Defensin-like peptide-2 from platypus venom: member of a class of peptides with a distinct structural fold

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The venom of the male Australian duck-billed platypus contains a family of four polypeptides of approx. 5 kDa, which are referred to as defensin-like peptides (DLPs). They are unique in that their amino acid sequences have no significant similarities to those of any known peptides; however, the tertiary structure of one of them, DLP-1, has recently been shown to be similar to β -defensin-12 and to the sodium neurotoxin peptide ShI (*Stichodactyla helianthus* neurotoxin I). Although DLPs are the major peptides in the platypus venom, little is known about their biological roles. In this study, we determined the three-dimensional structure of DLP-2 by NMR spectroscopy, with the aim of gaining insights into the natural function of the DLPs in platypus venom. The DLP-2 structure was found to incorporate a short helix that spans residues 9–12, and an antiparallel β -sheet defined by residues 15–18 and 37–40. The overall fold and cysteine-pairing pattern of DLP-2 were found to be similar to

those of DLP-1, and hence β -defensin-12; however, the sequence similarities between the three molecules are relatively small. The distinct structural fold of the DLP-1, DLP-2, and β -defensin-12 is based upon several key residues that include six cysteines. DLP-3 and DLP-4 are also likely to be folded similarly since they have high sequence similarity with DLP-2. The DLPs, and β -defensin-12 may thus be grouped together into a class of polypeptide molecules which have a common or very similar global fold. The fact that the DLPs did not display antimicrobial, myotoxic, or cell-growth-promoting activities implies that the nature of the side chains in this group of peptides is likely to play an important role in defining the biological function(s).

Key words: β -defensin-12, disulphide pairing pattern, NMR spectroscopy, peptide fold.

INTRODUCTION

The male duck-billed platypus, *Ornithorhynchus anatinus*, is peculiar amongst mammals in having a functional venomous spur on each hind limb [1,2]. Knowledge of the evolutionary origin and the natural function of the platypus venom apparatus is limited [1–6]. There have been many hypotheses drawn about the function of the spurs, and the prevailing view is that the apparatus is used as both an offensive and a defensive weapon [7]. The spurs are used offensively against other male platypuses to assert dominance, especially during the mating season [3,8]. It has also been observed that platypuses use the spurs to ward off potential enemies such as dogs and humans. Human envenomation is not rare and has been described as causing excruciating pain followed by profound swelling of local tissue [6].

Platypus venom contains several protein components with a wide range of sizes from 4–6 kDa [9–13] (Figure 1). Two of these components, namely the 4.2 kDa C-type natriuretic peptide (OvCNP) [10–12] and the 13 kDa nerve growth factor protein (OvNGF) [11], have some similarities to proteins from other sources. The OvCNP causes relaxation of the rat uterus, histamine release in the mast cell, and tissue oedema, while OvNGF has been implicated in the pain-producing activity of the venom.

Besides the above two proteins, there are several protein components which are unique in that their primary structures do

not share strong resemblance with any proteins listed in the SwissProt database [9–11]. Many of these proteins are in fact present in much larger amounts than the OvCNP and OvNGF proteins. Among these is a group of four peptides of approx. 5 kDa which are referred to as defensin-like peptides (DLPs) [9] (Figures 1 and 2). These peptides are named as such because the three-dimensional fold of one of its member peptides, DLP-1, has a strong similarity to β -defensin-12 [14,15], although the amino acid sequence similarity is weak. Unlike β -defensin-12, DLP-1 does not show any apparent antimicrobial activity, and its role in the venom is still unknown. Detailed analysis of the DLP-1 and β -defensin-12 structures reveals that although their secondary and tertiary structures are similar, the locations of the hydrophobic and hydrophilic cationic residues, which are known to be important in the activity in β -defensin-12, are significantly different in the two molecules [9].

