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D-Amino acid residue in the C-type natriuretic peptide from the venom of the mammal, *Ornithorhynchus anatinus*, the Australian platypus

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Abstract The C-type natriuretic peptide from the platypus venom (OvCNP) exists in two forms, OvCNPa and OvCNPb, whose amino acid sequences are identical. Through the use of nuclear magnetic resonance, mass spectrometry, and peptidase digestion studies, we discovered that OvCNPb incorporates a D-amino acid at position 2 in the primary structure. Peptides containing a D-amino acid have been found in lower forms of organism, but this report is the first for a D-amino acid in a biologically active peptide from a mammal. The result implies the existence of a specific isomerase in the platypus that converts an L-amino acid residue in the protein to the D-configuration.

Key words: Platypus venom; C-type natriuretic peptide; D-Amino acid; Toxin; Nuclear magnetic resonance; Posttranslational modification

1. Introduction

Peptides containing D-amino acids are commonly associated with proteins produced by phylogenetically lower forms of organism such as bacteria and yeast. It has long been accepted that such peptides are not present in higher order organisms including mammals. In recent years, however, there have been several reports of the existence of peptides that contain D-amino acids in multicellular organisms such as frogs [1–3], molluscs [4–6], crustaceans [7,8], and spiders [9,10]. These unusual peptides in metazoans are thought be produced post-translationally, requiring a specific isomerase (racemase) to convert them from the usual L- to the D-configuration [1,2]. It is of great interest to know if D-amino-acid-containing peptides or proteins are present in higher order organisms such as mammals.

The venom of the Australian platypus contains a C-type natriuretic peptide (OvCNP) that has been shown, by reverse-phase high performance liquid chromatography (RP-HPLC), to exist in two forms, OvCNPa and OvCNPb [11,12] (Fig. 1). OvCNP is the most biologically active peptide yet identified in platypus venom. This 39-residue peptide shares high sequence similarity with mammalian C-type natriuretic peptides [13] and has been implicated in the ability of the venom to relax rat uterine smooth muscle, cause oedema and promote mast cell histamine release. Until this study, the basis for the different chromatographic behaviour of the two forms of OvCNP remained unclear, but there was strong evidence from earlier studies to suggest that structural heterogeneity was localised in the region encompassing the first 11 residues of the N-terminus [11]. It was hypothesised that the heterogeneity may be caused by proline *cis–trans* isomerism since there are three proline residues in this N-terminal part of the peptide. This argument was weak because the two forms do not reversibly interconvert and the synthetic protein always elutes with the same retention time as OvCNPa.

Following the determination of the conformations of OvCNPa in aqueous solution and in sodium dodecyl sulphate micelles [14], we performed nuclear magnetic resonance (NMR) experiments to explain the difference between the two forms of OvCNP. In this paper, we demonstrate for the first time that a biologically active peptide that contains a D-amino acid residue exists in a mammal, the Australian platypus.

2. Materials and methods

2.1. Separation and purification of peptides

Separation of the platypus venom components was achieved by using RP-HPLC on a GBC HPLC system with LC 1110 pumps controlled by a DP 800 work station (Victoria, Australia). Details of this procedure are described elsewhere [15]. The RP-HPLC findings of De Plater and co-workers [11,12] showed that the amount of OvCNPa in the whole venom was about half that of OvCNPb. For the particular venom sample used in this study, it was found that the OvCNPa was substantially less than OvCNPb. Thus, only OvCNPb was purified for NMR study while OvCNPa was chemically synthesised. Several RP-HPLC experiments were performed in order to obtain sufficient OvCNPb for the NMR experiments. The OvCNPb NMR sample contained ~0.5 mg of peptide in 0.30 ml of 90% H_2O/10% D_2O and pH 3.

2.2. Synthesis of OvCNPa and D-Leu2-OvCNP

Both of these peptides were synthesised manually on a 0.50 mmol

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Abbreviations: NMR, nuclear magnetic resonance; OvCNP, *Ornithorhynchus* venom C-type natriuretic peptide; OvCNPa, *Ornithorhynchus* venom C-type natriuretic peptide A; OvCNPb, *Ornithorhynchus* venom C-type natriuretic peptide B; LAP, leucine amino peptidase; HPLC, high performance liquid chromatography; RP-HPLC, reverse-phase high performance liquid chromatography; DLP, defensin-like peptide; DLP-2, defensin-like peptide-2; DLP-4, defensin-like peptide-4; TOCSY, total correlation spectroscopy

