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Biochemical and monolayer characterization of Tunisian snake venom phospholipases

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ABSTRACT

The present study investigated the kinetic and interfacial properties of two secreted phospholipases isolated from Tunisian vipers'venoms: *Cerastes cerastes* (CC-PLA2) and *Macrovipera lebetina transmediterranea* (MVL-PLA2). Results show that these enzymes have great different abilities to bind and hydrolyse phospholipids. Using egg-yolk emulsions as substrate at pH 8, we found that MVL-PLA2 has a specific activity of 1473 U/mg at 37 °C in presence of 1 mM $CaCl_2$. Furthermore the interfacial kinetic and binding data indicate that MVL-PLA2 has a preference to the zwitterionic phosphatidylcholine monolayers (PC). Conversely, CC-PLA2 was found to be able to hydrolyse preferentially negatively charged head group phospholipids (PG and PS) and exhibits a specific activity 9 times more important (13 333 U/mg at 60 °C in presence of 3 mM $CaCl_2$). Molecular models of both CC-PLA2 and MVL-PLA2 3D structures have been built and their electrostatic potentials surfaces have been calculated. A marked anisotropy of the overall electrostatic charge distribution leads to a significantly difference in the dipole moment intensity between the two enzymes explaining the great differences in catalytic and binding properties, which seems to be governed by the electrostatic and hydrophobic forces operative at the surface of the two phospholipases.

1. Introduction

Snake venoms are complex mixtures of biological actives molecules [1], among them phospholipase A2 enzymes (EC 3.1.1.4) are the best-characterized components. Snake venoms phospholipases A2 are classified into two groups [2]. The secreted phospholipase A2 from Viperidae snake venoms fall under group II. They are generally Ca^{2+} dependant enzymes that catalyze the hydrolysis of the sn-2 fatty acid bond of phospholipids to release free fatty acids and lysophospholipids. These are small proteins (13–14 KDa), containing 120–125 amino acid residues and 7 disulfide bridges. The amino acid sequences of over 280 snake venom PLA2 enzymes have been determined (A database of snake venom PLA2 enzymes is available at http://sdmc.lit.org.sg/Templar/DB/snaketoxin_PLA2/index.html). Their structural comparison indicates that they share 40–99% identity in their amino acid sequences and hence significant similarity in their three dimensional folding [3]. They have a partially conserved structure that defines the PLA2 fold [4]. Despite this similarity, sPLA2 exhibit a wide variety of pharmacological properties such as anticoagulant, platelet aggregation inhibiting [5], bactericidal [6], anti-HIV [7], antimalarial and antiparasitic [8], antitumor [9] and antiangiogenic effects [10].

Generally there is a tendency to correlate pharmacological activities of a PLA2 enzyme to its phospholipid hydrolyzing ability. The in vitro studies can provide excellent information about the ability of the enzyme to hydrolyze specific phospholipids based on its

Abbreviations: PL, phospholipids; sPLA2, secreted phospholipase A2; CC-PLA2, *Cerastes cerastes* venom phospholipase A2; MVL-PLA2, *Macrovipera lebetina transmediterranea*; 1,2 DLPC, l-2 dilauroyl-sn-glycero-3-phosphatidylcholine; 1,2 DLPE, l-2dilauroyl-sn-glycero-3-phosphatidylethanolamine; 1,2 DLPG, l-2 dilauroyl-sn-glycero-3-phosphatidylglycerol; 1,2 DLPS, l-2dilauroyl-sn-glycero-3-phosphatidylserine.

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head group specificity and/or fatty acyl group selectivity. It also helps determining the catalytic efficiency of the enzyme [4]. An interesting approach to study protein–lipid interactions is the use of lipid monolayers spread at the air/water interface [11]. It is a useful tool for comparing the relative surface activities of various proteins or in the case of a given protein its interactions with various lipid monolayers spread over an aqueous subphase of a given composition.

Two Tunisian Viperidae snakes are known and have been well studied: the first one is *Macrovipera lebetina* and the second one is *Cerastes cerastes*. Both of them contain non cytotoxic sPLA2. *Macrovipera lebetina* venom sPLA2 (MVL-PLA2) has a molecular mass of 13626.64 ± 12 Da with an isoelectric point of 4.69 [12].

Cerastes cerastes venom sPLA2 called CC-PLA2 is an acidic enzyme which has a molecular weight of 13737.52 Da and reported to be the first glycosylated phospholipase A2 purified from snake venom with a rate of glycosylation of 2.5% [13]. The two enzymes showed anti-tumoral activities identified by their inhibition of adhesion and migration of tumoral cell lines. These pharmacological effects are dose dependent and do not depend on their enzymatic activities [13,14]. Thus, the authors showed that CC-PLA2 present a high enzymatic activity. However, to our knowledge, enzymatic activity of MVL-PLA2 was not reported. The aim of this work is to characterize CC-PLA2 and MVL-PLA2 with respect to their biochemical and interfacial properties in the same conditions. Moreover, for further comparison, 3-D structure models of the two enzymes are performed in an attempt to establish a structure-function relationship.

