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Purification, characterization, crystallization and preliminary X-ray crystallographic analysis of two novel C-type lectin-like proteins: Aall-A and Aall-B from *Deinagkistrodon acutus* venom

Gufeng Xu,[a,b] Maikun Teng,[a,b] Liwen Niu,[a,b]* Peng Liu,[c] Yuhui Dong,[c] Qun Liu,[d] Qingqiu Huang[d] and Quan Hao[d]

[a]Key Laboratory of Structural Biology, Chinese Academy of Sciences, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, People's Republic of China, [b]Departments of Molecular and Cell Biology, School of Life Sciences, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, People's Republic of China, [c]Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Beijing 100039, People's Republic of China, and [d]MacCHESS, Cornell High Energy Synchrotron Source, Cornell University, Ithaca, NY 14853, USA

Correspondence e-mail: lwniu@ustc.edu.cn

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Aall-A and Aall-B, two novel heterodimeric snake-venom C-type lectin-like proteins (sv-CLPs), were purified from the venom of *Deinagkistrodon acutus* from Anhui, China. Strikingly, both these proteins can localize on and congregate human erythrocytes, instead of aiming at the common targets of sv-CLPs such as platelet glycoproteins, von Willebrand factors, coagulant factors *etc.* The crystals of Aall-A belong to space group $P2$, with unit-cell parameters a = 105.2, b = 56.2, c = 108.7 Å, β = 100.5°, and diffract to 2.0 Å resolution, while the crystals of Aall-B belong to space group $P2_12_12_1$, with unit-cell parameters a = 36.8, b = 56.5, c = 149.2 Å, and diffract to 2.2 Å resolution. To our knowledge, this is the first report of sv-CLPs with this unique function and of their preliminary crystallographic analysis.

1. Introduction

Members of the superfamily of snake-venom Ca^{2+}-dependent (C-type) saccharide-specific lectins and their homologues, C-type lectin-like proteins (sv-CLPs), constitute one of the few primary components of Viperidae and Crotalidae venoms.

The members of the snake-venom C-type lectin superfamily that cannot bind saccharides are named C-type lectin-like proteins. They are normally devoid of enzymatic activity and alternatively bind targets that play essential roles in thrombosis and haemostasis. These important targets include coagulation factor IX/X (Atoda & Morita, 1989; Sekiya *et al.*, 1993; Chen & Tsai, 1996; Xu *et al.*, 2000), platelet membrane glycoproteins, such as GpIb, GpVI and $\alpha_2\beta_1$-integrin (Peng *et al.*, 1991; Taniuchi *et al.*, 1995; Kawasaki *et al.*, 1995), von Willebrand factor (vWF; Andrews *et al.*, 1989; Hamako *et al.*, 1996) and α-thrombin (Zingali *et al.*, 1993; Castro *et al.*, 1998). The crystal structures of many sv-CLPs have been reported (Mizuno *et al.*, 1997, 1999, 2001; Fukuda *et al.*, 2000, 2002; Sen *et al.*, 2001; Hirotsu *et al.*, 2001; Murakami *et al.*, 2003; Horii *et al.*, 2003; Jasti *et al.*, 2004; Huang *et al.*, 2004) and recently the crystal structure of the galactose-specific C-type lectin RSL from the rattlesnake *Crotalus atrox* has also been reported (Walker *et al.*, 2004). All these studies show that the structures of this superfamily are exceedingly worthy of attention. Moreover, new proteins of the sv-CLP superfamily are of great interest, especially if they possess unique features and constitute a new type.

Here, we report the crystallization of two novel sv-CLPs, Aall-A and Aall-B from *Deinagkistrodon acutus* venom, which target human erythrocytes. Details of their purification, characterization, crystallization and preliminary crystallographic analysis are provided below.

