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Conformational study of insect adipokinetic hormones using NMR constrained molecular dynamics

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Summary

Mem-CC (pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2), Tem-HrTH (pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-NH_2) and Del-CC (pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-Asn-NH_2) are adipokinetic hormones, isolated from the corpora cardiaca of different insect species. These hormones regulate energy metabolism during flight and so are intimately involved in an insect's mobility. Secondary structural elements of these peptides and the N7 analogue, [N7]-Mem-CC (pGlu-Leu-Asn-Tyr-Ser-Pro-Asn-Trp-NH_2), have been determined in dimethylsulfoxide solution using NMR restrained molecular mechanic simulations. The neuropeptides were all found to have an extended structure for the first 4 residues and a β-turn between residues 4–8. For Tem-HrTH and Del-CC, asparagine (N7) which is postulated to be involved in receptor binding and/or activation, projects outward form the β-turn. Mem-CC does not have an asparagine at position 7 while, for [N7]-Mem-CC, the N7 sidechain folds inside the β-turn preventing its interaction with the receptor.

Introduction

Insects form one of the largest groups of multicellular organisms inhabiting our planet. They are widely distributed and generally highly mobile. Important in this regard is their ability to fly. Insect flight metabolism is under hormonal control via the AKH/RPCH (adipokinetic hormones or red pigment-concentrating hormones). These hormones regulate the hydrolysis of fat stores of triacylglycerides to diacylglycerides and their subsequent transport to muscle cells. They are also involved in the regulation of carbohydrate and proline metabolism. More than 30 different AKH/RPCH hormones have now been isolated [1, 2]. Distinguishing features of these hormones include that they are all octa-, nona- or deca-peptides with blocked N-(pyroglutamate) and C-(amidate) termini, and that they display a high degree of homology.

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Recently, the neurohormones Del-CC and Tem-HrTH have been isolated from the corpora cardiaca of the blister beetle species, *Decaptoma lunata* [3]. Previously, Tem-HrTH was isolated from the corpora cardiaca of the tenebrionid beetles, *Tenebrio molitor* and *Zophobas rugipes* [4]and of the cockroach *Polyphaga aegyptiaca* [5]. A related neuropeptide, Mem-CC has been isolated from the corpora cardiaca of various beetles of the large superfamily Scarabaeoideae, *Melolontha melolontha*, *Geotrupes stercorosus*, *Pachnoda marginata* and *Pachnoda sinuata* [2]. The primary sequences of these peptides are shown in Figure 1. Mem-CC is quite unique for this family in that it is one of the few family members with an acidic aspartate at position 7 and a tyrosine residue at position 4 instead of the typical phenylalanine.

Relatively minor substitutions in the amino acids at positions 4 and 7 have a marked effect on the activity of these peptides in the locust assay measuring lipid mobilization. Substitution of phenylalanine for tyrosine results in a 30-fold reduction in activity, while substituting aspartate for asparagine results

NEUROHORMONE	SEQUENCE	ACTIVITY
Tem-HrTH :	pE - L - N - **F4** - S - P - **N7** - W - NH_2	(very active)
[N7]-Mem-CC:	pE - L - N - **Y4** - S - P - **N7** - W - NH_2	(poorly active)
Mem-CC:	pE - L - N - **Y4** - S - P - **D7** - W - NH_2	(almost inactive)
Del-CC	pE - L - N - **F4** - S - P - **N7** - W - G - N - NH_2	(very active)
Emp-AKH	pE - V - N - **F4** - T - P - **N7** - W - NH_2	

Figure 1. Primary sequence of some adipokinetic hormones with varying biological activity in the locust bioassay.

in a further 5-fold loss in activity. Tem-HrTH has an ED_{50} of 7pmol, [N7]-Mem-CC, the [Y4,N7] analogue has a drastically reduced potency with an ED_{50} value of 203 pmol, whereas Mem-CC [Y4,D7] does not give a typical dose-response curve and even a 1000 pmol dose elicits only 50% of the maximal possible response [6].

Since AKH/RPCH hormone receptors have not been isolated and are only poorly characterized [7], a study of the solution conformation of these peptides is of interest. CD studies indicate that the peptides do not have a preferred conformation in aqueous solution but can become structured in other solvents [8]. The peptides are only sparingly soluble in water and so DMSO was chosen as the solvent for nmr studies. While this is not ideal, recently we have shown [9] with the gonadotropin releasing hormones, which are structurally very similar to the AKH hormones, that there is a correlation between the preferred structure of the peptide in DMSO solution and its ability to bind to its receptor. Similarly, Tsikaris et al. [10] have determined the mAbSRYD-bound structure of IASRYDQL in water and the free octapeptide structure in DMSO and found them to be very similar. We have therefore used the success of Tsikaris [10–14] and others [15, 16] to study these peptides in DMSO-d^6 solution using nmr restrained molecular dynamics. It must be pointed out however, that there are examples where different structures are found in water and DMSO.

Materials and methods

Sample preparation

The neuropeptides were synthesized by Dr R. Kellner of Merck KGaA, Darmstadt, Germany. This was achieved using the standard protocol for solid phase peptide synthesis employing Fmoc-amino acid chemistry. The synthetic peptides were taken up in acetonitrile/water solution and purified using HPLC in the presence of 0.1% TFA as solvent. The samples were then dried and weighed.

