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Keywords

major, toxin, from, Australian, Common, Brown, Snake, hexamer, unusual, gas, phase, dissociation, properties, CMMB

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The Major Toxin from the Australian Common Brown Snake is a Hexamer with Unusual Gas-phase Dissociation Properties

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Short title: A protein with unusual gas phase properties

Keywords: Protein; mass spectrometry, asymmetric dissociation; electrospray; phospholipase

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Abstract

Asymmetric dissociation of multiply charged proteins assemblies has been frequently reported. This phenomenon, which relies on the dissociation of one or more highly charged monomers, has been shown to provide insights into the structure and organization of large monodisperse and polydisperse assemblies. Here, the process of asymmetric dissociation is investigated using the multi-subunit protein, textilotoxin, which has unusually high structural constraints on its monomers due to multiple disulfide linkages. Initially, it is shown that, contrary to previous reports, textilotoxin is made up of 6, rather than 5 subunits. Furthermore, the hexamer exists as two isoforms, one of which is substantially more glycosylated. Gas-phase dissociation studies on the hexamers reveal the subunit stoichiometry of each isoform to be $(A/B)_2C_2D_{2a}$ and $(A/B)CD_{2a}D_{2b}$, where A and B are subunits of very similar mass and D_{2a}, D_{2b} refer to differentially glycosylated dimers of the D subunit. The mechanism of dissociation was unusual, as rather than one subunit being largely removed prior to sequential dissociation of a second, the process was predominantly concurrent for the two smallest subunits. Furthermore, a small proportion of the dissociated species was observed to be a noncovalently associated dimer. A comparison of dissociation pathways for two neighboring charge states of the same textilotoxin isoform demonstrates that, in agreement with previous reports, variations in quaternary structure are responsible for the distinct charge states of a protein.

Introduction

Textilotoxin is the major presynaptic neurotoxin in the venom of the Australian common brown snake (*Pseudonaja textilis*)[1]. Although these types of toxin possess phospholipase A_2 activity, there is no direct correlation between enzyme activity and toxicity[2]. Textilotoxin is reportedly a pentamer[2,3] and as such is structurally the most complex of any known snake venom neurotoxin. It has recently been postulated that this family of snakes (Elapidae) have utilized protein oligomerization as an effective evolutionary tool to improve the effectiveness, hence lethality of their toxins[4]. The most recent study into the subunit sequence and stoichiometry of textilotoxin was published some 15 years ago[3], with the earliest data appearing in 1983[5]. Coulter et al. (1983) observed either a pentamer or hexamer, depending on the method of analysis used. Subsequent studies have assumed, based purely on the number subunits which can be purified, that textilotoxin is pentameric[2,3,6], consisting of subunits A, B, C and a D_2 dimer.

In the years since these studies were published, the technique of nanoelectrospray-ionization mass spectrometry (nanoESI-MS) has evolved into a powerful tool for the interrogation of native oligomeric protein structure[7-12]. Consequently, the use of nanoESI-MS for the unequivocal assessment of the oligomeric state of textilotoxin formed a primary aim of the current work. In addition to this, it was of considerable interest to use this unique oligomer of phospholipase A_2 based monomers to investigate their gas-phase dissociation behaviour. As each subunit of textilotoxin contains the stable multi-disulfide-bridged protein phospholipase A_2 scaffold, there would be expected to be considerable restraints on unfolding during gas-phase activation events. In fact previous

studies demonstrate considerable conformational inflexibility in the phospholipase A_2, resulting in very narrow charge state distributions and highly symmetrical dissociation of a non-specific homodimer[13]. In the present study, textilotoxin has been used to further explore the effects of conformational inflexibility on gas-phase dissociation in a multi-subunit system.

Materials and Methods

Protein:- Textilotoxin purified from the venom of the Australian common brown snake (*Pseudonaja textilis*), was purchased from Venom Supplies Pty Ltd, Tanunda, South Australia. Prior to mass spectrometry, the protein was buffer exchanged by loading 500 μg onto a Superdex 200 10/30 size exclusion chromatography (GE Healthcare) column pre-equilibrated with 200 mM ammonium acetate. Fractions containing textilotoxin were pooled and concentrated to 8 μM using Vivascience Vivaspin500 (Sartorius) centrifugal concentrating devices with a 10 kDa molecular weight cut-off. The protein was frozen at -20°C until required.

