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Conformational study on a representative member of the AKH/RPCH neuropeptide family, Emp-AKH, in the presence of SDS micelle*

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Abstract. Emp-AKH is a member of the large adipokinetic hormone (AKH) family of peptides. This peptide family appears to occur in the corpora cardiaca of all insect species and its members are involved in regulating substrate mobilisation. The secondary structure of Emp-AKH has been studied in the presence of sodium dodecyl sulfate micelles by comparing data obtained from Nuclear Magnetic Resonance and molecular dynamics simulations. The lowest energy conformer obtained in this study has a turn consisting of residues 5–8 and a tail consisting of the first five residues.

INTRODUCTION

Peptides of the large adipokinetic hormone (AKH) family regulate energy metabolism during periods of high energy demand in insects and are involved in the mobilisation of lipids, carbohydrates and proline (see Gäde, 1996; Gäde & Auerswald, 1998). Such peptides are synthesised and stored in intrinsic neurosecretory cells of the retrocerebral corpora cardiaca and have been found in all major orders of insects. Structurally the peptides are characterised by an amino acid length of 8 to 10 residues, a pyroglutamyl residue blocking the N-terminus, a C-terminus blocked by an amidation and aromatic amino acids at least at positions 4 (Phe, mostly, and Tyr) and 8 (Trp), but molecules with 3 aromatic amino acids are also known (see Gäde, 1996, 1997). The majority of the members of this family are uncharged; only three contain a negatively charged Asp residue at position 7 (Gäde et al., 1997).

The sequence of a member of AKH family, the Emp-AKH peptide from the praying mantis, *Mantis religiosa* (Mantodea: Mantidae), is given below:

pGlu–Val–Asn–Phe–Thr–Pro–Asn–Thr–NH_2

Adipokinetic Hormone/Red Pigment-Concentrating Hormone family peptides are assumed to operate by interacting with specific receptor molecules located in the membranes of the target fat body cells. However, information on peptide/receptor interaction is scarce (see Ziegler et al., 1995). Nevertheless, many studies have investigated structure/activity relationships based on robust bioassays such as lipid or carbohydrate mobilisation to indirectly evaluate the interaction of the AKH/RPCH peptide with its receptor (see, for example, Gäde, 1996, 1997; Gäde et al., 1997). There are, however, other physico-chemical and mathematical methods available to study the question of which functional features may be important on specific parts of such a peptide. Such methods, nuclear magnetic resonance (NMR) or circular dichroism (CD) spectroscopy, make it possible to get information on the molecular conformation of a peptide and these data, coupled to a rigorous modelling approach, can eventually give rise to the preferred molecular structure which may be adopted by the peptide under investigation. AKH/RPCH peptides have been subjected to various structure prediction and molecular modelling studies (Stone et al., 1978; Wheeler et al., 1990) and have been analysed in aqueous solution (no clear ordered conformation), at low temperature in ethanediol/aqueous buffers (P II extended conformation) and in interaction with SDS micelles (beta-structure; Cusinato et al., 1998), but also in dimethylsulfoxide using NMR (beta sheet conformation from residues 1–5 and beta turn encompassing residues 5–8; Zubrzycki & Gäde, 1994).

The present study continues our work on the conformation of a typical octapeptide member of the AKH/RPCH family, the molecule Emp-AKH which has been found in the praying mantis' corpus cardiacum (Gäde, 1991). Here, we make use of the NMR technique in combination with formed SDS micelles. We are confident that such experimental conditions may mimic the natural membrane environment more closely and possibly mirror interactions taking place in vivo.

MATERIAL AND METHODS

The peptide Emp-AKH was custom-synthesised on a solid phase synthesiser using conventional F-moc chemistry. SDS-d_{25} (99% D atom, M.W. 297) was purchased from Cambridge Isotope Laboratories and used without further purification. The studied sample contained 3 mM Emp-AKH and 90 mM SDS-d_{25} in 90% H_2O/10% D_2O solution (equalling a 3% SDS-d_{25}

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solution). All NMR experiments were carried out on a Bruker AM-X500 spectrometer operating at 500 MHz. The temperature was regulated with the accuracy of ±0.1 K by the Bruker temperature-controlling device.

All spectra were collected with the carrier frequency coinciding with water resonance, which was used to calibrate chemical shifts. 1D spectra were collected at three elevated temperatures 283 K, 288 K, and 293 K respectively. The data were processed on a Bruker X 32 station using the Bruker UXNMR programme. Double Quantum Filtered (DQF)-COSDY (Rance et al., 1983) spectrum was recorded with 90° pulse of 6.5 ms. Total Correlation Spectroscopy (TOCSY) (Bax & Davis, 1985) was carried out using MLEV-17 (Levitt & Freeman, 1981) composite pulse cycling. The spectrum was collected with 90° pulses of 7 ms (high power) and 28.2 ms (attenuation of 14 dB) at a mixing time of 80 ms. Nuclear Overhauser Enhancement Spectroscopy (NOESY) (Jeener et al., 1979) was performed at mixing times of 30, 60, and 80 ms with 90° presaturation pulse during relaxation and mixing time. All spectra were recorded using time proportional phase increments (TPPI). Acquisition parameters were kept constant for all experiments, with spectral width of 6024.10 Hz, dwell time of 83.0 ms, presaturation delay of 1.3 s and 8 dummy scans. For all spectra, 2K data points were used in F2, and 512 increments were acquired in F1, that led to the matrix 2K × 1K after zero filling.