Four DLPs have been identified in platypus venom thus far. DLP-2 was found to have 36% sequence identity with DLP-1. Despite this low similarity, it was hypothesized that the two peptides would be folded in the same fashion because, in both molecules, six disulphide residues are spaced similarly in the sequence, and important amino acid stretches such as 14–17 and 37–40, which incorporate most of the antiparallel β -strands in DLP-1, are also present in DLP-2. It is interesting to note that five out of the six cysteine residues of DLP-1 and DLP-2 can be

Abbreviations used: DLP, defensin-like peptide; DQF, double-quantum-filtered; NOE, nuclear Overhauser enhancement; RMSD, root-mean-square deviation; 2D, two-dimensional; OvCNP, *Ornithorhynchus* venom C-type natriuretic peptide; OvNGF, *Ornithorhynchus* venom nerve growth factor.

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Figure 1 Reverse-phase HPLC chromatogram of complete platypus venom

Peaks of components that eluted before DLP-3 were identified as non-proteinaceous. Abbreviations: KLP, Kunitz-type protease inhibitor-like protein; GDLP, guanosine diphosphate-dissociation inhibitor-like protein.

readily aligned with those of β -defensin-12. However, the sequence similarity of the two DLPs with β -defensin-12 are small, being 28 % for DLP-1 and 21 % for DLP-2.

The other two DLPs in platypus venom are more closely related to DLP-2 than to DLP-1. DLP-4 is the most recently identified peptide and it has an identical amino acid sequence and the same molecular mass as DLP-2, but it elutes with a different retention time in reverse-phase HPLC experiments. On the other hand, DLP-3 is shorter than DLP-2 with a few amino acid deletions and substitutions. It is interesting in that it has only two pairs of cysteine residues instead of three. Since the identification of the protein components in the venom is far from complete, it is possible that other DLP peptides will be discovered in the venom.

In this study, we first report the determination of the solution structure of DLP-2, a 42 residue peptide which has some sequence similarities to DLP-1 and β -defensin-12. The obtained structure was then analysed and compared with the structures of these two peptides. We also used the results to speculate on the tertiary structures of DLP-3 and DLP-4. It is expected that this study will help illuminate the role of DLPs in platypus venom.

MATERIALS AND METHODS

Separation, identification and purification

Venom components were fractionated using reverse-phase HPLC on a GBC system (Melbourne, Vic., Australia) with LC 1110

pumps controlled by a DP 800 workstation. Details of this procedure are described elsewhere [9]. DLP-4 was sequenced by Edman degradation using an Applied Biosystems 494 Procise HT and cLC pulsed-liquid protein sequencers, and its molecular mass was determined using a VG Analytical TOFSPEC matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry instrument and a saturated solution of α -cyano-4-hydroxycinnamic acid (in 40 % $\text{CH}_3\text{CN}/0.1$ % trifluoroacetic acid) as matrix.

The DLP-2 used in the NMR experiments was isolated from whole crude venom and venom gland extracts. Several reverse-phase HPLC experiments were performed in order to obtain sufficient DLP-2 for the NMR analysis. HPLC fractions containing DLP-2 were pooled, freeze-dried, and subsequently purified by centrifugal membrane-filtration using an Amicon YM-10 tube (Millipore). NMR samples contained approx. 1.0 mM DLP-2 in 0.35 ml total volume at pH 3.6.

NMR spectroscopy

NMR experiments were performed with the sample at either 10 °C or 25 °C on a Bruker AVANCE-600 DRX spectrometer (Karlsruhe, Germany) using a 5 mm ^1H inverse probe. All two-dimensional (2D) NMR spectra were acquired in the phase-sensitive mode using time-proportional phase detection [16]. Homonuclear 2D spectra that were recorded included: double-quantum filtered (DQF) COSY [17], with the phase-cycle modified for fast recycle times [18]; TOCSY [19], with MLEV spin-lock periods of 30 ms, 70 ms and 90 ms; and NOESY [20], with mixing times of 200 ms, 250 ms and 300 ms. Solvent suppression in TOCSY and one NOESY experiment was achieved by using the WATERGATE [21] pulse sequence, while that in DQF and other NOESY experiments was achieved by low-power irradiation at the water resonance frequency during the relaxation delay in the transients (1.3–1.4 s) and during the mixing period in NOESY experiments. ^1H - ^2H exchange experiments were carried out by reconstituting the freeze-dried sample with $^2\text{H}_2\text{O}$, acquiring a series of one-dimensional experiments for 30 min, and then acquiring a short-time 2D NOESY spectrum. Slowly exchanging amide protons were interpreted as being hydrogen-bond donors. Spectra were processed using XWIN-NMR software (Bruker).

