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Crystallization and preliminary X-ray studies of flavocetin-A, a platelet glycoprotein Ib-binding protein from the habu snake venom

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Flavocetin-A (FL-A) is a platelet glycoprotein Ib-binding protein, a high molecular mass oligomer (149 kDa) of C-type lectin-like subunits α and β isolated from the habu snake venom. Purified FL-A crystallized in the tetragonal space group $I4$ with unit-cell dimensions $a = b = 121.0$, $c = 63.2$ Å. The crystals diffract to at least 2.4 Å resolution. The structure has been solved by molecular replacement using the crystal structure of factors IX/X-binding protein (PDB code 1ixx) as a search model. The asymmetric unit contains one heterodimer, showing that FL-A is a novel tetradimer $(\alpha\beta)_4$ composed of four heterodimers related by a crystallographic fourfold axis.

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1. Introduction

Platelet aggregation is an essential cellular interaction which occurs after external trauma to the vasculature. The binding of plasma glycoprotein von Willebrand factor (vWF) to platelet membrane-receptor glycoprotein (GP) Ib–IX–V complex initiates the contact adhesion of platelets to exposed subendothelium and plays a crucial role in haemostasis (Sakariassen *et al.*, 1979). Therefore, molecular agents which can selectively block the interaction between vWF and GPIb–IX–V receptor would be useful as antithrombotic agents with possible therapeutic potential.

The GPIb–IX–V receptor is a multimeric molecule consisting of GPIbα (M_r = 145 kDa), GPIbβ (M_r = 22 kDa), GPIX (M_r = 17 kDa) and GPV (M_r = 82 kDa). Among these components, the α-subunit of GPIb is considered to be a structurally and functionally essential region of the receptor (López *et al.*, 1998). Recently, a number of snake-venom proteins which interact with the GPIb α subunit of the receptor have been isolated and characterized (for a review, see Fujimura *et al.*, 1996). They include alboaggregin-B from *Trimeresurus albolabris* (Peng *et al.*, 1991), echicetin from *Echis carinatus* (Peng *et al.*, 1993), agkicetin from *Agkistrodon acutus* (Chen & Tsai, 1995), tokaracetin from *Trimeresurus tokarensis* (Kawasaki *et al.*, 1995), jararaca GPIb-binding protein from *Bothrops jararaca* (Kawasaki *et al.*, 1996) and mamushigin from *Agkistrodon halys blomhoffii* (Sakurai *et al.*, 1998). Most of these proteins bind to the GPIb α-subunit and consequently inhibit the vWF-mediated platelet aggregation under high shear-stress conditions. Conversely, alboaggregin-B is known to induce aggregation of platelets (Peng *et al.*, 1991).

These venomous proteins are $\alpha\beta$ heterodimers with a low molecular mass of approximately 30 kDa, are composed of disulfide-linked α and β subunits and belong to group VII of the C-type lectin family (Drickamer, 1993). While biochemical and structural characterization of the low molecular mass C-type lectin-like proteins from snake venoms has proceeded, little is known about the high molecular mass venomous proteins. Convulxin (CVX), a collagen-like platelet-aggregating protein (72 kDa), was isolated from *Crotalus durissus terrificus* venom and the cDNA was cloned (Leduc & Bon, 1998). CVX is considered to be a trimer $(\alpha\beta)_3$ of C-type lectin-like heterodimers, although there is no structural evidence to support this and the organization of the subunits is not known. Flavocetin-A (FL-A), a platelet GPIb-binding protein antagonist, was isolated from the venom of habu snake *Trimeresurus flavoviridis* and characterized (Taniuchi *et al.*, 1995). FL-A functions as a competitive blocker of vWF-binding in platelet aggregation. Among the C-type lectin-like proteins found so far in snake venoms, FL-A is the highest molecular mass oligomer (149 kDa) composed of heterodimers consisting of C-type lectin-like α (16.5 kDa) and β (13.7 kDa) subunits. FL-A is known to bind to human platelets with high affinity ($K_d = 0.35 \pm 0.13$ nM), at 21 500 ± 1760 binding sites per platelet (approximately 25 000 copies of GPIb per platelet), compared with the low molecular mass proteins, and to inhibit the vWF-mediated platelet aggregation (Taniuchi *et al.*, 1995). It is likely that FL-A interacts with a region of the GPIb α-subunit at or in close proximity to the vWF-binding site.

