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Subunit structure and inhibition specificity of α-type phospholipase A_2 inhibitor from *Protobothrops flavoviridis* ☆

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Abstract

The α-type phospholipase A_2 inhibitor (PLIα) in the plasma of the Habu snake, *Protobothrop flavoviridis*, was shown to be a trimer of two homologous subunits, PLIα-A and PLIα-B, each of which contains one C-type lectin-like domain (CTLD). Since one molecule of trimeric PLIα binds stoichiometrically to one molecule of *P. flavoviridis* acidic phospholipase A_2 (PLA_2), the trimeric structure is critical for its inhibitory activity. Hydrophobic chromatography separated the purified *P. flavoviridis* PLIα into four different trimeric subspecies, A^3-PLIα, A^2B-PLIα, AB^2-PLIα, and B^3-PLIα, with different combinations of the two subunits. The trimeric PLIα could be reconstituted from the purified subunits, and the four different trimeric subspecies were formed through random association of the two subunits. The inhibitory activity of the PLIα-A homotrimer (A^3-PLIα) was more specific than that of the PLIα-B homotrimer (B^3-PLIα). This difference in inhibitory properties between the two homotrimers was probably caused by the amino acid differences at residues 10–37.
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Keywords: Phospholipase A_2; Phospholipase A_2 inhibitor; Inhibition specificity; Snake plasma; Trimeric structure; C-type lectin-like domain

1. Introduction

Snake venoms are complex mixtures of pharmacologically active proteins. A number of phospholipase A_2 (PLA_2) isozymes are generally included in these venoms. Elapid and hydrophid snake venoms contain group I PLA_2s; and viperid and crotalid snake venoms contain group II PLA_2s. These snake venom PLA_2s exhibit a wide variety of pharmacological effects including neurotoxicity and myotoxicity (Kini, 2003). Venomous snakes have three distinct types of PLA_2-inhibitory proteins (PLIα, PLIβ, and PLIγ) in their blood to protect themselves from leakage of their own venom PLA_2s into the circulatory system (Ohkura et al., 1997; Lambeau and Lazdunski, 1999; Dunn and

☆ *Ethical statement*: This study does not contain animal experiments or clinical studies.

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Broady, 2001). The α-type PLA_2 inhibitor (PLIα) was first purified by Kihara (1976) from the serum of the Habu snake, *Protobothrops flavoviridis* (renamed from *Trimeresurus flavoviridis* according to the present taxonomy). We further characterized *P. flavoviridis* PLIα (Kogaki et al., 1989) and found that it was a glycoprotein consisting of two distinct subunits, each of which contained a C-type lectin-like domain exhibiting significant homology to serum mannose-binding protein and lung-surfactant apoprotein (Inoue et al., 1991). PLIαs have only been identified in the blood of *Viperidae* snakes, such as *P. flavoviridis*, *Gloydius brevicaudus* (renamed from *Agkistrodon blomhoffii siniticus*) (Ohkura et al., 1993), *Bothrops asper* (Lizano et al., 1997), *Cerrophidion godmani* (Lizano et al., 2000), *Bothrops moojeni* (Soares et al., 2003), and *Atropoides nummifer* (Quiros et al., 2007), whereas a PLIα homolog (PLIα-LP) lacking PLA_2-inhibitory activity was identified in the blood of the nonvenomous snake *Elaphe quadrivirgata* (Okumura et al., 2003). All but *P. flavoviridis* PLIα are homomultimers composed of a single subunit. *B. asper* PLIα (BaMIP), *C. godmani* PLIα (CgMIP-II), and *B. moojeni* PLIα (BmjMIP) were suggested to be composed of four or five subunits (Lizano et al., 1997, 2000; Soares et al., 2003), whereas *G. brevicaudus* PLIα, *A. nummifer* PLIα (AnMIP), and *E. quadrivirgata* PLIα-LP were proved to be homotrimers (Ohkura et al., 1993, 2003; Quiros et al., 2007). Since *P. flavoviridis* PLIα contains equimolar amounts of two distinct subunits, PLIα-A and PLIα-B, one molecule of intact PLIα was expected to be a tetramer composed of two molecules of PLIα-A and two molecules of PLIα-B (Inoue et al., 1991).

In the present study, we clarified the subunit composition of *P. flavoviridis* PLIα and found the inhibitor to be trimeric. Furthermore, we also reconstituted homotrimers from the purified subunits and showed that the reconstituted PLIα-A and PLIα-B homotrimers had different inhibition spectra toward various snake venom PLA_2s.

2. Materials and methods

2.1. Materials

PLIα was purified from the blood plasma of the Habu snake *P. flavoviridis* by sequential chromatography on Sephadex G-200, Q-Sepharose, and Blue Sepharose columns as described previously (Kogaki et al., 1989). PLA_2s from various sources were purified or purchased as described earlier (Inoue et al., 1997). The molar concentrations of the pure proteins were determined spectrophotometrically by using the extinction coefficients calculated from their amino acid sequence data (Gill and von Hippel, 1989).

2.2. Chemical cross-linking

The purified *P. flavoviridis* PLIα in 50 mM Hepes buffer (pH 7.5, ionic strength of 0.2) was treated with bis(sulfosuccinimidyl)-suberate (BS^3; Pierce Biotechnology Inc., Rockford, IL, USA) for 3 h at room temperature. The reaction was terminated by the addition of SDS-PAGE sample buffer, and then the mixture was heated at 100 °C for 5 min and analyzed by SDS-PAGE followed by silver staining.

