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Purification, crystallization and preliminary X-ray analysis of the disintegrin contortrostatin from *Agkistrodon contortrix contortrix* snake venom

Natalia Moiseeva,[a] Stephen D. Swenson,[b] Francis S. Markland Jr[b] and Robert Bau[a]*

[a]Department of Chemistry, University of Southern California, Los Angeles, CA 90089, USA, and [b]Department of Biochemistry and Molecular Biology, University of Southern California School of Medicine, Los Angeles, CA 90033, USA

Correspondence e-mail: bau@usc.edu

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Disintegrins are cysteine-rich RGD-containing peptides that block tumor-cell implantation and angiogenesis. Contortrostatin, a homodimeric disintegrin (64 residues in each chain) from southern copperhead snake venom, has been purified to homogeneity and crystallized. Initial attempts at crystallization led to a form grown from polyethylene glycol (PEG), which crystallizes in the orthorhombic space group $C222_1$, with unit-cell parameters a = 57.39, b = 139.55, c = 78.98 Å, and diffracts to a resolution limit of 3.2 Å. Very recently, a new crystalline form of the title protein has been obtained grown from ammonium sulfate [$(NH_4)_2SO_4$] as a precipitant having a space group of $P2_12_12_1$, with unit-cell parameters a = 37.52, b = 59.93, c = 121.37 Å. These improved crystals diffract to a resolution limit of 1.7 Å.

1. Introduction

Contortrostatin (CN), a protein isolated from the venom of the southern copperhead snake *Agkistrodon contortrix contortrix* (Trikha *et al.*, 1994), is a member of the disintegrin family of polypeptides. The disintegrins are small disulfide-rich RGD-containing (Arg-Gly-Asp recognition sequence-containing) peptides that bind to another class of proteins, integrins, which are intimately involved in cell-adhesion processes. One of the reasons for studying these molecules is the finding that compounds that block integrins inhibit cancer-cell implantation. Contortrostatin inhibits tumor progression and angiogenesis *via* multiple mechanisms, which makes it advantageous as an anticancer agent.

Disintegrins have been characterized and purified from many snake venoms (Dennis *et al.*, 1990; Scarborough *et al.*, 1993) and originally were distinguished by their ability to inhibit platelet aggregation. Most disintegrins are monomers and some of them have been structurally analyzed by NMR spectroscopy (Saudek *et al.*, 1991; Adler & Wagner, 1992). During the past few years, there have been at least three reports of the crystallization and preliminary diffraction data of disintegrins: applaggin (Arni *et al.*, 1999), schistatin (Tomar *et al.*, 2001), piscivostatin and acostatin (Fujii *et al.*, 2002), all of which are either heterodimeric or monomeric proteins. In contrast, contortrostatin is homodimeric. Thus far, a full X-ray structure analysis has not been reported for any disintegrin.

Contortrostatin has been isolated and purified by a four-step HPLC procedure involving hydrophobic interaction HPLC, two steps of C_{18} reverse-phase HPLC and cation-exchange HPLC (Trikha *et al.*, 1994). The amino-acid sequence of contortrostatin has been determined recently (Zhou *et al.*, 2000). Like other members of the disintegrin family, each subunit of contortrostatin has an RGD site and the cysteine alignment is conserved.

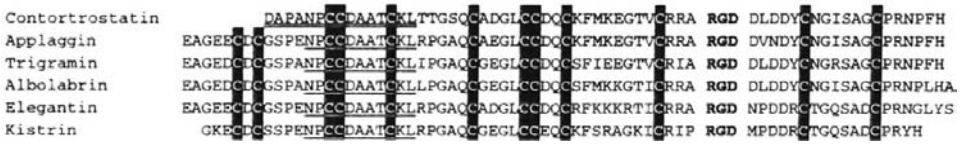

Figure 1
Sequence alignment of contortrostatin with other disintegrins [figure reprinted from Zhou *et al.*, 2000, copyright (2000) with permission from Elsevier Science. Note that contortrostatin possesses an eight-amino-acid amino-terminal truncation, which includes two cysteine residues that are normally involved in intrastrand disulfide-bond formation. These missing cysteines leave two unpaired cysteines in the remaining polypeptide chain and are thought to be involved in the formation of two intermolecular disulfide bonds between two identical chains to form the homodimeric protein.