Table 1
Summary of data-collection statistics for the native FL-A crystal.

Resolution range (Å)	60–2.5 (2.61–2.5)
Number of unique reflections	15040 (1631)
Completeness (%)	94.3 (82.2)
Multiplicity	3.3 (1.9)
$I/\sigma(I)$	16.9 (3.2)
$I > 3\sigma(I)$ (%)	78.4 (37.3)
$R_{\rm merge}$†	0.067 (0.30)

† $R_{\rm merge} = \sum\sum |I(h)j - \langle I(h)\rangle| / \sum\sum I(h)j$, where $I(h)$ is the measured diffraction intensity and the summation includes all observations.

This function must be closely related to the structure of FL-A. However, the three-dimensional structures of the GPIb-binding proteins are not known. Moreover, it even remains unclear what the exact oligomeric number of FL-A is. Is it a tetramer, pentamer or hexamer? Is polymerization and organization of the subunits essential for the high affinity for the GPIb α subunit? To answer these questions, we have studied the molecular structure of FL-A by the X-ray diffraction method. This is the first platelet glycoprotein-binding protein to be studied at the structural level.

2. Methods and results

FL-A was isolated and purified from the venom of *T. flavoviridis* using a modified version of a method described previously (Taniuchi *et al.*, 1995). The crude venom was dissolved in buffer *A* (50 m*M* Tris–HCl pH 8.0, 0.1 *M* NaCl) and applied to a Sephacryl S-200 HR gel-filtration column (Pharmacia). Subsequently, the eluent was chromatographed using Q- and S-Sepharose Fast Flow ion-exchange columns (both from Pharmacia). The purified protein was kept in buffer *A* at 277 K. For crystallization, the protein was concentrated to 10–40 mg ml^{-1} with a Millipore apparatus (Ultrafree-MC 10000 NMWL filter unit). Protein concentrations were estimated spectroscopically by absorbance at 280 nm, assuming an A_{280} of 1.0 for a 1.0 mg ml^{-1} solution. Crystallization trials were employed with the hanging-drop vapour-diffusion technique at 293 K. Reservoir solutions were initially screened using the sparse-matrix sampling reagents of Jancarik & Kim (1991) from Hampton Research (Crystal Screen I). Typically, droplets of 4 µl were prepared on siliconized coverglasses by mixing 2 µl of protein solution and an equal volume of the reservoir solution and were vapour-equilibrated against 1 ml reservoir solution.

Crystals initially grew as small prisms of longest dimensions 0.1 mm within a week under condition #24 of the Crystal Screen I kit (2-propanol was used as a precipitant). Further refinement to obtain fewer larger crystals in a droplet was undertaken using ethanol, methanol, acetone, dimethyl sulfoxide and 2-methyl-2,4-pentanediol (MPD) as well as 2-propanol as precipitants within the pH range 4.0–9.0 in the presence of various additives. The best crystals were obtained after three weeks in a reservoir solution consisting of 8.5%(*v*/*v*) MPD in 0.1 *M* sodium acetate buffer (pH 4.6) containing 10 m*M* $CaCl_2$ as an additive. The protein concentration was 20 mg ml^{-1}. The crystals grew to 0.6 mm in the longest dimension. Fig. 1 shows the typical crystal form of FL-A.

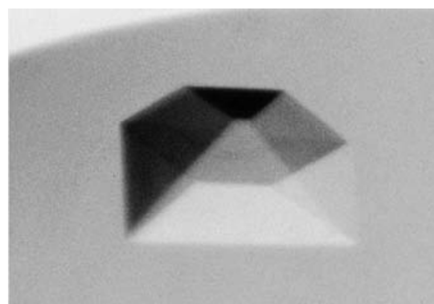

Figure 1
Crystal of flavocetin-A from the venom of *T. flavoviridis*. The crystals grow as prisms of maximum dimensions 0.3 × 0.6 × 0.6 mm.

