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Purification, crystallization and preliminary X-ray crystallographic analysis of agkaggregin, a C-type lectin-like protein from *Agkistrodon acutus* venom

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A snake-venom C-type lectin-like protein, agkaggregin, has been isolated from *Agkistrodon acutus* venom. Agkaggregin has an apparent molecular mass of about 28 kDa and consists of two different types of subunits, an α-subunit (~15 kDa) and a β-subunit (~14 kDa). Agkaggregin has the ability to induce platelet aggregation at concentrations of the order of nanomoles. The agkaggregin crystals grew for nearly a year by hanging-drop vapour diffusion and belong to the *I*222 space group, with unit-cell parameters a = 64.75, b = 74.21, c = 133.24 Å. One asymmetric unit contains one $\alpha\beta$ heterodimer, corresponding to a volume-to-mass ratio of 2.795 Å^3 Da^{-1}. Agkaggregin may exist in two association forms: an $\alpha\beta$ heterodimer and a dimer of $\alpha\beta$ heterodimers that associates during the long process of crystallization.

1. Introduction

The snake-venom C-type lectin-like proteins, members of the C-type lectin superfamily (Drickamer, 1999), are normally devoid of enzymatic activity and bind proteins that play essential roles in thrombosis and haemostasis. They are widely used to investigate the physiological functions of the target proteins involved, to detect the interactions with their targets and the subsequent effects of these interactions such as anticoagulation and platelet activity. These can be illustrated by experimental results from two examples: botrocetin (a vWF-binding protein from *Bothrops jararaca*; Andrews *et al.*, 1989; Sugimoto *et al.*, 1991; Dong *et al.*, 2001) and convulxin (a platelet membrane glycoprotein VI binding protein from *Crotalus durissus terrificus*; Polgar *et al.*, 1997; Jandrot-Perrus *et al.*, 1997; Andrews & Berdt, 2000).

Four snake-venom C-type lectin-like protein structures and one protein–substrate complex structure have been determined by X-ray diffraction analysis: habu factor IX/X binding protein and habu factor IX binding protein isolated from *Trimeresurus flavoviridis* (Mizuno *et al.*, 1997, 1999), flavocetin-A from *T. albolabris* (Fukuda *et al.*, 2000), botrocetin from *B. jararaca* (Sen *et al.*, 2001) and the complex of factor X binding protein from *A. acutus* with the Gla domain of factor X (Mizuno *et al.*, 2001). These structures are helpful in understanding the interactions between various snake-venom C-type lectin-like proteins and their targets (whole forms or architectural features for specific binding). For example, there are many hydrophilic, hydrophobic and water-molecule-mediated hydrogen-bonding interactions widely involved in the binding of factor X binding protein to its target protein according to the crystal structure of the complex (Mizuno *et al.*, 2001); the crystal structure of flavocetin-A shows a cyclic tetradimer which could account for the binding to its target protein GP Ibα with high affinity (Fukuda *et al.*, 2000).

The C-type lectin-like proteins from *A. acutus* venom have been studied in several laboratories. For example, there are publications for the purification and sequencing of a factor X binding protein (Atoda *et al.*, 1998), the functional characterization and sequencing of agkicetin, a platelet GP Ib-binding protein (Chen & Tsai, 1995), and the partial N-terminus sequencing and physiological characterization of factor X-inhibitor (Cox, 1993). All of those reported proteins associate into the form of $\alpha\beta$ heterodimers, although they possess specific targeting properties. On the other hand, the activities of subunits in some snake-venom C-type lectin-like proteins have been detected. Unlike native convulxin, reduced convulxin does not activate platelets and only affects collagen-induced platelet aggregation (Polgar *et al.*, 1997). Similar effects appear in the reduced form of the subunit of agkicetin and the reduced and modified forms of the β-subunit of jararaca GP Ib binding protein; they have mostly lost the biological functions of the native forms (Chen & Tsai, 1995; Kawasaki *et al.*, 1996). However, the reduced and modified forms of the β-subunit of echicetin retains about 25% of the original inhibitory activity towards platelet agglutination (Peng *et al.*, 1994). Some snake-venom C-type lectin-like proteins and their subunits

can maintain normal three-dimensional structures with reducing agents of certain concentrations. These implications are very useful for the work reported here. In order to partly overcome the difficulties in protein crystallization, the samples for crystallization have been pretreated with a reducing agent. The purpose of this paper is to describe studies on agkaggregin, a C-type lectin-like protein from *A. acutus* venom that appears to possess an ability to induce human platelet aggregation, including its chromatographic purification with a reducing agent, crystallization and preliminary X-ray diffraction analysis.