NMR experiments

All NMR experiments were conducted at 298 K on a Varian Unity 400 MHz spectrometer equipped with a 5 mm inverse detection probe. The hydrophobic nature of the peptides rendered them poorly soluble in aqueous solution, so all studies were conducted in DMSO-d_6 solution. The neuropeptides were dissolved in ∼0.75 ml of DMSO-d_6(99.9 atom % D, Aldrich Chemical Company) yielding an approximately 4mM solution. No saturation of the residual water signal was employed in 2D spectral acquisition. All data processing and integration was conducted on a Sun data workstation employing VNMR software (Varian Associates). 2D NOESY spectra [17] were acquired at several mixing times with the best results being obtained with a mixing period of 200 ms. NOESY spectra of small peptides generally exhibit weak intensities at small mixing times and, as a result, longer mixing times were used. 512 t_1 values of 32 transients were recorded, linear predicted in T1 and zero filled to 2K × 2K with the use of the sinebell and shifted sinebell window functions. Baseline corrections were applied in both dimensions. Various methods of integrating the cross peaks were investigated but finally, the standard VNMR software was used.

Calculation of restraints

^{1}H and $^3J_{N\alpha}$ coupling constants have been published before [18]. The internuclear distance restraints, r_{ij}, were estimated from NOESY data using the Isolated Spin Pair Approximation [ISPA] [19] which is represented by the following equation

$$r_{\text{ref}}/r_{ij} = (\sigma_{ij}/\sigma_{\text{ref}})^{1/6}, \quad (1)$$

where $\sigma_{ij}$is the peak volume, σ_{ref} is the reference peak volume for two geminal protons and r_{ref} is set to 1.77 Å. Because of the long mixing times used, before

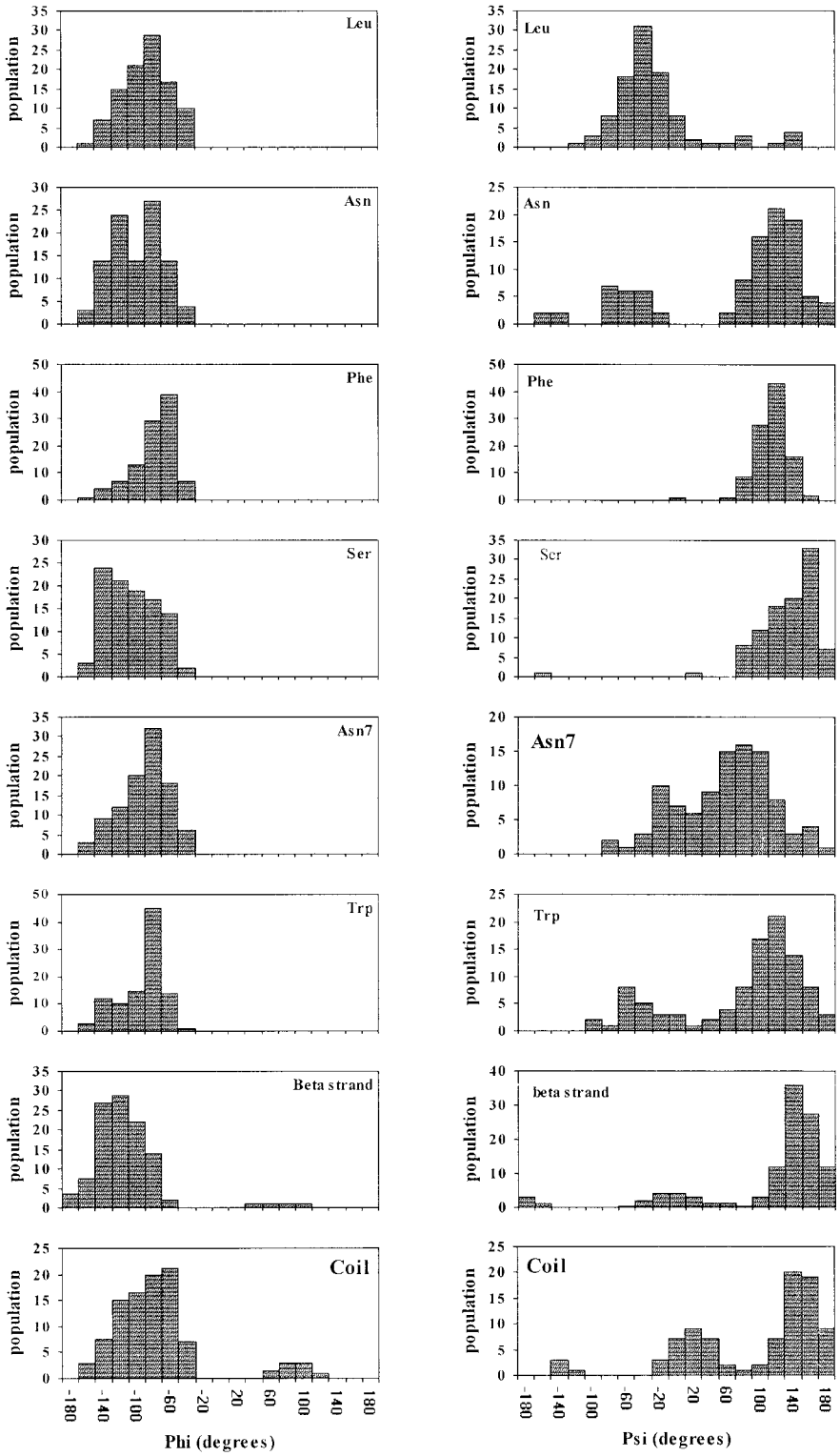

Figure 2. Histograms showing the distribution of ϕ and φ from a 2 ns simulation of Tem-HrTH. The coil and β strand data were taken from Smith et al. [29].