Structure calculations

All 2D spectra were analysed using the XEASY program [22]. Distance constraints were derived from NOESY spectra recorded at 25 °C with a mixing time of 300 ms. This ultimately yielded

Figure 2 Primary structures of DLPs and β -defensin-12

The amino acid sequences are aligned to show maximum correspondence between them. Similarities are shown relative to DLP-2 and DLP-4; identical residues are boxed and conservative substitutions are faint. The disulphide-bonding pattern determined for DLP-2 from the NMR data is shown below the numbered sequences (dashed lines). The secondary structure of DLP-2, as determined in the current study, is shown above the sequences (helix denoted as cylinder and β -strand denoted as arrow). The diagram was drawn using ALSCRIPT [42].

699 non-redundant inter-proton distance constraints, which were automatically assigned upper-distance limits based on the corresponding cross-peak intensities or volumes. To allow for conformational averaging and errors in volume integration, a correction of 0.3 Å (0.03 nm) was added to all upper-distance bounds.

Twenty four ϕ dihedral angle constraints were derived from $^3J_{\text{NH}\alpha}$ coupling constants obtained by inverse Fourier transformation of in-phase multiplets from 2D NOESY spectra, using the program INFIT [23]. The ϕ angles were constrained to $-60 \pm 30^\circ$ for $^3J_{\text{NH}\alpha} < 5$ Hz; $-60 \pm 40^\circ$ for $^3J_{\text{NH}\alpha}$ 5–6 Hz; $-120 \pm 30^\circ$ for $^3J_{\text{NH}\alpha}$ 8–9 Hz; and $-120 \pm 20^\circ$ for $^3J_{\text{NH}\alpha}$ 9 Hz. Hydrogen-bond constraints were deduced from ^1H – ^2H exchange experiments and the preliminary calculated structures. The latter constraints were introduced only in the final stages of the structure calculations and were given upper distance-limits of 2.2 Å for NH_i to O_j and 3.2 Å for N_i to O_j . The disulphide linkages were deduced from preliminary structures by measuring distances between sulphur atoms and checking the orientation of the cysteine side chains.

The torsion-angle dynamics program, DYANA [24], was used at all stages of the structure calculations prior to refinement. The NOAH protocol [25,26] was used in calculating preliminary structures and in assigning non-intra-residue and non-sequential NOESY cross-peaks. After obtaining the majority of the NOESY assignments, a standard simulated annealing procedure was applied. In the final structure calculations, 4800 DYANA structures were generated from random starting conformations. The 'best' 120 structures, with the lowest NOE violations, were then refined by simulated annealing with X-PLOR [27]. The simplified all-hydrogen force field was used, and the covalent geometry was constrained using the standard X-PLOR parameters. Interatomic distances and dihedral angles were constrained by experimental energy terms ($k_{\text{noe}} = 50 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$, $k_{\text{dihedral}} = 200 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$), which remained constant throughout the calculations. Initial atomic velocities were set from the Maxwell distribution at 1000 K, in which all non-bonded interactions were ignored, allowing the atoms to pass through each other (repel = 0.9, $C_{\text{rep}} = 0.005 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$). Simulated annealing was then performed by gradually 'cooling' the system to 100 K over 20 ps of simulated time, during which C_{rep} was increased linearly to $4 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$ while 'repel' was decreased to 0.75. Restrained gradient-minimization of 40 cycles was performed in the final stage of the protocol.

The coordinates for the ensemble of 20 structures have been deposited with the Brookhaven Protein Data Bank with accession code 1D6B.

RESULTS AND DISCUSSION

Structure determination by NMR

Standard procedures were implemented in assigning NMR resonances [28]. The amide-proton signals of DLP-2 at 298 K and pH 3.6 were generally well resolved, making the resonance assignment process straightforward. Most of the signals from the backbone amide protons were intense, except for those of Ser¹² and Tyr³¹ that were also broad. Spectra were also obtained at 283 K to aid in resolving several overlapping peaks and in confirming other resonance assignments.

