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Purification, crystallization and preliminary X-ray diffraction analysis of an acidic phospholipase A₂ with vasoconstrictor activity from *Agkistrodon halys pallas* venom

Phospholipases A₂ (PLA₂s) are the major component of snake venoms and exert a variety of relevant toxic actions such as neurotoxicity and myotoxicity, amongst others. An acidic PLA₂, here named AhV_aPA, was purified from *Agkistrodon halys pallas* venom by means of a three-step chromatographic procedure. AhV_aPA migrated as a single band on SDS-PAGE gels, with a molecular weight of about 14 kDa. Like other acidic aPLA₂s, AhV_aPA has high enzymatic activity. Tension measurements of mouse thoracic aortic rings remarkably indicated that AhV_aPA could induce a further contractile response on the 60 mM K⁺-induced contraction, with an EC₅₀ of 369 nmol l⁻¹. Rod-shaped crystals were obtained by the hanging-drop vapour-diffusion method and diffracted to a resolution limit of 2.30 Å. The crystals belonged to space group *P*222, with unit-cell parameters *a* = 44.27, *b* = 68.39, *c* = 81.54 Å.

1. Introduction

Phospholipases A₂ (PLA₂s; EC 3.1.1.4) catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position, releasing lysophospholipids and fatty acids (Kini, 2003). The PLA₂ superfamily has been divided into 15 groups and many subgroups, and includes six distinct types of enzymes, namely the secreted PLA₂s (sPLA₂s), the cytosolic PLA₂s (cPLA₂s), the Ca²⁺-independent PLA₂s (iPLA₂s), the platelet-activating factor acetylhydrolases (PAF-AHs), lysosomal PLA₂ and a recently identified adipose-specific PLA₂ (Duncan *et al.*, 2008). Snake venoms are one of the most abundant sources of sPLA₂s (dos Santos *et al.*, 2011).

Snake-venom PLA₂s display a wide range of isoelectric points from acidic to highly basic. These isoforms differ significantly in catalytic properties and pharmacological activities. The acidic PLA₂s with an Asp residue at position 49 show the highest *in vitro* catalytic activity towards conventional substrates; they do not exhibit neurotoxic activity, but rather antiplatelet aggregation, antibacterial, pro-inflammatory and myotoxic activities (Fuly *et al.*, 2000; Fernández *et al.*, 2010; Nunes *et al.*, 2011; Saikia *et al.*, 2011; Teixeira *et al.*, 2011). Nevertheless, the basic isoforms appear to have acquired the highest toxicity, especially in the case of neurotoxic and myotoxic enzymes (Ponce-Soto *et al.*, 2009). In addition to the classic pharmacological activities, recent studies showed that atratoxin, an acidic PLA₂ from *Naja atra* venom, is able to inhibit A-type K⁺ currents in dissociated rat DRG neurons (Hu *et al.*, 2008).

In this work, we report the crystallization and preliminary X-ray diffraction analysis of AhV_aPA isolated from the venom of *Agkistrodon halys pallas*, which presents high catalytic activity towards egg-yolk phospholipids as the substrate. The present study shows that AhV_aPA also has a vasoconstrictor effect on mouse thoracic aortic rings. This indicates that AhV_aPA may have effects on the ion channels or receptors in the plasma membrane of vascular smooth-muscle cells.

2. Materials and methods

2.1. Purification

AhV_aPA was purified from lyophilized crude venom of *A. halys pallas* (Tunxi Snakebite Institute, Anhui, People's Republic of China)

using the following three-step chromatographic procedure at 287 K (Fig. 1). Lyophilized *A. halys pallas* venom (1.0 g) was dissolved in 25 ml buffer *A* (20 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 6.0) and centrifuged at 18 800g for 15 min to remove the insoluble portion. The supernatant was applied at a rate of 1 ml min^{-1} onto a CM-Sepharose Fast

Figure 1
The purification of AhV_aPA using a three-step chromatographic protocol. (a) CM-Sepharose cation-exchange chromatography. (b) DEAE-Sepharose anion-exchange chromatography. (c) Superdex 75 molecular-exclusion chromatography. The inset shows the SDS-PAGE gel image. Lane *P*, AhV_aPA; lane *M*, molecular-mass markers (labelled in kDa).

Table 1

Data-collection and reduction statistics.

Values in parentheses are for the highest resolution shell.

Space group	<i>P</i> 222
Unit-cell parameters (Å)	<i>a</i> = 44.27, <i>b</i> = 68.39, <i>c</i> = 81.54
Wavelength (Å)	0.91940
Resolution limits (Å)	50.0–2.30 (2.38–2.30)
No. of observed reflections	57523
Unique reflections	11621
Completeness (%)	98.1 (99.6)
$\langle I/\sigma(I) \rangle$	15.0 (2.6)
$R_{\text{merge}}^{\dagger}$ (%)	11.5 (49.1)
Multiplicity	5.0 (4.3)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of an individual reflection *hkl* and $\langle I(hkl) \rangle$ is the average intensity for this reflection.