ISPA derived distances could be employed in structure calculations, allowance had to be made for spin diffusion. This was done using the relaxation matrix program MORASS [Multispin Overhauser Relaxation Analysis and Simulation] which uses the atomic coordinates of a molecule to simulate its NOESY spectrum at a given mixing time [20]. The experimental and theoretical NOESY volumes are then merged to obtain a new set of internuclear distance restraints. From these new restraints a new minimized molecular structure is calculated. The whole process is repeated until there is convergence of the experimental and theoretical NOESY volumes. Upper and lower bounds for the restraint distances were obtained from an analysis of the experimental error in the NOESY volumes.

Torsion angles θ and hence, the ϕ polypeptide backbone dihedral angle were calculated from literature $^3J_{NH}$ coupling constants [18] and Bystrov's Karplus equation [21]:

$$^3J_{N\alpha} = 6.4\cos^2\theta - 1.4\cos\theta + 1.9, \quad (2)$$

where $\theta = |\phi - 60°|$

Molecular modelling

Calculations were performed on a Silicon Graphics Indigo workstation using Discover3 [22], CHARMm [23] and MACROMODEL [24] software. Using MACROMODEL, a Monte Carlo search of conformational space was performed while with DISCOVER3 and CHARMm software a simulated annealing protocol similar to Young et al. [25] was used to generate 100 structures (Scheme 1, bcl source code archived as supplementary material). A cluster analysis of the 100 structures was performed based on the RMS value for superimposing backbone atoms. Structures with a maximum backbone RMS < 1.0 were grouped into the same family. In the case of the CHARMm runs, randomizing the backbone torsion angles generated random starting structures. This protocol, while giving the same results as the Young protocol, is preferred as the conformational search is restricted to realistic regions of conformational space. Although different computer programs and different protocols were used, they all gave essentially the same results and hence only the results from DISCOVER3 using the cvff forcefield are reported here.

Absence of $H_{\alpha i}$-$H_{\alpha(i+1)}$ nOe contacts in the NOESY spectra confirmed that all the peptide bonds were in the trans conformations and were maintained as such during the calculation. nOe cross peak between $Ser_{\alpha H}$ and the δCH_2 protons of proline indicated that the trans form of proline was dominant and was modelled as such.

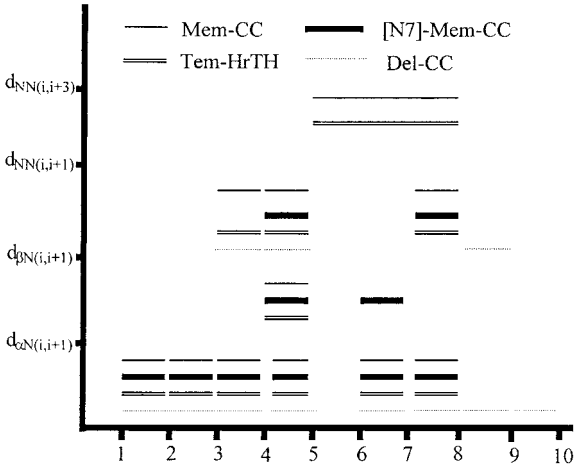

Figure 3. NOE connectivity patterns.

Global backbone rmsd values were determined for the ten lowest energy structures of each family using the program MOLMOL [26]and the R-factor [27] calculated to compare theoretical nOe volumes with experimental results using the program MORASS [20].

Molecular dynamic calculations were performed using DISCOVER3 at 298K on a *NVT* ensemble. Typically, dynamics were initialized for 1 psec and then resumed for a further 2 ns with a step length of 1 fs. Structures were collected every 10 ps. To simulate the effect of DMSO, a dielectric constant of 4 was used.

Results

^{1}H proton assignments and $^3J_{NH}$ coupling constants have been reported before [18]. Results from a 2 ns molecular dynamics simulation of Tem-HrTH in DMSO are presented in Figure 2 in the form of ϕ and φ distribution diagrams. From these results ensemble averaged $^3J_{NH\alpha}$ coupling constants have been calculated and compared with measured coupling constants in Table 1.

The nOe derived interproton distances (Table 2, Figure 3), together with the calculated torsion angles were used as restraints in the molecular mechanics search of conformational space. There are relatively few restraints per residue and many of these are sequential. One reason for this was spectral overlap in the NOESY spectra. Where a cross peak could not be uniquely assigned to a particular proton pair, it was

Scheme 1. Simulated annealing protocol.

