Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of jararhagin, a metalloproteinase/disintegrin from *Bothrops jararaca* snake venom

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Received 19 January 2001
Accepted 10 April 2001

Jararhagin is a toxic protein, isolated from the venom of the snake *Bothrops jararaca*, which is composed of a metalloprotease domain coupled to a disintegrin/cysteine-rich domain. It induces local haemorrhage owing to the proteolytic digestion of the basement membrane of capillaries. Jararhagin also cleaves the $\alpha_2\beta_1$ integrin on the surface of platelets, thereby acting as a potent inhibitor of collagen-induced platelet aggregation. Crystals of jararhagin were obtained by the vapour-diffusion technique at 273 K in 200 m*M* sodium acetate, 100 m*M* cacodylate buffer pH 6.5 and 30% PEG 8000. Diffraction data have been obtained to a resolution of 2.8 Å from a single frozen crystal, which belonged to space group $P2_12_12_1$ with unit-cell parameters a = 73.7, b = 100.3, c = 133.4 Å. The asymmetric unit contains two jararhagin molecules and has a solvent content of 45%. A molecular-replacement solution has been obtained using a homology-built model based on the crystal structure of acutolysin, a haemorrhagic zinc metalloproteinase from the venom of the snake *Agkistrodon acutus*; attempts are under way to locate the remaining domains.

1. Introduction

Metalloproteinase/disintegrin-like cysteine-rich proteins (MDC) comprise a new class of mammalian metalloproteinases with a potential regulatory role in cell–cell and cell–matrix adhesion and signalling. Several lines of evidence have shown the involvement of MDC proteins in a variety of important physiological processes such as sperm–egg fusion (Blobel *et al.*, 1992), myoblast fusion (Yagami-Hiromasa *et al.*, 1995) and tumour-necrosis factor (TNF) processing (Black *et al.*, 1997). MDC proteins have a mosaic structure with several domains, including an N-terminal enzymatically active Zn^{2+} binding sequence containing domain (metalloproteinase), an adhesive disintegrin-like domain and a cysteine-rich domain of unknown function. Most members of this family also have transmembrane and cytoplasmatic domains. The catalytic domain of MDC proteins seems to be related to the release of the extracellular domains of many cell-surface proteins (Black *et al.*, 1997; Kamiguti *et al.*, 1996). On the other hand, the disintegrin domain is likely to interact with specific integrins on the plasma membrane and could be responsible for triggering membrane fusion between cells. The disintegrin domain of MDC proteins is highly related to soluble snake-venom integrin ligands called disintegrins. These are short cysteine-rich RGD-containing peptides found in many Viperidae and Crotalidae snake venoms (Niewiarowski *et al.*, 1994). Disintegrins strongly inhibit fibrinogen-dependent platelet aggregation by specifically binding the platelet surface fibrinogen receptor integrin IIb/IIIa. Despite the similarity of the disintegrin domain, the RGD sequence itself is replaced by an E/DCD sequence in most MDC proteins, suggesting that the latter could interact with different types of integrins.

Besides the isolated disintegrins, the snake venom often also contains members of the MDC family in which the disintegrin domain is coupled to metalloproteinase and cysteine-rich domains. Jararhagin is a high molecular-weight MDC protein isolated from the venom of the snake *B. jararaca* (Paine *et al.*, 1992) which induces strong local haemorrhage owing to the proteolytic digestion of the basement membrane of capillaries. Jararhagin is also a potent inhibitor of collagen-induced platelet aggregation (Kamiguti *et al.*, 1997) and this effect is mainly a consequence of the proteolytic cleavage of the $\alpha_2\beta_1$ integrin on the platelet surface (Kamiguti *et al.*, 1996) or alternatively of the blockage of its interaction with collagen (Moura-da-Silva *et al.*, 2001). Recently, it was demonstrated that the disintegrin-like domain of alternagin, a homologous MDC protein from *B. alternatus* venom, also inhibits collagen-mediated cell adhesion, suggesting a functional role of this domain in target binding (Souza *et al.*, 2000). Structure–

Table 1
Crystal data and data-collection statistics.

Values for the highest resolution shell (2.87–2.80 Å) are shown in parentheses.

