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Inhibitory activities of the heterotrimers formed from two α-type phospholipase A_2 inhibitory proteins with different enzyme affinities and importance of the intersubunit electrostatic interaction in trimer formation

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ABSTRACT

α-type phospholipase A_2 inhibitory protein (PLIα) isolated from the serum of the venomous snake *Glyoidius brevicaudus*, *Gb*PLIα, is a homotrimer of subunits having a C-type lectin-like domain. The serum protein from nonvenomous snake *Elaphe quadrivirgata*, *Eq*PLIα-LP, is homologous to *Gb*PLIα, but it does not show any inhibitory activity against PLA_2s. When a mixture of denaturant-treated monomeric forms of *Gb*PLIα and *Eq*PLIα-LP was used to reconstitute their trimers, no significant amounts of heterotrimers composed of *Gb*PLIα and *Eq*PLIα-LP subunits could be formed. On the other hand, when a mixture of denaturant-treated monomeric forms of *Gb*PLIα and the recombinant chimeric *Eq*PLIα-LP, *Eq*13*Gb*37*Eq*, in which the residues 13–36 were replaced by those of *Gb*PLIα, was used to reconstitute their trimers, significant amounts of their heterotrimers were observed. Furthermore, when a mixture of denaturant-treated monomeric forms of *Eq*PLIα-LP and the recombinant chimeric *Gb*PLIα, *Gb*13*Eq*37*Gb*, in which the residues 13–36 were replaced by those of *Eq*PLIα-LP, was used, significant amounts of their heterotrimers were observed. By comparison of the respective inhibitory activities of the heterotrimeric subspecies, it was suggested that the inhibitory activity of the trimer was governed by one subunit with the highest activity, and not affected by the number of these subunits. The intermolecular electrostatic interactions between Glu23 and Lys28 of *Gb*PLIα were also suggested to be important in stabilizing the trimeric structure. The importance of the electrostatic interaction was supported by the less stability of the homotrimeric structure of a mutant *Gb*PLIα with a single amino acid substitution, *Gb*PLIα(K28E).

1. Introduction

Venomous snakes have three distinct types of phospholipase A_2 (PLA_2) inhibitory proteins (PLIα, PLIβ, and PLIγ) in their blood so as to protect themselves from the leakage of their own venom PLA_2s into the circulatory system [1–3]. PLIα is a 75-kDa trimeric glycoprotein of 20-kDa subunits having a C-type lectin-like domain (CTLD), which is homologous to those of collectins, such as serum mannose-binding protein and lung surfactant apoproteins [4]. CTLDs have been identified in a variety of proteins that interact with protein ligands without carbohydrate binding activity [5]. Besides PLIα, the M-type PLA_2 receptor and surfactant apoprotein A are the CTLD-containing proteins having PLA_2 binding activity [6–8]. PLIα has only been identified in the blood of *Viperidae* snakes, such as Habu *Protobothrops flavoviridis* [9], Chinese mamushi *Gloydius brevicaudus* [10], *Bothrops asper* [11], *Cerrophidion godmani* [12], *Bothrops moojeni* [13], and *Atropoides nummifer* [14], whereas PLIα homolog (PLIα-LP) lacking PLA_2 inhibitory activity was identified in the blood of the nonvenomous snakes *Elaphe quadrivirgata* [15] and *Elaphe climacophora* [16]. *P. flavoviridis* and *G. brevicaudus* PLIαs inhibit specifically the group II acidic PLA_2s from their own venom [17] and *B. asper*, *C. godmani*, *B. moojeni*, and *A. nummifer* PLIαs inhibit the group II basic myotoxic PLA_2s from their own venom. Since one molecule of trimeric PLIα binds stoichiometrically to one molecule of PLA_2, the trimeric structure of PLIα is critical to its inhibitory activity [18]. By constructing the chimeric proteins composed of *G. brevicaudus* PLIα (*Gb*PLIα) and *E. quadrivirgata* PLIα-LP (*Eq*PLIα-LP), the residues important in the binding and inhibition of PLA_2 were mapped on region 13–37, which were expected to be located in the helical neck region of the trimeric PLIα molecule [19]. Recently, we have shown that *P. flavoviridis* PLIα was composed of four different trimeric subspecies of two homologous subunits, PLIα-A and PLIα-B, and these four different trimers could be reconstituted through random association of the purified two subunits [18].