2. Material and methods

2.1. Chemicals

NaCl, $CaCl_2$, Tris–HCl, Ethylene Diamine Tetra Acetic acid (EDTA), Sodium taurodeoxycholate (NaTDC), ethylene glycol tetra acetic acid (EGTA)were purchased from Sigma–Aldrich (St. Quentin-Fallavier, France). Chloroform supplied from SDS (Peypin, France) was used as spreading solvent.

2.2. Phospholipids

l-2dilauroyl-sn-glycero-3-Phosphatidylcholine (1,2 DLPC), l-2 dilauroyl-sn-glycero-3-Phosphatidylethanolamine (1,2 DLPE),l-2 dilauroyl-sn-glycero-3-Phosphatidylglycerol (1,2 DLPG), l-2 dilauroyl-sn-glycero-3-Phosphatidylserine (1,2 DLPS) were purchased from Avanti Polar Lipids. All substrates are used without further purification.

2.3. Phospholipases A2

CC-PLA2 and MVL-PLA2 were purified from Two Tunisian Viperidae snakes: *Cerastes cerastes* and *Macrovipera lebetina* as described previously by Zouari-Kessentini et al. [13] and Bazaa et al. [12] respectively.

2.4. Determination of protein concentration

Protein concentration was measured spectrophotometrically according to the Bradford method [15], using BSA as a standard.

2.5. Evaluation of the level of phospholipase activity

Phospholipase activity was measured titrimetrically with a pH-stat Metrohm (Switzerland), using egg-yolk emulsions as substrate in the presence of different concentrations of NaTDC (0–10 mM) and $CaCl_2$ (0–10 mM). With the pH-stat method, phospholipase activity is measured on a mechanically stirred emulsion. Free fatty acids released with time are neutralized by adding titrated NaOH (0.1 N) in order to maintain the pH at a constant end point value. CC-PLA2 was used at a concentration of 0.3 mg/ml and MVL-PLA2 at a concentration of 0.86 mg/ml. One unit of phospholipase activity corresponds to 1 μmole of fatty acid liberated per minute under standard conditions.

2.6. Effect of temperature on phospholipases activities and stabilities

The CC-PLA2 (0.3 mg/ml) and MVL-PLA2 (0.86 mg/ml) activities were determined at different temperatures (25–60 °C) at pH 8.5. The thermal stability was studied by incubating each enzyme at various temperatures (25–90 °C) for 30 min and measuring, after centrifugation, the residual activity under standard assay condition, using egg-yolk emulsion as substrate.

2.7. Monomolecular film technique for kinetic measurements on sPLA2

The monolayer study was performed as described previously by Pattus et al. [16]. Prior to each experiment, the Teflon trough used to form the monomolecular film was cleaned with water before being gently brushed with distilled ethanol and washed again with tap water. The aqueous subphase contained 10 mM Tris–HCl, pH 8, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA with all phospholipases tested. The buffer was prepared with double distilled water and filtered through a 0.22 μm Millipore filter. Any residual surface-active impurities were removed before each assay by sweeping and suction of the surface. Kinetic experiments were performed at room temperature with a KSV-2200 barostat (KSV Helsinki) and a "zero-order" Teflon trough equipped with a mobile Teflon barrier, which was used to compensate for the substrate molecules removed from the film by enzyme hydrolysis, thus maintaining the surface pressure constant. The latter was measured using a Wilhelmy plate (perimeter 3.94 cm) attached to an electro-balance, which was connected in turn to a microprocessor controlling the movements of the mobile barrier. The subphase of the reaction compartment was continuously agitated with a 2-cm magnetic stirrer moving at 250 rpm. The enzyme solution (1–2 μl) was injected through the film over the stirrer with a Hamilton syringe. The surface area of the reaction compartment was 120 cm^2 and its volume was 120 ml. The reservoir compartment was 148 mm wide and 249 mm long. Hydrolysis of phospholipid monolayers by sPLA2 results in the formation of lysophospholipid and free fatty acid. As these products are more water-soluble than the native phospholipid, they desorb from the monolayer, which results in a surface pressure decrease. Thus, phospholipase activity was monitored as change in surface pressure at constant total area. Activities were expressed as the number of moles of substrate hydrolysed by unit time and unit surface of the reaction compartment of the "zero-order" trough for appropriate sPLA2 concentration. All the experiments were carried out at 37 °C.

2.8. Molecular modeling of CC-PLA2 and MVL-PLA2

Entries with accession numbers P0CAR9 and B5U6Z2 corresponding to CC-PLA2 and MVL-PLA2, respectively were fetched from the UniProt database. The complete mature forms of the proteins were used for the modeling. The homology models of MVL-PLA2 and CC-PLA2 were obtained by submitting the sequences to the Phyre2 server [17] using profile searching and Hidden Markov models to identify structural homologous of the protein target [18]. Models were refined in two stages of energy minimization using CHARMM molecular modeling package [19]. The best returned