Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Insights on the structure of native CNF, an endogenous phospholipase A_2 inhibitor from *Crotalus durissus terrificus*, the South American rattlesnake

Q1 Consuelo Latorre Fortes-Dias [a,*], Paula Ladeira Ortolani [a], Carlos Alexandre H. Fernandes [b], Kelli Roberta Lobo [a], Lutiana Amaral de Melo [a], Márcia Helena Borges [a], Wallance Moreira Pazin [c], Mário de Oliveira Neto [b], Roberto Morato Fernandez [b], Marcos Roberto M. Fontes [b,*]

[a] *Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias (FUNED), Rua Conde Pereira Carneiro 80, CEP 30510-010, Belo Horizonte, MG, Brazil*
[b] *Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista, UNESP, Botucatu, SP, Brazil*
[c] *Departamento de Física e Matemática, Faculdade de Filosofia Ciências e Letras, USP, Ribeirão Preto, SP, Brazil*

ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 28 April 2014
Accepted 1 May 2014
Available online xxxx

Keywords:
Phospholipase A_2 inhibitor
PLI
Phospholipase A_2
PLA_2
Crotalus
Quaternary structure

ABSTRACT

Q6 Several snake species possess endogenous phospholipase A_2 inhibitors (sbPLIs) in their blood plasma, the primary role of which is protection against an eventual presence of toxic phospholipase A_2 (PLA_2) from their venom glands in the circulation. These inhibitors have an oligomeric structure of three to six subunits and have been categorized into three classes (α, β and γ) based on their structural features. SbγPLIs have been further subdivided into two subclasses according to their hetero or homomeric nature, respectively. Despite the considerable number of sbγPLIs described, their structures and mechanisms of action are still not fully understood. In the present study, we focused on the native structure of CNF, a homomeric sbγPLI from *Crotalus durissus terrificus*, the South American rattlesnake. Based on the results of different biochemical and biophysical experiments, we concluded that, while the native inhibitor occurs as a mixture of oligomers, tetrameric arrangement appears to be the predominant quaternary structure. The inhibitory activity of CNF is most likely associated with this oligomeric conformation. In addition, we suggest that the CNF tetramer has a spherical shape and that tyrosinyl residues could play an important role in the oligomerization. The carbohydrate moiety, which is present in most sbγPLIs, is not essential for the inhibitory activity, oligomerization or complex formation of the CNF with the target PLA_2. A minor component, comprising no more than 16% of the sample, was identified in the CNF preparations. The amino-terminal sequence of that component is similar to the B subunits of the heteromeric sbγPLIs; however, the role played by such molecule in the functionality of the CNF, if any, remains to be determined.

1. Introduction

Snake venoms are amongst the richest sources of phospholipases A_2 (PLA_2, EC 3.1.1.4), a widespread superfamily of structurally related enzymes that hydrolyze glycerophospholipids in lysophospholipids and free fatty acids. Based on molecular and biochemical criteria, secretory PLA_2 ($sPLA_2$) has been classified into 15 different groups [1]. Snake venom PLA_2 belongs to groups I and II, which also encompasses pancreatic and inflammatory $sPLA_2$.

The $sPLA_2$s in snake venoms may exert their deleterious actions as monomeric or multimeric toxins with at least one catalytically active subunit. A well-known example of a multimeric $sPLA_2$ is the crotoxin (Ctx), a β-neurotoxin [2], which is the major toxic component of the venom of the South American rattlesnake, *Crotalus durissus terrificus*. Ctx has a heterodimeric structure formed by an acidic, non-enzymatic subunit (CA) and a basic, enzymatically active counterpart (CB) [3] that are strongly bound by non-covalent interactions. CA acts as a chaperone, preventing the non-specific adsorption of CB to membrane structures other than its physiological target, thereby enhancing the pharmacological potency and, consequently, the lethal effect of CB [4]. The structural and functional activities of Ctx have been recently reviewed by [5].

Aiming, primarily, at a physiological protection against the eventual presence of venom gland contents in their circulating blood, several snake species have been provided with PLA_2 inhibitors, generally referred to as sbPLIs (snake blood phospholipase A_2 inhibitors). During the last two decades, a growing number of sbPLIs has been described for several venomous and non-venomous snake species [6]. These inhibitors have an oligomeric structure of, at least, three subunits and, based on known mammalian protein domains, they have been categorized into three structural classes (α, β and γ). Members of these three classes can be concomitantly present in a single snake species.