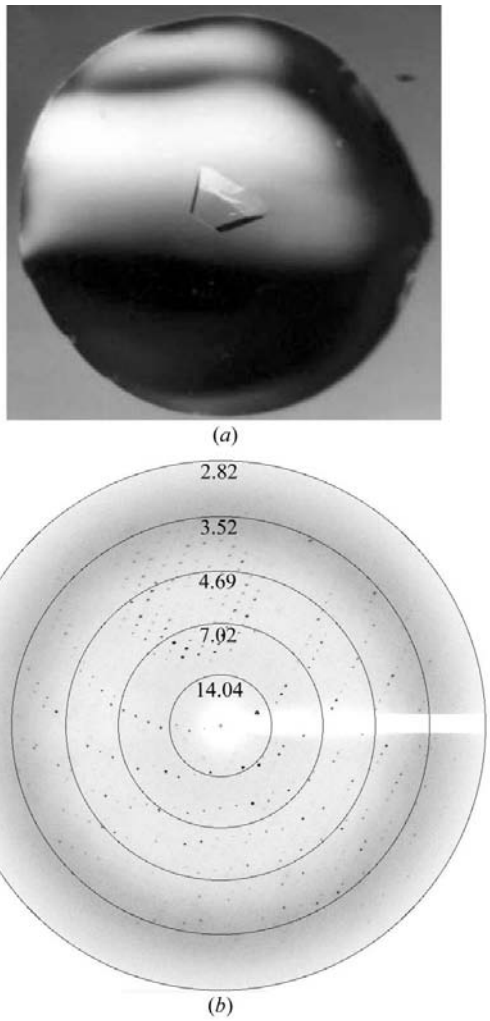

Figure 2
(*a*) Initial sample of contortrostatin crystals (type 1) grown from polyethylene glycol as precipitant and (*b*) their diffraction pattern, which only extends to a resolution of about 3.2 Å. Typical dimensions of this type of contortrostatin crystals are 0.2 × 0.1 × 0.05 mm.

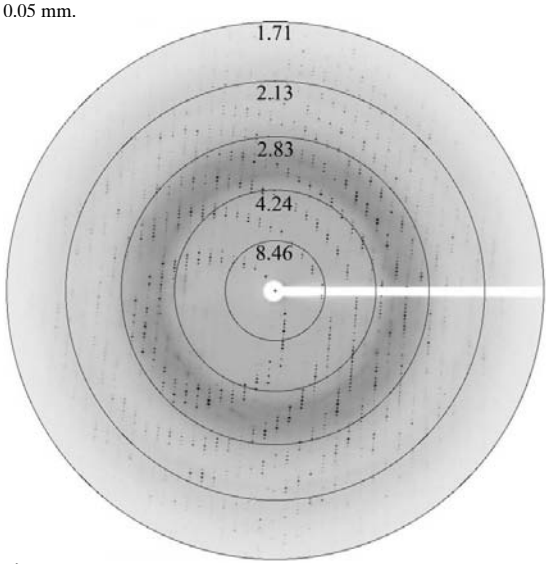

Figure 3
The diffraction pattern from our new contortrostatin crystal (type 2), grown from ammonium sulfate as a main precipitant, which diffracts up to a resolution of 1.7 Å. Typical dimensions of this type of contortrostatin crystals are 0.35 × 0.15 × 0.1 mm.

However, despite its high sequence homology with other disintegrins, a distinguishing feature of contortrostatin is the existence of an eight-amino-acid amino-terminal truncation relative to other disintegrins (see Fig. 1); the deleted portion includes two cysteine residues that are normally involved in intra-chain disulfide-bond formation. These missing cysteines leave two unpaired cysteines in the remaining polypeptide chain and are thought to be involved in the formation of two intermolecular disulfide bonds between two identical chains to form the homodimeric protein. It is not known if this intermolecular linkage takes place in a parallel or antiparallel fashion.