In the present study, we created the heterotrimers of PLIα composed of two different subunits derived from the recombinant *Gb*PLIα, *Eq*PLIα-LP, and *Gb*PLIα-*Eq*PLIα-LP chimera homotrimers,

Abbreviations: PLIα, α-type phospholipase A_2 inhibitory protein; PLA_2, phospholipase A_2; CTLD, C-type lectin-like domain; PLIα-LP, PLIα-like protein; GndHCl, guanidine hydrochloride; SPR, surface plasmon resonance

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which showed different affinities for *G. brevicaudus* acidic PLA_2, and investigated the inhibitory activities of the heterotrimers in order to estimate the contribution of each subunit to the total inhibitory activity as a trimeric PLA_2 inhibitory protein.

2. Materials and methods

2.1. Materials

G. brevicaudus venom were obtained from Japanese Snake Institute (Gunma, Japan). *G. brevicaudus* acidic PLA_2 was purified from the venom as described previously [17].

2.2. Expression of the recombinant PLIα in E. coli

The cDNA of *G. brevicaudus* PLIα (*Gb*PLIα) was cloned and inserted into pET-16b (Invitrogen, Carlsbad, CA, USA) as described previously [20]. The *E. quadrivirgata* PLIα-LP (*Eq*PLIα-LP), chimeric PLIα (*Gb*13*Eq*37*Gb* and *Eq*13*Gb*37*Eq*), and mutated *Gb*PLIα(K28E) expression plasmids were constructed as described previously [19]. The expression plasmid DNAs were used to transform competent *E. coli* strain BL21(DE3)pLysS (Invitrogen). The single colony of *E. coli* BL21 (DE3), harboring each expression vector, was inoculated into 5 ml of Luria-Bertani (LB) medium containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol and grown at 37 °C overnight. Then, 1 ml of culture was transferred to 2 l of LB medium containing carbenicillin and chloramphenicol in a jar fermenter (model MDL500, Marubishi, Tokyo, Japan) at 27 °C. When the absorbance at 600 nm reached 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM. After further cultivation for 4 h, the culture was centrifuged and the cells were washed with 50 mM Tris–HCl buffer (pH 8.0) and sonicated on ice. The inclusion body pellet was obtained by centrifugation at 25,000×*g* for 20 min at 4 °C after washing with 50 mM Tris–HCl buffer containing 4% Triton X-100.

2.3. Preparation of the recombinant PLIα

The obtained pellet was solubilized in 50 mM Tris–HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride (GndHCl) and 0.1 M NaCl. The mixture was then centrifuged at 15,000×*g* for 5 min to remove any remaining insoluble particles. The solution was then dialyzed against 50 mM Tris–HCl buffer (pH 8.0) containing 3 M GndHCl and 1 mM EDTA and the protein was refolded by stepwise dialysis against the same buffer containing 2 M GndHCl, then against the buffer containing 1 M GndHCl and 0.5 M arginine hydrochloride. After the further dialysis against the buffer containing 0.5 M arginine hydrochloride, the protein sample was finally dialyzed against 50 mM Hepes buffer (pH 7.5) containing 1 mM EDTA and NaCl to give an ionic strength of 0.2. The insoluble unfolded protein was removed by centrifugation at 15,000×*g* for 10 min. The folded trimeric PLIα was further purified by gel filtration chromatography on a Superdex 200 pg column (HiLoad 16/60, GE Healthcare, Buckinghamshire, England), which had been equilibrated with 50 mM Hepes buffer (pH 7.5, ionic strength of 0.2). From 0.5 to 3 mg of the folded trimeric recombinant PLIα preparations could finally be obtained from 2 l of the bacterial culture. No disulfide-bonded oligomers could be detected by SDS-PAGE of the preparations under non-reducing conditions. Furthermore, no free thiol groups could be detected in the folded PLIα preparations using Ellman's reagent, 5, 5′-dithiobis (2-nitrobenzoaic acid).

