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CONFORMATIONAL STUDY ON AN INSECT NEUROPEPTIDE OF THE AKH/RPCH-FAMILY BY COMBINED ^{1}H NMR SPECTROSCOPY AND MOLECULAR MECHANICS

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Peptides of the AKH/RPCH family are mainly involved in influencing energy metabolism in insects,i.e.,regulating carbohydrate and/or lipid breakdown in the fat body. We have studied the solution conformation of a member of this family, the peptide Emp-AKH from praying mantis. It has been characterized by use of two dimensional nuclear magnetic resonance spectroscopy and molecular modelling. The proton spectrum of the Emp-AKH peptide was assigned by sequential assignment procedure. Proton-proton distances were derived from the volumes of cross-peaks in two dimensional nuclear Overhauser enhancement spectra. The three dimensional structure was built using Evans & Sutherland molecular modelling station. Our data indicate that the Emp-AKH peptide has adopted a ß-sheet structure for amino acids 1 to 5 and a ß-turn for amino acids at positions 5 to 8. The type of the turn appears to be a Non-specific ß-turn.

Peptides of the adipokinetic hormone/red pigment-concentrating hormone family (AKH/RPCH-family) have been found in all major orders of insects and in some crustaceans [1]. They are synthesized and stored in small neurosecretory organs of these arthropods. These organs, called corpora cardiaca, are comparable to the vertebrate neurosecretory hypothalamus and hypophysis system. In insects, these peptides are mainly involved in influencing energy metabolism, e.g. regulating carbohydrate and lipid breakdown in the fat body [2].

The structural features of peptides of the AKH/RPCH family are as follows: they are 8 to 10-mers (mostly octapeptides), which are blocked by a pyroglutamyl residue at the N-terminus and by an amidated C-terminus. Furthermore, aromatic amino acids reside at positions 4 (Phe or Tyr) and 8 (Trp). All members are uncharged with the exception of two which contain a negatively-charged aspartic acid residue at position 7 [1, 2].

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Abbreviations: AKH, adipokinetic hormone; Emp, *Empusa pennata*; 1D, one dimensional; 2D, two dimensional; 3D, three dimensional; NMR, nuclear magnetic resonance; (TNDQ)COSY, double quantum filtered cosy experiment using transmitter for presaturation during presaturation period; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy.

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It is believed that the AKH/RPCH peptides bind to specific receptors on the membrane of fat body cells to exert their biological effect. As yet no direct receptor studies have been performed. However, structure-activity relationships were studied by measuring lipid or carbohydrate mobilization in locusts and cockroaches respectively, using either the naturally-occurring (see for example: [3, 4, 5]) peptides (bioanalogues) which are similar but not identical, or single amino acid replacement analogues in order to determine the importance of the termini and side chains [6, 7]. From these studies, it became clear that especially the termini and the aromatic residues at positions 4 and 8 are important for interaction with a putative receptor.

We are interested in the relation between the activity of AKH/RPCH-family peptides and their 3-dimensional structure. Conformational information, however, is scarce. Application of the secondary structure calculations of Chou and Fasman [8] to the locust adipokinetic decapeptide (acronym: Lom-AKH-I) predicted that residues 5-8 (Thr-Pro-Asn-Trp) would form a ß-bend [9]. Using the ß-turn prediction algorithm of Wilmot and Thornton [10], type-I ß-turns were predicted for a number of AKH/RPCH-family members [11]. Furthermore, circular dichroism (CD) studies revealed that whereas no identifiable secondary structure emerged in aqueous solution, the addition of sodium dodecylsulfate (SDS) resulted in spectra characteristic of a type-I ß-turn. This was only observed for peptides containing a Pro residue at position 6 [11].