Step 1.	Minimization random starting structure. 100 cycles steepest descent until max deriv <0.01. Scale nonbond = 1e-5, dihedral = 1e-6, NOE = 1e-6.
Step 2.	Mimimization. 1000 cycles BFGS until max deriv. <0.01.
Step 3.	Dynamics. Initialize at 700 K for 10000 steps of 1.0 fs. Scale nonbond, dihedral, NOE = 0.001
Step 4.	Resume dynamics at 700 K for 20 ps. Scale nonbond = 0.016, dihedral, NOE = 1.0
Step 5.	Minimization. 100 cycles steepest descent until max deriv <0.01.
Step 6.	Mimimization. 1000 cycles BFGS until max deriv. <0.01.
Step 7.	Annealing. Decrease temperature stepwise from 700 K to 300 K. Initialize 2 ps, resume dynamics 2 ps at each temperature. Scale nonbond = 0.25.
Step 8.	Resume dynamics at 300 K for 25 ps.
Step 9.	Minimization 100 cycles steepest descent, 1000 cycles BFGS. Scale factors = 1.0.
Step 10.	Archive final structure.
Step 11.	Repeat steps 1 to 10 until 100 structures collected.

Table 1. Homonuclear coupling constants $^3J_{NH}$ (Hz) for Tem-HrTH measured and calculated from dynamic simulations in DMSO. Standard deviations are given in parentheses. Literature values for a random coil model are taken from Smith et al. [29].

	Leu	Asn	Phe	Ser	Asn7	Trp
Exptl	8.10	7.85	8.11	7.32	7.85	7.85
DMSO	7.47 (2.09)	7.99 (1.50)	7.07 (1.81)	8.06 (1.48)	7.56 (1.76)	7.78 (1.43)
Literature	7.1	7.4	7.5	6.7	7.4	6.9

not used to restrain the molecule. However, in the analysis of the final structures, MORASS calculated nOe volumes for overlapping peaks were compared with experimental data. For each peptide, minimized structures were generated and clustered into families based on their backbone rmsd's. Of the 100 structures generated, no structures were discarded and a cluster analysis grouped all the conformations into only five families. Of these, the dominant family for Tem-HrTH, contained 73 structures (Figure 4) with an average energy value of 134 kcal mol^{-1}. The average restraints energy for this family was 3 kcal mol^{-1}, the mean global backbone rmsd for ten random structures was 0.69±0.38 Å and the R-factor was 0.40. This indicates good overall agreement between experimental and theoretical data. However, not all of the specified nOe contacts could be satisfied. Violation were noted between $F4_{\beta H}$–$S5_{NH}$, $S5_{\beta H}$–$S5_{NH}$ and $N3_{\beta H}$-$N3_{NH}$. Such violations are commonly observed for oligopeptides that adopt a rather flexible structure [28].

The results of the restrained molecular dynamics calculation and analysis of the conformers of Del-CC are summarized in Figure 5. Two dominant conformations comprising a total of 84 structures were found. The major family comprised 58 structures with an average energy value of 105 kcal mol^{-1} and the minor family 26 structures of average energy 117 kcal mol^{-1}. The mean global backbone rmsd value for ten random conformers was 0.74±0.30 Å and the *R*-factor 0.39, suggesting good agreement between experimental and theoretical nOe values. Common inter-residue restraint violations were observed for $G9_{\alpha H}$-$N10_{NH}$. This may be related to the conformational flexibility of the dihedral angles of the glycine residue. The serine β protons exhibit an intraresidue nOe violation to its amide proton.

Cluster analysis of the simulated annealing results for Mem-CC also yielded two families of structures (Figure 6). 65 Structures superimposed to give one family (147 kcal mol^{-1}, rmsd = 0.58±0.30, R = 0.34) while 32 structures (149 kcal mol^{-1},

Table 2. Internuclear distance restraints (Å) calculated from nOe volume measurements using MORASS [20]. The uncertainty is given in parentheses.

Residue	Peptide			
	Tem-HrTH.	Del-CC	Mem-CC	Mem-Anal
$pE_{\alpha H}$ - $L2_{NH}$	2.60 (20)	2.50 (25)	2.50 (20)	3.00 (20)
$L2_{\alpha H}$ - $N3_{NH}$	2.30 (20)	2.15 (15)	2.30 (20)	2.30 (20)
$N3_{\alpha H}$ - $Y,F4_{NH}$	2.40 (30)	2.45 (25)	2.20 (20)	2.30 (20)
$Y,F4_{\alpha H}$ - $S5_{NH}$	2.10 (20)	2.55 (35)	2.10 (20)	2.15 (15)
P6αH - $D,N7_{NH}$	2.55 (35)	2.20 (20)	2.50 (30)	2.30 (20)
D,N7αH - $W8_{NH}$	2.40 (30)	2.25 (25)	2.90 (20)	2.5 (20)
W8αH - $G9_{NH}$		2.45 (25)		
G9αH - $N10_{NH}$		3.70 (50)		
Y,F4βH - $S5_{NH}$	3.55 (55)		3.35 (35)	3.45 (45)
$N3_{NH}$ - $Y,F4_{NH}$	3.25 (25)	3.20 (30)	3.00 (40)	
$Y,F4_{NH}$ - $S5_{NH}$	2.60 (40)	2.40 (30)	2.75 (15)	2.40 (20)
$D,N7_{NH}$ - $W8_{NH}$	2.60 (30		3.20 (20)	2.70 (20)
$W8_{NH}$ - $G9_{NH}$		2.95 (25)		
$S5_{NH}$ - $W8_{NH}$	2.70 (30)		2.90 (40)	
S5αH - P6δH	2.35 (45)	2.65 (65)	2.90 (70)	2.15 (25)
Y,F4βH - $Y,F4_{NH}$	4.00 (80)	3.00 (50)	2.80 (40)	2.85 (35)
D,N7βH - $D,N7_{NH}$	3.25 (35)	2.75 (45)	3.50 (50)	3.40 (60)
N3βH - $N3_{NH}$	2.30 (40)		2.80 (50)	3.25 (45)
S5βH - $S5_{NH}$	2.50 (50)	2.65 (35)	2.60 (30)	2.75 (35)
W8βH1 - W8βH2		1.77 (10)		
G9βH - $G9_{NH}$		3.10 (40)		
$W8_{NH}$ - W8βH			3.45 (35)	
$N7_{NH}$ - P6βH				3.70 (20)