2.3. Direct binding of *P. flavoviridis* acidic PLA_2 to PLIα

Trimeric PLIα (0.17 nmol) was incubated for 1 h at room temperature with 0, 0.25, or 0.5 nmol of *P. flavoviridis* acidic PLA_2 in 200 μl of 50 mM Tris–HCl buffer (pH 7.5, ionic strength of 0.2) containing 0.05% (w/v) Tween 20. The mixture was then applied to a Superose 12 HR10/30 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), which had been equilibrated with the same buffer, and eluted at a flow rate of 0.4 ml/min. The fractions containing the PLIα–PLA_2 complex were analyzed by reversed-phase HPLC.

2.4. Reversed-phase HPLC for separation of the subunits

The two subunits of *P. flavoviridis* PLIα, PLIα-A, and PLIα-B were separated by reversed-phase HPLC as described previously (Inoue et al., 1991). PLIα was applied to a Vydac C4 column (The Separations Group, Hesteria, CA, USA) or a Cosmosil 5C4-AR-300 column (Nacalai Tesque, Kyoto, Japan) that had been equilibrated with 0.1% trifluoroacetic acid. The subunits were eluted with a linear gradient of acetonitrile, from 0% to 48%, in 0.1% trifluoroacetic acid. The fractions containing PLIα-A and PLIα-B were separately collected and lyophilized.

2.5. Hydrophobic chromatography

The purified *P. flavoviridis* PLIα was loaded onto a Protein Pak G-Butyl column (Millipore, Milford,

MA, USA) that had been equilibrated with 0.1 M phosphate buffer containing 1.7 M ammonium sulfate (pH 7.5). After the column had been washed with the same buffer, the adsorbed proteins were eluted with a linear decreasing concentration gradient of ammonium sulfate, from 1.7 to 0 M. The obtained fractions were analyzed by reversed-phase HPLC.

2.6. Reconstitution of the trimeric PLIα

The lyophilized subunits were dissolved in 50 mM Hepes buffer (pH 8.0) containing 6 M guanidine–HCl and then allowed to stand overnight at 4 °C. Then the solutions were dialyzed against Hepes buffer (pH 8.0) at 4 °C or room temperature for 3 h. In order to avoid protein aggregation, we kept the final concentrations of the subunits to less than 2 nmol/ml. The inhibitory activities toward *P. flavoviridis* acidic PLA_2 recovered were measured and expressed as values relative to the value for the purified PLIα at the same concentration.

The reconstituted PLIα-A and PLIα-B homotrimers were further purified by using a HiTrap Blue column (GE Healthcare Bio-Sciences). The column was equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 2 mM EDTA. After the reconstituted proteins had been applied to the column, the column was washed with the same buffer, and the homotrimers were then eluted with the same buffer containing 0.3 M NaCl.

2.7. PLA_2 assays

PLA_2 activities were measured fluorometrically by using 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (β-py-C_{10}-HPC; Molecular Probes, Eugene, OR, USA) as the substrate, according to the method of Radvanyi et al. (1989) with some modifications as described previously (Inoue et al., 1997). The activities of PLA_2s from various sources were measured in the presence of various concentrations of the PLIα-A and PLIα-B homotrimers (10^{-12}–10^{-6} M). Values of the apparent inhibition constant (K_i^{app}) were determined by non-linear least-squares analysis of the relative PLA_2 activity, as described previously (Okumura et al., 1999).

3. Results and discussion

3.1. Trimeric PLIα binds to a single PLA_2 molecule

Earlier we reported that PLIα of *G. brevicaudus* (renamed from *A. blomhoffii siniticus*) is a homotrimer of a single type of subunit (Ohkura et al., 1993). In order to clarify the multimeric structure of *P. flavoviridis* PLIα, we treated the purified PLIα with a bifunctional cross-linking reagent, BS^3, and then analyzed the products by SDS-PAGE. As shown in Fig. 1A, the subunits of PLIα could be cross-linked to yield dimers and trimers, but no higher degree of polymerization was observed. Therefore, like *G. brevicaudus* PLIα, the purified *P. flavoviridis* PLIα was found to be a trimer.

As shown in Fig. 1B, the formation of complexes between *P. flavoviridis* PLIα and *P. flavoviridis* acidic PLA_2 was investigated by gel filtration on a Superose 12 column. When 0.17 nmol of trimeric

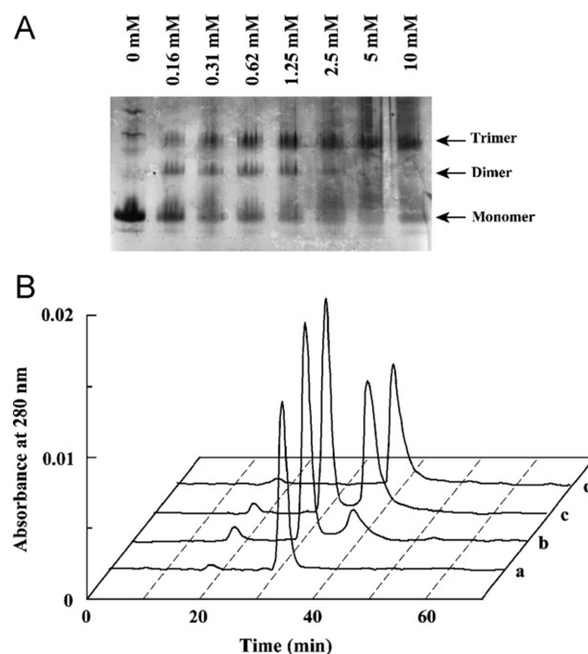

Fig. 1. Trimeric PLIα binds to a single PLA_2 molecule. (A) Chemical cross-linking of PLIα. Aliquots of the inhibitor were treated with various concentrations of bis(sulfosuccinimidyl)suberate as indicated. The cross-linked PLIαs were analyzed by SDS-PAGE. (B) Gel filtration of a mixture of *P. flavoviridis* acidic PLA_2 and PLIα on a Superose 12 column. The column had been equilibrated with Hepes buffer containing 0.05% (w/v) Tween 20 (pH 7.5, $\mu = 0.2$). (a) 0.17 nmol of trimeric PLIα; (b) mixture of 0.17 nmol of trimeric PLIα and 0.25 nmol of acidic PLA_2; (c) mixture of 0.17 nmol of trimeric PLIα and 0.5 nmol acidic PLA_2; and (d) 0.25 nmol of acidic PLA_2.