Preliminary structure calculations were performed with DYANA [24] implementing the NOAH automatic structure calculation scheme [25,26]. This protocol led to a moderately well-defined global fold with identification of approx. 90% of all

NOESY cross peaks. The validity of the assignments obtained by NOAH were checked, and further NOESY cross-peak assignments were manually performed by analysing the 2D data and the calculated medium resolution structures.

Although no disulphide bonds were included during the early stages of the calculations, the resulting medium-resolution structures did show the defined cysteine pairing configurations of DLP-2 to be Cys⁹–Cys³⁹, Cys¹⁶–Cys³² and Cys²⁴–Cys⁴⁰. Direct NOE cross-peaks, which were evident between the H β of the six cysteine residues, assisted considerably in orientating the cysteine side chains in the calculation, allowing the cysteine pairs to be identified with ease.

After using the NOAH protocol, the standard simulated-annealing procedure in DYANA was used to improve the 'quality' of the structures; thus an iterative cycle of calculation, structure analysis, and constraint revision was performed. The hydrogen-bonding pairs were deduced only during the later stages of the calculations, and were included as conformational constraints.

A total of 715 non-redundant inter-proton distance constraints, 24 dihedral-angle constraints, and 16 hydrogen-bond constraints, were used to obtain the final DLP-2 structure. The NOE distance constraints consisted of 172 long-range, 95 medium-range, 190 sequential, and 242 intraresidue constraints. In the final round of calculations, 120 out of 4800 structures that had been generated in DYANA were refined using the simulated annealing protocol in X-PLOR [27]. The 'best' 20 X-PLOR structures with the lowest total 'energies' were used as representatives of the DLP-2 solution structure. None of these structures had NOE violations greater than 0.2 Å, or dihedral-angle violations greater than 2°. Table 1 contains a summary of the structural characteristics of the ensemble of 20 DLP-2 structures.

Description of the structure of DLP-2

The DLP-2 structure incorporates a poorly defined N-terminal region of up to five residues, as indicated by the low backbone angular order parameters for ϕ and ψ angles, and high root-mean-square difference(s) (RMSD) from the mean structure. The well-defined region of the molecule, which encompasses residues 6–42, had angular parameters (S) greater than 0.86 in all cases, and the mean global backbone RMSD, with respect to the mean structure, was 0.46 ± 0.13 Å. In contrast, the mean global backbone RMSD was 1.28 ± 0.35 Å when the backbone of the whole molecule was considered. Figure 3 shows the ensemble of 20 calculated structures superimposed over the backbone of the 'well-defined' region (residues 6–42). Some parts of the well-defined region are clearly seen in Figure 3 to be better defined than other parts, for example residues 14–19, 25–27, and 37–42.

PROMOTIF analysis [29] of the ensemble of 20 DLP-2 structures revealed that the main secondary structural elements in DLP-2 consist of a helical conformation over residues 9–12, and an antiparallel β -sheet over residues 15–18 and 37–40 (Figure 4A). Thus the DLP-2 structure begins with a flexible five-residue N-terminus. Residues 7–8 are situated close to residues 30–31, with the backbone NH of Gln⁷ forming a hydrogen bond with the carbonyl of Tyr³¹. This configuration almost creates a small parallel β -sheet in this part of the molecule. In nine out of the 20 calculated structures, residues 9–12 formed a short α -helix; in one structure this formed a 3_{10} helix, while in 10 of the structures this region incorporated a series of three β -turns situated at residues 8–11, 9–12, and 10–13. The dearth of medium-range cross-peaks in the NOESY spectrum was responsible for the lack of consistent definition in this part of the molecule. Residues 13–14 connected this helix-like configuration to the first strand of

Table 1 Structural statistics for the ensemble of 20 DLP-2 structuresValues given as means $\pm$ S.D.