Wavelength (Å)	1.544
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 73.7, b = 100.3, c = 133.4
Resolution (Å)	20.0–2.8
V_M (two molecules per asymmetric unit) (Å^3 Da^{-1})	2.24
Percentage solvent	45
R_{merge}† (%)	7.0 (27.0)
Completeness (%)	91.7 (90.5)
No. of observations	64333
No. of unique reflections	23061
Reflections with $F > 3\sigma$ (%)	79 (42)

† $R_{merge} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$.

function relationships of MDC proteins are of great interest owing to their potential application in thrombosis and adhesion research as well as for anti-metastatic drug design.

In this paper, we report the crystallization and preliminary crystallographic characterization of jararhagin. Crystal structures are currently available for the isolated N-terminal metalloproteinase domain from the TNF α-converting enzyme as well as for homologous metalloproteinases which lack the remaining disintegrin and cysteine-rich domains (Gomis-Ruth *et al.*, 1994; Zhang *et al.*, 1994; Gong *et al.*, 1998; Maskos *et al.*, 1998). Furthermore, NMR structures are also available for several isolated RDG disintegrins in solution (Adler *et al.*, 1991; Saudek *et al.*, 1991). This is the first report of the crystallization of a complete member of the metalloproteinase/disintegrin class of proteins; its full structure determination should permit us to address questions concerning the inter-relationships between the domains, including their relative orientations, which may be important for the control of their biological activity.

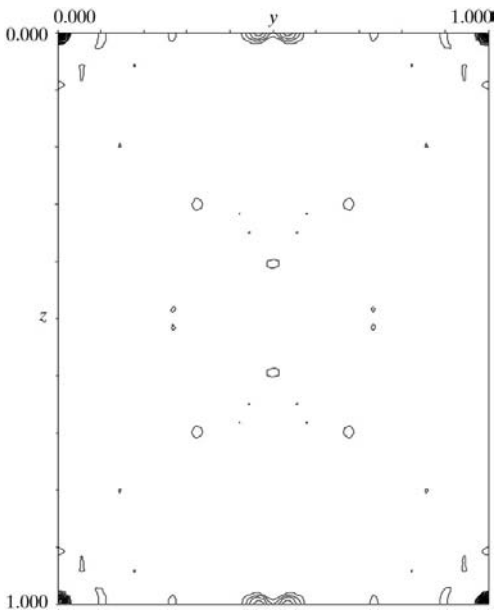

Figure 1
The u = 0 section of the native Patterson function as drawn viewed along the u axis showing a large (15σ) peak corresponding to a non-crystallographic translation of approximately b/2.

2. Methods

2.1. Purification and crystallization

Jararhagin was purified as previously described (Paine *et al.*, 1992) with minor modifications. Briefly, *B. jararaca* venom was first fractionated on an FPLC phenyl Superose (HR 5/5) column equilibrated with 1.2 *M* amonium sulfate, 20 m*M* Tris–HCl buffer pH 7.4. The most hydrophobic fraction which eluted from the column during an inverse linear gradient of 20 ml total volume from 1.2 to 0 *M* ammonium sulfate contained both haemorrhagic and proteolytic activities. This peak was pooled and applied to a mono-Q column equilibrated in 20 m*M* Tris–HCl pH 6.8 and eluted with a linear salt gradient from 0 to 1 *M* in a total volume of 20 ml. The second peak to elute from the ion-exchange column corresponded to jararhagin and was homogeneous as determined by SDS–PAGE, which gave an estimated molecular weight of 52 kDa. Inhibition of the enzymatic activity of jararhagin with the metalloproteinase inhibitor POL 647 (Gomis-Ruth *et al.*, 1998) was achieved by incubation of the enzyme with the inhibitor in an enzyme:inhibitor molar ratio of 1:50.

A protein solution containing jararhagin at a concentration of 8.5 mg ml^{-1}, 50 m*M* sodium phosphate pH 6.0, 250 m*M* NaCl and POL 647 (1:50) was screened for crystallization conditions at 273 K using the hanging-drop vapour-diffusion method and employing Hampton Research Crystal Screen I factorial solutions (Jancarik & Kim, 1991). In a typical crystallization experiment, 3 µl of protein solution was mixed with the same volume of reservoir solution and allowed to equilibrate against 500 µl of the latter. Microcrystals were obtained under several conditions, but crystals suitable for X-ray diffraction were only obtained under condition No. 28 (200 m*M* sodium acetate, 100 m*M* cacodylate buffer pH 6.5, 30% PEG 8000). Attempts to refine the crystallization conditions did not result in a significant improvement in crystal size, even though crystals of similar habit were also obtained with a reduced PEG concentration (27%).