Nanoelectrospray mass spectrometry:- All spectra were acquired on an Ultima hybrid quadrupole time-of-flight mass spectrometer (Waters UK, Ltd.) and calibrated using a cesium iodide spectrum acquired on the same day. Sample was introduced into the vacuum region of the instrument by electrospraying 2 μl of the textilotoxin stock from a gold coated borosilicate nanoelectrospray (nanoESI) capillary (prepared in-house). Conditions for the acquisition of MS spectra were as follows: capillary voltage, 1.5 kV; cone gas, 150 Lh^{-1}; sample cone, 140 V; RF lens 1 energy, 80 V; collision cell, 4 V; collision cell gas pressure 1.5 x 10^{-2} mbar; source and desolvation temperature of 20 °C. Tandem MS (MS/MS) experiments were performed using the same conditions except that the voltage applied to the collision cell was stepped up as denoted on the z-axes in figure 3. MS/MS spectra in figure 2 were acquired at 30 V and 40 V applied to the collision cell, respectively. Spectra were acquired over the range 1,000 to 15,000 *m/z* and the resultant data were calibrated prior to twofold smoothing with a 16 channel window using the mean algorithm in the MassLynx software (Waters/Micromass).

Results and Discussion

Textilotoxin has previously been reported to consist of 4 distinct subunits, A, B, C, and D, the last of which forms a covalently linked dimer[3]. Based only on sequence data, and in the absence of any quaternary structural studies, it was assumed that the active toxin was a pentamer ($ABCD_2$) of mass 70,551 Da. In the present study, native state nanoESI-MS has been used to more closely investigate the quaternary architecture of this multi-subunit phospholipase.

A typical native state mass spectrum of textilotoxin was found to exhibit a group of peaks within the range 4,400 - 5,500 *m/z* (Fig. 1). Interestingly, these peaks did not arise from a single assembly of textilotoxin subunits, but rather two, with similar charge state distributions but differing *m/z* values. This implied that textilotoxin contains a mixture of two assemblies of dissimilar total mass. The charge state series for the respective species are marked in italic and bold (Fig. 1), and from these values the mass of each assembly was calculated to be 83,770 ± 22 Da (TxI) and ~87,000 Da (TxII) respectively (Table 1). These values are significantly higher than the previously reported sequence sum value of 70,551 Da for a pentameric assembly[3].

A comparison of the observed masses versus the summed sequence masses revealed that TxI and TxII were 13,219 Da and 16,736 Da greater in mass than the sequence summed pentamer. Considering that glycosylation has been reported for subunit D_2[3], and that native state MS retains some residual water which contributes to the observed mass, this increase could readily be accounted for by the inclusion of an extra C subunit (13,001

Da) in the case of TxI. The additional mass observed for TxII however, was not so readily attributable as no subunits have a sequence mass within 2,500 Da of this value. On closer inspection however, it was observed that the difference in mass between the D_2 dimer and subunit A is 16,083 Da, which, with the abovementioned glycosylation and hydration readily accounts for this discrepancy. It should be pointed out that D_2 could replace either subunit A or B within this hexameric species, and this added heterogeneity may be responsible for the broader peaks observed for TxII compared with TxI. Furthermore, heterogeneity in the glycosylation state of subunit D_2 would also contribute to peak broadening.

