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Crystal structure of a novel myotoxic Arg49 phospholipase A_2 homolog (zhaoermiatoxin) from *Zhaoermia mangshanensis* snake venom: Insights into Arg49 coordination and the role of Lys122 in the polarization of the C-terminus

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Abstract

The venom of *Zhaoermia mangshanensis*, encountered solely in Mt Mang in China's Hunan Province, exhibits coagulant, phosphodiesterase, L-amino acid oxidase, kallikrein, phospholipase A_2 and myotoxic activities. The catalytically inactive PLA_2 homolog referred to as zhaoermiatoxin is highly myotoxic and displays high myonecrotic and edema activities. Zhaoermiatoxin possesses a molecular weight of 13,972 Da, consists of 121 amino-acid residues cross-linked by seven disulfide bridges and shares high sequence homology with Lys49-PLA_2s from the distantly related Asian pitvipers. However, zhaoermiatoxin possesses an arginine residue at position 49 instead of a lysine, thereby suggesting a secondary Lys49→Arg substitution which results in a catalytically inactive protein. We have determined the first crystal structure of zhaoermiatoxin, an Arg49-PLA_2, from *Zhaoermia mangshanensis* venom at 2.05 Å resolution, which represents a novel member of phospholipase A_2 family. In this structure, unlike the Lys49 PLA_2s, the C-terminus is well ordered and an unexpected non-polarized state of the putative calcium-binding loop due to the flip of Lys122 towards the bulk solvent is observed. The orientation of the Arg-49 side chain results in a similar binding mode to that observed in the Lys49 PLA_2s; however, the guadinidium group is tri-coordinated by carbonyl oxygen atoms of the putative calcium-binding loop, whereas the Nζ atom of lysine is tetra-coordinated as a result of the different conformation adopted by the putative calcium-binding loop.

Keywords: *Zhaoermia mangshanensis*; Snake venom; Arg49-phospholipase A_2; Lys49-phospholipase A_2; Myotoxin; Crystal structure

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1. Introduction

Envenoming from snakebites is a major neglected problem of the 21st century. Besides numerous fatalities, it results in a significantly large number of victims who survive with permanent physical and psychological sequelae mostly due to the local tissue-damaging effects of snake venoms (Gutiérrez et al., 2006). Among the three distinct groups of snake venom proteins that are responsible for the direct induction of myonecrosis, the most important ones from pitviper venoms are group II phospholipase A_2 (PLA_2, EC 3.1.1.4) myotoxins. These have been divided into two main subgroups based primarily on the presence of a conserved aspartic acid residue at position 49 in the catalytically active Asp49-PLA_2 enzymes, or a lysine in homologues (Lys49-PLA_2s; reviewed in Arni and Ward, 1996; Ownby et al., 1999; Murakami and Arni, 2003) that are considered to be catalytically inactive due to their inability to bind the cofactor Ca^{2+}, which in turn prevents the stabilization of the tetrahedral intermediate observed in the Ca^{2+}-dependent catalytic reaction promoted by Asp49-PLA_2s (Van den Berg et al., 1989).

Although catalytically inactive, Lys49-PLA_2s are truly multi-functional venom proteins that are highly expressed in pitviper venoms. They display inflammatory properties and induce edema, an activity for which alternative mechanisms have been proposed in the absence of phospholipid hydrolysis or mobilization of arachidonic acid (Lomonte et al., 1993, 1994a, b; Teixeira et al., 2003; Zuliani et al., 2005), and exhibit broad antibacterial activities (Páramo et al., 1998; Santamaría et al., 2005). Recently, Lys49-PLA_2s have been implicated in the inhibition of the vascular endothelial growth factor and its receptor system, which plays a central role in angiogenesis (Yamazaki et al., 2005). However, from a clinical point of view, the highly potent induction of myonecrosis by Lys49-PLA_2s is most significant. This important phenomenon, its mechanisms and their possible inhibition have received considerable attention. For example, strategies to elucidate the structural determinants for the myotoxicity of Lys49-PLA_2s have included chemical modification (Andrião-Escarso et al., 2000), sequence comparison (Selistre de Araujo et al., 1996; Ward et al., 1998), charge distribution (Kini and Iwanaga, 1986; Kini and Evans, 1989), hydrophobicity profile (Kini and Iwanaga, 1986), synthetic peptide (Lomonte et al., 1994b; Núñez et al., 2001) and site-directed mutagenesis (Ward et al., 1995, 2002) studies, and compounds such as suramin, heparin, heparin-like glycosaminoglycans, related polyanions and polyethylene glycol derivatives have been used to effectively inhibit the activity of myotoxic Lys49-PLA_2s both *in vitro* and *in vivo* (Murakami et al., 2005, 2007).

Apart from the large group of Lys49-PLA_2s from pitvipers, a few other variants replacing Asp49 at this generally highly conserved position in snake venom group II PLA_2s have been reported, e.g., Ser49-PLA_2s from the venoms of two true vipers (Krizaj et al., 1991; Polgar et al., 1996) and Asn49-PLA_2s from two Asian pitvipers (Pan et al., 1998; Tsai et al., 2004) that probably originated from at least two independent Asp49→Asn substitutions within the subgroup of basic Arg6-Asp49-PLA_2s (Mebs et al., 2006). Recently, a novel Arg49 PLA_2 analog (zhaoermiatoxin, Mebs et al., 2006) was reported from the venom of the Mt. Mang Viper, *Zhaoermia mangshanensis* (Zhao and Chen, 1990), a rare pitviper known only from a single mountain range in China's Hunan Province. This Arg49-PLA_2 is the major toxin of *Z. mangshanensis* (formerly *Trimeresurus mangshanensis*, *Ermia mangshanensis*; see Gumprecht and Tillack, 2004) and induces edema and myonecrosis in mice in the absence of catalytic activity upon phospholipids. Sequence comparison of zhaoermiatoxin reveals that the novel toxin possesses most of the residues of the Lys49-PLA_2 subgroup, displays more than 80% identity to two Lys49-PLA_2s from two distantly related Asian pitvipers, and is rooted within a comprehensive sample of Lys49-PLA_2s in phylogenetic analyses (Mebs et al., 2006). In this context, zhaoermiatoxin as a member of the Lys49-PLA_2 subgroup, and its Arg49 substitution as a secondary Lys49→Arg substitution does not alter its catalytic inactivity.

We present the first crystal structure of an Arg49 phospholipase A_2 homolog isolated from *Z. mangshanensis* venom at 2.05 Å resolution, which possesses a well-ordered C-terminus and an unexpected non-polarized state of the putative calcium-binding loop tetra-coordinating by guanidinium group of Arg49 and with Lys122 oriented towards the bulk solvent.

2. Materials and methods

2.1. Crystallization and X-ray data collection

The purification of zhaoermiatoxin was carried out using gel-filtration chromatography followed by