2. Methods and results

2.1. Purification of Aall-A and Aall-B

1 g of crude venom from *D. acutus* (purchased from Huangshan Institute of Snakes, Anhui, China) was dissolved in 20 ml 20 m*M* Tris–HCl buffer pH 8.0 and centrifuged at 12 000 rev min^{-1} at 277 K for 15 min. The supernatant was applied onto a DEAE-Sepharose Fast Flow column (Amersham Pharmacia, Sweden) pre-equilibrated with the same buffer. A linear gradient of 0–0.2 *M* NaCl was utilized for initial isolation of the crude venom. A CM-Sepharose column (Amersham Pharmacia, Sweden) pre-equilibrated with 50 m*M* sodium citrate buffer pH 5.0 was then used with a linear gradient of 0–0.2 *M* NaCl for further isolation. Final purification of Aall-A and Aall-B was performed using Mono Q (Amersham Pharmacia, Sweden) with a non-linear pH gradient from 20 m*M* Tris–HCl pH 8.0 buffer to 20 m*M* acetic acid/sodium acetate buffer pH 5.6.

2.2. Protein characterization

2.2.1. Molecular weights. The molecular weights of Aall-A and Aall-B and their subunits were first determined by 15% SDS–PAGE without and with reducing agent. Both proteins showed a band of 27 kDa and a band of approximately 15 kDa under non-reducing and reducing conditions, respectively, indicating that Aall-A and Aall-B are both

disulfide-linked dimeric proteins. For more accurate determination of the molecular weights of Aall-A and Aall-B we used MALDI–TOF MS, which gave values of 30 266 Da for Aall-A and 30 305 Da for Aall-B.

2.2.2. N-terminal sequencing. Aall-A and Aall-B were reduced and *S*-pyridylethylated according to the method of Atoda *et al.* (1991) and N-terminal sequencing was performed using a Procise 491 protein sequencer (Applied Biosystems, USA). N-terminal sequence analysis of one of the subunits of Aall-A and Aall-B resulted in the same sequence, GSCCP PGWST YDQYC YQGFN, revealing homology with other sv-CLPs. Several attempts using the other subunit of approximately the same weight failed with both Aall-A and Aall-B.

2.2.3. Erythrocyte-function assay. Human blood of all four types (O^+, AB^-, A^+, B^+) was defibrinogenated, washed and then suspended as a 2% solution. 0.1 ml of this suspension was placed on a silicone oil-treated cover slip (2.2 × 2.2 cm) and mixed with 10 µl of sample solution (5 µg µl^{-1}) at room temperature. The results were observed with the naked eye and it was observed that both Aall-A and Aall-B can congregate human erythrocytes of all four types (O^+, AB^-, A^+, B^+).

Purified Aall-A and Aall-B were dialyzed against PBS overnight and then linked with NHS-Rhodamin (Pierce, Ireland) according to the standard protocol from the manufacturer. A 1:1000 dilution of washed human erythrocytes was fixed with paraformaldehyde in PBS at 277 K for 1 h and then blocked with 5% milk in PBS for 30 min. After incubation with 1:100 diluted Rhodamin-treated proteins for another 30 min, the erythrocyte samples were examined on an Axiovert 200 microscope and it was observed that both Aall-A and Aall-B can localize on the human erythrocyte surface (Fig. 1).

