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Solution conformations of an insect neuropeptide: Crustacean cardioactive peptide (CCAP)

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ABSTRACT

The solution structure of crustacean cardioactive peptide (CCAP), a cyclic amidated nonapeptide neurohormone, was studied using molecular dynamics techniques, with constraints derived from NMR studies in water and water/dodecylphosphocholine micellar medium. This peptide, found in various invertebrates, has the primary sequence Pro[1] Phe[2] Cys[3] Asn[4] Ala[5] Phe[6] Thr[7] Gly[8] Cys[9] NH_2, with an intramolecular disulfide bridge between the two cysteine residues. In aqueous solution the peptide was found to have a type(IV) β-turn between residues 5–8. In a water/decane biphasic medium a type(IV) β-turn between residues 3 and 6 and two classic γ-turns between residues 4–6 and 7–9, were found. Analysis of the ^1H and ^{13}C NMR chemical shifts data showed that the model free S^2 order parameter of the residues varied between 0.65 and 0.9. The molecular dynamic root mean square fluctuations of structural ensembles of the backbone varied between 0.5 and 2.2 with the central residues showing the least fluctuations.

1. Introduction

Crustaceans and insects are the most well-known and conspicuous members of the phylum Arthropoda. As with all other Metazoa, they utilize peptidergic neurosecretions as the major system for cellular communication. Neurosecretory cells synthesize and release a range of neuropeptides which regulate diverse processes such as metabolism, development and growth, reproduction and behavior. A large number of these neuropeptides have been identified in Arthropods [12,15] and many of their modes of action and function have been established, [13,15] yet, not much is known about arthropod neuropeptide secondary structure.

The focus of this study is the crustacean cardioactive peptide (CCAP), which is an amidated nonapeptide (Pro[1] Phe[2] Cys[3] Asn[4] Ala[5] Phe[6] Thr[7] Gly[8] Cys[9] NH_2) cyclized by an intramolecular disulfide bridge between the cysteine residues at positions 3 and 9 [35]. CCAP was first isolated and sequenced from the pericardial organs of the shore crab, *Carcinus maenas*: it was found to have a stimulatory effect on the heart rate [35]. Authentic CCAP has been identified in other crustaceans and in a number of insect species (for reviews, see [10,16]). In insects, genes have been identified or predicted which encode the precursor for CCAP and a G-protein-coupled receptor for CCAP from the fruitfly *Drosophila melanogaster* is known [16].

The immunocytochemical localization of CCAP in a wide number of cells and organs from crustaceans and insects has been studied, and its pattern of distribution suggests that this peptide has multiple and diverse neurohormonal and neuromodulatory functions [10,16]. It has a cardioacceleratory and myotropic effects in crustaceans, stimulates contraction of heart, hindgut and oviduct in insects, is a releasing factor for metabolic hormones in the migratory locust, inducts the motor program for ecdysis behavior in insects and is involved in the stimulation of insect midgut enzymes [31]. Having such vital and diverse actions, CCAP is a good candidate for the bio-rational design of peptidomimetic insecticides which are highly selective, safe for humans [14], stable and nonpolluting [24].

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Abbreviations: DPC, dodecylphosphocholine; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt; TSP, 3-(trimethylsilyl)-propionic acid-D4 sodium salt; DMSO, dimethyl sulfoxide; 2D, two-dimensional; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; ppm, parts per million; ppb, parts per billion; MD, molecular dynamics; ISPA, isolated spin pair approximation; MD_RMSD, molecular dynamic root mean square deviation; GPCR, G-protein coupled receptor.

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A key step for the development of such peptidomimetics is the elucidation of the preferential or active conformations of the parent compound. This knowledge may lead to a greater understanding of the peptide–receptor interactions and, subsequently, to the design of receptor specific, peptidomimetic, agonists and antagonists.

It is now recognized that G-protein coupled receptor (GPCR) regulated neuropeptides utilize a membrane-associated pathway to receptor binding and activation [36,37]. For this reason we have studied the peptide CCAP in water and in a membrane mimetic solution of dodecylphosphocholine (DPC) micelles. As the reactivity of this neuropeptide is dependent upon its conformational state, the aim of this study was to determine and compare the conformations of CCAP in water and the membrane-mimetic medium using NMR restrained molecular modeling [25].

2. Experimental methods

2.1. NMR experiments

2.1.1. Sample preparation

CCAP was purchased from BACHEM (Peninsula) Laboratories (San Carlos, CA, USA). Its purity was checked by HPLC and NMR and found to be >95%.

The NMR sample for the aqueous studies was prepared in 50 mM 9:1, v/v H_2O–D_2O phosphate buffer solution (pH 4.55) at a peptide concentration of 3.0 mM. Lower pH is necessary to prevent fast proton exchange which would hamper measurement of all NMR data related to amide NH protons (chemical shifts, J-couplings, temperature coefficients, NOEs). DSS was used as an internal reference at 0.00 ppm.