* Corresponding authors.
E-mail addresses: consuelo.latorre@funed.mg.gov.br (C.L. Fortes-Dias), mrfontes@ibb.unesp.br (M.R.M. Fontes).

http://dx.doi.org/10.1016/j.bbapap.2014.05.001
1570-9639/

The αPLIs have a C-type lectin domain, whereas the distinguishing feature in the βPLIs is the occurrence of leucine-rich repeats, known as LRR. The members of these three classes can be concomitantly PLIs, which in turn, are composed of two structural units of highly conserved tandem repeats of half cysteines known as three-finger motifs [7]. Based on the current understanding, the last class comprises the highest number of sbPLIs that are, typically, oligomers of glycosylated and non-glycosylated subunits. Based on the identity of their subunits, Lizano et al. [8] proposed a sub-classification into heteromeric (subclass I) or homomeric (subclass II) sbMembers of these three classes can be concomitantly PLIs.

A gamma sbγPLI from *C. d. terrificus* snakes, named CNF (for an acronym of *Crotalus* neutralizing factor) has been extensively studied by the authors of this paper, leading to important conclusions on its mechanism of action. CNF is able to displace CA from the native Ctx complex and to bind tightly to CB, thus forming a stable CNF.CB complex. CNF.CB is devoid of any PLA_2 activity and may be considered as reminiscent of the interaction of Ctx with its target receptor at the pre-synaptic neuromuscular junctions [9,10]. Native CNF is an oligomer of glycosylated and non-glycosylated single subunits of 24 kDa and 20 kDa [9] and has been assigned to subclass II of sbγPLIs [8]. However, fundamental questions remain to be answered such as the number of subunits in the oligomer, the role of glycosylation in the inhibitor functionality and its homomeric character. These issues are not exclusive to CNF but can be extended to most sbγPLIs. Although oligo/polymeric structures have been assigned to all of these inhibitors, the number of forming subunits, in most cases, remains undetermined. Regarding glycosylation, the ability to bind or to inhibit phospholipases A_2 was solely demonstrated for homologous recombinants with no carbohydrates from *Python* and *Protobothrops* inhibitors [11,12]. In addition, the fact that a second subtype of gamma inhibitor was isolated from a cDNA library from *Protobothrops flavoviridis* snake liver [13] made the homomeric character of the subclass II sbγPLIs questionable. The translated protein is similar to the subunit B of heteromeric sbγPLIs.

In the present study, we focused on the native structure of CNF, the sbγPLI from *C. d. terrificus* snakes, in an effort to increase our understanding of oligomerization, role of glycosylation and subunit composition.

2. Materials and methods

2.1. Purification of CNF and crotoxin

Heparinized blood plasma from *C. d. terrificus* snakes was obtained from the Serpentarium of Fundação Ezequiel Dias, depending on the availability of specimens. The procedure followed the protocol approved by the Committee for Ethics in Animal Use of the Fundação Ezequiel Dias (CEUA FUNED 022/2012). Native CNF was purified in two steps, *i.e.*, ion exchange in DEAE-Sephacel [14] followed by a hydrophobic interaction chromatography on a Hitrap Phenyl FF (GE HealthCare) [7], starting with plasma from different *C. d. terrificus* specimens. All the final preparations consistently displayed the expected 24 kDa and 20 kDa protein bands on SDS-PAGE [15], under variable ratios. These bands correspond to the glycosylated and the non-glycosylated monomers, respectively, in native CNF. The non-glycosylated monomer is always present at much lower proportion than the glycosylated monomer.

Crotoxin (Ctx) was purified from whole *C. d. terrificus* snake venom by classical gel filtration on Sephadex G75 [16].