2. Contortrostatin crystallization and data collection

Initial crystallization conditions were based on a sparse-matrix screen (Jancarik & Kim, 1991). The hanging-drop vapor-diffusion method (Ducruix & Giegé, 1992) was used with 1 µl of protein sample (concentration $\simeq$ 10 mg ml^{-1}) and 1 µl of precipitant. Crystals appeared in more than half of the 50 initial conditions tried, but with varying quality. After refining the initial conditions in a systematic way, it was found that thin plate-like crystals of reasonable diffraction quality could be obtained under the following conditions: 20% PEG 8000, citrate–HCl buffer pH 5.5, 0.2 *M* magnesium acetate. Subsequently, it was found that the use of PEG MME 5000 instead of PEG 8000 as the main precipitant led to an increase in the size and thickness of crystals. Eventually, a medium-resolution (3.2 Å) data set was collected at Stanford Synchrotron Radiation Laboratory (SSRL) beamline 7-1 on these crystals, with space group $C222_1$ and unit-cell parameters a = 57.39, b = 139.55, c = 78.98 Å. One of the resulting crystals (hereafter referred to as type 1 crystalline form) and its diffraction pattern is shown in Fig. 2.

However, in order to resolve some important structural details (*i.e.* the conformation of the RGD loops), it was necessary to improve the crystal quality. Very recently, we have found new crystallization conditions which extend the diffraction limits of contortrostatin crystals dramatically from 3.2 to 1.7 Å resolution. By experimenting with different crystallization conditions, it was found that the new contortrostatin crystals (type 2), grown using $(NH_4)_2SO_4$ as the main precipitant [conditions: 1.8 *M* $(NH_4)_2SO_4$, Tris–HCl buffer pH 8.5] diffract extremely well. This crystal form of contortrostatin has space group $P2_12_12_1$, with unit-cell parameters a = 37.52, b = 59.93, c = 121.37 Å. The diffraction pattern of a type 2 contortrostatin crystal (resolution 1.7 Å) is shown in Fig. 3. X-ray diffraction data were collected from a flash-frozen crystal at beamline 9-1 of the SSRL. 12–15% glycerol added to the well solution was used as cryoprotectant. Some of the X-ray data-collection statistics for both crystalline forms are summarized in Table 1.

3. Preliminary X-ray analysis

The X-ray diffraction data of the contortrostatin crystals were processed and integrated using *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Given the dimeric nature of contortrostatin, the type 1 (*C*-centered orthorhombic) contortrostatin crystals contain either two or three dimers per asymmetric unit, corresponding to a solvent content of 58 or 37% and a calculated Matthews coefficient V_M of 2.9 or 1.9 Å^3 Da^{-1}, respectively, with both values being within the range typically observed in protein crystals (Matthews, 1968). Analysis of the self-rotation function calculated with *GLRF* (Tong & Rossman, 1990) did not reveal any significant peaks at κ = 180°, suggesting either the absence of non-crystallographic symmetry or, alternatively, a near-superposition of the crystallographic and non-crystallographic twofold rotation axes.

Attempts at solving the contortrostatin structure by the molecular-replacement technique are currently under way. However, in the preliminary crystallographic data analyses of applaggin and schistatin it was found that molecular-replacement methods, in which the NMR solution structure of a monomeric disintegrin was used as a search model, were not effective in phasing the X-ray data (Arni *et al.*, 1999; Tomar *et al.*, 2001). Consequently,

Table 1
Selected X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell, which was 3.25–3.19 Å for the type 1 crystal form and 1.73–1.70 Å for the type 2 crystal form.

Crystals	Type 1 (*C*-centered orthorhombic)	Type 2 (primitive orthorhombic)
X-ray source	SSRL BL 7-1	SSRL BL 9-1
Wavelength (Å)	1.08	0.775
Total No. of reflections	14158	70599
No. of unique reflections	5139	27378
Space group	$C222_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 57.39, b = 139.55, c = 78.99	a = 37.52, b = 59.93, c = 121.37
Unit-cell volume (Å^3)	∼633000	∼273000
Resolution (Å)	3.19	1.70
Completeness (%)	92.6 (86.5)	88.8 (38.9)
Reflections with $I > 3\sigma(I)$ (%)	79.0 (43.8)	76.2 (73.8)
$R_{\rm merge}$	0.099 (0.532)	0.051 (0.176)
$V_{\rm M}$ (Å^3 Da^{-1})	1.9–2.9	2.5
Molecules (dimers) per asymmetric unit	2–3	2

we are also currently trying various heavy-atom methods, such as MIR and MAD, to solve the contortrostatin structure. Our preliminary results indicate that contortrostatin crystals grown in the presence of different lanthanide ions, Ln^{3+}, diffract as well as native contortrostatin crystals. Initial analysis of the data from these derivative crystals suggests that they are potentially good heavy-metal derivatives. The search for other suitable heavy-metal derivatives is continuing.

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