In an attempt to more clearly understand the subunit architecture of these two textilotoxin assemblies, a series of tandem mass spectrometry (MS/MS) experiments was performed. In such experiments, the ions responsible for a particular charge state peak in the MS spectrum (Fig. 1) are isolated and subjected to collision-induced dissociation (CID) in order to separate one or more individual subunits from the assembly. Figure 2 presents MS/MS data obtained by CID of the most abundant ions of TxI (17^+) and TxII (18^+). Immediately obvious for both assemblies of textilotoxin is that, at a collision energy where the abundance of the parent ion was reduced by fifty percent, all species of subunit have, to a varying degree, been able to be dissociated from the parent oligomer. This diverse dissociation pattern permits a ready identification of the subunits incorporated within each textilotoxin assembly. The most abundant monomer dissociated from TxI was subunit C (Fig. 2A), which is indicative of two C subunits being constituents of the proposed hexamer. This subunit was observed to have a mass of 13,002 ± 2 Da, which is

in agreement with the published sequence mass[3] (Table 2). Subunits A and B were found to be 97 Da and 126 Da less in mass than the published values (Table 2), however, considering the complicated chemical and enzymatic procedures used to characterize these subunits[3], it is highly likely that minor errors were included in the originally published sequences. The least abundant dissociated species was found to be the D_2 dimer (designated D_{2a}), reflecting its greater mass, thus lower propensity to dissociate at the same collision energy as the smaller monomeric subunits. The mass of the D_{2a} dimer was calculated to be 29,864 Da, 58 Da less than the published sequence mass (Table 2).

Definitive information regarding the subunit composition of the TxI assembly could be found in the stripped oligomer region of the spectrum between 5,000 and 6,500 *m/z* (Fig 2A). This region contains ions from TxI oligomers which have been stripped of both mass and charge due to the removal of a subunit during the CID process. For TxI it was possible to identify four distinct ion series in this region, corresponding to four species of stripped oligomer. The most abundant ions arose from a pentamer of mass 70,819 Da due to the loss of subunit C. This pentamer, designated species F [$(A/B)_2CD_{2a}$], consisted of two A or B subunits, one C subunit and one D_2 dimer (Table 2). This assignment confirms that the composition of the original TxI hexamer is indeed $(A/B)_2C_2D_2$. Due to the similarity in mass of subunits A and B, they have been used interchangeably in all subsequent calculations.

In an analogous fashion to the characterization of pentamer F, it was possible to assign the ion series labeled G [$(A/B)C_2D_{2a}$], H [$(A/B)_2C_2$] and I [$(A/B)CD_{2a}$] to stripped

oligomers which were complementary in mass to the dissociated monomeric and dimeric subunits (Fig. 2A and Table 2). As expected an oligomer (G) stripped of subunit A/B was present, as was an oligomer stripped of the D_2 dimer (H). A surprising result of this experiment was the dissociation of a dimer consisting of subunits A/B and C. As this species was not observed in a spectrum of chemically dissociated TxI (10% formic acid, 40% acetonitrile), it was assumed that the subunits were not disulfide linked (spectrum not shown). This is unusual, as it has previously been observed that subunits from multimeric assemblies are dissociated sequentially to yield first an *n-1* stripped oligomer, followed by *n-2* and in some cases *n-3*[14-17]. The peaks for this species are labeled E [(A/B)C] and the complementary tetramer labeled I. The peak labeled with an asterisk corresponds to the 16^+ charge state of TxI and arises from the stripping of a positive charge from the 17^+ charge state of TxI via dissociation of an ammonium ion[18].

The subunit composition of TxII was investigated using the same CID approach as described for TxI. Figure 2B shows the dissociated subunits and residual stripped oligomers for TxII derived from CID of the 18^+ charge state of the proposed hexamer (Fig. 1). Similar to TxI, subunit C was the monomer removed by CID to the greatest extent, followed by subunits A/B. In contrast to TxI, there appeared to be two species of D_2 dimer present in TxII. Signal from ions corresponding to the D_{2a} dimer observed in figure 2A are clearly present at the same *m/z* with similar peak shape. However, two additional broad peaks can also be observed centered around 3000 *m/z* and 3350 *m/z*, which correspond to the 10+ and 9+ charge states of a ~30 kDa protein. This species, designated D_{2b} most likely is a D_2 dimer with greater heterogeneity in its glycosylation, as

evidenced by the broader peaks when compared to the D_{2a} dimer. This additional glycosylation would also explain the broader peaks of the TxII hexamer charge states in figure 1. As with TxI, it was possible to assign charge states to the complementary stripped oligomers labeled J, K and L (Fig. 2B and Table 2).