2.2. Gel filtration chromatography

Gel filtration chromatography was performed in an Akta Purifier 10 (GE HealthCare) with Protein-Pak 300 SW (Waters, Millipore Co.), Superose 6 HR10/30, Superose 12 HR10/30 and Superdex 200 HR10/30 (GE HealthCare) columns, using 0.1 M sodium phosphate pH 7.0 as elution buffer. Each column was previously calibrated with the following reference proteins acquired from Sigma Co: aprotinin (A1153), bovine serum albumin (Sigma A7517), cytochrome C (C3131), concanavalin A (C2010), ovalbumin (A7642), and RNase (R6513). Catalase, ferritin and thyreoglobulin from an old kit (Pharmacia Fine Chemicals, 170441-01) complemented the range of reference molecular masses. Firstly, each protein was loaded individually to guide their unequivocal identification in the elution profiles of the protein mixture. Calibration curves were constructed by the linear least squares regression method using the Graph Prism® 6.0 for Mac OS X (GraphPad software Inc., La Jolla, California). Horseradish peroxidase (HRPO P6782, Sigma Co.) and Ctx (prepared in our laboratory) were run as controls of a glycosylated and a non-glycosylated protein, respectively.

2.3. Cross-linking assays

BS^3 (Bis[sulfosuccinimidyl]suberate, Pierce) or glutaraldeyde (25% EM grade, Merck) was added in increasing concentrations (1 to 10 mM) to a fixed amount of CNF (native or enzymatically deglycosylated) in 20 mM HEPES pH 7.5, achieving a final concentration of 6.7 mg/ml of the inhibitor. A control sample was prepared using the same concentration of CNF but omitting the BS^3. In addition, a control experiment was performed with 6.7 mg/ml of bovine serum albumin (B2518 from Sigma Co.) in the presence of increasing concentrations of BS^3 (1 to 10 mM), in order to check for non-specific reactions of the cross-linker. The mixtures were incubated in the dark for 3 h at room temperature before subjecting them to the analysis by SDS-PAGE on 8–25% gradient Phast® gels (GE HealthCare) under non-reducing conditions. The CNF molecular mass (in kDa) was calculated by interpolation, using the calibration curves obtained with protein markers and expressed as mean ± SD of two independent experiments per cross-linker ($n = 4$).

The cross-linking experiment was repeated by incubating increasing concentrations of native CNF with 10 mM of BS^3. The final concentrations of CNF were 6.7, 16.7 and 33.3 mg/ml. A control sample was prepared with 6.7 mg/ml of CNF but omitting the BS^3. The cross-linked samples were analyzed by SDS-PAGE, as before.

2.4. Dynamic light scattering

Dynamic light scattering (DLS) measurements with native CNF (2.5 mg/ml in 10 mM ammonium formate pH 6.5) were carried out at 4 °C (277 K), 10 °C (283 K) and 18 °C (291 K) on a DynaPro Titan equipment (Wyatt Technology). The average values of one hundred measurements were analyzed with the Dynamics DynaPro version 6.10 software.

2.5. Small angle X-ray scattering

Small angle X-ray scattering (SAXS) data for CNF was collected on the D02A-SAXS2 beam line at the Brazilian Synchroton Light Laboratory (LNLS). CNF samples were prepared in 20 mM ammonium formate pH 6.5 at the concentrations of 1 and 5 mg/ml (41.7 μM and 208.4 μM, respectively). The samples were centrifuged for 30 min at 23,500 ×g at 4 °C to remove potential aggregates. The radiation wavelength was set to 1.48 Å and a 165 nm MarCCD detector was used to record the scattering patterns. The sample-to-detector distance was set to 1000 mm to obtain the range of the scattering vector q from 0.013 to 0.33 $Å^{-1}$, where q is the magnitude of the q-vector defined by $q = 4\pi \sin\theta/\lambda$ (2θ is the scattering angle). Two successive frames, each of 300 s duration, were recorded for each sample to monitor radiation damage and beam stability. After buffer scattering subtraction, protein SAXS patterns were integrated using Fit2D software [17]. The radius of gyration, R_g, was computed from the Guinier equation [18] and by indirect Fourier transform method using the Gnom package [19]. The distance distribution p(r) also was calculated using the Gnom, and the maximum diameter, D_{max}, was obtained. The molecular mass estimation was calculated using the SAXS Mow web tool [20].

Please cite this article as: C.L. Fortes-Dias, et al., Insights on the structure of native CNF, an endogenous phospholipase A_2 inhibitor from Crotalus durissus terrificus, the South American..., Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbapap.2014.05.001