rmsd $= 1.14 \pm 0.43$, $R = 0.46$) formed the second family. Analysis of [N7]-Mem-CC structures also gave rise to two families (Figure 7) of 55 structures (114 kcal mol^{-1}, rmsd $= 0.95 \pm 0.39$, $R = 0.37$) and 23 structures (150 kcal mol^{-1}, rmsd $= 1.02 \pm 0.58$, $R = 0.58$) respectively.

Discussion

Before any meaningful discussion of the restrained molecular dynamics results can be made, it is necessary to comment on the use of restraints, calculated assuming a single conformation, when clearly these small peptides will be rapidly interconverting between multiple conformations. Smith et al. [29, 30] have shown that, even though the backbone torsion angles of these peptides are likely to be undergoing considerable fluctuations, there may still be some overall conformational preference. In order to check this preference, unrestrained molecular dynamics calculations were performed on Tem-HrTH. The results, given in the form of torsion angles ϕ and φ population distributions (Figure 2), indeed, show that, while the peptide is flexible, the different residues show a preference for particular torsion angle ranges. The ϕ torsion angle of the central serine residue shows little preference in the range $-140 < \phi < -60$, while tryptophane, asparagine(7), phenylalanine and leucine show distinct preferences for $\phi = -80$, -80, -60 and $-80°$ respectively. These results can be compared to those obtained from an analysis of 85 protein structures [30]. For α helical structures a narrow distribution ($-65 < \phi < -61°$), while for β strands ($-140 < \phi < -100°$) and random coil ($-140 < \phi < -60°$) much broader distributions are found. Perhaps more informative is the distribution of φ torsion angles. The central serine and phenylalanine have φ distributions similar to that of a beta strand, while tryptophane and asparagine(3) are more closely aligned to a random coil distribution. Asparagine(7) appears unique in that φ shows no preference within

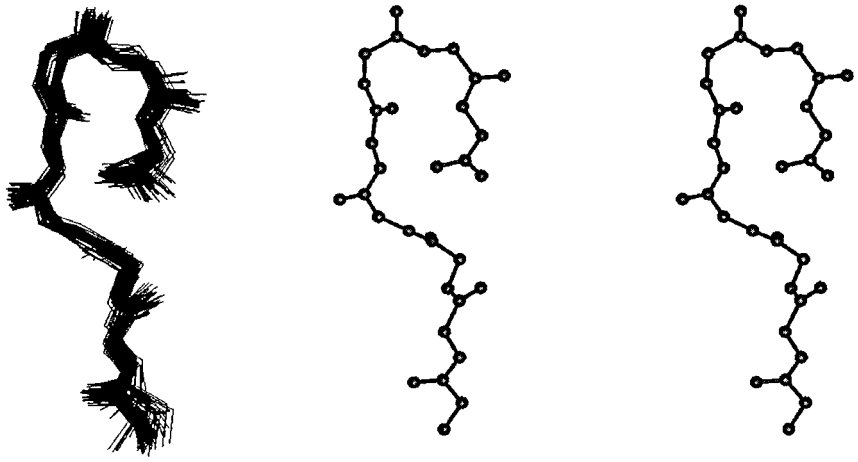

Figure 4. Overlay of most predominant Tem-HrTH families and stereo view of lowest energy conformer.

Figure 5. Overlay of 84 structures of Del-CC grouped into 2 families.

Figure 6. Overlay of 97 structures of Mem-CC grouped into 2 families.

the range $-20<\varphi<120°$. For leucine the most populated φ torsion angle is $-40°$. This is unusual as one would expect leucine, which is near the beginning of the peptide strand, to be more flexible and have a random coil structure. Presumably, the pyroglutamate restricts the motion of this residue. Of interest is the distinctly different distributions of asparagine in positions 3 and 7. The position of Asp7 next to proline and the terminal tryptophan give it far more flexibility than Asp3 which is three residues from the terminus. This trend is continued, with residues 4 and 5 exhibiting the least flexibility. The results for unrestrained, molecular dynamics over a 2 ns time period indicate that Tem-HrTH is flexible in solution but that it also shows some non-random structure.