Micellar solutions were prepared by sonication of DPC-d_{38} (Cambridge Isotopes, 98.6% *d*) in a 50 mM 90% H_2O/10% D_2O phosphate buffer solution. At a concentration of 180 mM, each micelle consisted of approximately 50 DPC molecules. CCAP was dissolved in this solution to give a final concentration of 2.6 mM. Trimethylsilylpropionate (TSP) was added to the sample as a chemical shift reference (0.00 ppm).

2.1.2. NMR

2.1.2.1. Water. Gradient enhanced 2D NOESY, TOCSY and HSQC spectra were acquired on a Bruker Avance DRX 500 NMR spectrometer using an inverse multinuclear, single-axis gradient probe at 275 K. A combination of water flip-back sequence, radiation damping suppression using weak bipolar gradients during t_1, and an excitation sculpting module for further reducing the residual water signal were used for solvent suppression in both TOCSY and NOESY experiments [11]. NOESY spectra were acquired with mixing times of 150 and 180 ms while the TOCSY spectrum was recorded with a mixing time of 64 ms at an average spin-lock power of 8.33 kHz. The DIPSI-2 [33] mixing sequence was flanked by simultaneously applied spin-lock and gradient pulses [8,21] to obtain pure absorption signals for coupling constant measurements. A band-selective QUIET-NOESY experiment [45,49] including Watergate [29] for solvent suppression was also performed to focus on the NOE correlations between the α-protons and side chain protons. For band-selective excitation of the 2.7 ± 2 ppm region, a pulse width modulated DANTE train [30] was implemented within a double pulsed field gradient spin-echo (DPFGSE) sequence [38], prior to t_1 and in the middle of the mixing period, to quench the undesirable cross-relaxation and spin diffusion pathways. Temperature coefficients for amide protons were determined by fitting a linear curve to their chemical shifts measured at 5 K intervals between 275 and 300 K. All spectra were processed and analyzed with the Bruker software XWINNMR.

2.1.2.2. DPC. The NOESY and the TOCSY experiments were run on a Bruker 600 MHz spectrometer using an inverse multinuclear, single-axis gradient probe at 285 K. The mixing time was 100 ms for the TOCSY experiment and varied between 80 and 150 ms for the NOESY experiments. Data were processed using NMRPipe [9] and analyzed using Sparky [17].

2.1.3. NMR analysis

The resonance assignments of CCAP were obtained from two-dimensional NMR experiments using the conventional assignment strategy [48]. NOESY cross-peak volumes were determined using the volume integration method and converted into distance bounds using the isolated spin pair approximation (ISPA) [18,41]. A reference distance of 1.78 Å was used for the geminal proton–proton distance of the β-methylene groups of the proline residue and/or the α-protons of the glycine residue. The lower bound distances were calculated by subtracting 10% of the originally determined distance. The upper bound distances were obtained by adding 20% to the ISPA distances. In the case of the aqueous solution data, spin diffusion was taken into account using the MARDIGRAS approach [6].

In water, dihedral angle restraints were obtained from $^3J_{HNH\alpha}$ coupling constants using Pardi's Karplus equation [28].

2.2. Molecular dynamics

The GROMACS molecular dynamics engine and analysis modules [3,23,42] were used for all the simulations. Simulations were performed using the OPLS force field [19] with the peptide bonds in the trans configuration. This configuration was confirmed by dynamic simulations and NMR data from a QUIET-NOESY experiment which showed no sign of sequential Hα–Hα NOE correlations, a necessary condition for a *cis* configuration. An *in vacuo* conformational search was carried out using a simulated annealing protocol in which the peptide was allowed to move for 1 ns at 600 K using a time step of 2 fs and the neighborhood list updated every 10 fs. Non-bonded interactions were treated with a switch function operating between 10 and 12 Å. The temperature of the system was coupled to an external bath using a time constant of 0.1 ps [1]. Snapshots of the dynamics were collected every 5000 steps. The collected structures were cooled from 600 to 300 K in 1 ns and then energy minimized. This gave a total simulation time of 101 ns. Cluster analysis using the linkage algorithm [3,23,42] was performed on the final collection of 100 structures.

2.2.1. Water

The starting structure, obtained from the preliminary, vacuum, conformational search described above, was solvated with 5789 water molecules in a cubic simulation box (5.6 nm × 5.6 nm × 5.6 nm). The system was energy minimized followed by a 500 ps position restrained dynamics simulation at 300 K where the peptide atoms were restrained using the SHAKE algorithm [3,23,42]. A 50 ns molecular dynamics simulation at 300 K was then run with structures collected every 100 ps. NOE-based distance restraints were applied to the peptide continuously through the simulation using a force constant of 2000 kJ mol^{-1} nm^{-2}. The peptide was represented by the OPLS-AA force field [19] while the water molecules were treated using the SPC model [2]. The integration time step was 2 fs and the neighborhood list was updated every 10 fs. Non-bonded interactions were treated with a switching function operating between 10 and 12 Å. The temperature of the system was controlled by coupling to an external temperature bath using a 0.1 ps time constant [1].