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scale using 2-(1*H*-benzotriazol-1-yl-)1,1,3,3-tetramethyluronium hexafluorophosphate activation of Boc-amino acids with in situ neutralisation chemistry as previously described [16]. Following HF cleavage, the reduced peptides were isolated and purified using RP-HPLC using a C4 column. Disulphide formation was carried out in 100 mM ammonium acetate (pH 7.5) and the final purified products were isolated by preparative RP-HPLC as follows: fractions were collected, based on monitoring the column effluent at 230 nm. Those fractions shown by electrospray mass spectrometry analysis to contain solely the target peptide were combined and lyophilised. The OvCNPa NMR sample was prepared by dissolving 4.8 mg of the lyophilised peptide in 0.37 ml of 90% H_2O/10% D_2O, resulting in a final protein concentration of 3.1 mM and pH 3.0; the D-Leu2-OvCNP NMR sample was prepared in the same fashion but the sample contained 3.0 mg (2.0 mM) of peptide.

2.3. NMR spectroscopy

All NMR experiments were performed at 25°C on a Bruker AVANCE-600 DRX spectrometer using a 5-mm ^{1}H inverse probe. Two-dimensional (2D) NMR spectra were acquired in phase-sensitive mode using time-proportional phase detection [17]. The homonuclear 2D spectra that were recorded for the OvCNPa and OvCNPb were total correlation spectroscopy (TOCSY) [18], with spin-lock periods of 60 ms, and nuclear Overhauser enhancement spectroscopy [19] with mixing times of 200 ms. Double-quantum-filtered scalar correlated spectroscopy [20] spectra, with a phase cycle modified for fast recycle times [21], were recorded for OvCNPa to aid in resonance assignment. Solvent-signal suppression was achieved either by presaturation or by using the WATERGATE [22] pulse sequence. Spectra were processed using XWIN-NMR software (Bruker) and were analysed using the program XEASY [23].

2.4. Digestion by leucine aminopeptidase (LAP)

The NMR samples used previously were also used in this part of the study. The peptide digestion was begun by simultaneously mixing 20–50 μl of the peptide samples, 500 μl of 50 mM Tris pH 8.5, and 50 μl of 1 mg/ml LAP. The mixtures were then maintained at 25°C for a specified period after which the OvCNPa sample was injected into an Phenomenex Jupiter HPLC C18 column attached to a Thermoquest LCQ classic mass spectrometer (San Jose, CA, USA). The same procedure was then applied to the sample that contained OvCNPb. Since the HPLC run took approximately 45 min, the second sample, OvCNPb, underwent an extra period of digestion by LAP before the analysis.

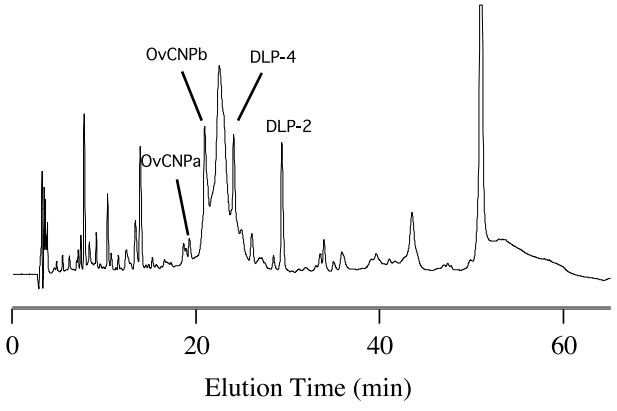

Fig. 1. RP-HPLC chromatogram of complete platypus venom. The venom contains many protein components which are unique and are not found elsewhere in Biology. The labelled peaks are from peptides that exist in two isomeric forms. OvCNPa, *Ornithorhynchus* venom C-type natriuretic peptide A; OvCNPb, *Ornithorhynchus* venom C-type natriuretic peptide B; DLP-2, defensin-like peptide 2; DLP-4, defensin-like peptide 4.