2.4. Reconstitution of the heterotrimeric PLIα

Equimolar amounts (about 5 nmol) of two molecular species of the homotrimeric recombinant PLIαs were mixed in 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 6 M GndHCl. The mixture was dialyzed against the same buffer containing 3 M GndHCl. The denaturant was gradually removed by stepwise dialysis against the buffers containing decreased concentrations of GndHCl. The reconstituted PLIαs were finally dialyzed against 50 mM Tris–HCl buffer (pH 8.0). When the reconstituted preparations were analyzed on Superdex 200 column, the main peak appeared at the position of trimeric form and no peaks at the position of monomeric form.

2.5. Separation of the heterotrimeric PLIα

Reconstituted PLIα was separated on a MonoQ column (HR5/5, GE Healthcare), which had been equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The adsorbed proteins were eluted with the same buffer containing a linear concentration gradient of NaCl, from 0 to 2 M.

2.6. PLA_2 inhibitory activity of heterotrimeric PLIα

PLA_2 inhibitory activity was estimated on the basis of the decrease in the PLA_2 activity which was assayed fluorometrically with 1-palmitoyl-2-(10-pyrenyldecanoyl)-sn-glycero-3-phosphorylcholine (10-Pyrene PC, Cayman Chemical, Ann Arbor, MI, USA) as a substrate according to the methods of Radvanyi et al. [21] with slight modifications described previously [10]. The concentrations of each heterotrimer were adjusted to 9.5×10^{-7} M in 50 mM Hepes buffer (pH 7.5, ionic strength 0.2). After the addition of *G. brevicaudus* acidic PLA_2 solution to the substrate solution (in 50 mM Hepes buffer containing 10 mM $CaCl_2$ and 0.1% bovine serum albumin), the rate of fluorescence increase at 398 nm (with excitation at 345 nm) by the PLA_2 activity was continuously monitored. Then, an aliquot of the sample solution was added to the reaction mixture, and the degree of inhibition was estimated by the difference in the rate of fluorescence increase before and after the addition of the sample solution.

2.7. Binding analysis by surface plasmon resonance

Bindings of *Gb*PLIα(K28E) to *G. brevicaudus* acidic PLA_2 were analyzed with a Biacore X system (GE Healthcare) as described previously [19]. Various concentrations of *Gb*PLIα(K28E) were injected at a flow rate of 10 μl/min at 25 °C with running buffer (50 mM Hepes buffer (pH 7.5, ionic strength of 0.2) containing 0.05% Tween 20, and 1 mM EDTA. Analysis of the association and dissociation curves was performed with the BIAevaluation 3.0 software using the 1:1 L binding model with drifting base line. During the fitting process, the dissociation rate constant (k_{diss}) and the maximum net increase of response units (RU_{max}) were used as global fitting parameters.

2.8. Edmundson wheel analysis

A Schiffer and Edmundson wheel analysis [22] was performed by means of GENETYX ver. 6 (Genetyx, Tokyo, Japan).

2.9. Fluorescence measurements

Tryptophyl fluorescence spectra of *Gb*PLIα and K28E in the presence of GndHCl were measured at 25 °C with a Hitachi model 850 fluorescence spectrophotometer. The final concentration of PLIα was adjusted to 1.5×10^{-7} M in 20 mM *N*-tris-(hydroxymethyl)-2-aminoethane sulfonic acid (TES) buffer (pH 7.5) containing various concentrations of GndHCl. The excitation wavelength was 290 nm. The fluorescence of a reference solution of *N*-acetyl-L-tryptophanamide was measured just before and again after the measurement of the sample solution so as to correct for small instrumental fluctuations.