Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Initial structural analysis of an $\alpha_4\beta_4$ C-type lectin from the venom of *Crotalus durissus terrificus*

M. T. Murakami,[a] L. Watanabe,[a] L. M. Gava,[a] S. P. Zela,[a] A. C. O. Cintra[b] and R. K. Arni[a]*

[a]Department of Physics, IBILCE/UNESP, R. Cristovão Colombo 2265, CEP 150054-000, São Jose do Rio Preto-SP, Brazil, and [b]Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP, CEP 14040-903, Ribeirão Preto-SP, Brazil

Correspondence e-mail: arni@df.ibilce.unesp.br

Received 2 July 2003
Accepted 22 July 2003

Convulxin, an $\alpha\beta$ C-type lectin, is a potent platelet activator isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus*. It is a 26.5 kDa $\alpha\beta$ heterodimer consisting of two homologous disulfide-linked chains. The crystals belong to space group $I4$, with unit-cell parameters $a = b = 131.61$, $c = 121.85$ Å, and diffraction data were collected to 2.7 Å. The structure was solved by molecular replacement and the asymmetric unit contains two $\alpha\beta$ heterodimers, each of which forms a disulfide-linked cyclic $\alpha_4\beta_4$ tetramer in the unit cell. These $\alpha_4\beta_4$ tetramers are stacked to form a large solvent channel.

1. Introduction

Thrombus formation involves platelet adhesion receptors, glycoproteins Ib-IX-V and VI, which bind von Willebrand Factor (vWF) and collagen. Platelet adhesion is mediated by the receptor glycoprotein (GP), which plays a key role in haemostasis. Adhesion of activated platelets to sites of vascular injury is initiated by the interaction of vWF, which is exposed on the subendothelium, with the platelet GPIb. These interactions then initiate intracellular signals which lead to degranulation, significant elevation of cytosolic Ca^{2+} levels and activation of integrin and GPIIb-IIIa which binds vWF or fibrinogen and mediates platelet aggregation (Andrews *et al.*, 1996).

A number of snake-venom proteins which are haemostatically active and interfere with the interaction of vWF and platelet GPs have been characterized (Fujimara *et al.*, 1996). Some of these inhibit coagulation factors and platelet components. These proteins affect vWF–platelet GPIb-V-IX. Echicetin (Peng *et al.*, 1993), alboaggregin-B and agglucetin (Wang & Huang, 2001) bind GPIbα, while rhodocetin interacts with GPIa-IIa (Wang *et al.*, 1999) and convulxin (CVX) interacts with GPVI (Prado-Franceschi & Brazil, 1981). However, alboaggregin-A (Asazuma *et al.*, 2001) and alboluxin bind both GPVI and GPIbα (Andrews *et al.*, 1996).

CVX, a C-type lectin (Drickamer, 1993) is a potent platelet-aggregation inducer (Vargaftig *et al.*, 1980; Francischetti *et al.*, 1997; Polgár *et al.*, 1997; Jandrot-Perrus *et al.*, 1997) isolated from the venom of the Brazilian rattlesnake *Crotalus durissus terrificus*. This protein is a disulfide-linked heterodimer composed of two homologous subunits CVXα (13.9 kDa) and CVXβ (12.6 kDa) (Leduc & Bon, 1998). These subunits display significant homology to the carbohydrate-recognition domain (CRD) of the C-type lectin family, but lack the consensus sequences for both carbohydrate and Ca^{2+} binding (Leduc & Bon, 1998). Binding of CVX is not inhibited by α-thrombin, fibrinogen, ADP, RGDS peptides or adrenaline. The mechanism of CVX-mediated activation of platelets has been shown to be dependent on Ca^{2+} but independent of galactose or mannose, fibrinogen, ADP and cyclooxygenase (Vargaftig *et al.*, 1983). In human platelets, collagen binds to GPIa-IIa and GPVI and it has been proposed that CVX binds to the collagen receptor GPVI with high affinity (K_d = 30 p*M*; Prado-Franceschi & Brazil, 1981).

CVX has been suggested to exist as a trimer ($\alpha_3\beta_3$) in solution with a molecular weight of between 72 and 80 kDa (Niedergang *et al.*, 2000). We have crystallized CVX and solved the structure by molecular-replacement methods. In the crystal structure, the asymmetric unit contains two $\alpha\beta$ heterodimers, resulting in the presence of two cyclic $\alpha_4\beta_4$ tetramers in the unit cell. An interchain disulfide bridge between the C-terminus of the α-subunit of one heterodimer and the N-terminus of the β-subunit of a neighbouring heterodimer stabilize each $\alpha_4\beta_4$ tetramer, indicating that this protein exists as an $\alpha_4\beta_4$ tetramer in solution, like flavocetin (Fukuda *et al.*, 2000).