PLIα (equivalent to 0.5 nmol of PLIα subunits) and 0.25 nmol of acidic PLA_2 were separately applied to the column, they were eluted as single peaks at 30.5 and 38.1 min, respectively, corresponding to their apparent molecular masses of 72 and 16 kDa. However, when the mixture was applied to the column, the height of the latter peak decreased and that of the former peak increased, suggesting the formation of a stable complex between PLIα and PLA_2. The peak height of the PLIα–PLA_2 complex did not increase further when gel filtration of the mixture containing 0.17 nmol of PLIα and 0.5 nmol of PLA_2 was done, indicating that the complex was saturated with respect to PLA_2. The reversed-phase HPLC analysis of the PLIα–PLA_2 complex showed that the molar ratio of PLA_2 to trimeric PLIα was 0.96, suggesting that three subunits of PLIα bind to one molecule of acidic PLA_2. Therefore, the trimeric structure of PLIα is most likely to contribute the inhibitory activity toward PLA_2. The apparent molecular mass (75 kDa) of the complex estimated from its retention time of 30.3 min on a Superose 12 column was nearly equal to that of the PLIα trimer (72 kDa). On the complex formation, PLA_2 molecule might be buried deep in the central pore formed on the trimerization of PLIα, as proposed previously (Okumura et al., 2005).

3.2. Subunit composition of *P. flavoviridis* PLIα

As described previously (Inoue et al., 1991), *P. flavoviridis* PLIα is composed of two homologous subunits, PLIα-A and PLIα-B. Since the purified PLIα contained equimolar amounts of the subunits, the purified PLIα might consist of two molecular

Table 1
Renaturation of *P. flavoviridis* PLIα subunits

PLIα subunit	6 M guanidine HCl treatment	Renaturation temperature (°C)	Recovery of the inhibitory activity[a] (%)
PLIα-A	–	25	9.6
	+	4	10.9
	+	25	40.2
PLIα-B	–	25	6.4
	+	4	0.0
	+	25	33.3

[a]Recovery was calculated on the basis of the inhibitory activity of intact PLIα.

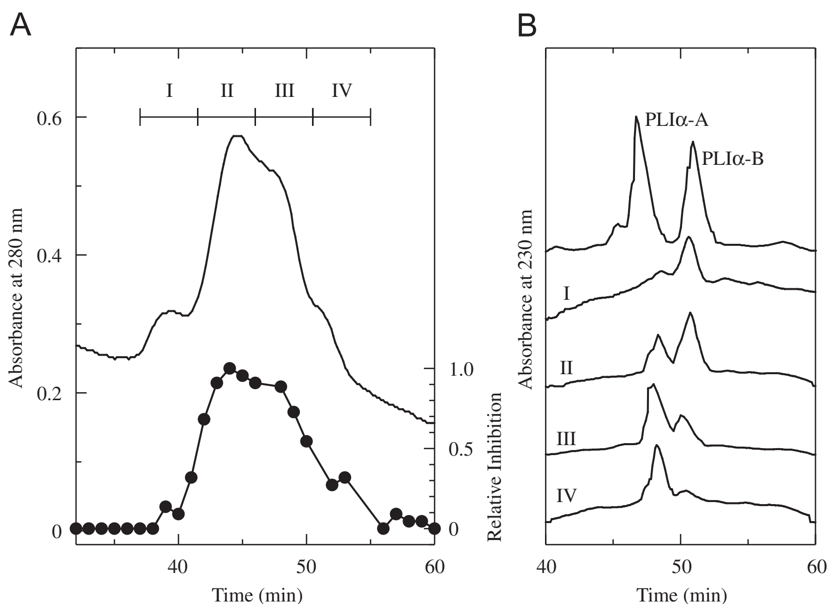

Fig. 2. Separation of four molecular species of trimeric PLIα. (A) Hydrophobic chromatography of the *P. flavoviridis* PLIα on a Protein Pak G-Butyl column. A PLIα sample containing 1.7 M ammonium sulfate was applied to a column that had been equilibrated with 0.1 M phosphate buffer (pH 7.5) containing 1.7 M ammonium sulfate. The column was washed with the same buffer, and then eluted with a linear decreasing concentration gradient of ammonium sulfate, from 1.7 to 0 M. ●, relative inhibitory activity toward *P. flavoviridis* acidic PLA_2. (B) Reversed-phase HPLC analysis of the intact PLIα and its four fractions separated by hydrophobic chromatography. The respective samples were applied to a Cosmosil 5C4-AR-300 column, and then eluted with a linear concentration gradient of acetonitrile, from 24% to 48%, in the presence of 1% trifluoroacetic acid.

subspecies of homotrimers, the PLIα-A homotrimer (designated as A^3-PLIα) and PLIα-B homotrimer (designated as B^3-PLIα), or four molecular subspecies additionally including a heterotrimer composed of two PLIα-A and one PLIα-B subunit (designated as A^2B-PLIα), and a heterotrimer composed of one PLIα-A and two PLIα-B subunits (designated as AB^2-PLIα). In order to determine the number of subspecies of *P. flavoviridis* PLIα, we attempted to separate further the purified PLIα by means of various kinds of column chromatographies, but most methods could not separate the subspecies and gave only one broad peak, probably reflecting the heterogeneity of the glycosidic chains