Figure 7. Overlay of 78 structures of [N7]-Mem-CC grouped into 2 families.

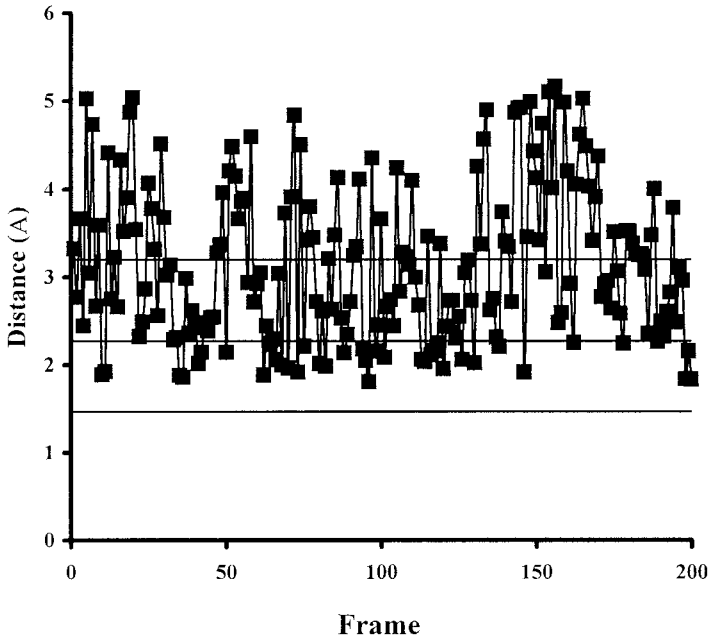

Figure 8. Variation in PheNH-LeuCO internuclear distance during a 2 ns dynamic simulation of Tem-HrTH. The distance criteria of Jeffrey [34] for weak and moderate hydrogen bonds are indicated.

The molecular dynamics simulation results for Tem-HrTH can also be compared with experimental results. Table 1 gives the measured and calculated $^3J_{NH\alpha}$ coupling constants together with literature values for a random coil model. With the exception of phenylalanine, for which the calculated and observed coupling constants differ by 1 Hz, the other coupling constants are in close agreement. In comparison with literature random coil coupling constants, the observed results are all higher than the literature results. This is markedly so for serine where the difference is 3 Hz. Generally serine has a much lower coupling constant because, in the $g^-\chi^1$ rotamer, which is essentially forbidden in an α helix, its polar side-chain oxygen can hydrogen bond to the adjacent main-chain NH [29].

It has long been known [31–33] that, for peptides of limited flexibility, a chemical shift temperature coefficient of $<3 \times 10^{-3}$ (ppm/K) for an amide proton is indicative of a solvent inaccessible proton involved in H-bonding. Alternatively, a temperature coefficient $>5 \times 10^{-3}$ (ppm/K) is indicative of a solvent accessible proton which is not involved in intra-molecular H-bonding. For small flexible peptides the situation is less clear in that hydrogen bonds may be continually being formed and broken. Orlewski et al. [13] have illustrated this using dynamic simulation. In our case, all the amide temperature coefficients are between 2×10^{-3} and 4×10^{-3} (ppm/K), indicating the presence of partial (time averaged) hydrogen bonds. Figure 8 shows the simulation results for the internuclear distance between PheNH-LeuO. Jeffrey [34] classifies an H• • •B bond length of 1.5–2.2 Å to be a moderate hydrogen bond while a H• • •B bond length of 2.2–3.2 Å is classified as weak. With this classification a weak hydrogen bond exists for 56% of the simulation and a moderate hydrogen bond for 15% of the time.

These results, together with the agreement between the calculated and observed coupling constants, lends some justification to the use of nOe and dihedral restraints in a simulated annealing search of conformational space. The practical consequences of this is a decrease in the time required for the search or alternatively, to perform a more detailed search of a limited region of space. In the final minimization, the restraints energies are all <5% of the total energy so that there is no large forcing potential. If restraints are removed during the latter stages of the search there is no significant change in the final structure.

Inter-residue distance data suggest that, since only sequential $d_{\alpha N}$ contacts are present for the first three residues of the peptides, the first portion exists mainly in an 'extended' form. However, for residues 3 and 4,

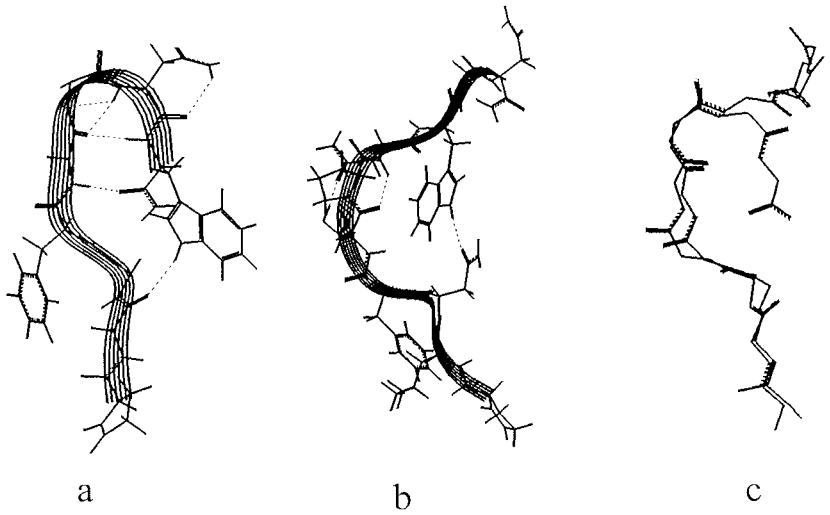

Figure 9. Representative conformers of most predominant family showing some hydrogen bonding and the backbone as a ribbon structure; (a) Tem-HrTH and (b) Del-CC. (c) Overlay of Tem-HrTH and Del-CC.