As a first step to investigating the conformation of AKH/RPCH-family peptides more vigorously, we examined the secondary structure of a neuropeptide from a praying mantis. This octapeptide (acronym:Emp-AKH; pGlu-Val-Asn-Phe-Thr-Pro-Asn-TrpNH$_2$) was isolated and sequenced from two species of mantids [12]. We chose Emp-AKH as our first peptide for closer inspection for the following reasons: (1) it is a typical uncharged octapeptide member of the family containing the Pro^6 residue; (2) it is almost as active as the native peptide in the cockroach bioassay [13] and only about 5-fold less active than the native peptide in the locust bioassay [14] and, most importantly, (3) we had large quantities of the synthetic peptide available for pilot studies.

MATERIALS AND METHODS

The peptide was custom synthesized on a solid phase peptide synthesizer using conventional F moc chemistry. The crude peptide was purified be preparative HPLC (reversed-phase, C-18 column) and checked by amino acid analysis, automated sequencing and analytical HPLC.

DMSO-d$_6$ was purchased from Aldrich and used without further purification. The sample used for NMR structure determination contained 3 mM Emp-AKH peptide in DMSO-d$_6$ solution. The use of DMSO as a solvent is fully justified in the light of the previously presented results [15, 16]. All NMR experiments were carried out on a Varian-400 MHz spectrometer. The temperature of the sample was fixed at 30 °C. 2D NMR spectra were recorded non-spinning while 1D spectra were recorded with the sample spinning. A double quantum filtered cosy pulse sequence [17] using transmitter for presaturation during presaturation period- (TNDQ)COSY, Total correlation spectroscopy-TOCSY, homo J resolved 2D spectroscopy [18, 19] and nuclear Overhauser enhancement spectroscopy-NOESY spectra were collected using a acquisition matrix of 1024x128 at a spectral width of 4499.9 Hz. All spectra were run in a phase sensitive mode with the following acquisition and processing parameters. The relaxation delay, mixing time, number of transient and total acquisition time for the (TNDQ)COSY spectrum were equal to 0.500, 0.030, 512, 28.9 hours respectively. Data were processed using the following set of parameters in F1 : gaussian window with center at 0.002 sec and width 0.002 sec, and sine bell 0.029 sec and zero filling in F1 to 512. For the TOCSY spectrum the following parameters were used: relaxation delay 0.50 sec, mixing time 0.030 sec, number of transients 512 with the total acquisition time equal to 26.3 hours. Spectrum in F1 dimension was processed using gaussian apodization equal to 0.022 sec and sine bell square equal to 0.028, and zero filling in F1 to 512. NOESY spectra were collected with mixing times equal to 100, 200, and 300 ms. The NOESY spectrum with the mixing time equal to 200 ms was processed in F1 dimension using sine bell equal to 0.010 sec with zero filling in F1 to 512. Homo J resolved 2D experiment spectrum was processed in F1 dimension as follows: sine bell equal to 0.640 sec and zero filling in F1 to 512. Chemical shifts refer to the DMSO set at 2.5ppm. The 3.3 ppm residual water signal was suppressed using continuous decoupler power during relaxation and evolution time, and mixing time for the TOCSY and the NOESY spectrum respectively. NOESY intensities were measured using the volume integration function of the instrumental software.

The intervening torsion angles were calculated according to the Karplus equation: $^3J_{NH\alpha}=6.4\mathrm{x}\cos^2\theta-1.55\mathrm{x}\cos\theta+1.35$ [20].

The 3D model of the structure was built using the molecular design and analysis program from Biodesign Inc., installed on an Evans & Sutherland Molecular Modelling Station connected to a VAXstation 3200.

The linear structure of the peptide was built using the standard set of L-amino acids. C-and N-terminal amino acids, i.e. Glu and Trp, were modified in order to obtain pyroglutamic acid at the N-terminus and the amidated C-terminus. Using the DGEOM program [21] twenty different structures, based on the distance constraints obtained from NOESY spectrum and torsion angles from homo J resolved 2D spectrum, were created. From those the three with lowest distance violation factor were chosen. After this step the DMSO molecules were introduced and energy minimization was performed three times using the Conjugate-Gradient Algorithm over 100 steps at each times. The dihedral angles and distance constraints were given high weighting factors in order to stabilize the backbone of the molecule. The spatial position of side chains of all amino acids was establish by a conformational energy search. After this stage the energy minimization was performed over 50 steps for the whole structure in a DMSO solution.