2. Materials and methods

2.1. Materials

The dried crude venom of *A. acutus* was obtained from the southern mountains of Anhui Province, China. DEAE-Sepharose and CM-Sepharose were obtained from Pharmacia (Uppsala, Sweden). The series of PEG MMEs (molecular-weight ranges from 2000 to 20 000 Da) were purchased from Fluka Co. (Switzerland). Standard proteins for the estimation of molecular weight were produced by Shanghai Institute of Biochemical Technology Co. (Shanghai, China). Other reagents and chemicals were of analytical grade from commercial sources.

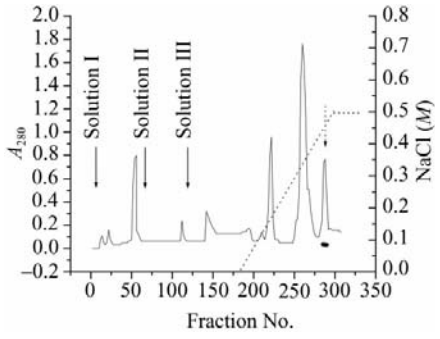

Figure 1
CM-Sepharose column chromatography with β-mercaptoethanol (a reducing agent) of agkaggregin. The protein fraction containing agkaggregin was desalted and ultrafiltered to a volume of 20 ml and then applied to a CM-Sepharose column (1.4 × 40 cm) pre-equilibrated with 20 m*M* sodium citrate pH 5.0 at a flow rate of 120 ml h^{-1}. The column was sequentially eluted with 200 ml solution I (20 m*M* sodium citrate pH 5.0), 500 ml solution II (20 m*M* sodium citrate solution pH 5.6) and 300 ml solution III (20 m*M* sodium citrate pH 5.6 containing 10 m*M* β-mercaptoethanol). The column was further washed with 800 ml of NaCl solution with a linear gradient from 0 to 0.5 *M*. The elution was monitored at 280 nm. The absorbance at 280 nm and the elution of the salt gradient are shown by a solid line and a dashed line, respectively. The last effluent peak, indicated by a solid bar, contains the protein agkaggregin.

2.2. Estimation of molecular weights

Conventional SDS–PAGE was carried out to estimate the molecular weight using 15% polyacrylamide gel concentration. The samples were pretreated in 2.5%(*w*/*v*) SDS with 5%(*v*/*v*) β-mercaptoethanol (reducing conditions) or without β-mercaptoethanol (non-reducing conditions) according to the experimental requirements.

2.3. Assays for platelet aggregation

Healthy human blood was obtained from voluntary donors and extracted into plastic tubes containing 3.8%(*w*/*v*) sodium citrate in a volume ratio of 1:10 (anticoagulant: sample). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation at 100*g* for 8 min and at 1000*g* for 15 min, respectively. According to the method of the manufacturer, the assays for platelet aggregation were performed with PRP samples using a TYXN-961 aggregometer made in Shanghai, China.

2.4. Preparation of samples for characterization and crystallization

The isolation of the agkaggregin samples for crystallization and characterization was carried out by two-step ion-exchange chromatography. Firstly, crude venom (1.5 g) was dissolved in 50 ml solution containing 20 m*M* Tris–HCl pH 8.2 at 277 K for 2 h. The insoluble materials were removed by centrifugation (3000*g*) at 277 K for 20 min. The supernatant was applied to a DEAE-Sepharose column (1.6 × 40 cm) pre-equilibrated with the buffer mentioned above. The effluent was monitored at 280 nm and adjusted to a flow rate of 134 ml h^{-1}. The column was eluted with a linear gradient (made up of a mixture of 400 ml pre-equilibrate solution and 400 ml solution containing 0.25 *M* NaCl buffered with 20 m*M* Tris–HCl pH 8.2) and sequentially eluted with a solution of 0.5 *M* NaCl. The effluent was collected using the rate of one tube per 3 min. The chromatographic peak containing agkaggregin was pooled. Secondly, a CM-Sepharose column with β-mercaptoethanol (a reducing agent) (see Fig. 1) was used for further purification of the protein fraction containing agkaggregin from the first chromatography. The purified samples were desalted and ultrafiltered to a concentration of 10 mg ml^{-1} for crystallization and characterization.