(a)		
Distance restraints		
Intraresidue ($i-j = 0$)	242	
Sequential ($ i-j = 1$)	190	
Medium-range ($ i-j \leq 5$)	95	
Long-range ($ i-j > 5$)	172	
Hydrogen bonds	16	
Total	715	
Dihedral-angle restraints		
ϕ	24	
(b)		
Mean RMSD		
From experimental restraints		
NOE ($\text{\AA}$)	0.013 $\pm$ 0.001	
Dihedrals (deg.)	0.08 $\pm$ 0.05	
From idealised covalent geometry*		
Bonds ($\text{\AA}$)	0.00137 $\pm$ 0.00008	
Angles (deg.)	0.307 $\pm$ 0.003	
Impropers (deg.)	0.106 $\pm$ 0.006	
(c)		
Mean energies ($\text{kcal} \cdot \text{mol}^{-1}$)		
$E_{\text{NOE}}^\dagger$	6.44 $\pm$ 0.95	
$E_{\text{cdih}}^\dagger$	0.01 $\pm$ 0.02	
E_{vdW}	0.08 $\pm$ 0.08	
E_{bond}	1.29 $\pm$ 0.16	
E_{improper}	0.66 $\pm$ 0.08	
E_{angle}	17.59 $\pm$ 0.30	
E_{total}	26.07 $\pm$ 1.23	
(d)		
Ramachandran plot analysis (PROCHECK [43])		
Most favoured region	49.9 $\pm$ 4.7	
Additionally allowed region	40.4 $\pm$ 5.9	
Generously allowed region	9.1 $\pm$ 4.4	
Disallowed region	0.6 $\pm$ 1.1	
(e)		
Atomic RMSD ($\text{\AA}$) ‡		
	Versus mean	Pairwise
Backbone atoms (6–42)	0.46 $\pm$ 0.13	0.65 $\pm$ 0.18
Heavy atoms (6–42)	1.00 $\pm$ 0.12	1.41 $\pm$ 0.21

* Idealized geometry as defined by the CHARMM force field within X-PLOR.

 † Final values of the square-well NOE and dihedral-angle potentials calculated with force constants of 50 $\text{kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$ and 200 $\text{kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$ respectively. ‡ Atomic differences given as average RMSD against mean coordinate structure (mean) and average RMSD of all pairwise structures (pairwise). Values given as means $\pm$ S.D.

the antiparallel β -sheet, as defined by residues 15–18. The ϕ dihedral angle of residue 13 had a characteristic positive value near 90°, suggesting a reverse turn [30]. The helix axis was seen to be almost parallel to the second β -strand.

The presence of an antiparallel β -sheet formed by residues 15–18 and 37–40 is consistent with the results of the ^1H – ^2H exchange experiment which provided evidence of the hydrogen-bonded pairs as follows: NH Val¹⁵–CO Cys⁴⁰, NH Cys⁴⁰–CO Val¹⁵, NH Arg¹⁷–CO Lys³⁸, and NH Lys³⁸–CO Arg¹⁷. The antiparallel β -sheet appeared to be twisted and connected by a large loop defined by residues 19–36 that surrounds the second β -strand formed by residues 37–40. This gives the impression that the second strand (or the C-terminus) is trapped in the middle of the molecule, surrounded by the first strand on one side and by the large loop on the other (Figure 4B). The loop defined by residues 19–36 incorporates four β -turns, 18–21 (type I), 21–24 (type IV), 27–30 (type IV), and 34–37 (type IV). The turn at residues 27–30 forms a distinct bulge on the loop. The C-terminus is bent near residue 41 and points towards the first β -strand.

The disulphide conformation of Cys⁹–Cys³⁹ was right-handed in 19 out of 20 of the calculated structures, with a χ_{ss} value of $86 \pm 4^\circ$, while that of Cys²⁴–Cys⁴⁰ was left handed in 14 out of the 20 structures with a χ_{ss} value of $-72 \pm 7^\circ$. The conformation of the Cys¹⁶–Cys³² disulphide bond was poorly defined, although direct NOE interactions between the H β of the cysteine pairs were observed experimentally, so they were included as constraints in the structure calculations. The DLP-2 molecule has a large hydrophobic region that spans most of the N-terminus and the bulge on the large loop. Hydrophobic residues in this region include Ile¹, Met², Phe³, Phe⁴, Ala⁸, Cys⁹, Trp¹⁰, Pro²⁶, Met²⁷, Ala²⁸, Trp²⁹, and Cys³⁹. With the exception of Cys⁹ and Cys³⁹, which are buried in the interior of the molecule, all these hydrophobic residues have their side chains orientated towards the surface of the molecule, thus exposing them to the solvent.

