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Insights into the subunit arrangement and diversity of paradoxin and taipoxin

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ABSTRACT

Paradoxin and taipoxin are neurotoxic phospholipases from the inland and coastal species of Australian taipan. Despite their relatively high sequence homology of 70% and 84% for the acidic and basic chains respectively, they differ substantially in reported assays of neurotoxicity. This study provides the first characterisation of paradoxin, which like taipoxin, is a trimer at physiological pH. More broadly, these toxins were found to be composed of a more diverse range of subunits than previously recognised, including newly discovered γ_{TPx} isoforms, which give rise to an additional, major conformation of TPx.
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1. Introduction

Snake venom is a predominantly proteinaceous mixture, which incorporates phospholipase A_2 (PLA_2) enzyme(s) as a major constituent (Burke and Dennis, 2009). These enzymes can exist as monomers or multimers (Doley and Kini, 2009), which are capable of inducing a range of cytotoxic effects, such as; neurotoxicity, myotoxicity, cardiotoxicity and antiplatelet activity (Chow et al., 1998; Montecucco et al., 2008; Sribar et al., 2014). The binding targets of PLA_2 have yet to be identified, and the structure-function relationship of these toxins is not well understood (Hodgson and Wickramaratna, 2002; Montecucco et al., 2008). However, models have been proposed whereby PLA_2 binds to specific cell surface proteins in order to disrupt cellular membranes (Montecucco et al., 2008; Sribar et al., 2014; Tonello et al., 2012).

PLA_2 found in snake venom is capable of forming potent multimeric complexes (Doley and Kini, 2009; Montecucco and Rossetto, 2008), with heteromeric PLA_2 species containing one active subunit, whose toxicity is augmented by additional inactive subunits (Doley and Kini, 2009; Montecucco and Rossetto, 2008). It has been hypothesised that the inactive subunits within these complexes increase the binding affinity of PLA_2 for its cellular targets (Montecucco and Rossetto, 2008), and studies have shown that the subunit isoforms of multimeric PLA_2 can have different pharmacological activities (Cendron et al., 2012; Faure et al., 1993). Of the PLA_2 tested so far, the heteromeric species from *Oxyuranus microlepidotus* (paradoxin), *Oxyuranus scutellatus* (taipoxin) and *Pseudonaja textilis* (textilotoxin) seem to be the most potent neurotoxins in mouse models (Hodgson and Wickramaratna, 2002).

Paradoxin (PDx) and taipoxin (TPx) are hetero-trimers, composed of three unique subunits ($\alpha\beta\gamma$), which have similar amino acid profiles (Fohlman, 1979; Fohlman et al., 1976). PDx and TPx inhibit nerve twitches in chick-biventer models at comparable rates and concentrations, however, of the PLA_2 tested, PDx exhibits greatest neurotoxicity in mice (Hodgson and Wickramaratna, 2002; Hodgson et al., 2007). The structural similitude of these toxins, yet variance in specific toxicity, suggests they are ideal targets to help elucidate the structure function relationship of venom PLA_2. However, to date, the quaternary topography and range of subunit isoforms within each complex are yet to be fully described.

Over the last two decades, electrospray ionisation mass spectrometry (ESI-MS) has become a powerful tool for interrogating proteins, particularly the quaternary architecture of large multisubunit assemblages (Nesvizhskii et al., 2003; Schmidt et al., 2003; van Duijn, 2010), as well as investigating the non-covalent

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features of protein–protein interactions (Dodds et al., 2011). These techniques have been used previously to describe the quaternary architecture of textilotoxin, a hexameric PLA_2 from the brown snake (Aquilina, 2009). In the present study, the native subunit configuration and subunit complexity of PDx and TPx have been probed using MS.

2. Materials and methods

2.1. Materials

Lyophilised whole venom was a gift from Venom Supplies Pty Ltd (Tanunda, South Australia). All reagents used were of analytical grade unless specified. Ammonium acetate (NH_4OAc), caesium iodide (CsI), acetonitrile (ACN), guanidinium hydrochloride (GuHCl) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, U.S.A).

2.2. Size exclusion chromatography (SEC) and reverse phase HPLC experiments

O. microlepidotus and *O. scutellatus* lyophilised whole venom was dissolved in 200 mM NH_4OAc and loaded onto a Superdex 200 10/300 size exclusion column (GE Healthcare, Uppsala, Sweden), which was equilibrated with 200 mM NH_4OAc (pH 7.0). Samples were stored at −20 °C until required for testing. HPLC fractionation of the toxins into component subunits was performed as described elsewhere (Cendron et al., 2012) with the following exceptions; Samples where dissociated with GuHCl for 1 h prior to loading on column; HPLC fractions were reduced in volume using an SC-100 Savant Speed Vac (Waltham, Massachusetts, USA) and frozen at −20 °C until required for MS.