2.5. Crystallization

Using the method of hanging-drop vapour diffusion at room temperature (about 293 K), crystals of agkaggregin suitable for X-ray diffraction could be harvested from mother-liquor solution (5 mg ml^{-1} protein concentration containing 10% Li_2SO_4) equilibrated against a reservoir solution consisting of 20% Li_2SO_4. No reducing agent was included in the crystallization experiment. After much patience, crystals with sizes of 0.6 × 0.2 × 0.05 mm (see Fig. 2) appeared nearly a year later.

Table 1
Data collection and processing statistics from an agkaggregin crystal.

Values in parentheses are for the highest resolution shell (2.85–2.80 Å).

Space group†	*I*222
Unit-cell parameters (Å)	a = 64.75, b = 74.21, c = 133.24
Resolution range (Å)	30–2.8
Observations	15329
Independent reflections	7655
R_{merge}‡	0.117 (0.324)
Completeness§ (%)	93.3 (80.0)

† The correct space group was confirmed by the method of molecular replacement and preliminary structural refinement. ‡ $R_{merge} = \sum_h \sum_j |I(h)_j - \langle I(h)\rangle| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the observed intensity of the jth reflection and $\langle I(h)\rangle$ is the mean intensity of reflection h. § Completeness to the number of possible reflections.

2.6. X-ray diffraction data collection and preliminary analysis

X-ray diffraction data collection from one crystal of agkaggregin was performed at room temperature using a MAR Research image-plate system (diameter 300 mm) mounted on an X-ray generator with a graphite monochromator and sealed copper-target tube at our laboratory. The working tube voltage and current were 40 kV and 50 mA, respectively. A total of 120 imaging frames were recorded at a 200 mm crystal-to-detector distance with a 1° oscillation angle and 300 s exposure time per frame (data not shown). The diffraction data was

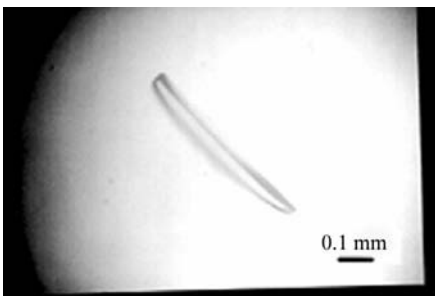

Figure 2
Photomicrograph of a single crystal of agkaggregin formed in the solution containing Li_2SO_4 (maximum dimensions of 0.6 × 0.2 × 0.05 mm).

processed using the software packages *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The final statistics for data collection and processing are summarized in Table 1.

The phasing problem was solved by the method of molecular replacement using the program *AMoRe* (Navaza, 1994). The structural model of homologous flavocetin-A (PDB code 1c3a) was chosen as the search model without any substitution of amino-acid residues, partly because the complete sequence of agkaggregin has not yet been determined precisely. The rotation search was performed using a sphere radius of 30 Å and an angular step size of 2.5° within the resolution range 20–3.5 Å. A translation search and rigid-body refinement were performedand gave a unique solution with a correlation coefficient of 32.2% and an *R* factor of 48.0%. After several cycles of preliminary refinement using the program package *X-PLOR* (Brünger, 1992), the *R* factor and free *R* factor fell to 28 and 37%, respectively.

3. Results and discussion

A snake-venom protein, agkaggregin, has been purified from *A. acutus* venom using two-step chromatography. For crystallization, the reducing agent β-mercaptoethanol was added in the last step of chromatography (Fig. 1). Agkaggregin is a member of the family of snake-venom C-type lectin-like proteins, as shown by the crystallographic results of molecular replacement and preliminary refinements, as the three-dimensional structure of agkaggregin is similar to that of the homologous flavocetin-A. Agkaggregin has an apparent mass of about 28 kDa and consists of two different types of subunits, an α-subunit (~15 kDa) and a β-subunit (~14 kDa) as estimated by SDS–PAGE (Fig. 3), and possesses an ability to induce platelet aggregation at concentrations of the order of nanomoles (Fig. 4), despite being isolated using a reducing agent in the course of preparation. All of these properties are similar to those of other snake-venom C-type lectin-like proteins (Andrews & Berdt, 2000).