Two highly charged regions exist in the calculated DLP-2 structures; these are situated near or within the β -turns along the large loop. The first region, which includes Arg¹⁷, Asp¹⁸, Lys¹⁹, Glu²¹, and Arg²², is relatively large and encompasses part of the first β -strand and two β -turns (residues 18–21 and 21–24). The second region is located at the end of the large loop and encompasses one β -turn (residues 34–37) and incorporates Glu³³, Arg³⁵, and Lys³⁸. Both of these charged regions of DLP-2 are very hydrophilic since they also include polar residues such as Asn, Gln, and Ser. The region defined by residues 10–13, 29–31, and 41–42 are moderately hydrophilic.

Comparison of DLP-2 with β -defensin-12

The superposition of the backbone atoms of residues 7–42 in DLP-1 and DLP-2 (Figure 5A) yielded an RMSD value of 1.1 $\text{\AA}$, clearly showing that the tertiary structures of the two molecules are very similar, notwithstanding the fact that their sequence similarity is only 36%. This similarity of folding is manifest in the disulphide-bond pairing patterns of Cys⁹–Cys³⁹, Cys¹⁶–Cys³², and Cys²⁴–Cys⁴⁰ that are identical in the two DLPs. Moreover, the N-termini of DLP-1 and DLP-2 are both flexible, while the C-termini that incorporate residues 40–42 are bent in the same manner.

The secondary structural elements found in DLP-2 are similar to those in DLP-1 (Figure 5B); the helix, antiparallel β -sheets, and β -turns that are present in DLP-1 [9] were also evident in DLP-2. A subtle difference is that the helix in many of the calculated DLP-2 structures was an α -helix, spanning residues 9–12, while in DLP-1 it is a 3_{10} helix that spans residues 10–12. This may not be a 'real' difference since this region of the molecule was not as clearly defined in the calculated structures of DLP-2 as it was in DLP-1. The similarity in the secondary structures of DLP-1 and DLP-2 is also manifest in the results

Figure 3 Ensemble of 20 DLP-2 structures

Stereo view of the ensemble of 20 DLP-2 structures superimposed to show the best fit over the backbone atoms of residues 6–42 of the mean-coordinate structure. Only the N, C, and C α atoms of the backbone are displayed.

Figure 4 Schematic drawing of DLP-2 structure

Schematic drawing of DLP-2 showing the locations of the disulphide bonds (yellow), β -strands (cyan), and α -helix (red and yellow). The two Figures are related by approx. 90° rotation about the 'virtual' vertical axis. The program MOLMOL [44] was used to generate the Figures.

of ^1H – ^2H exchange experiments that showed a similar pattern of exchange rates for the amide protons.

The major difference between the structural features of DLP-1 and DLP-2 was only revealed when the nature of the side chains on the different regions of the molecules were emphasized. Specifically, in DLP-2 the N-terminus up to residue 6 is highly hydrophobic, while in DLP-1 only the first two residues are hydrophobic. The surface region which encompasses residues 8, 10 and 31 in DLP-2 is moderately hydrophobic, but it is highly basic in DLP-1. DLP-2 has a large continuous hydrophobic surface region incorporating the N-terminus up to residue 11 and the hydrophobic bulge in the large loop, but these features are

not evident in DLP-1. These differences between DLP-1 and DLP-2 are illustrated in the surface representation of the two molecules in Figure 6.

Despite a sequence similarity of only 21%, DLP-2 and β -defensin-12 have similar global folds (Figures 5C and 5D). As with DLP-1, the two antiparallel β -strands of DLP-2 closely match those of β -defensin-12, but the latter is longer by one residue. Furthermore, part of the large loop in DLP-2 can be superimposed well on the third antiparallel β -strand that contains the β -bulge in β -defensin-12. However, a computer-based structure comparison using the DALI algorithm [31] showed that the degree of 'structural similarity' between DLP-2 and β -defensin-12 is less than that between DLP-1 and β -defensin-12 [9].