RESULTS AND DISCUSSION

NMR studies were performed in a DMSO solution at 30°C. All proton resonances, except N-H of the Trp ring, whose chemical shift is expected to be greater than 10 ppm, were assigned by analyses of (TNDQ)COSY and TOCSY spectra following the method proposed by Wüthrich [22]. The identification of spin systems for Pro, Thr, and Val were performed by subtraction of the (TNDQ)COSY spectrum from the TOCSY spectrum which resulted in arrangement of cross-peaks specific for spin systems of these amino acids. We have experienced some difficulties with accurate establishment of amide chemical shifts belonging to the pGlu, Val, Phe, Trp, and Asn^3, Thr, Asn^7 group respectively. In order to overcome this problem we have carefully examined the homo J resolved 2D spectrum in amide region. The final set of all chemical shifts for the $\mathrm{C}^{\alpha}\mathrm{H}$ protons as well as for side chain protons is listed in Table 1.

Table 1

Chemical shifts of proton resonances of Emp-AKH peptide at 30°C in DMSO

Amino acid residue	Chemical shifts (ppm)			
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others
pGlu	7.78	4.14	2.08 2.04	γCH_2 2.22 $\gamma' CH_2$ 2.13
Val	7.89	4.18	1.94	$\gamma' CH_3$ 0.79
Asn	8.11	4.53	2.53 2.39	NH 7.38 NH 6.94
Phe	7.86	4.56	3.02 2.82	3,5H 7.20 4H 7.17 2,6H 7.14
Thr	8.09	4.36	3.88	γCH_3 1.11 OH 4.68
Pro		4.32	1.84 1.90	γCH_2 1.63 $\gamma' CH_2$ 1.74 σCH_2 3.69 $\sigma' CH_2$ 3.55
Asn	8.07	4.46	2.57 2.42	NH 7.39 NH 6.94
Trp	7.87	4.38	3.20 2.96	4H 7.55 6H 7.05 7H 7.33 5H 6.97 2H 7.10 NH 6.92 NH 7.40

It has been shown that a regular secondary structure can be characterized by a pattern of short sequential distances between amides, C^{α}Hs, and a set of coupling constants [23]. Coupling constants can be used as an unambiguous proof for the existence of a secondary structure only in the case of a α-helix. The sequential $d_{\alpha N}$ distance in a ß-structure is equal to 2.2 Å, whereas in α- helices equal to 3.4 Å. In order to identify the type of a secondary structure which is adopted by Emp-AKH, we have defined that a set of $d_{\alpha N}$ connectivities shorter than 2.2 Å indicates a ß-structure. As a criteria for the prediction of a ß-turn the set of the following NOE connectivities was established: $d_{NN}(i, i+1)$ between residues 2->3, and 3->4, $d_{\alpha N}$ sequential connectivities between residues 1, 2, 3, and 4.

Medium-range (i, i+2) NOE connectivity can be treated as definite proof for the existence of a ß-turn in the studied structure. In order to establish the specific type of ß-turn the examination of coupling constant for residues 2 and 3 of the turn must be performed.

To support the explanation of our experimental data, we used the Chou and Fasman algorithm [24] in order to predict the secondary structure of Emp-AKH. The results indicate the following main features: (1) the expected secondary structure will be a ß-structure and (2) a group of four amino acids (Thr, Pro, Asn, and Trp) have the highest probability of forming a ß-turn. The p_t value for this cluster of amino acids is equal to $8.21*10^{-4}$ whereas the average probability of the occurrence of a ß-turn is equal to $0.75*10^{-4}$. It was also observed that $<P_t>$ was greater than 1 and greater than $<P_\alpha>$ and $<P_\beta>$ which is in agreement with the rules of this algorithm. We also used Wilmot-Thornton algorithm to predict the type of ß-turn. The preferable turns were type I and a Non-specific ß-turn, whereas a type-II turn was rejected by this prediction.