2.2. Data collection

For cryoprotection experiments, crystals were rapidly soaked in solutions containing 200 m*M* sodium acetate, 100 m*M* cacodylate buffer pH 6.5, 30% PEG 8000 and 15%(*v*/*v*) glycerol. Cryocooling was performed by mounting single crystals in cryoloops (Hampton Reserch, CA, USA) followed by immediate immersion in a nitrogen-gas stream at 100 K. X-ray diffraction data collection using a cryocooled crystal was carried out at the Brazilian National Synchroton Laboratory (LNLS). A total of 105 images were collected using the oscillation method. Individual frames consisted of a 0.7° oscillation angle measured for 170 s with the crystal-to-detector distance set to 200 mm. Data were processed and integrated using *DENZO* and scaled using *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Autoindexing and consideration of systematically absent reflections revealed that the crystals belong to space group $P2_12_12_1$, with typical unit-cell parameters a = 73.7, b = 100.3, c = 133.4 Å. With the assumption of two molecules of jararhagin in the asymmetric unit, a Matthews parameter value (Matthews, 1968) of 2.24 Å^3 Da^{-1} is obtained, with a corresponding solvent content of approximately 45%. Both these values are within the expected ranges for typical protein crystals. Processing of the 64 333 measured reflections led to 23 061 unique reflections with an R_{merge} of 7% (Table 1). The crystals diffracted to 2.6 Å resolution, but qualitatively the data set is only useful to 2.8 Å.

No significant peak was observed in the self-rotation function corresponding to κ = 180°. This indicates an absence of non-crystallographic twofold rotational symmetry or alternatively a coincidence of the crystallographic and non-crystallographic rotation axes. Evidence for the former came from the native Patterson function (Fig. 1) calculated with data in the resolution range 20–2.8 Å, which showed a large peak (15$\sigma_{r.m.s.}$) at u = 0.0, v = 0.47, w = 0.0, corresponding to a non-crystallographic translation of approximately b/2.

Molecular-replacement studies were undertaken using the program *AMoRe*

(Navaza, 1994) employing a homology-built model for the metalloproteinase domain of jararhagin. The latter was based on the crystal structure of acutolysin (PDB code 1bud; Gong *et al.*, 1998), a haemorrhagic zinc metalloproteinase from the venom of *A. acutus* with which jararhagin shares 56% sequence identity. Data in the resolution range 20–2.8 Å were used. A solution was obtained for the rotation and translation functions assuming two molecules in the asymmetric unit, leading to a final correlation coefficient of 0.34 after rigid-body refinement. The second-best solution yielded a correlation coefficient of 0.27, demonstrating a clear contrast with the first, increasing confidence that the latter is the correct solution even though the overall correlation coefficient is rather low. This is most probably because of the fact that the search model represents less than 50% of the total molecule. Evidence that the solution is correct is derived from the fact that the two *AMoRe* solutions are related by a non-crystallographic translation consistent with the native Patterson function described above. The solution leads to acceptable crystal packing but leaves large voids along the planes parallel to the *x* axis. The C-termini of the metalloproteinase domains points towards these voids, which are presumably filled by the disintegrin and cysteine-rich domains, leading to sufficient contacts for the maintenance of the crystal lattice (Fig. 2).

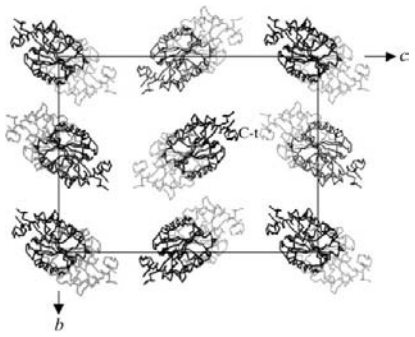

Figure 2
Crystal packing as viewed along the *x* axis. Voids in the structure can be seen between molecules where the C-terminal disintegrin and cysteine-rich domains are expected to be located. The two crystallographically independent molecules of the asymmetric unit are shown in different tones of grey and can be seen to be related by a translation of approximately *b*/2.

Density-modification and refinement techniques are currently being employed in attempts to locate the remaining parts of the structure.

We thank Dr G. Laing (Liverpool School of Tropical Medicine, Liverpool, England) and V. Politi (Department of Biology, University 'Roma TRE', Rome, Italy) for kindly supplying the POL inhibitor. We also thank the Brazilian National Synchrotron Laboratory (LNLS) for access to the Protein Crystallography (PCr) beamline. This work was supported by FAPESP, FINEP (PRONEX) and CNPq.

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