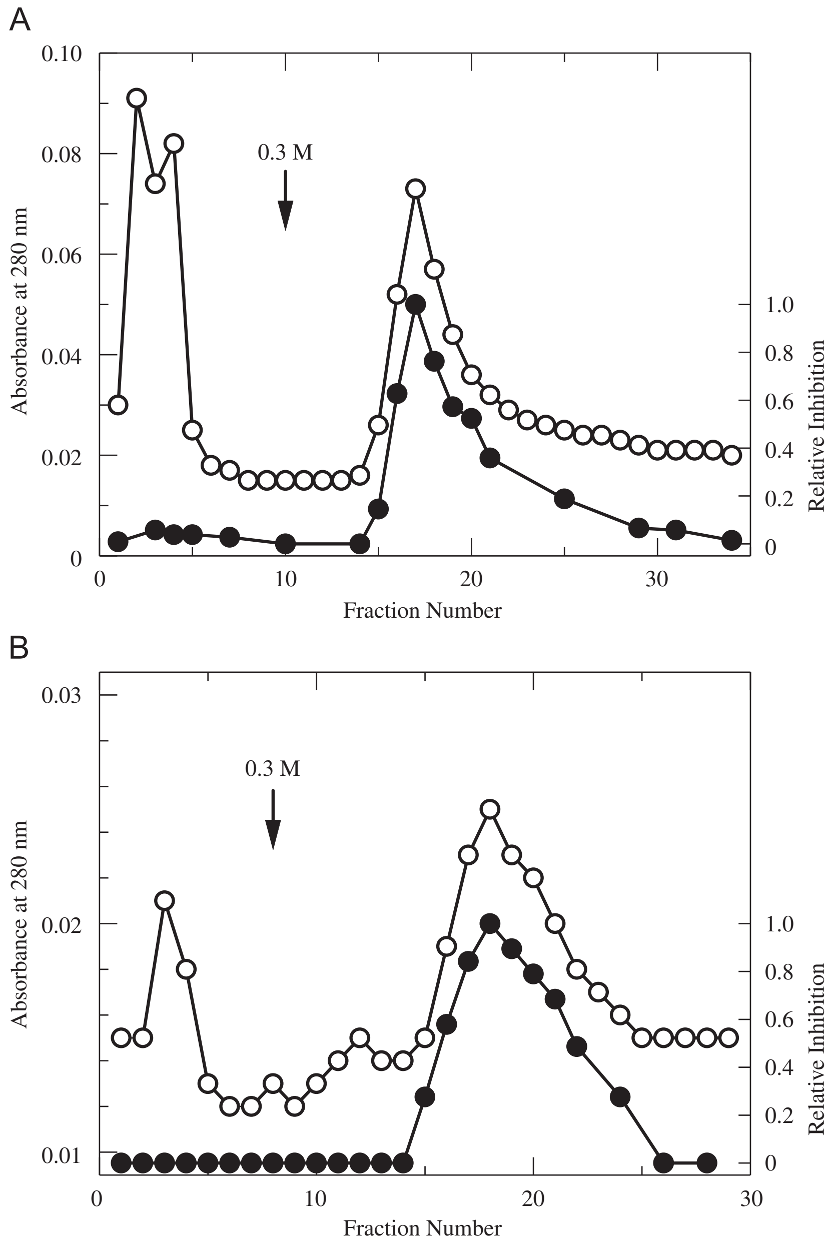

Fig. 3. HiTrap Blue column chromatography of the reconstituted PLIα-A and PLIα-B homotrimers. The reconstituted homotrimers of PLIα-A (A) and PLIα-B (B) were applied to a column that had been equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 2 mM EDTA. The adsorbed proteins were eluted with the same buffer containing 0.3 M NaCl. ○, absorbance at 280 nm; ●, relative inhibitory activity toward *P. flavoviridis* acidic PLA_2.

of the subunits. As shown in Fig. 2A, hydrophobic chromatography on a Protein Pak G-Butyl column could successfully separate the purified PLIα into four fractions (I, II, III, and IV). When the fractions were analyzed by reversed-phase HPLC, fractions I, II, III, and IV were found to correspond to B^3-PLIα, AB^2-PLIα, A^2B-PLIα, and A^3-PLIα, respectively (Fig. 2B). Since the peak areas of fractions I, II, III, and IV were in the ratio of about 1:3:3:1, trimerization of the PLIα-A and PLIα-B subunits seemed to have occurred randomly.

3.3. Reconstitution of PLIα

Lyophilized PLIα-A and PLIα-B subunits, each of which had been separated from the purified *P. flavoviridis* PLIα by reversed-phase HPLC, were used to reconstitute the two homotrimers, A^3-PLIα and B^3-PLIα. As shown in Table 1, when the lyophilized subunits were directly dissolved in 50 mM Hepes buffer (pH 8.0), the recovery of the inhibitory activity was less than 10%. But, when the subunits were dissolved in 50 mM Hepes buffer (pH 8.0) containing 6 M guanidine–HCl, followed by dialysis against 50 mM Hepes buffer at room temperature, the recovery of the inhibitory activity was approximately 40%. Therefore, the guanidine–HCl–denatured PLIα subunits were found to be preferable for the reconstitution. Moreover, the reconstitution of PLIα was found to be sensitive to temperature, since the recovery of the inhibitory activity decreased on dialysis at 4 °C. However, rapid recovery of the inhibitory activity was observed when the dialysis temperature was raised from 4 °C to room temperature (data not shown).

Through reconstitution from the respective subunits described above, we could obtain two homotrimers, A^3-PLIα and B^3-PLIα. The reconstituted preparations seemed to contain inactive misfolded proteins and their aggregates, judging from the 40% recovery of the inhibitory activity, and thus further purification was required. Therefore, the reconstituted A^3-PLIα and B^3-PLIα preparations were separately applied to a HiTrap Blue column. As shown in Fig. 3, the misfolded proteins having no inhibitory activity flowed through the column, whereas the reconstituted homotrimers were specifically adsorbed to the column and eluted with the buffer containing 0.3 M NaCl.