Flow column (GE Healthcare) pre-equilibrated with the same buffer. After the unbound fractions had been washed out, a gradient of 0–0.4 M NaCl in buffer *B* (20 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ pH 8.0) was used. The unbound fractions in peak 1 were collected, dialyzed against buffer *C* (50 mM Tris–HCl pH 8.0) and applied onto a DEAE-Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with buffer *C*. The column was eluted at a flow rate of 1 ml min^{-1} using an NaCl gradient (0–0.4 M). The fractions corresponding to peak 4 were collected and purified on a Superdex 75 column (GE Healthcare) pre-equilibrated with buffer *D* (20 mM Tris–HCl pH 8.0, 200 mM NaCl) and eluted at a flow rate of 1 ml min^{-1} . Fractions containing AhV_aPA (peak 4) were collected and dialyzed against deionized water to remove salt. The purity of the protein was identified by SDS-PAGE. Protein concentration was determined using the BCA Protein Assay (Thermo Scientific).

2.2. Crystallization

AhV_aPA was concentrated to 25 mg ml^{-1} in buffer *E* (5 mM Tris pH 7.5, 50 mM NaCl). Crystallization experiments were performed at 287 K by the hanging-drop vapour-diffusion method with drops consisting of 1 μl protein solution and 1 μl reservoir solution, using Hampton Research crystallization screens to screen for initial crystallization conditions. Microcrystals were observed within one week using a precipitant solution consisting of 20% PEG 4000, 0.1 M Tris pH 8.0. Subsequent screening was performed by varying the pH and the PEG 4000 concentration. After optimization, crystals of high

Figure 2
Crystals of AhV_aPA.

quality were obtained using 18% PEG 4000, 0.1 M Tris pH 8.0 and were used for data collection (Fig. 2).

2.3. X-ray diffraction analysis

All diffraction data were obtained from a crystal cryoprotected in 18% PEG 4000, 0.1 M Tris pH 8.0, 25% (v/v) glycerol. The crystal was directly flash-cooled in a stream of cold nitrogen gas at 100 K. A set of diffraction data was collected at 100 K on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF) and was subsequently processed to 2.30 Å resolution using *HKL-2000* (Otwinowski & Minor, 1997). The cutoff at 2.30 Å resolution was chosen because in our judgment the R_{merge} in the shells above this cutoff was too high for these data to be included. The statistics of the data processing are shown in Table 1.

2.4. Enzymatic assays

The enzymatic activity of AhV_aPA was detected according to the conventional method, using egg-yolk phospholipids as the substrate (Joubert & Taljaard, 1980). One egg yolk was suspended in 1 l physiological saline. 0.2 ml of this suspension was mixed with 0.8 ml 0.1 M Tris pH 8.0 with subsequent addition of 0.1 mg enzyme and was then incubated at 303 K. One unit of PLA₂ activity was defined as the amount of protein which produced a decrease of 0.01 in the absor-

bance between 5 and 10 min at 740 nm. The specific activity was expressed as units per milligram of protein.

2.5. Isolation of thoracic aorta and tension measurement

All animal experiments were conducted in accordance with NIH publication No. 8523. Isolated thoracic aortic strip segments for tension measurements were prepared as described previously (Ye *et al.*, 2004). Briefly, five-week male ICR mice were killed using CO₂ gas. The thoracic aorta was then rapidly dissected free and placed in Krebs–Henseleit solution (58 mM NaCl, 64.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 25.2 mM NaHCO₃, 11.1 mM glucose). At room temperature (298 K), the adhering perivascular tissue was carefully removed and the descending thoracic aorta was cut into 2 mm rings. The vessels were mounted onto two thin stainless steel holders in 0.5 ml organ baths containing Krebs–Henseleit solution at 310 K and continuously bubbled with a gas mixture consisting of 95% O₂ and 5% CO₂ to maintain a pH of 7.4. 500–550 mg passive tension was applied to the vessel to produce the optimal resting tension. After an equilibration period of 1 h, the vessel was contracted twice using 60 mM K⁺ solution (58 mM NaCl, 64.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 25.2 mM NaHCO₃, 11.1 mM glucose). After a subsequent wash, the vessels were again precontracted with 60 mM K⁺ solution in order to achieve sustained contractions. AhV_aPA was then added in a cumulative fashion to the bath to obtain the concentration-response curve. The contractile response to AhV_aPA was expressed as a percentage of the 60 mM K⁺-induced tone.