2.3. Crystallization

Lyophilized Aall-A and Aall-B dissolved in distilled water at a concentration of 15 mg ml^{-1} were screened using the typical hanging-drop vapour-diffusion method at room temperature using Crystal Screens I and II (Hampton Research, USA). Briefly, 1.0 µl protein solution (10 mg ml^{-1}) was mixed with 1.0 µl of the screening agent and equilibrated against 400 µl reservoir solution at room temperature. Small or needle-shaped crystals of Aall-A and Aall-B appeared after one week under a wide range of conditions (I-10, I-15, I-28, I-41, II-26 and II-38 for Aall-A, and I-10, I-18, I-28, I-37, I-46, II-4, II-14, II-26, II-30, II-37 and II-38 for Aall-B). Condition 26 of Crystal Screen II [30%(*w*/*v*) PEG MME 5000, 100 m*M* MES pH 6.5, 200 m*M* $(NH_4)_2SO_4$] was carefully refined using varying reservoir solution and additive solution concentrations with a linear concentration gradient of Aall-A from 10 to 20% and the best single crystals of Aall-A (0.3 × 0.2 × 0.05 mm) were finally obtained using 12.0 mg ml^{-1} protein mixed with 14.5%(*w*/*v*) PEG MME 5000, 100 m*M* MES pH 6.5, 80 m*M* $(NH_4)_2SO_4$, 0.2% NaN_3 at 293 K after three weeks (Fig. 2*a*). Similarly, we obtained the best single crystals of Aall-B (0.15 × 0.08 × 0.03 mm) using 10.0 mg ml^{-1} protein mixed with 12.0%(*w*/*v*) PEG MME 5000, 100 m*M* MES pH 6.5, 0.2% NaN_3 at 293 K after one week (Fig. 2*b*).

2.4. Data collection

Diffraction data from crystals of Aall-A were collected at MacCHESS beamline F1, Cornell University, Ithaca, USA (λ = 0.9160 Å). Aall-A crystals were mounted in loops after stepwise soaking in crystallization solution containing an additional 30%(*v*/*v*) glycerol. The crystals were frozen in a stream of liquid nitrogen at 100 K. Data sets were collected using a MAR345 IP detector in 1.0° oscillation steps over a 180° range and were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

Diffraction data from crystals of Aall-B were collected at beamline 3wla, Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, People's Republic of China (λ = 0.8883 Å). Aall-B crystals were mounted in thin-walled Lindemann glass capillaries. Data sets were collected using a MAR345 IP detector in 1.0° oscillation steps over a 100° range at 289 K and were processed using *marFLM*5.0 (Bartels & Klein, 2000).

Data-collection statistics for Aall-A and Aall-B are shown in Table 1.

2.5. Preliminary X-ray crystallographic analysis

The crystals of Aall-A belong to the monoclinic space group *P*2 and diffract to 2.0 Å resolution. It is most probable that four or five Aall-A molecules are located in the asymmetric unit, with a V_M value of 2.61 or 2.09 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 53 or 41%, respectively. The crystals of Aall-B belong to the orthorhombic space group $P2_12_12_1$ and diffract to 2.2 Å resolution. We assume that only one Aall-B molecule is located in the asymmetric unit and the V_M

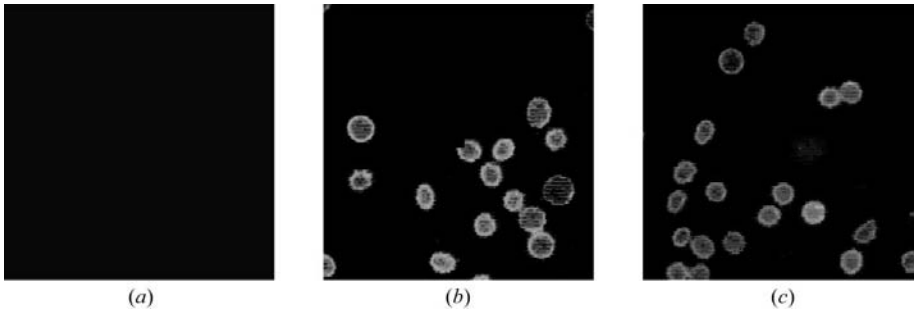

Figure 1
Aall-A and Aall-B localize on the human erythrocyte surfaces. 0.4% paraformaldehyde-fixed human erythrocytes directly stained with Rhodamin-linked BSA (*a*), Aall-A (*b*) and Aall-B (*c*). Samples were examined on a ZEISS Axiovert 200 microscope and pictures were collected with *Axiovision* software.