Structural implications

Considering the fact that DLP-2, DLP-1, and β -defensin-12 are folded similarly, it is reasonable to postulate that the unique fold shared by these peptides is due to the conserved residues in their amino acid sequences. As mentioned above, there are only a few of these equivalent residues; they are Cys⁹, Gly¹⁴, Val¹⁵, Cys¹⁶, Cys³², Lys³⁸, Cys³⁹, and Cys⁴⁰ in all three molecules, and Lys²⁵ and Met²⁷ in DLP-2, which are conservatively substituted as Arg and Ile, in DLP-1 and β -defensin-12 respectively. It is clear that the cysteine residues that are paired in the order 1–5, 2–4, and 3–6 in all three molecules play a crucial role in achieving and/or maintaining the distinct tertiary structure. Also, most of the conserved residues (including the cysteines) are involved in hydrogen bonding with other residues.

It was shown previously that the disulphide-connectivity pattern of 1–5, 2–4, and 3–6 of the DLPs is also found in other toxic peptides, such as the sea anemone peptides ShI, ATX-II and AP-A [32–34], and the rattlesnake myotoxin α [35,36]. The global fold of DLP-1 was shown, using the DALI algorithm, to have some weak similarities to ShI. Superposition of the structurally equivalent backbone atoms showed that the β -strands

Figure 5 Comparison of DLP-2, DLP-1 and β -defensin-12 structures

(A) DLP-2 and DLP-1 structures superimposed over N, C, and C α atoms of the backbone of residues 7–42. DLP-2 and DLP-1 residues are shown in light grey and black respectively. (B) Same as diagram in (A) except drawn in MOLMOL [44]. (C) DLP-2 and β -defensin-12 structures superimposed over N, C, and C α atoms of the backbone of residues 8–23, 24–27, and 30–41 of DLP-2, and residues 4–19, 21–24, and 25–36 of β -defensin-12. DLP-2 and β -defensin-12 residues are shown in light grey and black respectively. (D) Same as diagram in (C) except drawn in MOLMOL [44].

in the antiparallel β -sheet in both molecules coincide well, although the primary structures are significantly different. This finding, together with the similarity to DLP-2, underscores the importance of the cysteine-pairing pattern as an indicator of the specific tertiary structure.

Possible structures of DLP-3 and DLP-4

Due to the high level of sequence similarity between DLP-2, DLP-3, and DLP-4, it is likely that they are all folded in a similar manner. One possible difference between the folds of DLP-2 and DLP-4, which are isomers, is that their cysteines are paired in different ways. This is plausible because the six cysteines are relatively close to each other and in particular the last two cysteines of DLP-2 and DLP-4 are adjacent in the sequence, thus enabling interchange of the disulphide bonds without significantly altering the main part of the tertiary structure. The latter exchange phenomenon is exemplified by β -defensin-12 and NP-2, which have similar global folds but their disulphide pairings are quite different, being 1–5, 2–4 and 3–6 in β -defensin-12 and 1–6, 2–4, 3–5 in NP-2 [15]. Disulphide-pairing patterns such as 1–4, 2–5, 3–6, are also conceivable in the DLPs: the first cysteine is relatively close to the fourth one situated in the large loop, while the second cysteine that is located on the first β -strand is also near the fifth cysteine that is located in the adjacent second β -strand. Although some of these disulphide-pairing patterns are possible, the fact that the 1–5, 2–4, and 3–6 pattern is found in

DLP-1, DLP-2, β -defensin-12, ShI, and myotoxin *a* implies that it is probably more stable than other possible combinations.

DLP-3 lacks the third and the fifth cysteines that correspond to Cys²⁴ and Cys³⁹ in DLP-2 (Figure 2). Since the disulphide-pairing pattern in DLP-2 is 1–5, 2–4, and 3–6, this implies that the four cysteines in DLP-3 could be paired differently from those in DLP-2. Assuming that the global folds of DLP-2 and DLP-3 are similar, the likely disulphide pairing in DLP-3 is therefore 1–6 and 2–4, which is similar to that in NP-2. However, it is important also to consider the fact that DLP-3 lacks the residue corresponding to Asn³⁶ in DLP-2, so that the sixth cysteine may be the one that is actually missing and not the fifth. This line of reasoning leads to the proposal that the cysteine pairing is 1–5 and 2–4.

