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Crystallization and preliminary crystallographic studies of dimeric disintegrins from the venom of two *Agkistrodon* snakes

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Piscivostatin (PVS) and acostatin are members of the disintegrin family of platelet-aggregation inhibitors found in snake venoms and are dimeric disintegrins which play different roles to the monomeric disintegrins. The crystals of PVS belonged to the $P2_12_12_1$ space group, with unit-cell parameters $a = 34.3$, $b = 54.4$, $c = 115.9$ Å, and diffracted to 2.0 Å resolution. The crystals of acostatin belonged to the $P2_1$ space group, with unit-cell parameters $a = 55.4$, $b = 69.3$, $c = 63.5$ Å, $\beta = 111.7^\circ$, and diffracted to 2.8 Å resolution.

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1. Introduction

Disintegrins represent a family of platelet-aggregation inhibitors found in various snake venoms. They are of low molecular weight (49–84 amino acids), are cysteine-rich and contain (Arg/Lys)-Gly-Asp [(R/K)GD] tripeptides that inhibit fibrinogen binding to the glycoprotein IIb/IIIa (GPIIb/IIIa) complex. They also inhibit binding of other ligands to (R/K)GD-dependent integrins on the surface of cells (*e.g.* endothelial cells, tumour cells and fibroblasts). Disintegrins have been reported to be unique and potentially useful tools not only for investigating cell–matrix and cell–cell interactions, but also for the development of antithrombotic and antimetastatic agents in terms of their antimigration activities against certain tumour cells.

Like disintegrins, the haemorrhagins represent another family of snake-venom proteins that interfere with haemostasis and cell–cell adhesion. Haemorrhagins are snake-venom metalloproteinases (SVMPs) and some subclasses in this family have a disintegrin or disintegrin-like domain in addition to the metalloprotease domain. It has been suggested that the disintegrins are formed by proteolytic processing of the homologous precursors of the PII class of SVMP composed of pre-, prometalloproteinase and disintegrin domains (Au *et al.*, 1991; Bjarnason & Fox, 1994).

Not only snakes but also other species produce proteins containing disintegrin-like domains. ADAMs (a disintegrin and metalloproteinase) are cell membrane-anchored proteins that contain a disintegrin-like domain. Wolfsberg & White (1996) listed 13 different ADAMs that have been identified in human, monkey, rabbit, rat, guinea pig and bovine tissues and two in *Xenopus* and *Caenorhabditis elegans*. It has been suggested that the disintegrin domain of the guinea pig sperm surface protein fertilin-β (one of the ADAMs) interacts with integrin $\alpha6\beta1$ (suggested egg receptor for sperm) and plays an important role in fertilization (Schlöndorff & Blobel, 1999).

Disintegrins can be divided into three classes according to molecular size: short (49–51 amino acids), medium (70–73 amino acids) and long (83–84 amino acids). More recently, new types of (dimeric) disintegrins have been discovered, such as EC3 (Marcinkiewicz, Calvete, Marcinkiewicz *et al.*, 1999), EMF-10 (Marcinkiewicz, Calvete, Vijay-Kumar *et al.*, 1999) and contortrostatin (Trikha *et al.*, 1994; Zhou *et al.*, 2000). Piscivostatin (PVS) represents a new heterodimeric disintegrin containing (R/K)GD peptides (Okuda & Morita, 2001). PVS is constituted of 65 and 68 amino acids with an RGD sequence in the α-chain and a KGD sequence in the β-chain. Acostatin is a protein similar to PVS (D. Okuda *et al.*, unpublished results). The amino-acid sequences of both proteins have a high degree of identity to each other (approximately 96%) and other snake-venom disintegrins (70–80%). These proteins exist as a dimer (16 kDa) under non-reducing conditions and as two monomers (8 kDa) under reducing conditions on SDS–PAGE. Dimeric disintegrins such as PVS and acostatin have a different effect in ADP-induced platelet aggregation from that of the monomeric disintegrins such as trimestatin (Okuda & Morita, 2001). To clarify the different properties of dimeric and monomeric disintegrins, we have crystallized PVS and acostatin and obtained data sets to 2.0 and 2.8 Å resolution, respectively.