As shown in Fig. 4, when the reconstituted homotrimers, A^3-PLIα and B^3-PLIα, were separately applied to a Protein Pak G-Butyl column, they were eluted as single peaks at 52 and 38 min, respectively, in agreement with the retention times expected from the data for the purified PLIα. When a mixture of equimolar amounts of the reconstituted A^3-PLIα and B^3-PLIα was incubated overnight at room temperature and then applied to the column, no peaks for the two heterotrimers, A^2B-PLIα and AB^2-PLIα, could be detected, indicating that the subunit exchange between the two homotrimers did not occur under normal conditions. On the contrary, when a mixture of equimolar amounts of the guanidine–HCl-denatured PLIα-A and PLIα-B was reconstituted by dialysis and then applied to the column, four peaks appeared, the elution profile being similar to that for the purified PLIα.

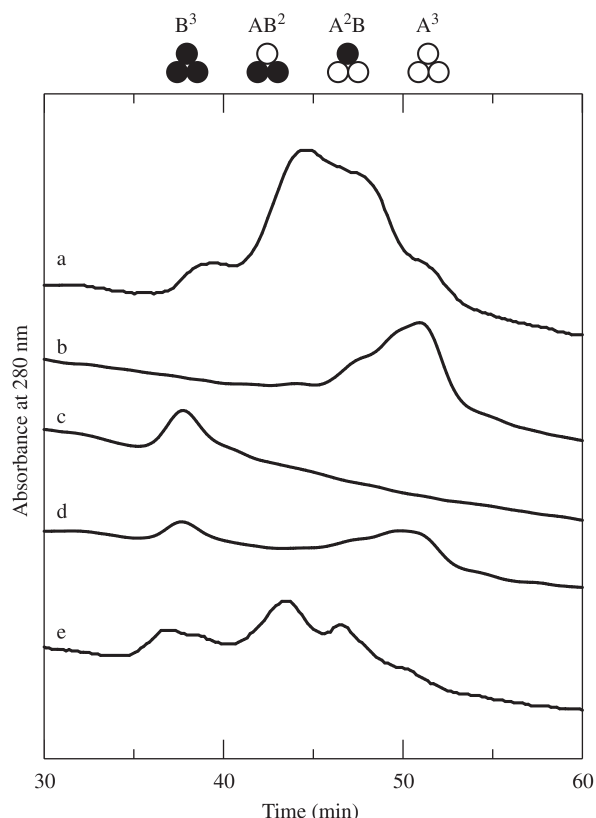

Fig. 4. Hydrophobic chromatography of the reconstituted PLIα on a Protein Pak G-Butyl column. The samples were applied to a column that had been equilibrated with 0.1 M phosphate buffer (pH 7.5) containing 1.7 M ammonium sulfate, and the adsorbed proteins were then eluted with a linear decreasing concentration gradient of ammonium sulfate, from 1.7 to 0 M. (a) Intact PLIα; (b) reconstituted A^3-PLIα; (c) reconstituted B^3-PLIα; (d) mixture of the reconstituted A^3-PLIα and B^3-PLIα; and (e) the sample reconstituted from a mixture of equimolar amounts of PLIα-A and PLIα-B.

Therefore, on constitution, the denatured PLIα-A and PLIα-B subunits formed trimeric PLIα randomly through the same inter-subunit interaction. Since the PLIα-A and PLIα-B genes have been reported to be expressed only in the liver (Nobuhisa et al., 1997a), these subunits are probably synthesized equally and simultaneously in the hepatocytes of *P. flavoviridis*, and assembled randomly to generate trimeric PLIα in the process of plasma protein secretion.

3.4. Inhibition specificity of homotrimeric PLIα

Fig. 5 shows the typical inhibitory activities of the reconstituted PLIα homotrimers toward various snake venom PLA_2s. Table 2 summarizes their apparent inhibition constants (K_i^{app}) with respect to various PLA_2s. Both A^3-PLIα and B^3-PLIα inhibited *P. flavoviridis* acidic PLA_2 with apparent inhibition constant (K_i^{app}) values of around 3.7 nM. Unexpectedly, A^3-PLIα strongly inhibited

