding to the particulate fraction of rabbit aorta. Competition binding assays were as described in section 2, using 5 nM ^{125}I-SRTX-b and various concentrations of SRTX-b and SRTX-d.

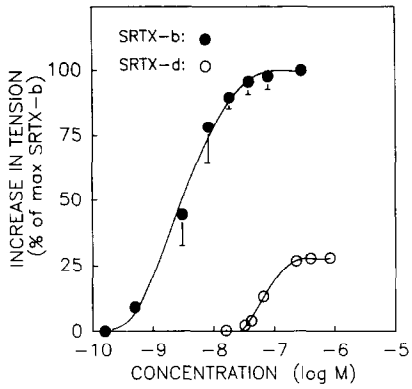


Fig.3. Contractile dose response of rabbit aorta strips to cumulatively applied SRTX-b (mean ± SE, n = 10) and SRTX-d (n = 2). Responses are expressed as % of maximal contraction induced by SRTX-b.

concentration-dependent contraction, which was weaker than the contraction induced by SRTX-b by one to two orders of magnitude (fig.3). The corresponding ED_{50} values (i.e. the cumulative doses that induce half maximal responses) for SRTX-b and SRTX-d were about 5 nM and 90 nM respectively; the maximum isometric tension (± SD) induced by SRTX-d was 0.4 ± 0.01 g. This is about 26% of the maximum tension induced by SRTX-b or norepinephrine (see also [7]).

It is thus evident that SRTX-d is a considerably weaker vasoconstrictor than either SRTX-a, SRTX-b or ET-1 [7]; it is also less toxic by more than one order of magnitude, yet its affinity to binding sites in the rabbit aorta is similar to that of SRTX-b. It should be stressed here again that SRTX-d differs from SRTX-b in two substitutions, which apparently do not affect binding in the aorta, but cause considerably lower lethality and vasoconstrictor potency. A similar trend in activity is shown by SRTX-c and, to a certain degree, also by ET-3, which share the Thr^2/Ser substitution (in addition to several others) ([9] and unpublished results). We conclude that it is the change in position 2 which is most probably responsible for the differences observed between the weak Thr^2 peptides and the others, as it is the only substitution common to all of them and found in none of the others.

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