2.3. Mass spectrometry analysis of native subunits

All spectra were acquired using a Synapt HDMS time-of-flight mass spectrometer (Waters, Manchester, UK), and calibrated using a CsI spectrum acquired on the same day. Samples were introduced into the vacuum region of the instrument by electrospraying a 2 μL solution from gold coated borosilicate nanoelectrospray (nanoESI) capillaries (prepared in-house). Conditions for the acquisition of MS spectra were as follows (unless otherwise stated): capillary voltage, 1.5 kV; sample cone, 140 V; extraction cone, 4 V; trap collision 12.5 V; transfer collision 25 V; collision cell gas pressure, 1.16×10^{-2} mbar; backing gas, 4.0×10^{0} mbar. All protein samples were suspended in 200 mM NH_4OAc prior to spraying.

2.4. Collision induced dissociation (CID) of trimer peaks

For these experiments, *m/z* values corresponding to the 13^+ charge states of the trimer species were subjected to CID. For each of these charge states, the trap and transfer collision energies were increased from 12.5 to 30 V and 25–55 V in increments of 5 V with 10 scans at each increment, with a low mass resolution of 4.7 and a high mass resolution of 15.0.

3. Results and discussion

PLA_2 toxins are abundant proteins in elapid snake venom and are readily purified from the crude secretions. In this study, we performed SEC of *O. microlepidotus* and *O. scutellatus* whole venoms, which were separated into four and five distinct size-based fractions respectively (Fig. 1 from the Data in Brief paper). SDS-PAGE analysis of the sample corresponding to the A^{280} maximum of peak 2 (Fig. 1 inset from the Data in Brief paper) resolved a distribution of proteins with apparent molecular masses similar to those reported for monomeric PLA_2 toxins (Cendron et al., 2012; Fohlman, 1979). These data are in agreement with previous studies, in which PDx and TPx were found to elute as constituents of the second SEC peak, and to which the remainder of this study is confined.

PDx and TPx have been reported to be functional heterogeneous trimers, therefore we used MS to examine the protein population of peak 2, under conditions known to preserve the non-covalent interactions responsible for protein quaternary structure (Aquilina et al., 2013; Sobott et al., 2002), (Fohlman, 1979; Fohlman et al., 1976). A mass spectrum of *O. microlepidotus* isolate contained four distinct charge state series in the range 1700 to 4300 *m/z* (Fig. 1). Three of these, clustered in the range 1700 to 2800 *m/z*, corresponded to proteins of mass 13,345, 13,895 and to 19,309 Da; the same range of masses reported for the analogous subunits of TPx (Cendron et al., 2012; Fohlman, 1979). Within this *m/z* region, the species of lowest mass (13,345 Da) was tentatively identified as β_{PDx}, as it was closest in mass to the corresponding β_{TPx} (Cendron et al., 2012; Lind, 1982).

The charge states corresponding to a 19,309 Da protein were relatively broad compared to others in this *m/z* region, which is typical of glycosylated protein (Aquilina, 2009). The apparent glycosylation and similarity in mass to γ_{TPx} suggested that this was the corresponding subunit of PDx (Fohlman et al., 1977, 1976). By a process of elimination, the remaining species within this *m/z* range (13,895 Da) was assumed to be α_{PDx}. The similarity in mass between this protein, and that reported for α_{TPx}, would support this assumption (Cendron et al., 2012).

The most abundant ion series in the spectrum, between 3500 and 4300 *m/z*, corresponded to a protein of mass ~46,577 Da, accompanied by a series of low abundance ions arising from a 46,100 Da protein. The substantial mass and dominant ion intensity of the 46,577 Da species indicated that this was a non-covalent assemblage, the partial dissociation of which had given rise to the individual subunits observed at lower *m/z*. The aggregate of our observed masses for the α, β, and γ subunits as a 1:1:1 trimer is 46,549 Da—in good agreement with the major species in the spectrum.

Similar to PDx, the mass spectrum of TPx (Fig. 2) contained three distinct charge state series in the monomer region, corresponding to proteins of approximate mass 13,260, 18,500 and 19,260 Da. Closer inspection of the charge state peak at ~2200 *m/z* (Fig. 2, inset) revealed it to be distinctly triplet in nature (Fig. 3, inset), arising from two additional proteins with masses of 13,230 and 13,310 Da. The masses detected in this study have been previously observed by Cendron et al. (2012), and are similar to the sequence masses of β_{TPx1} (13,236 Da) and β_{TPx2} (13,313 Da) (Cendron et al., 2012; Lind, 1982). The 18,500 Da protein is consistent with the published mass of a γ-subunit (Fohlman et al., 1977). Also detected within this spectrum were charge states arising from proteins of mass 19,160 and 19,260 Da, the broadness of which is indicative of glycosylation (Aquilina, 2009).

Between 3400 and 4300 *m/z*, two ion series were evident, arising from proteins of molecular weight 45,640 and 46,380 Da, both of which have been reported for TPx (Fohlman et al., 1976). Interestingly, the difference in mass between these large proteins matched the difference in mass between γ_{TPx} and the 19,260 Da species identified in this spectrum. For this reason, the 19,260 and 46,380 Da proteins have been tentatively labelled γ_{TPx2} and TPx_2 respectively.

To confirm our preliminary assignment of the toxin trimers, we performed tandem MS (MS/MS) experiments on each of the 13^+ charge states observed above. The resulting dissociation of PDx

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