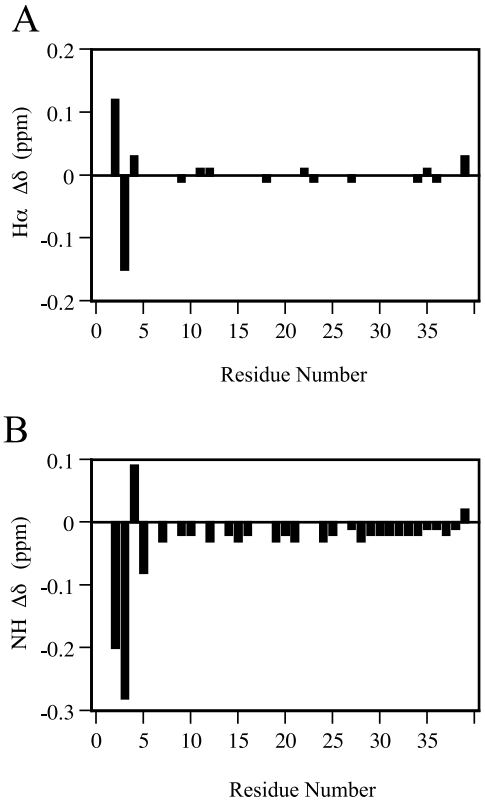

Fig. 2. Proton NMR chemical shift differences between OvCNPa and OvCNPb. A: Hα chemical shift difference. B: NH chemical shift difference. Values were obtained by subtracting the chemical shifts of the proton resonances of OvCNPb from those of OvCNPa. The Hα of Lys17 of OvCNPb and the NH of Lys17 of OvCNPa and OvCNPb were not detected in the NMR spectra so were not included in the plot.

3. Results and discussions

NMR experiments on both OvCNPa and OvCNPb showed that chemical shifts, or positions of proton signals, in three proline residues in OvCNPb were similar to those of OvCNPa thus disproving the hypothesis that the heterogeneity is caused by proline *cis–trans* isomerism [11]. Surprisingly, we discovered that there were significant differences between the chemical shifts of some protons of residues 1–5 in the OvCNPa and OvCNPb. The discrepancy was more pronounced in the Hα and NH chemical shift values of residues 2 and 3, suggesting that the structural difference between the two forms is localised in the region that spans residues 2 and 3 (Fig. 2).

What causes the difference between OvCNPa and OvCNPb? It is worth noting that De Plater and co-workers had previously shown that there is a peculiar conformation or a 'modified' amino acid in the section of OvCNPb near His3 and Asp4, because the peptide bond between the two residues was not cleaved by endoproteinase Asp-*N* [11]. The only plausible explanation for this outcome that is consistent with the NMR results is that a D-amino acid exists at positions 2 or 3 (or both) in OvCNPb. NMR alone cannot unambiguously prove the existence of a D-amino acid or even pinpoint its exact location in the sequence of residues. Other experimental techniques were needed to establish this unusual peptide conformation, if it really existed.

The possibility of a D-amino acid residing in the first few

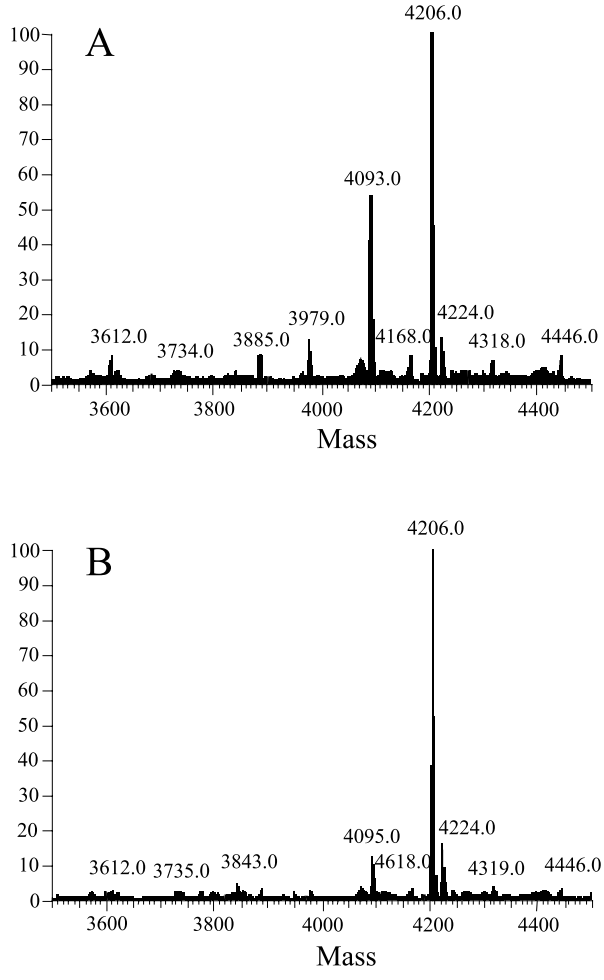

Fig. 3. Mass spectra of OvCNPa and OVCNPb digested with LAP. A: OvCNPa after digestion for 1 h. B: OvCNPb after digestion for 1.75 h. Peaks with molecular masses of 4206, 4093, and 3979 units correspond to proteins that were uncleaved, cleaved by one N-terminal residue, and cleaved by two N-terminal residues, respectively.