Crystals were mounted in thin-walled glass capillaries with a small volume of reservoir solution. X-ray diffraction measurements were performed at 293 K at the BL-6B beamline of the Photon Factory at the High Energy Accelerator Research Organization, Tsukuba Science City, Japan. The wavelength used was 1.00 Å. The incident beam, focused by double bent mirrors, was collimated to 0.1 mm in diameter. Intensity data were collected on 400 × 800 mm imaging plates (Fuji Film) using the Weissenberg camera for macromolecules with a radius of 573 mm (Sakabe, 1991) and the oscillation method with 6° rotation per frame. The crystals diffracted at least to 2.4 Å resolution. These diffraction data were digitized with a Rigaku image analyzer. The digital data were then processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

A complete native data set was collected to 2.5 Å resolution using a single crystal of FL-A. Table 1 shows a summary of the X-ray diffraction data statistics. The autoindexing procedure using *DENZO* indicated that the crystals belong to the tetragonal crystal system with unit-cell dimensions $a = b = 121.0$, c = 63.2 Å, and the systematic absence rule showed that the space group is $I4$. The final data set had a merging R factor of 0.067. The completeness of the data (15040 unique reflections) was 94.3% (60–2.5 Å resolution) and 82.2% in the outermost resolution shell (2.61–2.5 Å). We estimated the number of molecules or heterodimers in the asymmetric unit (Matthews, 1968). Assuming one or two heterodimer(s), the V_m value is calculated to be 3.7 or 1.9 Å^3 Da^{-1}, respectively.

Recent sequence studies of FL-A (Shin *et al.*, 1999) have revealed that the α and β subunits have high sequence identity to subunits *A* and *B* of a heterodimeric protein, coagulation factors IX/X-binding protein (IX/X-bp) from *T. flavoviridis* venom (Atoda *et al.*, 1991; Atoda & Morita, 1993), having 58 and 52% identity, respectively. This indicates that the three-dimensional structure of the heterodimer of FL-A is very similar to that of IX/X-bp, whose crystal structure was determined at 2.5 Å resolution (Mizuno *et al.*, 1997). Therefore, we attempted to solve the crystal structure of FL-A by molecular-replacement methods using the program *AMoRe* (Navaza, 1994). The search model was a heterodimer constructed with the program *HOMOLOGY* in the package *InsightII* (Molecular Simulation Inc.) using the sequence of FL-A and the atomic coordinates of IX/X-bp (PDB code 1ixx). The N-terminal residues 1–2 and the C-terminal residues 132–135 of the α-subunit and the corresponding residues 1–3 and 123–125 of the β-subunit were then removed. A rotation search with a sphere radius of 30 Å and an angular step size of 2.5° was carried out in the resolution range 20–3.5 Å and led to a single significant peak. Subsequent translation-function searches showed ten peaks above 18.9% of the correlation coefficient. After 30 cycles of rigid-body refinement in the resolution range 20–3.5 Å, a unique solution was obtained with a correlation coefficient of 31.7 and an R factor of 51.1%. A $2F_o - F_c$ map computed after rigid-body refinement showed that the β-subunit was correctly modelled, but the α-subunit was required to rotate by approximately 10° relative to the β-subunit around the linker region to fit the electron density. This suggests a domain movement. Similar observations have been reported recently in the comparison between the structures of IX/X-bp and factor IX-binding protein (Mizuno *et al.*, 1999). The model was then adjusted manually, yielding an R factor of 46.5% after rigid-body refinement. The

molecular packing in the tetragonal space group *I*4 using the graphical software *QUANTA* (Molecular Simulation Inc.) showed that four heterodimers are related by a crystallographic fourfold symmetry parallel to the *c* axis, and that the heterodimer comfortably contacts with adjacent subunits. This molecular model forms a cyclic tetramer $(\alpha\beta)_4$ containing a large internal solvent channel along the fourfold axis. This is consistent with the relatively large solvent content of 67% in the crystal, as calculated from the V_m of 3.7 $\text{Å}^3\,\text{Da}^{-1}$. Further steps of model building and refinement are in progress.

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