2. Material and methods

2.1. Protein purification

The purification method for PVS has been described previously (Okuda & Morita, 2001)

and that for acostatin was basically the same as that for PVS except for the source of the snake venom. The lyophilized venoms of *A. piscivorus piscivorus* (water moccasin) for PVS and *A. contortrix contortrix* (copperhead) for acostatin were purchased from Kentucky Reptile Zoo (KY, USA). The crude venom was dissolved in 50 m*M* Tris–HCl buffer pH 8.0 and insoluble materials were discarded by centrifugation. The supernatant was fractionated by gel filtration using Superdex 75 pg. Fractions were assayed for inhibitory activity on ADP-induced aggregation of fresh human platelets. The active fractions were pooled and purified by the successive use of SP-Sepharose High Performance and reverse-phase HPLC columns. The yields of PVS and acostatin were 6.9 and 5.2 mg, respectively, from 1 g of venom. The MW of PVS and acostatin on SDS–PAGE were 16 kDa under non-reducing conditions and 8 kDa under reducing conditions, respectively. The masses of the proteins were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager-DE, Applied Biosystems). The averaged molecular weight of PVS and acostatin were 14 197.0 and 13 642.3 Da, respectively. The purity was estimated to be >98% by 16% polyacrylamide gel electrophoresis with staining by Coomassie Brilliant Blue.

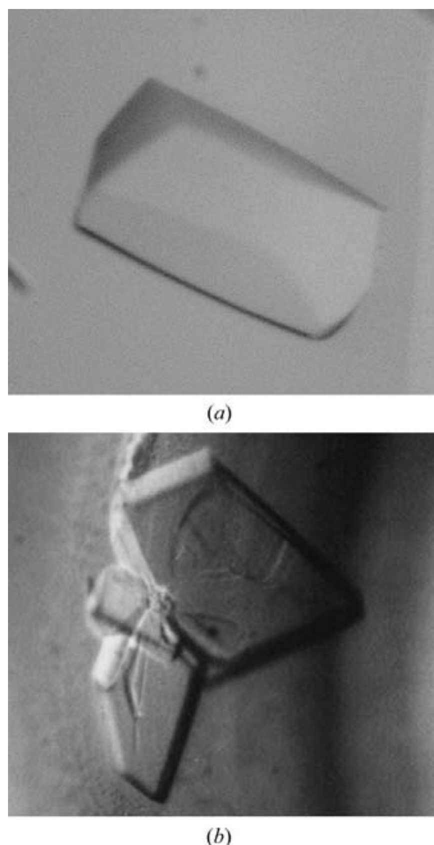

Figure 1
Crystals of dimeric disintegrins grown by the microbatch technique. Photographs of (*a*) PVS and (*b*) acostatin crystals.

2.2. Crystallization

Lyophilized PVS was dissolved in distilled water at a concentration of 15.0 mg ml^{-1}. Crystallization conditions were screened by the microbatch technique using Crystal Screen kits 1 and 2 (Hampton Research, USA). Typically, a 0.5 µl drop of protein solution was mixed with 1.5 µl of the screening agent. In some conditions (1-2, 1-6, 1-18, 1-29, 1-40 and 1-41), we obtained small or needle crystals of PVS. These conditions were refined and single crystals of PVS (0.2 mm in each dimension) were finally obtained using 12.0 mg ml^{-1} protein and 1.4 *M* sodium formate at 293 K (Fig. 1*a*). Similarly, we obtained thin plate crystals of acostatin in some conditions in the initial screening (1-10, 1-22, 1-36, 1-41, 2-30, 2-37 and 2-38). After refinement of these conditions, crystals of acostatin (of dimensions 0.3 × 0.3 × 0.01 mm) were obtained using 7.5 mg ml^{-1} protein, 6%(*w*/*v*) polyethylene glycol 8000 (PEG 8K) and 75 m*M* Tris–HCl pH 8.5 at 293 K (Fig. 1*b*).

2.3. Data collection

Diffraction data from native crystals were obtained at beamline BL41-XU, SPring-8, Harima, Japan (λ = 1.00 Å). PVS crystals were mounted in thin-walled quartz capillaries and were flash-frozen in a nitrogen stream at 100 K (Rigaku, Japan). Acostatin crystals were mounted in nylon loops (Hampton Research, USA) after five steps of soaking in a cryoprotectant solution (up to 30% PEG 8K) added to the crystallization solution. The crystals were cooled in a nitrogen-gas stream at 100 K (Rigaku, Japan). Native data sets were collected using a MAR CCD area detector in 2.0° oscillation steps over a 360° range. All data sets were processed using *DPS*/*MOSFLM* (Rossmann & van Beek, 1999). The data-collection statistics are shown in Table 1.