In order to establish the exact secondary structure of Emp-AKH peptide we have examined NOESY spectra for the existence of short sequential and medium range NOE connectivities with the result described below.

NOE intensities were correlated with an intermolecular distance using the $r_{ij}=r_{ref}(a_{ref}/a_{ij})^{1/6}$ relationship, where r_{ref} and a_{ref} refer to the fixed distance between methylene protons and the intensity of the cross-peak respectively. The NOESY spectrum of Emp-AKH exhibits negative values in the C^αH-NH region. The vicinal coupling constants, NH-C^αH, read from the amide split observed in the homo J resolved 2D spectrum resulted in values greater than 7 namely, 8.66Hz, 7.72Hz, 8.09Hz, 7.72Hz, 8.09Hz for Val, Asn^3, Phe, Thr, Asn^7, and Trp respectively. This can support the assumption that the investigated peptide can adopt a ß-extended structure [25], but it cannot be unambiguously stated. Therefore, the exact verification of the suspected secondary structure can be done on the bases of NOESY connectivities.

It was observed that the average sequential distance constraint was of the order of 2.3 Å. Such observation is in agreement with previously established data for a formation of a ß-structure [23]. The existence of a ß-turn in Emp-AKH is evident by presence of $d_{\alpha N}$(i, i+2) and $d_{\alpha N}$(i, i+3) medium range connectivities . The exact type of the turn can be established on the basis of the observation of distance constraints [$d_{\alpha N}$(i, i+2), $d_{\alpha N}$(i, i+3)] and coupling constants which are characteristic for one of four types of ß-turns.

Because of very close chemical shifts of NH protons and, and therefore, overlapping of NOE cross-peaks the exact evaluation of d_{NN} distance constraints was practically impossible. Hence, the final establishment of the exact type of the turn could be done only on the basis of the C^αH-NH connectivities and vicinal coupling constants. Observable sequential and medium range NOE connectivities $d_{\alpha N}$(i, i+2), $d_{\alpha N}$(i, i+3), and $d_{\alpha N}$(i+1, i+2) where "i" is a Thr, "i+1" is a Pro, "i+2" is a Asn^7, and "i+3" is a Trp, indicate that the turn adopted by Emp-AKH is a Non-specific ß-turn. The estimation of coupling constant for the position "i+1" is impossible because the second amino acid involved in a turn is Pro which lacks the N-H group. In this case the decision about the type of a turn had to be taken on the basis of coupling constant for the "i+2" position and this also indicates that the studied ß-turn will be a Non-specific ß-turn. Based on this two independent values, i.e.,distance constraints and coupling constant, we feel confident that the adopted turn will be a Non-specific turn. As supporting evidence for the proposed structure the temperature coefficients of amide proton resonances were used. These resonances are widely used as indicators of a spatial orientation of amide protons. Protons involved in intramolecular hydrogen bonding or shielded from the solvent will have significantly lower temperature coefficient then those fully accessible to the solvent molecules. The large temperature coefficients (1.0-1.5) ppb°C^{-1} for the backbone amide protons of pGlu, Val, Asn^3, Thr, and Asn^7 were observed, whereas significantly lower coefficients (0.5-0.75ppb)°C^{-1}·were observed for N-terminus amide, side chain of Asn^3, and backbone amide of Phe protons. This suggest that the latter amide protons are involved in intramolecular hydrogen-bonds or are shielded from the solvent. We are fully aware that, in order to established the most reliable structure one must also examined the NOE connectivities for side chains of all amino acids. Unfortunately this region was so crowded on our NOESY spectrum and so many peaks were overlapped that we were not able to say with full confidence that the volume of integrated peak is reliable. Therefore, we have used a combination of two methods, NMR spectroscopy and molecular mechanics, in order to establish a probable structure of the studied peptide. The molecular model of the Emp-AKH peptide, built on the basis of the distance constraints and using the procedure outlined in Materials and Methods, is presented in Fig. 1. The model indicates that the Emp-AKH peptide in DMSO solution adopts a ß-structure, with sequential distance constraints which seem to be close to those listed for regular antiparallel ß-sheet [23], and has a Non-specific tight ß-turn at position 5 in the amino acid chain. The spatial arrangement of side chains was evaluated as described in Materials and Methods. It can also be observed that the secondary structure is stabilized by three hydrogen bonds which was confirmed by both, the NMR spectroscopy and molecular modelling. The solvent accessible surface of the Emp-AKH neuropeptide has a very distinctive hydrophobic domain which is created by side chains of Val, Phe and Trp. This domain could be involved in peptide-membrane interactions. The Asn^7 involved in the formation of the ß-turn will create a hydrophilic microsphere which may be important for peptide-receptor interaction. Hence, on the basis of molecular modelling we conclude that the region of the ß-turn could be directly involved in hormon-receptor interactions. Moreover, interactions between amino acids of the turn with the receptor have most probably electrostatic character. The structure presented here is one of the few we have created using the outlined approach. Here we have decided to show the one with the lowest overall energy minimum.