Protein crystallization is controlled by many factors. This paper gives an example of obtaining suitable crystals for X-ray diffraction analysis by pre-treating protein samples with a reducing agent. Our previous efforts mainly focused on the crystallization of an agkaggregin sample that was obtained without any reducing agent in the course of the preparation mentioned above; not only did the sample appear to have an apparent molecular mass of about 51 kDa containing two kinds of subunits (of about 14 kDa) estimated by SDS–PAGE under non-reducing conditions (data not shown), but the sample also often aggregated at low ionic concentration (*e.g.* less than 0.1 *M* NaCl). Unfortunately, suitable crystals for X-ray diffraction analysis could not be produced after many crystallization trials; the harvested crystals from such a sample were needle-shaped or highly mosaic (data not shown). In this paper, the pre-treatment of protein samples with reducing agent in the course of preparation has been demonstrated to be successful and might be a general approach to overcoming the obstacle(s) for crystallization. A similar experimental measure has also been reported in a recent publication (Habel *et al.*, 2001), in which the reducing agent DTT has been utilized to pre-treat the protein samples in order to shorten the trial time. Our group has made use of the reducing agent β-mercaptoethanol to decrease the mosaicity of the crystals. As both agkaggregin and the protein crystallized by Habel and coworkers comprise two different kinds of subunits associating into a form of polymer, it may in some cases be effective for the crystallization of polymeric proteins to treat the samples with reducing agent according to the trial goal.

The result of the SDS–PAGE (Fig. 5) is of interest. The sample for crystallization has the association form of an $\alpha\beta$ heterodimer. However, the crystalline protein associates into another form with an apparent molecular mass of about 52 kDa, appearing to be a dimer of $\alpha\beta$ heterodimers. It is very possible that certain reactions have occurred during the long crystallization process. The crystal possesses a reasonable volume-to-mass ratio of 2.795 $Å^3\ Da^{-1}$ based on the molecular mass of 28 kDa (Matthews, 1968), corresponding to one $\alpha\beta$ heterodimer per asymmetric unit; therefore, the postulated reactions might have happened between two adjacent $\alpha\beta$ heterodimers during crystallization. It is not clear to us why the crystals of agkaggregin took so long to grow and how the inclusion of reducing agent in the course of preparation might have affected the crystallization. It is worth pointing out that most snake-venom C-type lectin-like proteins associate into a $\alpha\beta$ heterodimer form consisting of homologous α- and β-subunits. All of the sequenced heterodimeric proteins contain one interchain disulfide bond and three intrachain disulfide bonds in each subunit. Besides the basic form of the $\alpha\beta$ heterodimer, other association forms have also been found in snake-venom C-type lectin-like proteins. Apart from alboaggregin-A, a platelet GP Ibα binding protein from *T. albolabris* (Kowalska *et al.*, 1998) consisting of four different subunits (α, β, γ and δ), flavocetin-A (a platelet GP Ibα binding protein from *T. flavoviridis*) and convulxin (a platelet GP

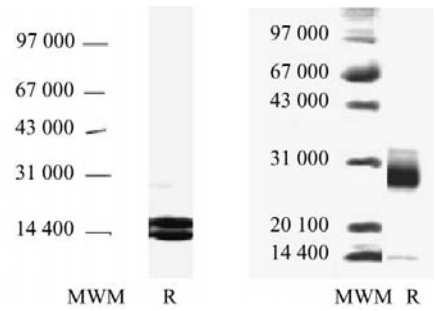

Figure 3
SDS–PAGE of agkaggregin under reducing conditions (left, R) and non-reducing conditions (right, NR) (with molecular-weight markers, MWM).

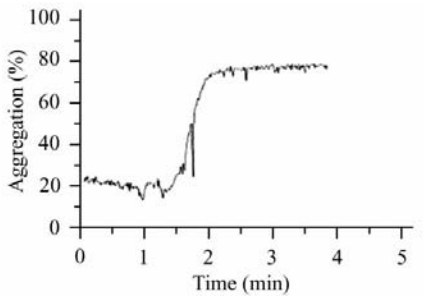

Figure 4
Human platelet aggregation induced by agkaggregin. 10 µl of agkaggregin solution was added to 200 µl of human platelet-rich plasma pre-incubated at 310 K for 3 min. The final concentration of agkaggregin was 5 µg ml^{-1} (179 n*M*).