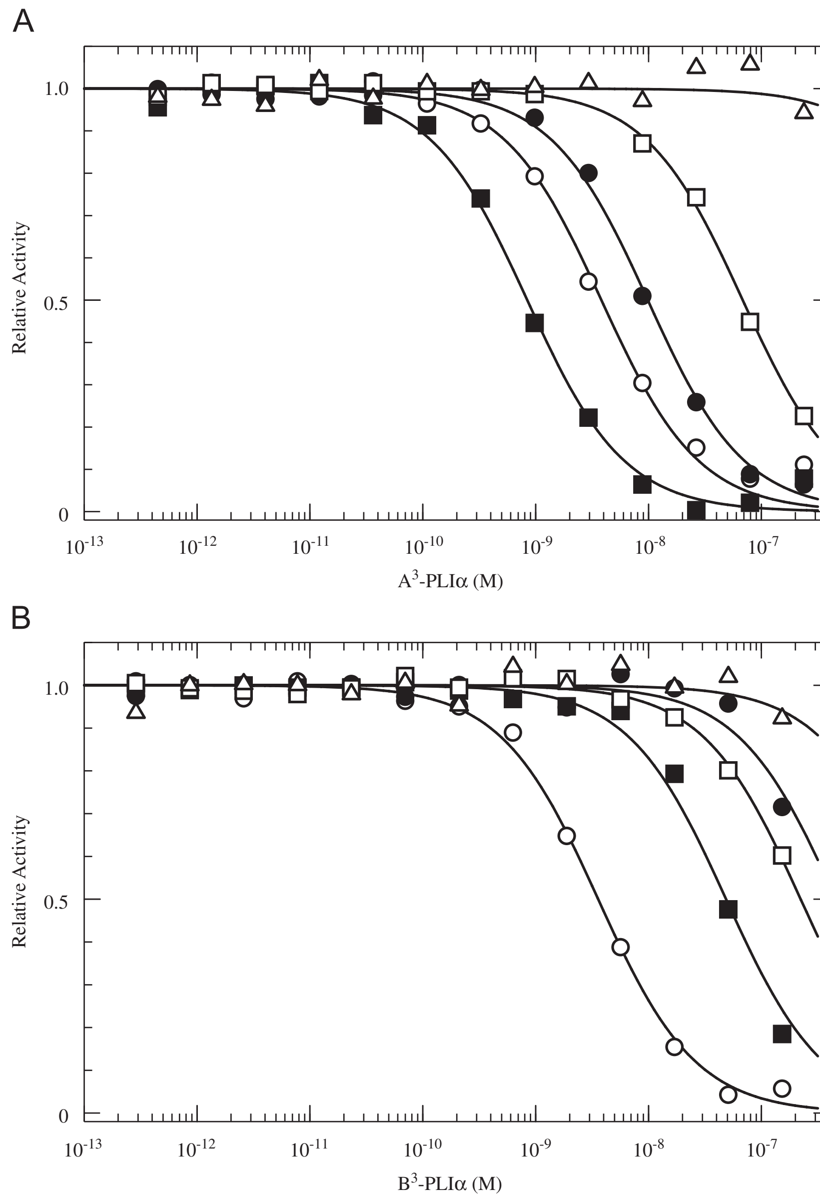

Fig. 5. Inhibition of the enzymatic activities of PLA_2s from various sources by A^3-PLIα and B^3-PLIα. PLA_2 activity was measured fluorometrically with β-py-C_{10}-HPC as a substrate in the presence of various concentrations of the inhibitors. ○, *P. flavoviridis* acidic PLA_2; ●, *G. brevicaudus* acidic PLA_2; □, *Daboia russelli russelli* PLA_2-III; ■, *G. blomhoffii* acidic PLA_2; △, *Naja kaouthia* CM-II.

Table 2
Apparent inhibition constants (K_i^{app}) of A^3-PLIα and B^3-PLIα with respect to various groups of PLA_2s

PLA_2		A^3-PLIα	B^3-PLIα
Group I PLA_2s			
Naja kaouthia	CM-II	>1000	>1000
Naja atra	PLA_2	>1000	>1000
Pseudechis australis	Pa-12A	>1000	918
Group II PLA_2s			
Protobothrops flavoviridis	Acidic PLA_2	3.78	3.56
	PL-X	>1000	395
Gloydius brevicaudus	Acidic PLA_2	9.62	458
	Neutral PLA_2	>1000	508
	Basic PLA_2	935	738
Gloydius blomhoffii	Acidic PLA_2	0.83	48.6
	Neutral PLA_2	>1000	285
	Basic PLA_2	819	213
Daboia russelli russelli	PLA_2-III	67.6	222
	PLA_2-V	>1000	321
Group III PLA_2			
Apis melifera	PLA_2	>1000	>1000

The apparent inhibition constant values (K_i^{app}) were determined by nonlinear least-squares analysis of the relative PLA_2 activities shown in Fig. 5.

G. blomhoffii acidic PLA_2 with a K_i^{app} value of 0.8 nM, which was 4.3-fold lower than that for *P. flavoviridis* acidic PLA_2. A^3-PLIα showed a narrow inhibition spectrum and inhibit only some of the group II acidic PLA_2s including *G. blomhoffii* acidic PLA_2, *P. flavoviridis* acidic PLA_2, *G. brevicaudus* acidic PLA_2, and *Daboia russelli russelli* (renamed from *Vipera russelli russelli*) PLA_2-III, whereas B^3-PLIα showed a rather broad inhibition spectrum. The inhibition spectrum of the purified intact PLIα, which had been determined in a previous study (Inoue et al., 1997), was an intermediate between those spectra of A^3-PLIα and B^3-PLIα. Therefore, the four subspecies of PLIα trimers generated through the random combination of the two subunits are expected to have inhibition spectra different from each other.

In contrast to the PLIαs from Asian *Viperidae* snakes, *G. blomhoffii*, and *P. flavoviridis*, the PLIαs from American *Viperidae* snakes, such as *B. asper*, *C. godmani*, and *A. nummifer*, showed inhibitory specificities toward group II basic PLA_2s rather than toward group II acidic PLA_2s (Lizano et al., 1997, 2000; Quiros et al., 2007). Furthermore, American *Viperidae* PLIαs inhibited the myotoxic activities of basic K-49 PLA_2 myotoxins, of which the PLA_2 activities were negligible due to the replacement of the catalytic Asp-49 with lysine. Nobuhisa et al. (1998, 1997b) reported that *P. flavoviridis* PLIα also bound to Lys-49 PLA_2 homologs, basic proteins I (BP-I) and II (BP-II) from *P. flavovidis* venom. Since BP-I and BP-II exhibit strong myotoxicity (Kihara et al., 1992), PLIα would function as an endogenous antitoxic protein. The PLIα-B subunit might bind to these Lys-49 PLA_2 homologs more preferentially than the PLIα-A one, judging from the electropherogram results showing that the GST-fused PLIα-B (PLI-V in the paper concerned), which had been bound to the immobilized BP-I on magnetic beads, exhibited a more intense band than GST-fused PLIα-A (PLI-IV) (Nobuhisa et al., 1998). Although BP-I and BP-II reportedly have low enzymatic activity (Liu et al., 1990), we could not detect any activity of BP-I and BP-II that had been highly purified by reversed-phase HPLC (data not shown). The reported low enzymatic activities of the samples might be due to trace contamination by Asp-49 PLA_2, as reported in the case of the *Agkistrodon piscivorus piscivorus* Lys-49 PLA_2 homolog (van den Bergh et al., 1989). Therefore, the effects of PLIα on BP-I and BP-II remain to be examined.