and residues 5 and 8 there are inter-residue $d_{NN}(i,i+1)$ and $d_{NN}(i,i+3)$ contacts, which suggest that some form of secondary structural elements exist for the last five residues. In general, the intensities of the sequential NH-$C_\alpha H_{(i,i+1)}$ nOe contacts were markedly stronger than the intraresidue NH-$C_\alpha H_{(i,i)}$ contacts suggesting the absence of a helical conformation in solution [31]. No CD studies are presently available for the peptides Tem-HrTH and Del-CC. However, previous CD studies [8, 35, 36] on *Locusta migratoria* neuropeptides containing a proline residues indicate the presence of a β structure in SDS solution.

Wilmot and Thornton [37], in their study of β type turns, have defined a β turn as existing when the distance between the $C_\alpha(i)$ and $C_\alpha(i+3)$ residue does not exceed 7 Å. In addition, a turn is considered to be four residues long with the possibility of an *i,i+3* {C=O (main chain) to NH} hydrogen bond. Using these criteria, the dominant conformations of all four peptides are found to have β turns between residues 4–8. These peptide segments also contain amino acid residues which are favoured for β turn types I and II, viz., residues D, P, N and S. Furthermore, the Y/F,S,P,D segment satisfies the interproton distances requirement $d_{\alpha N(1,4)}$ and $d_{NN(2,4)}$ for turn type I, 3.1-4.2 Å and 3.8 Å, respectively [38].

The dominant conformer of Tem-HrTH (Figure 9a) has eight hydrogen bond contacts. These contacts involve both the extended portion of the chain as well as the β turn. Four interactions involve the S5 residue and 3 contacts involve W8. Interactions between the S5 main chain C=O and W8:NH, S5 amide proton and W8:C=O, N7:NH with S5:C=O, the ring proton of W8 and L2: C=O and that of the terminal amide protons with the initial extended portion, stabilize the β turn.

Analysis of the dominant conformer of Del-CC (Figure 9b) indicates that there are fewer hydrogen bonding interactions than for Tem-HrTH. S5:C=O shows long range H-bond contact with the amide proton of N7. Figure 9c shows an overlay of Tem-HrTH and Del-CC. As expected, for the first 6 residues, both peptides have very similar structures. Thereafter, the presence of the additional residues, G9 and N10 of Del-CC results in the terminal amide contact with residue 4 being lost.

Analysis of the hydrogen bonding patterns for the Mem-CC conformers (Figure 10) shows that N3, S5, D7 and W8 all have hydrogen bonds involving both the backbone and side chains. A common hydrogen bonding interaction is that between the hydroxyl proton of the tyrosine side chain and the C=O group of pE1 which allows for the definition of the extended portion of the first four residues. The β turn of conformer B is stabilized by backbone/side chain hydrogen bonds involving the S5, D7 and W8 residues. The C-terminal backbone turn of conformer A is stabilized by a hydrogen bond interaction between the amide proton of W8 and the carbonyl group of S5 and an interaction between the terminal amide group and the side chain carboxy group of N3.

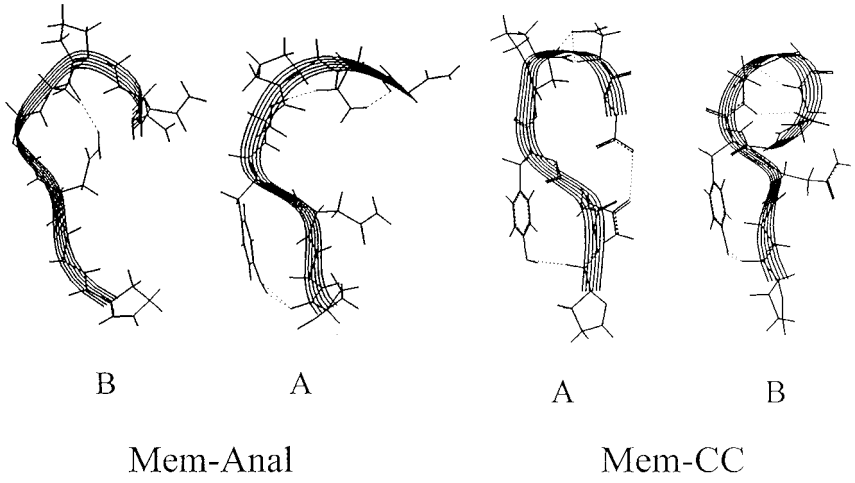

Figure 10. Representative conformers of the most predominant families of Mem-CC and [N7]-Mem-CC showing some hydrogen bonding and the backbone as a ribbon structure.