residues of OvCNPb was tested by digesting both OvCNPa and OvCNPb with LAP and then analysing the products formed afterwards by mass spectrometry. It was postulated that the digestion of the peptide by LAP would be hindered by the presence of the D-amino acid in the peptide molecule. We found that there was an about five-fold lower rate of digestion of OvCNPb, compared with OvCNPa. Fig. 3 shows the mass spectrum obtained after exposing OvCNPa and OvCNPb to LAP for 1 and 1.75 h, respectively. For OvCNPa, three major peptide peaks with molecular masses of 4206, 4093 and 3979 were present after 1 h of exposure to LAP, indicating significant amounts of remaining intact OvCNPa, and fragments cleaved by one and two amino acid residues from the N-terminus. Digestion of OvCNPb for 1.75 h resulted in a peak at 4093 mass units that was only $\sim$10% as intense as the main peak at 4206, while a peak at 3979 was not present at all. Considering that OvCNPb was exposed to LAP for longer than OvCNPa, we can readily state that the rate of digestion of OvCNPb by LAP is considerably less than that of OvCNPa. Thus, it is likely that a D-amino acid residue is present near the N-terminus of OvCNPb. The subsequent chiral chromatography experiment provided data that were in clear agreement with this.

To ultimately prove the existence of a D-amino acid in OvCNPb and to determine its exact location in the amino acid sequence, we chemically synthesised OvCNP analogues, containing D-amino acids at positions 1, 2 and 3. RP-HPLC showed that the D-Leu2 OvCNP analogue eluted with the same retention time (chromatogram position) as OvCNPb; thus it was concluded that a D-leucine was highly likely to be present at the second position of OvCNPb. The subsequent 1D and 2D NMR experiments finally proved beyond doubt that the D-Leu2 OvCNP analogue was indeed OvCNPb (Fig. 4).

The functional significance of the D-amino acid in OvCNP in the platypus venom is unclear at present. Experiments performed by De Plater et al. [11,12] did not reveal any difference in activity between the two isomers; the two forms had the same potency in elevating cGMP levels, and producing vasodilatation, in cultured vascular smooth muscle. However, OvCNP also causes oedema and mast cell histamine release, and may have other biological activities that are yet to be discovered. A more comprehensive pharmacological study is therefore warranted in order to compare the spectrum of activities of the two isoforms.

It is easy to speculate that the D-amino acid in OvCNPb is probably important in increasing its stability to protease degradation in the venom during storage in the gland prior to envenomation. It is worth noting that various peptides that contain D-amino acids in multicellular organisms are primarily found in toxins. Specifically, D-amino-acid-containing peptides have been discovered in sawfly larvae and these are implicated in the poisoning of predators that results from the ingestion of the larvae [24]. D-Amino-acid-containing peptides are found in the skin of the South American tree frog *Phyllomedusa sauvagei* [3], and tissues of the snail *Achatina fulica* [5]; such peptides from both species are biologically active while the corresponding L-forms are inactive [1]. It is clear that in these peptides, the D-amino acid plays crucial structural roles that define their biological activities.

The existence of a D-amino acid at position 2 in the residue sequence of a peptide is not entirely unique to the platypus. Such unusual peptides from frog skin and neuropeptides achatin I and fulicin from mollusc tissues all have a D-amino acid in the second position from the N-terminus [1–3,5,6]. This occurrence implies that an isomerase similar to those found in the above species may exist in the platypus venom gland. Since an isomerase that acts near the C-terminus of a peptide has been found in the venom of the spider *Agelenopsis aperta* [9,10], it would be logical to test for such an isomerase that operates near the N-terminus in the platypus venom. It will also be interesting to determine if other D-amino-acid-containing peptides are present in platypus venom. Furthermore, defensin-like peptide (DLP)-2, whose three-dimensional structure has recently been determined [25], has an isomer (DLP-4) with the same molecular mass. The basis for this structural heterogeneity is not yet known so it would be important to determine if a D-amino acid is present in one of the two DLPs.

D-Aspartate residues have been found in aged mammalian lens proteins, but their functional significance is unknown [26,27]. Their occurrence has been attributed to the 'natural' ageing process and is linked to the many posttranslational