3. Results

The crystals of PVS belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters *a* = 34.3, *b* = 54.4, *c* = 115.9 Å, and diffracted to 2.0 Å resolution. Assuming two PVS molecules in an asymmetric unit the V_M value is 1.9 $Å^3$ Da^{-1}, which is within the expected range (Matthews, 1968). This V_M value corresponds to a solvent content of approximately 35.6%.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	PVS	Acostatin
Wavelength (Å)	1.00	1.00
Resolution (Å)	38.0–2.0 (2.1–2.0)	34.0–2.8 (2.95–2.8)
Completeness (%)	91.3 (61.8)	96.1 (96.1)
R_{merge} (%)	7.4 (33.7)	5.6 (23.1)
Average $I/\sigma(I)$	8.6 (2.4)	9.8 (2.2)
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters		
a (Å)	34.3	55.4
b (Å)	54.4	69.3
c (Å)	115.9	63.5
β (°)		111.7
Unique reflections	14052	10711
Observed reflections	78837	20258
V_M ($Å^3$ Da^{-1})	1.9	2.77–2.1
Molecules per asymmetric unit	2	3–4

The crystals of acostatin belong to the monoclinic space group $P2_1$, with unit-cell parameters *a* = 55.4, *b* = 69.3, *c* = 63.5 Å, β = 111.68°, and diffracted to 2.8 Å resolution. Because of the decay, only the first 180° of data were used in scaling and merging. Assuming three or four acostatin molecules in an asymmetric unit the V_M value is 2.77 or 2.1 $Å^3$ Da^{-1}, respectively, both of which are within the expected range (Matthews, 1968). These V_M values correspond to solvent contents of approximately 40.8 and 55.6%, respectively.

Recently, we have determined the crystal structure of trimestatin, a 70 amino-acid residue member of the medium-size disintegrin subgroup which exists as a monomer in solution (Y. Fujii *et al.*, unpublished results). PVS and trimestatin have approximately 65–72% sequence identity, while acostatin and trimestatin have approximately 63–70% sequence identity. Molecular replacement was used for structural analyses of PVS and acostatin using the crystal structure of the monomeric disintegrin trimestatin as a search model.

Rotation–translation molecular replacement using the program *AMoRe* (Navaza, 1994) and the direct-rotation program *CNS* (Brunger *et al.*, 1998) did not provide any consistent solution over many search parameters. A complete six-dimensional search using the program *EPMR* (Kissinger *et al.*, 1999) and the program *Queen of Spades* (Glykos & Kokkinidis, 2000) also did not provide any consistent solution over many search parameters.

The disulfide-bond pattern of the heterodimeric disintegrin EMF-10 was established by a combination of N-terminal amino-acid

sequencing and mass spectrometry of fragments isolated by HPLC after degradation (Calvete *et al.*, 2000). Using the information of the two intramolecular disulfide-bond patterns, molecular models of PVS and acostatin were built and energy-minimized using the program *INSIGHT-II* (Molecular Simulations, Inc., San Diego, USA) to relieve short contacts. Further analysis with these dimeric disintegrin models using the programs *AMoRe*, *CNS*, *EPMR* and *Queen of Spades* is under way.

References

Au, L. C., Huang, Y. B., Huang, T. F., Teh, G. W., Lin, H. H. & Choo, K. B. (1991). *Biochem. Biophys. Res. Commun.* **181**, 585–593.

Bjarnason, J. B. & Fox, J. W. (1994). *Pharmacol. Ther.* **62**, 325–372.

Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.

Calvete, J. J., Jurgens, M., Marcinkiewicz, C., Romero, A., Schrader, M. & Niewiarowski, S. (2000). *Biochem. J.* **345**, 573–581.

Glykos, N. M. & Kokkinidis, M. (2000). *Acta Cryst.* D**56**, 169–174.

Kissinger, C. R., Gehlhaar, D. K. & Fogel, D. B. (1999). *Acta Cryst.* D**55**, 484–491.

Marcinkiewicz, C., Calvete, J. J., Marcinkiewicz, M. M., Raida, M., Vijay-Kumar, S., Huang, Z., Lobb, R. R. & Niewiarowski, S. (1999). *J. Biol. Chem.* **274**, 12468–12473.

Marcinkiewicz, C., Calvete, J. J., Vijay-Kumar, S., Marcinkiewicz, M. M., Raida, M., Schick, P., Lobb, R. R. & Niewiarowski, S. (1999). *Biochemistry*, **38**, 13302–13309.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Okuda, D. & Morita, T. (2001). *J. Biochem.* **130**, 407–415.

Rossmann, M. G. & van Beek, C. G. (1999). *Acta Cryst.* D**55**, 1631–1640.

Schlöndorff, J. & Blobel, C. P. (1999). *J. Cell. Sci.* **112**, 3603–3617.

Trikha, M., Rote, W. E., Manley, P. J., Lucchesi, B. R. & Markland, F. S. (1994). *Thromb. Res.* **73**, 39–52.

Wolfsberg, T. G. & White, J. M. (1996). *Dev. Biol.* **180**, 389–401.

Zhou, Q., Hu, P., Ritter, M. R., Swenson, S. D., Argounova, S., Epstein, A. L. & Markland, F. S. (2000). *Arch. Biochem. Biophys.* **375**, 278–288.