Fig. 6 compares the amino acid sequences of the PLIαs from *P. flavoviridis* and *G. brevicaudus* (Inoue et al., 1991; Ohkura et al., 1993). The amino acid sequences of the two subunits, PLIα-A and PLIα-B, of *P. flavoviridis* PLIα differ from each other by 15 amino acid residues, 11 of which are located between residues 10 and 37, which are encoded by exon 3 of the PLIα gene (Nobuhisa et al., 1997a). Previously, we reported that residues 13–36 of *G. brevicaudus* PLIα were located in the neck region of its trimeric structure, and were important for the inhibition of and binding to *G. brevicaudus* acidic PLA_2 (Okumura et al., 2005). Therefore, the difference in the inhibition spectra of A^3-PLIα and B^3-PLIα may be attributable to the difference in the amino acid residues in this region. Since the inhibitory properties of A^3-PLIα, which are characterized by its high specificity and strong preference for acidic PLA_2s, were similar to that of homotrimeric *G. brevicaudus* PLIα (Inoue et al., 1997), common structural properties, such as the positive charge of residue 22 and the negative charge of Glu-23, might contribute to their similar inhibitory properties. Further studies by use of site-directed mutagenesis will reveal the structural basis of the inhibitory specificity of PLIα.

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                                               1         10        20        30        40        50        60
(1) Protobothrops flavoviridis PLIα-A          HETDPDGQVMSSMIETLMFLQKEYANLRYAFMTVNNARSFGSGSERLYVSNKEIKTFEPL
(2) Protobothrops flavoviridis PLIα-B          HETDSDGQVMNSMIEALTELQEMIVNLRYAFLTVHKARSFGSGSERLYVSNKEIKTFEPL
(3) Gloydius brevicaudus PLIα                  HETDPDGHVLNSLMLIVMRLQREFSNLKDGFLTVHKARSFGSGSERLYVTNKEVGNFEGL

          70        80        90        100       110       120       130       140    147
(1)   KEICEEAGGHIPSPQLENQNKAFASVLERHNKAAYLVVGDSANFTNWAAGQPNEADGTCVKADTHGSWHSASCDENLLVVCEFYFIL
(2)   KEICEEAGGHIPSPQLENQNKAFANVLERHNKAAYLVVGDSANFTNWAAGQPNEADGTCVKADTHGFWHSASCDEKLLVVCEFYFIL
(3)   GEICRQAGGRIPSPQLKNQNKAFANVLERHNKEAYLVVGNSANFTNWAEGQPKKADGTCVKADTHGFWHSTSCDDNLLVVCEFYFIL
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Fig. 6. Comparison of the amino acid sequences of the two subunits of *P. flavoviridis* PLIα, PLIα-A and PLIα-B, with that of *G. brevicaudus* PLIα. The stippled boxes indicate the amino acids different from those of other sequences. The NCBI accession codes for these proteins are as follows: *P. flavoviridis* PLIα-A, P71555; PLIα-B, P71556; and *G. brevicaudus* PLIα, BAA86972.

Ohno et al. (1998) have proposed that snake toxin genes have developed through the process of accelerated evolution. *P. flavoviridis* venom gland PLA_2 isozymes are suggested to have evolved via accelerated evolution to gain new physiological activities (Nakashima et al., 1993). The exons of the PLA_2 genes have evolved with greater rates of substitution than introns and thus have generated accelerated amino acid substitutions in their protein-coding regions. Consequently, the venom PLA_2 isozymes have acquired different physiological activities. During the course of evolution, PLA_2 inhibitors would need to have changed their inhibitory specificities in order to match the accelerated substitutions of the venom PLA_2 isozymes. By comparison of the nucleotide sequences between PLIα-A and PLIα-B genes, Nobuhisa et al. (1997a) showed that nonsynonymous substitutions were almost four times as frequent as synonymous substitutions in exon 3 of the PLIα genes. The accelerated amino acid substitutions encoded by exon 3 of the PLIα gene could have caused the difference in inhibitory specificities between the two homotrimers, A^3-PLIα and B^3-PLIα, in the present study. Furthermore, the varieties of the combinations of the two subunits of PLIα would contribute to the inhibitory plasticity toward the accelerated evolution of venom PLA_2 isozymes. Venom PLA_2s and their plasma inhibitors have probably co-evolved with each other via accelerated evolution.

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References

Dunn, R.D., Broady, K.W., 2001. Snake inhibitors of phospholipase A2 enzymes. Biochim. Biophys. Acta 1533, 29–37.

Gill, S.C., von Hippel, P.H., 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. 182, 319–326.

Inoue, S., Kogaki, H., Ikeda, K., Samejima, Y., Omori-Satoh, T., 1991. Amino acid sequences of the two subunits of a phospholipase A_2 inhibitor from the blood plasma of *Trimeresurus flavoviridis*: sequence homologies with pulmonary surfactant apoprotein and animal lectins. J. Biol. Chem. 266, 1001–1007.

Inoue, S., Shimada, A., Ohkura, N., Ikeda, K., Samejima, Y., Omori-Satoh, T., Hayashi, K., 1997. Specificity of two types of phospholipase A_2 inhibitors from the plasma of venomous snakes. Biochem. Mol. Biol. Int. 41, 529–537.

Kihara, H., 1976. Studies on phospholipase A in *Trimeresurus flaoviridis* venom. III. Purification and some properties of phospholipase A inhibitor in Habu serum. J. Biochem. (Tokyo) 80, 341–349.