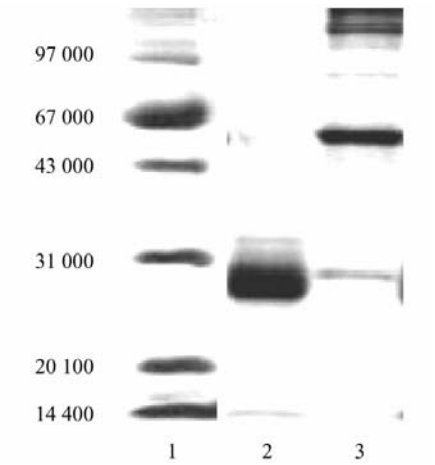

Figure 5
SDS–PAGE of agkaggregin before (lane 2) and after (lane 3) crystallization under non-reducing conditions (with molecular-weight markers, lane 1).

VI binding protein from *C. durissu terrificus*) could associate into a confirmed tetradimer $(\alpha\beta)_4$ and a postulated tridimer $(\alpha\beta)_3$ (Shin *et al.*, 2000; Fukuda *et al.*, 2000; Leduc & Bon, 1998). One of the primary structural features in flavocetin-A and convulxin is that there are eight cysteine residues in each subunit; an additional disulfide bridge can make further connections between the $\alpha\beta$ heterodimers containing seven ordinary disulfide bonds (Leduc & Bon, 1998; Shin *et al.*, 2000). Moreover, it has been confirmed by X-ray diffraction analysis that flavocetin-A is a cyclic tetradimer $(\alpha\beta)_4$ with an interchain disulfide bridge between the cysteine residues at the C-terminus of the α-subunit and those at the N-terminus of the β-subunit of the neighbouring $\alpha\beta$ heterodimer (Fukuda *et al.*, 2000). In agkaggregin crystals it is still unknown whether or not there is a similar situation. The sequencing of the amino-acid residues of agkaggregin and detailed structural refinement are being carried out and will provide useful information to understand those experimental results.

It has been found that the $\alpha\beta$ heterodimers isolated from different snake venoms normally possess different target proteins and therefore that these $\alpha\beta$ heterodimers can be grouped into four classes as follows: (i) factor X/IX binding proteins (Atoda & Morita, 1989; Atoda *et al.*, 1995; Sekiya *et al.*, 1993; Chen & Tsai, 1996), (ii) α-thrombin-binding protein/inhibitors (Zingali *et al.*, 1993; Castro *et al.*, 1998), (iii) vWF-binding proteins (Andrews *et al.*, 1989; Hamako *et al.*, 1996), and (iv) platelet GP binding proteins (Peng *et al.*, 1991; Taniuchi *et al.*, 1995; Andrews *et al.*, 1996; Kawasaki *et al.*, 1996; Fujimura *et al.*, 1995; Peng *et al.*, 1993; Sakurai *et al.*, 1998). For example, of the snake-venom C-type lectin-like proteins that possess the activity of inducing platelet aggregation, convulxin (Polgar *et al.*, 1997; Jandrot-Perrus *et al.*, 1997), 50 kDa alboaggregin (Andrews *et al.*, 1996) and alboaggregin-A (Peng *et al.*, 1992; Kowalska *et al.*, 1998) are confirmed to be GPIb-binding proteins, while botrocetin (Fujimura *et al.*, 1991; Sugimoto *et al.*, 1991) and bitiscetin (Hamako *et al.*, 1996) have the potential to bind vWF. It is still unclear why other proteins can induce platelet aggregation. Also, of the five snake-venom C-type lectin-like proteins whose crystal structures have been determined, botrocetin is the only protein with the ability to induce platelet aggregation. Unlike botrocetin, which exists in the form of a stable $\alpha\beta$ heterodimer (Sen *et al.*, 2001), agkaggregin has been shown in this paper to exist in two association forms, at least one of which possesses the ability to induce platelet aggregation. Therefore, determination of the agkaggregin structure will probably show the physical basis that accounts for the related biological functions.

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