As stated above, the dissociation of subunits from homogeneous multimeric proteins has been shown to follow the sequential pathway described by the following expression[10,16]:

$$n^{q} \rightarrow [n\text{-}1]^{q-x} + m_1^{x} \rightarrow [n\text{-}2]^{q-(x+y)} + m_1^{x} + m_2^{y} \rightarrow [n\text{-}3]^{q-(x+y+z)} + m_1^{x} + m_2^{y} + m_3^{z}$$

where n is the number of subunits in the oligomer, q is the number of charges on the oligomer, x, y and z are the number of charges carried by a monomer m after dissociation from the parent oligomer. Such a mechanism is usually characterized by one subunit carrying a disproportionate amount of charge, compared with its mass, upon dissociation[15,17].

The subunits of textilotoxin are unusually structurally stable due to the presence of seven disulfide bonds in each of them. Consequently it was of interest to investigate the gas phase dissociation properties of textilotoxin as a model conformationally restrained protein. A previous investigation using non-specific homodimers formed at high monomer concentration showed, as for multimeric proteins, asymmetric charge partitioning is the result of one of the monomers unfolding[13]. However, if the monomers were equally conformationally restrained by crosslinking, symmetric charge partitioning

was observed. textilotoxin represents a more complex and physiologically relevant system than non-specific homodimers, and the results of CID performed over a range of voltages applied to the collision cell are shown in figure 3.

For the 18^+ charge state of TxI CID was performed over a range of voltages applied to collision cell of 15 to 40 V. At 20 V, subunit C was found to be exclusively dissociated from the hexamer, accounting for 20% of the spectral signal intensity. As the voltage was increased, subunits A/B, and to a lesser extent, the D_{2a} dimer, were also found to dissociate from the parent hexamer. This pattern of dissociation is unusual for two reasons. Firstly, the dissociation of a subsequent species (A/B) was observed to begin at only a slightly higher voltage (25 V versus 20 V) than the first species (C). This differs from the sequential pattern described in the expression above, which is commonly observed for homo-oligomers, where the abundance of species m_1^x approaches 100 percent before species m_2^y is observed[14-17]. Secondly, in sequential dissociation the number of charges carried by successively removed monomers decreases such that for species m_1^x , m_2^y and m_3^z, the number of charges carried is $x > y > z$. This phenomenon occurs as CID is a symmetrical process with regards to surface area charge density[14-17], and there are fewer charges remaining on successive stripped oligomers for the monomers to remove. Clearly, for TxI no such sequential dissociation occurs as the number of charges on the dominant charge state of the dissociated species was observed to increase (C^{5+}, A/B^{6+}, D_{2a}^{8+}). Furthermore there was no evidence of a sequential *n-2* species in any of the CID spectra (region of spectra not shown). This unusual dissociation behavior may arise from the fact that, with 7 disulfides per subunit, they are simply

unable to unfold and give rise to the characteristic asymmetric (with respect to mass) dissociation. Hence, rather than dissociation arising from the unfolding of the least stable subunit in the assembly, it appears for TxI that the order of dissociation is based either on subunit mass, since the least massive subunit C is first to dissociate, or subunit topology. Consequently, subunit C is the most readily dissociated, followed by A/B then D_{2a} (Fig.3A, inset).

This CID process was repeated for the 17^+ charge state (Fig. 3B), and as expected for a less highly charged oligomer[13], the amount of energy required to affect dissociation was greater, with the first monomers being detected at 25 V, cf. 15 V for the 18^+ charge state. Compared with the CID of 18^+ ions, there was less discrimination between the onset and extent of dissociation of the C and A/B subunits. The most likely explanation for this more equitable dissociation pattern is that there is a structural difference between hexamers carrying 18 and 17 protons. Charge dependent structural variation of protein complexes has been observed previously, both in charge reduction and ion-mobility MS experiments[19,20]. In the case of TxI, it is most likely that the arrangement of monomers in the 18^+ charge state is somewhat different to that in the 17^+ charge state, such that subunits C and A/B are more equally activated and dissociated from the complex.
The average charge states for the C and A/B subunits was reduced from 5.10 and 6.22, to 4.86 and 5.92, respectively. Interestingly, the difference in average charge state between C and A/B was minimally changed from 1.12 to 1.06, supporting the proposal that mass rather than charge is the determining factor in the dissociation of highly structurally constrained proteins. Further evidence that the individual subunits are unable to unfold