In the structural analysis of [N7]-Mem-CC, only the data for conformer B satisfies the Cα(*i*) - Cα(*i*+3) distance requirements for a β turn. None of the conformers (Figure 10) satisfy the sequential and medium inter-proton distance requirements for turn types I or II. Fewer hydrogen bonding patterns are noted for the conformers of [N7]-Mem-CC as compared to Mem-CC (Figure 10) with Conformer B having hydrogen bonding interactions between the S5 and N3 residues. Conformer A of [N7]-Mem-CC has hydrogen bonding interactions which involve the extended portion of the peptide, i.e., that between the tyrosine side chain and the main chain C=O of pE1 as well as an interaction between the amide of L2 and the side chain atoms of N3. Additional hydrogen bonding interactions that stabilize the turn of conformer A include that between the side chain hydroxy proton of S5 and the side chain of N7.

The existence of a β turn in the dominant conformers of the four peptides is consistent with previous CD [8, 39] and computational [40–42] studies on AKH peptides. This characteristic β structure at the C terminal end of the peptides has been postulated to be important in the interaction of the hormones with their putative receptors. In contrast, however, the present results provide no evidence for a β sheet conformation as found for the first five residues of Emp-AKH [40]. The first four residues conform to the definition of 'extended structure' as proposed by Siligardi and Drake [38] for small linear peptides and as denoted for single peptide chains whose φ, ϕ dihedral angles are located in the allowed β region of the Ramachandran plot. Also, no evidence was found for an extended P_{II} conformation as recently proposed by Cusinato et al. [8] for *Locusta migratoria* peptides.

Studies which correlate the formation of a β structure in SDS micelles with high activity in the lipid mobilization assay [8], postulate that the β structure is highly favoured for receptor interaction. In the *Locusta migratoria* series of AKH peptides, Lom-AKH-I and Lom-AKH-III both have a β-turn conformation and show a high potency, while Lom-AKH-II, which does not form a β structure, has a lower activity. Sato et al. [43] have synthesized a series of hypertrehalosemic hormone analogues which incorporate a conformationally restricted β-turn mimetic. Their results indicate that a β-turn is necessary for bioactivity. With the exception of some of the [N7]-Mem-CC conformers, all four peptides in this study exhibit a β-turn between residues 4–8. In fact, between residues 1 and 8, the backbone structure of all four peptides is very similar. In the case of Del-CC and Tem-HrTH it is almost identical and these two peptides both have very high activity. This would imply that the extra 2 residues of Del-CC, which project away from the β-turn, do not hinder receptor binding.

The secondary structural elements of the dominant conformers of Mem-CC and [N7]-Mem-CC in comparison with Tem-HrTH and Del-CC indicate some interesting trends as regards primary sequence, biological activity (Figure 1) and solution conformation. From structure/activity correlations, it has been suggested that asparagine at position 7 is necessary for biological activity. The amide side chain of asparagine

is postulated to hydrogen bond to the putative protein receptor. Mem-CC, does not have asparagine at position 7 and hence its lack of biological activity is explained. However, [N7]-Mem-CC does have N7 and yet is still only poorly active. In this case, our results show, that the presence of tyrosine at position 4, causes a subtle change in the hydrogen bonding pattern of the predominant conformation of the molecule. This leads to the N7 side chain folding inside the β-turn. In this position, the amide group is unable to hydrogen bond to the receptor and hence the poor activity. For Tem-HrTH and Del-CC, in their predominant conformations, the amide side chain of N7 projects outwards from the β-turn and hence is free to interact with the receptor. The importance of an asparagine residue at position 7 has also been noted in structural studies on Emp-AKH [40]. Emp-AKH possesses a β turn involving an N7 residue which has been postulated to assist peptide-receptor interactions via its hydrophilic nature.

Besides the effect on the side chain orientation of N7, a change of Y4 for F4, influences the β structure of the peptide. Tem-HrTH has a relatively tight turn that is stabilized by hydrogen bonding within the molecule. In [N7]-Mem-CC, some of the hydrogen bonding is lost resulting in a weaker turn. A similar conclusion was reached in a study on a series of synthetic peptide analogues of the insect neuropeptide Lom-AKH I [36]. Here it was reported that substitutions in the area of residues 6 to 8 of adipokinetic hormones reduced biological activity because it apparently hindered the β turn at proline 6. It seems apparent from this study that the residues at positions 4 and 7 are crucial for favourable receptor interaction with the preferred residue being F4 and N7 as in the case of Tem-HrTH and Del-CC.

Conclusion

In summary, the solution conformations of four insect neuropeptides have been studied in DMSO-d_6 solution and their structures calculated using restrained molecular dynamics. The results indicate that the neuropeptides exist as a mixture of conformers but that there is a tendency for each neuropeptide to adopt a dominant conformer in solution. They all have very similar backbone structures from residues 1–8 with a β-turn. The results suggest that favourable interactions may occur between N7 and the receptor but that the orientation of N7 and the tightness of the β-turn are influenced by F4. These secondary structural changes may be responsible for the increased activity of Del-CC and Tem-HrTH relative to Mem-CC and [N7]-Mem-CC.

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