Roles of DLPs in the platypus venom

As mentioned in the Introduction, the platypus venom is not likely to be used to capture prey since its venom apparatus is absent from the female and it is situated on the hind legs away from the mouth. Also, because of its relatively low toxicity, compared to the snake venoms, platypus venom most probably is used to incapacitate and ward off enemies without necessarily inflicting permanent harm to them. Therefore it is unlikely that platypus venom includes highly toxic components like those in the snake venoms; the latter are clearly used to capture prey and include α - and β -neurotoxins, and proteases and phospholipases which aid in the pre-digestion of the prey.

The discovery of OvCNP and OvNGF in platypus venom provided important clues on the basis of action of the venom, as these peptides have significant sequence similarities to those found in other biological systems. Natriuretic peptides exist in tissues such as mammalian heart, brain, and endothelium, where they are involved in reducing blood pressure; and it has recently been discovered in the venom of green mamba snake (*Dendroaspis anguisceps*) [37]. The NGF, on the other hand, exists in mouse submaxillary glands and is also prevalent in the venom of a wide variety of snake venoms. However, it is worth emphasizing that the distinctly different location of the venom glands suggests an evolutionary/embryological relationship that is not straightforward, or that the peptide composition of the secreted solution may be the result of common evolution in various exocrine glands.

The existence of numerous protein and non-protein components is not peculiar to platypus venom. Snake venoms are also complex, but what makes the platypus venom unique is the presence of many novel components, such as the DLPs, whose physiological actions are unknown at present. We have performed various assays to assess the antimicrobial [9], myotoxic [38], haemolytic [39], and cell-promoting activities [40] of DLPs, however all of these tests gave negative results.

Determining the roles of the components has proven difficult, and this may be partly due to the activity being affected by the presence of other components, similar to some lipases which require a co-lipase for their activity to be manifest. Specifically, a venom component may act synergistically with others so that it may be inactive during a pharmacological/physiological assay when present alone. Note also that the platypus lives in an isolated environment in eastern Australia and therefore encounters very few species of animals its own size during its lifetime. Thus common biological assays may not be applicable to the platypus venom since the cells that are routinely used are obtained from tissues of species that may never be encountered by a platypus.

Figure 6 Comparison of DLP-2 and DLP-1 molecular surfaces

Molecular forms of DLP-2 (**A**) and DLP-1 (**B**) showing the charge distributions of one of their corresponding surfaces. Surfaces with positive and negative electrostatic potentials were drawn in blue and red respectively. These Figures were created using the standard charge settings in MOLMOL [44].

One possible role of the DLPs in the venom is that they may act synergistically with OvNGF to produce the pain that is such a prominent symptom of platypus envenomation. It has been speculated that NGF in some snake venoms potentiates the effect of other toxins by stimulating various cells of its victim, thus rendering the victim more susceptible to the toxins. Human NGF causes hyperalgesia and not pain itself [41]; hyperalgesia is also found with platypus envenomation. The suggestion that OvNGF causes pain is based on experiments using composite protein fractions of the venom. Since DLPs are abundant in platypus venom and DLP-3 co-elutes with OvNGF in reverse-phase HPLC, there is a distinct possibility that DLPs were present in all the samples of OvNGF that were used in experiments which elicited hyperalgesia.

Conclusion

Unlike the situation with most snake venoms, the existing knowledge on the nature of platypus venom is far from complete. Platypus venom has been shown to contain numerous novel peptides and proteins whose structures and functions require further investigation before a definitive understanding of the pharmacology of this mixture is arrived at. The biological roles of the constituents have been elusive partly due to complications introduced by 'synergistic effects' in the assays used, and partly because the constituents are unique and absent in the venoms of other animals.

The present study of the tertiary fold of DLP-2 is a step towards understanding the biological function of the DLPs which are the major peptides in platypus venom. The work not only revealed structural information on the DLPs but also provided useful data on the structures of β -defensin-12. The distinct fold shared by DLP-1, DLP-2, and β -defensin-12 can be attributed to a small number of amino acid residues in the sequence that appear to determine and maintain the form of the structural fold. These important residues include six cysteines that are paired in the order 1–5, 2–4, and 3–6. The

distinct structural scaffold of the DLPs is a configuration that has evolved to yield a small compact molecule. The findings presented here introduce a structural peptide core that could support different functional groups and hence display different pharmacological/physiological activities.

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