2. Methods

2.1. Protein purification

C. durissus terrificus crude venom was obtained in lyophilized form from a local serpentarium and 250 mg was dissolved in 5 ml of 20 m*M* Tris–HCl pH 8.0 buffer. This solution was centrifuged, filtered (0.4 μ*M*) and applied

to a benzamidine Sepharose (CL6B) column to remove gyroxin, a thrombin-like enzyme. The unbound fraction which contained CVX was collected and further purified on a Sephacryl S300 column. The sample was then concentrated to 10 mg ml^{-1} in Centricon microconcentrators (Amicon).

Silver-stained SDS–PAGE gels (9%) indicated the presence of a number of protein bands. Dynamic light-scattering (Dynapro 801) experiments conducted at 291 K indicated the presence of a single monodispersive population.

2.2. Crystallization

Crystallization trials were conducted using the vapour-diffusion technique, in which 1 µl of protein was mixed with an equal amount of the corresponding reservoir solution. Crystals were obtained when the protein was equilibrated against 100 m*M* sodium acetate buffer containing 200 m*M* $CaCl_2$ pH 4.6 and when 14% 2-methyl-2,4-pentanediol was used as a precipitant.

2.3. X-ray diffraction analysis

Monocromatic X-rays were produced by a Rigaku RU300 (Rigaku-Denki) rotating-anode generator operating at 50 kV and 80 mA equipped with Osmic mirrors and diffraction intensities were recorded using a MAR345 (MAR Research) imaging-plate detector. A single crystal with a maximum dimension of 0.1 mm (Fig. 1) was mounted in a cryoloop (20% glycerol), flash-frozen and diffraction data were collected at 100 K. Diffraction was observed to a maximum resolution of 2.2 Å; however, data beyond 2.7 Å were weak and were not included in the processing. Data were indexed with *DENZO* and scaled and reduced using *SCALEPACK* (Otwinowski & Minor, 1997) (Table 1).

3. Results

The SDS gels showed a ladder-like distribution, with a number of protein bands which can be attributed to different states of aggregation of the α- and β-subunits. The dynamic light-scattering experiment, however, indicated the existence of a monomodal distribution.

The crystals belong to the space goup *I*4, with unit-cell parameterers $a = b = 131.61$, $c = 121.85$ Å. Processing of the 244 151 measured reflections to 2.2 Å led to 25 792 unique reflections with an $R_{\rm merge}$ of 4.9% for data to 2.7 Å (20.7% in the last shell).

Assuming a molecular mass of 26.5 kDa per asymmetric unit, Matthews parameter ($V_{\rm M}$) values (Matthews, 1968) of 4.8 and 3.2 Å^3 Da^{-1} were obtained for the presence of two and three heterodimers per asymmetric unit, corresponding to solvent contents of 73.9 and 60.9%, respectively.

Molecular replacement was carried out using the program *AMoRe* (Navaza, 1994) using a model built based on the atomic coordinates of the C-type lectin flavocetin-A from the venom of *Trimeresurus flavoviridis* (Fukuda *et al.*, 2000; PDB code 1c3a). A solution was obtained for two molecules present in the asymmetric unit. Rigid-body refinement of this solution using data in the resolution range 30.0–3.0 Å resulted in a correlation coefficient of 41.5 and an *R* factor of 50.3%. Refinement with *CNS* (Brünger *et al.*, 1998) and model building using *TURBO-FRODO* resulted in an *R* factor of 27% ($R_{\rm free}$ = 33%) for data in the same resolution range.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last resolution shell.

Space group	*I*4
Unit-cell parameters (Å)	$a = b = 131.61$, $c = 121.85$
Maximum resolution (Å)	2.7
No. of unique reflections	25792
$R_{\rm merge}$† (%)	4.9 (20.7)
Completeness (%)	96.9 (99.3)
$V_{\rm M}$ (Å^3 Da^{-1})	4.8
No. of molecules per AU	2
$I/\sigma(I)$	15.5 (4.0)

† $R_{\rm merge} = \sum\sum I(h)_i - \langle I(h)\rangle / \sum\langle I(h)\rangle$, where I_h is the observed intensity of the *i*th measurement of reflection *h* and $\langle I(h)\rangle$ is the mean intensity of reflection *h* calculated after scaling.

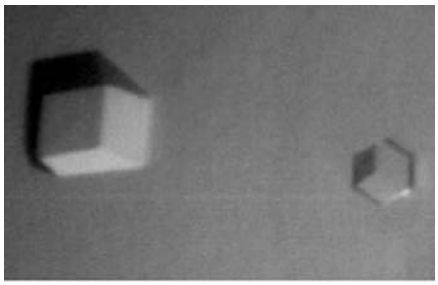

Figure 1
Photomicrograph of tetragonal crystals of CVX (maximum dimension 0.1 mm).