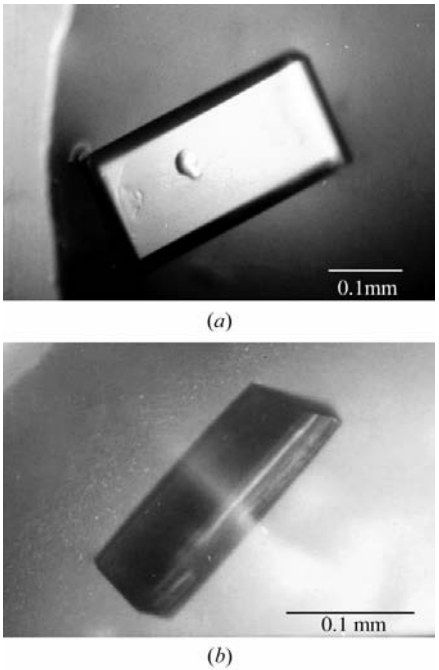

Figure 2
Crystals of Aall-A (*a*) and Aall-B (*b*).

Table 1
Statistics of diffraction data collection.

Values in parentheses are for the highest resolution shell.

	Aall-A	Aall-B
Space group	$P2$	$P2_12_12_1$
Unit-cell parameters		
a (Å)	105.22	36.79
b (Å)	56.21	56.48
c (Å)	108.73	149.21
β (°)	100.46	
No. observations	227104	71273
No. independent reflections	76473	16853
Resolution limits (Å)	30.00–2.01 (2.05–2.01)	26.44–2.18 (2.25–2.18)
$\langle I/\sigma(I)\rangle$	40.2 (10.3)	6.8 (2.3)
$R_{\rm merge}$† (%)	4.1 (16.6)	6.2 (32.8)
Completeness‡ (%)	90.8 (81.2)	99.2 (95.2)

† $R_{\rm merge} = \sum_h \sum_j |\langle I(h)\rangle - I(h)_j| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the jth observed reflection intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h. ‡ The completeness is the ratio of number of observed reflections to the number of possible reflections.

value is 2.56 Å^3 Da^{-1}, which corresponds to a solvent content of approximately 52%.

The phase problem of Aall-B was solved by molecular replacement using *AMoRe* (Navaza, 1994). After eliminating all water molecules, the structure of flavocetin-A (PDB code 1c3a) was chosen as the search model. A rotation search was performed with a radius of 30 Å and an angular step size of 2.5° within the resolution range 20–3.5 Å. Although the correlation coefficient (17.9%) and R factor (51.3%) of the initial solution are outside the expected range, it is indeed a correct solution, as the values for the next highest solution are far from these. Further model building was performed with the program *O* (Jones *et al.*, 1991) and after several steps of simulated-annealing and positional refinement as incorporated in the program *CNS* v.1.1 (Brünger *et al.*, 1998), the R factor and $R_{\rm free}$ (10% of reflections) dropped to 28% and 34%, respectively.

3. Discussion

N-terminal amino-acid sequence analysis and comparison with the sequences of other sv-CLPs show that Aall-A and Aall-B from *D. acutus* venom belong to the sv-CLP superfamily. Also, preliminary refinement analysis confirmed the structural homology between Aall-B and flavocetin-A. Since both Aall-A and Aall-B possess a novel erythrocyte-targeting function instead of the traditional sv-CLPs function of affecting the coagulant cascade or platelets, they must constitute a new type for this superfamily. However, the erythrocyte-targeting function of Aall-A and Aall-B differs from that of the saccharide-specific lectins found in snake venoms (data not shown), which can cross-link receptors and agglutinate erythrocytes directly (Hirabayashi *et al.*, 1991).

As they show a new type of sv-CLP function that has never been reported previously, the structures of Aall-A and Aall-B should be helpful for the explanation of their unique functions as well as for the expansion of our structural knowledge of the whole superfamily; their structures should also provide new insight into investigation of the fundamental strategy of how sv-CLPs have evolved.

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