AhV_aPA stock solutions were prepared in deionized water and stored at 253 K in small aliquots for not more than one week. The isometric tension was recorded on a polygraph (BL-420 System, BL-NewCentury, TME Technology, People's Republic of China).

2.6. Statistical analysis

The results of the enzymatic assays and tension measurements were expressed as mean ± standard deviation. In the concentration-response curves, the data were fitted to the sigmoidal dose-response equation with variable Hill slope $\{y = \text{bottom} + (\text{top} - \text{bottom})/[1 + 10^{(\log EC_{50} - C) \times \text{Hill slope}}]\}$, where C is the logarithm of the AhV_aPA concentration} using *Origin* software (<http://www.originlab.com/>).

3. Results and discussion

In the present work, AhV_aPA was isolated from *A. halys pallas* venom using a three-step chromatographic procedure (Fig. 1). The electrophoretic profile of AhV_aPA obtained by SDS-PAGE showed a single band of ~14 kDa under nonreducing conditions (Fig. 1c). The purified enzyme efficiently cleaved egg-yolk phospholipids with a specific enzymatic activity of $6.2 \pm 0.2 \times 10^4$ U mg⁻¹ (four independent measurements).

We examined the effects of AhV_aPA on mouse thoracic aortic ring contraction. The studies remarkably showed that AhV_aPA could induce a further contractile response on the 60 mM K⁺-induced contraction (Fig. 3a). The concentration-response curves were fitted to the sigmoidal dose-response equation and the obtained EC₅₀ and Hill slope were 369 nmol l⁻¹ and 2.43, respectively (six rings from six mice; Fig. 3b). 500 nmol l⁻¹ AhV_aPA induced a further contractile response of $21.1 \pm 2.1\%$ on the 60 mM K⁺ solution-induced contraction (eight rings from eight mice). Vascular tone is regulated by the channels in the plasma membrane of the vascular smooth-muscle cells, such as K⁺ channels, voltage-gated Ca²⁺ channels, store-operated Ca²⁺ (SOC) channels and stretch-activated cation (SAC)

Figure 3
Vasoconstrictor effect of AhV_aPA on mouse thoracic aortic strings. (a) Contractile response induced by 500 nmol l⁻¹ AhV_aPA on 60 mM K⁺-induced contraction. (b) Concentration-response curve.

channels. Calcium influx through voltage-gated Ca^{2+} , SOC and SAC channels provides the major source of activator Ca^{2+} used by resistance arteries and arterioles. In addition, Ca^{2+} release from the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) is involved in excitation–contraction coupling (Jackson, 2000). Natrin, a cysteine-rich secretory protein from *N. atra* venom, can specifically inhibit 60 mM K^{+} -induced contraction in isolated thoracic aorta by blocking the BK_{ca} channels (Wang *et al.*, 2005). Therefore, it is hypothesized that AhV_aPA may have effects on the ion channels or receptors in the plasma membrane of vascular smooth-muscle cells.

AhV_aPA crystallized in space group *P*222, with unit-cell parameters $a = 44.27$, $b = 68.39$, $c = 81.54$ Å. Calculations based on the protein molecular mass indicated the presence of two molecules in the asymmetric unit. This corresponds to a Matthews coefficient of $2.2 \text{ Å}^3 \text{ Da}^{-1}$, with an approximate solvent content of 44%. Using the structure of an acidic PLA_2 from *Deinagkistrodon acutus* venom (PDB entry 1ijl; 80% sequence identity; Gu *et al.*, 2002) as a search model, the phase problem was solved by molecular replacement with *MOLREP* (the resolution range was 33.8–2.5 Å and the *R* factor and correlation coefficient reported by *MOLREP* were 47.8% and 0.56, respectively; Vagin & Teplyakov, 2010). *REFMAC5* (Murshudov *et al.*, 2011) was used for rigid-body refinement of this solution in the resolution range 40.0–2.3 Å (excluding the 5% of reflections used for R_{free} calculation), resulting in an *R* factor of 38.7% and an R_{free} of 41.6%. Structure refinement and analysis are currently in progress. It is expected that our crystal structure will help us to understand the significant/relevant differences between these PLA_2 enzymes.

In conclusion, AhV_aPA isolated from *A. halys pallas* venom was crystallized and X-ray diffraction data were collected to 2.3 Å resolution. AhV_aPA has a high enzymatic activity, similar to other acidic aPLA_2 s. Like the natrin toxin from *N. atra* venom (Wang *et al.*, 2005), AhV_aPA was identified to have vasoconstrictor activity on mouse thoracic aortic rings. Further structural studies, including a structural comparison with other PLA_2 s, may provide insights into the molecular basis of its biological actions.

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