was obtained by comparing the ratio of their average charge to that of the parent ion. For both the 18^{+} and 17^{+} charge state CID, the A/B monomer charge state ratio was 0.35 whereas the C subunit ratio increased from 0.28 to 0.29. Such consistency in charge state ratio strongly supports the fact that no unfolding events occurred during the dissociation process.

In order to demonstrate the profound effect of structural constraint on the dissociation of multi-subunit proteins, a comparison of the CID spectra of TxI and the pentameric acute-phase reactant, serum amyloid P component (SAP) is shown in figure 4. SAP has a mass of 127.5 kDa[21], thus, for the major 24^{+} charge state, SAP has a lower surface charge density than TxI. The dissociation pattern of SAP however, is clearly dissimilar to that of TxI. Firstly, the SAP monomers exhibit a much broader envelope of charge states compared with the TxI monomers, indicating that an unfolding event has taken place. Secondly, this unfolding results in the classical asymmetric dissociation where one subunit carries with it a disproportionate amount of charge, in this case 46 percent. In the case of TxI, however, subunit C, the first and most abundantly dissociated subunit, carries only 29 percent of the total charge, indicating that greater native structure is maintained during the CID process.

NanoESI-MS has become an invaluable tool for investigating the stoichiometry and subunit architecture of multi-component protein assemblies [7-12]. The ability to preserve the integrity of non-covalent complexes in the transition from liquid to gas phases has provided new opportunities to gain structural data rapidly, and with the use of only

picomolar quantities of sample. In the current work it was effectively revealed that Tx exists in two hexameric isoforms comprising distinct combinations of the previously reported four subunits. Mass measurements permitted a preliminary assignment of subunits to each isoform, TxI and TxII, and a series of CID experiments confirmed these proposed arrangements. Furthermore, the unusually high level of disulfide bonding in the subunits led to unusual gas-phase dissociation behavior. These results suggest that for a protein made up of structurally constrained subunits, mass and subunit topology, rather than subunit stability, are the primary determinants of the order of subunit dissociation.

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Figure Legends

Figure 1: Mass spectrum of textilotoxin from Australian brown snake venom electrosprayed from 200 mM ammonium acetate, pH 7.0. Two distinct charge state series are evident corresponding to separate quaternary structures of the protein. The species of textilotoxin corresponding to the charge state series were designated TxI (italic labels) and TxII (bold labels).

Figure 2: MS/MS spectra of the major charge states observed for each species of textilotoxin in figure 1. (A) Spectrum shows the CID product ions of the 17^+ charge state of TxI. The signal arising from the dissociated monomers can be observed between 2,000 and 3,300 *m/z*, and the dimer ions between 3,700 and 4,500 *m/z*. Signal from the stripped oligomers occurs at *m/z* values greater than the parent ion. The peak marked with an asterisk is the 16^+ charge state of TxI, resulting from charge stripping of the parent ion. (B) Analogous to TxI, the product ions arising from CID of the TxII 18^+ charge state can be observed in *m/z* regions corresponding to monomers, dimers and stripped oligomers.

Figure 3: MS/MS spectra acquired over a range of voltages applied to the collision cell for, (A) the 18^+ charge state, and (B) the 17^+ charge state of TxI. In both cases, as the voltage was raised, the population of dissociated monomers, dimers and stripped oligomers was observed to increase. In the case of the 17^+ charge state in particular, the abundance of stripped oligomers began to decrease from 35 V. This was most likely due to the removal of a second monomer and subsequent poor transmission of the stripped

oligomers carrying a very low number of charges. Insets show the relative abundance of monomers A/B and C and the molecular ion of TxI.