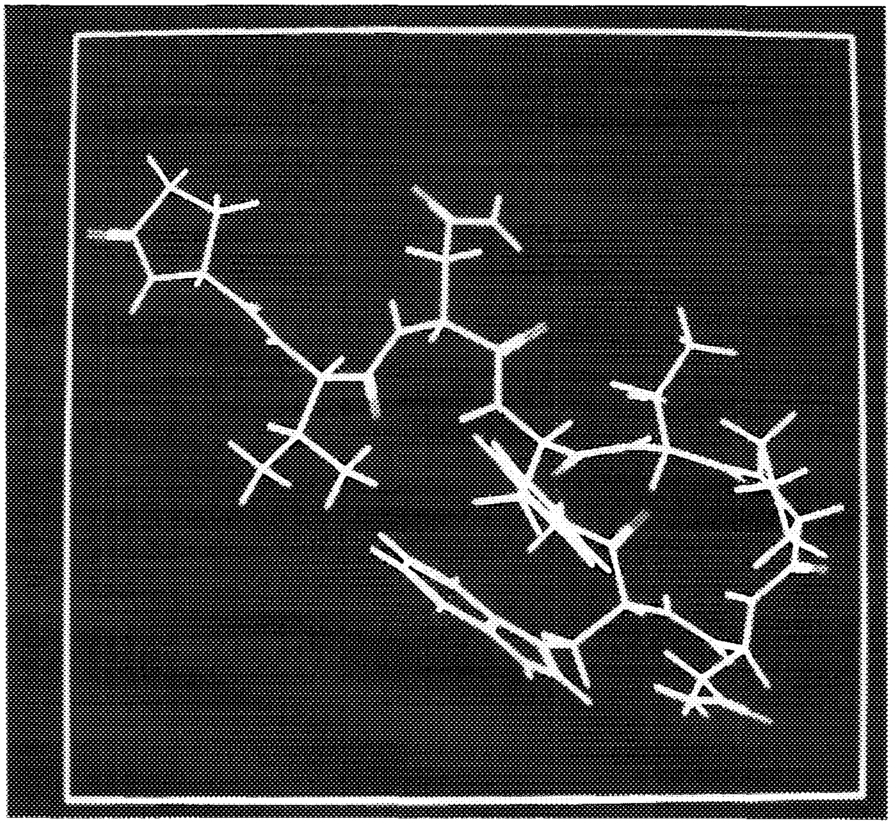

Figure 1.

The three dimensional model of the Emp-AKH neuropeptide. The model was built as described in materials and methods. The N-terminus is located at upper left.

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