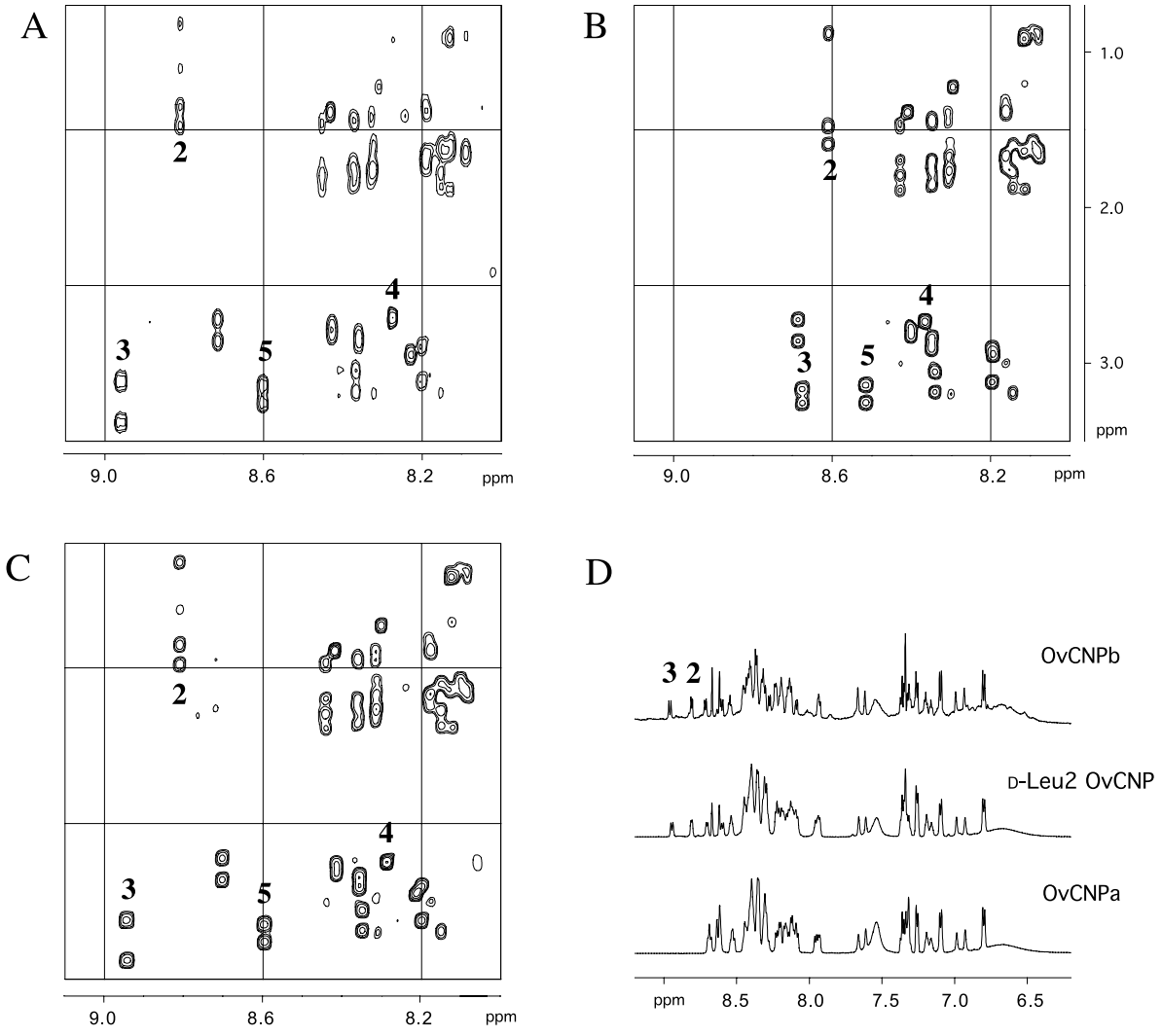

Fig. 4. Comparison of 2D TOCSY and 1D NMR spectra of various OvCNPs. 2D TOCSY sections of (A) native OvCNPb, (B) synthetic OvCNPa, and (C) D-Leu2-OvCNP. Proton amide cross-peaks of residues that are different in the two OvCNP forms are indicated by their sequence numbers placed below or above their corresponding cross-peaks. (D) Part of the 1D spectrum of the three peptides. Amide signals from Leu2 and His3 in the native OvCNPb are highlighted.

modifications that the long-lived α-crystallins can undergo [26]. Perhaps the most important development in the search for D-amino acids in mammalian metabolism and protein structures is the discovery of D-aspartate and D-serine as free amino acids in the mammalian nervous system [28,29]. D-Serine interacts with the glycine site of glutamate/*N*-methyl-D-aspartate receptor causing activation of the receptor [30].

4. Conclusion

This present study has shown for the first time that a D-amino-acid-containing biologically active peptide is present in a mammalian system. As discussed, this unusual peptide is likely to have an important functional role in platypus venom. It would not be surprising therefore that many of these types of protein/peptide isoforms will be discovered in other higher order organisms in the future. Clearly, D-amino acids are utilised more extensively in living systems than previously thought.

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