Kihara, H., Uchikawa, R., Hattori, S., Ohno, M., 1992. Myotoxicity and physiological effects of three *Trimeresurus flavoviridis* phospholipases A_2. Biochem. Int. 28, 895–903.

Kini, R.M., 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon 42, 827–840.

Kogaki, H., Inoue, S., Ikeda, K., Samejima, Y., Omori-Satoh, T., Hamaguchi, K., 1989. Isolation and fundamental properties of a phospholipase A_2 inhibitor from the blood plasma of *Trimeresurus flavoviridis*. J. Biochem. (Tokyo) 106, 966–971.

Lambeau, G., Lazdunski, M., 1999. Receptors for a growing family of secreted phospholipases A_2. Trends. Pharmacol. Sci. 20, 162–170.

Liu, S.Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S., Kihara, H., 1990. Purification and amino acid sequence of basic protein II, a lysine-49-phospholipase A_2 with low activity, from *Trimeresurus flavoviridis* venom. J. Biochem. (Tokyo) 107, 400–408.

Lizano, S., Lomonte, B., Fox, J.W., Gutierrez, J.M., 1997. Biochemical characterization and pharmacological properties of a phospholipase A_2 myotoxin inhibitor from the plasma of the snake *Bothrops asper*. Biochem. J. 326, 853–859.

Lizano, S., Angulo, Y., Lomonte, B., Fox, J.W., Lambeau, G., Lazdunski, M., Gutierrez, J.M., 2000. Two phospholipase A_2 inhibitors from the plasma of *Cerrophidion* (*Bothrops*)

godmani which selectively inhibit two different group-II phospholipase A_2 myotoxins from its own venom: isolation, molecular cloning and biological properties. Biochem. J. 346, 631–639.

Nakashima, K., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H., Ohno, M., 1993. Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A_2 isozymes. Proc. Natl. Acad. Sci. USA 90, 5964–5968.

Nobuhisa, I., Chiwata, T., Fukumaki, Y., Hattori, S., Shimohigashi, Y., Ohno, M., 1998. Structural elements of *Trimeresurus flavoviridis* serum inhibitors for recognition of its venom phospholipase A_2 isozymes. FEBS Lett. 429, 385–389.

Nobuhisa, I., Deshimaru, M., Chijiwa, T., Nakashima, K., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, S., Kihara, H., Ohno, M., 1997a. Structures of genes encoding phospholipase A_2 inhibitors from the serum of *Trimeresurus flavoviridis* snake. Gene 191, 31–37.

Nobuhisa, I., Inamasu, S., Nakai, M., Tatsui, A., Mimori, T., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, S., Kihara, H., Ohno, M., 1997b. Characterization and evolution of a gene encoding a *Trimeresurus flavoviridis* serum protein that inhibits basic phospholipase A_2 isozymes in the snake's venom. Eur. J. Biochem. 249, 838–845.

Ohkura, N., Inoue, S., Ikeda, K., Hayashi, K., 1993. Isolation and amino acid sequence of a phospholipase A_2 inhibitor from the blood plasma of *Agkistrodon blomhoffii siniticus*. J. Biochem. 113, 413–419.

Ohkura, N., Okuhara, H., Inoue, S., Ikeda, K., Hayashi, K., 1997. Purification and characterization of three distinct types of phospholipase A_2 inhibitors from the blood plasma of the Chinese mamushi, *Agkistrodon blomhoffii siniticus*. Biochem. J. 325, 527–531.

Ohno, M., Menez, R., Ogawa, T., Danse, J.M., Shimohigashi, Y., Fromen, C., Ducancel, F., Zinn-Justin, S., Le Du, M.H., Boulain, J.C., Tamiya, T., Menez, A., 1998. Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? Prog. Nucleic Acid Res. Mol. Biol. 59, 307–364.

Okumura, K., Inoue, S., Ikeda, K., Hayashi, K., 1999. cDNA cloning and bacterial expression of phospholipase A_2 inhibitor PLIα from the serum of the Chinese mamushi, *Agkistrodon blomhoffii siniticus*. Biochim. Biophys. Acta 1441, 51–60.

Okumura, K., Inoue, S., Ikeda, K., Hayashi, K., 2003. Identification and characterization of a serum protein homologous to α-type phospholipase A_2 inhibitor (PLIα) from a nonvenomous snake, *Elaphe quadrivirgata*. IUBMB Life 55, 539–545.

Okumura, K., Ohno, A., Nishida, M., Hayashi, K., Ikeda, K., Inoue, S., 2005. Mapping the region of the α-type phospholipase A_2 inhibitor responsible for its inhibitory activity. J. Biol. Chem. 280, 37651–37659.

Quiros, S., Alape-Giron, A., Angulo, Y., Lomonte, B., 2007. Isolation, characterization and molecular cloning of AnMIP, a new α-type phospholipase A_2 myotoxin inhibitor from the plasma of the snake *Atropoides nummifer* (Viperidae: Crotalinae). Comp. Biochem. Physiol. 146, 60–68.

Radvanyi, F., Jordan, L., Russo-Marie, F., Bon, C., 1989. A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. Anal. Biochem. 177, 103–109.

Soares, A.M., Marcussi, S., Stabeli, R.G., Franca, S.C., Giglio, J.R., Ward, R.J., Arantes, E.C., 2003. Structural and functional analysis of BmjMIP, a phospholipase A_2 myotoxin inhibitor protein from *Bothrops moojeni* snake plasma. Biochem. Biophys. Res. Commun. 302, 193–200.

van den Bergh, C.J., Slotboom, A.J., Verheij, H.M., de Haas, G.H., 1989. The role of Asp-49 and other conserved amino acids in phospholipases A_2 and their importance for enzymatic activity. J. Cell. Biochem. 39, 379–390.