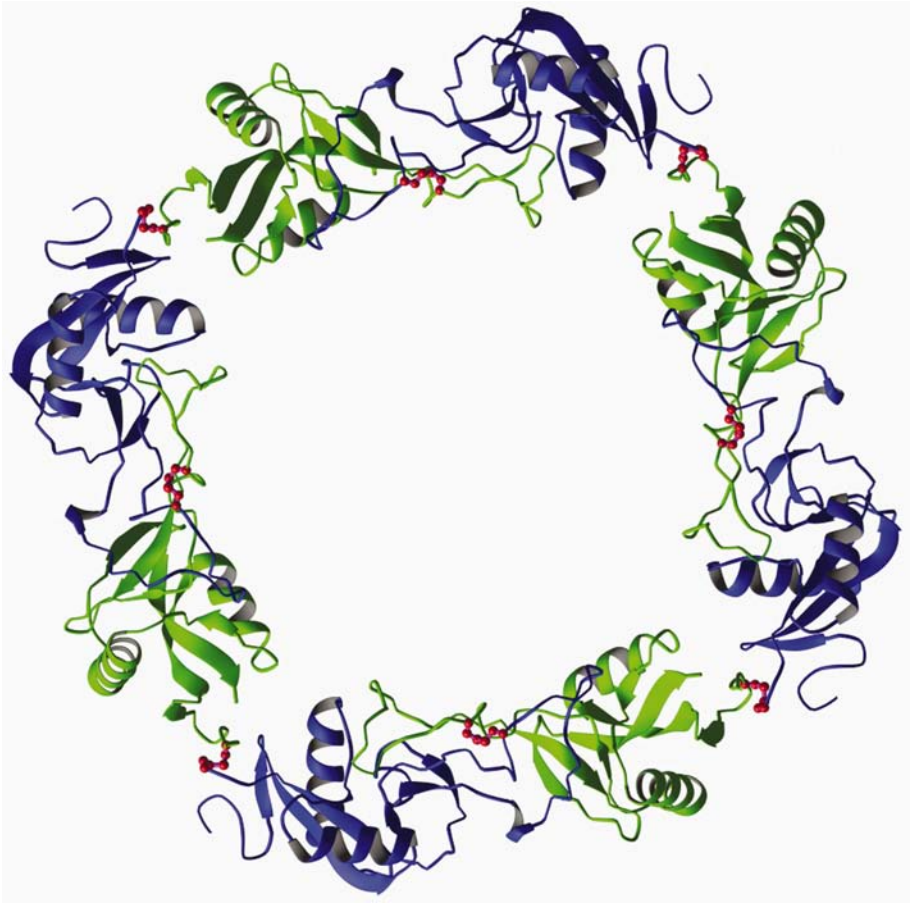

Figure 2
Ribbon representation of the tetrameric structure of CVX. The α- and β-subunits are shown in blue and green, respectively. The disulfide bonds (C^{α}, C^{β}, S^{γ}) stabilizing the $\alpha\beta$ heterodimers and the $\alpha_4\beta_4$ cyclic tetramer are shown in red. The figure was generated using *RIBBONS* (Carson, 1991).

The asymmetric unit contains two $\alpha\beta$ heterodimers. Each heterodimer is linked by a disulfide bridge formed between the α-subunit of one heterodimer and the β-subunit of a neighbouring heterodimer to form a cyclic $\alpha_4\beta_4$ tetramer (Fig. 2) analogous to the cyclic $\alpha_4\beta_4$ tetramer reported in the crystal stucture of flavocetin-A (Fukuda *et al.*, 2000). In contrast to the structure of flavocetin-A, which contains only one $\alpha_4\beta_4$ tetramer, the unit cell of CVX contains two $\alpha_4\beta_4$ tetramers. These cyclic tetramers are stacked on each other, forming a large solvent channel with a diameter of approximately 65 Å. These results indicate that CVX could also be present as an $\alpha_4\beta_4$ tetramer in solution and not as an $\alpha_3\beta_3$ protein (Leduc & Bon, 1998).

Higher resolution data will be collected at the Laboratorio Nacional de Luz Síncrotron (Campinas, Brazil) and structure refinement will be initiated.

This study was supported by grants from FAPESP/SMOLBNet and CNPq (Brazil). MTM and LW are recipients of FAPESP fellowships and LMG is the recipient of a CNPq fellowship. We are grateful to C. B. Oliva for expert assistence.

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