The 3D model of the structure of Emp-AKH was built using the standard set of L-amino acids. The C-and N-termini were modified in order to obtain pyroglutamic acid at the N-terminus and an amidated C-terminal Trp. The substructure coordinates were obtained using Geometry Optimisation procedure with the set of distance restraints obtained through the analysis of the NOE spectrum and $^3J_{HN\alpha}$ coupling constants. The distances were computed using the known distance between the geminal C beta protons of Phe and torsional constraints using Karplus equation. Results obtained at mixing times of 30, 60, and 80 ms were in good agreement. Spatial constraints obtained at the mixing time of 60 ms were used for the construction of the 3d model of Emp-AKH. In the next step, the structure was subjected to 10 ps of heating from 0 K to 1,000 K, and run at 1,000 K for 20 ps. At this point, the structures were selected at 1 ps intervals from the last 5 ps of the run. These structures were subjected to the two step energy minimisation consisting of 100 steps of Steepest Descend minimisation and Polak-Ribiere minimisation (Gill et al., 1981) procedure until the energy gradient was smaller than 0.001 kcal/mol Å. These structures were subjected to the Restrained Molecular Dynamics procedure (RMD) (Atkinson et al., 1994) consisting of 6.5 ps heating from 0 K to 500 K, 10 ps run at 500 K, 8 ps cooling from 500 K to 300 K and 10 ps run at 300 K. From the last run, 5 snapshots were taken at 1 ps intervals from the last 5 ps of the simulation. These structures were also subjected to the two-step energy minimisation method given above.

RESULTS

1D NMR spectra of the peptide Emp-AKH at three elevated temperatures (283 K, 288 K and 293 K) were examined in the presence of SDS. The broadening of peaks with the temperature increment due to increment of relaxation time and the interaction between SDS micelles and the peptide was observed. The analysis of these results allowed for choosing 283 K as the most suitable temperature to perform 2D NMR experiments.

A long mixing time of τ = 80 ms for the TOCSY experiment allowed for complete magnetisation transfer from amide protons through alpha- and beta-H's to the end of side chains of the amino acids. Assignments of frequencies of hydrogen atoms were performed using well-described methods based on the identification of scalar-coupling networks (Ohno et al., 1993). The (DQF)-COSY spectrum was used for confirmation of the assignment obtained by examination of the TOCSY spectrum. Also, a sequential NOE network was used along the peptide backbone, and protons were identified by intraresidual and interresidual connectivity between N(i)H and C alpha(i)H, and C alpha(i)H and N(i+1)H, respectively. The complete proton spin system assignment, in the presence of SDS-d_{25} micelles at the temperature of 283 K, is presented in Table 1. It is known that exposed protons, or those involved in conformational

TABLE 1. Chemical shiftes of proton resonances of Emp-AKH peptide in presence of SDS micelles at 20°C.

Amino acid residue	Chemical shifts (ppm)				
	$^3JH_{N\alpha}$ (Hz)	HN	CαH	CβH	Others
$_P$Glu			4.25	1.80	γCH_2 2.32 $\gamma' CH_2$ 1.81
Val	9.05	7.97	3.88	1.83	γCH_3 0.72
Asn	9.05	8.28	4.55	2.60 2.47	NH2 7.36
Phe	8.60	7.66	4.42	2.82 2.70	3,5H 7.21 4H 7.00 2,6H 6.90
Thr	9.05	7.77	4.24	8.76	γCH_3 0.53
Pro			3.76	1.35 1.20	$\gamma\gamma' CH_2$ 0.71 δCH_2 3.20 $\delta' CH_2$ 2.85
Asn	9.05	8.13	4.38	2.56 2.42	NH 7.33
Trp	8.60	7.16	4.47	3.08 3.03	NH 9.80 4H 7.25 6H 6.88 7H 7.33 5H 6.81 2H 6.99

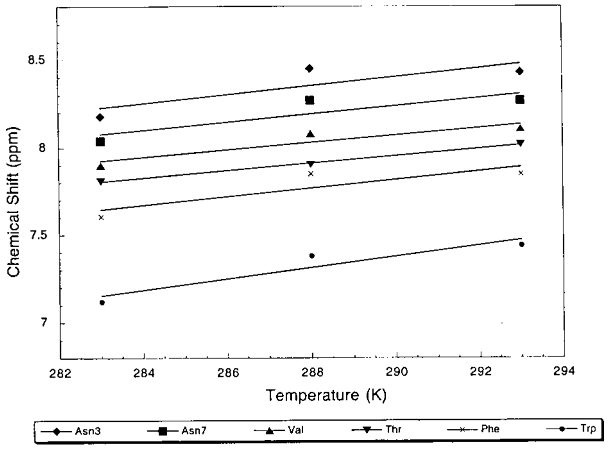

Fig. 1. Correlation between the chemical shift and the elevated temperature observed for amide protons of Asn3, Asn7, Val, Thr, Phe and Trp residues.

changes, show greater changes in chemical shifts during increasing temperature than the internal ones. This can be expressed in terms of temperature coefficients $\Delta\delta/\Delta T$ which are generally in the order of 10^{-3} ppm·K^{-1}. Thus, the temperature dependence of chemical shifts of the amide protons from the set of 1D spectra recorded at elevated temperature (283, 288 and 293 K) has been analysed in order to establish the position of these protons and possible hydrogen bonds. The profiles of NH chemical shifts versus temperature vary between 0.024 ppm·K^{-1} and 0.027 ppm·K^{-1} as shown in Fig 1. There is, unfortunately, no general consensus concerning the relationship of the temperature coefficient and the structure in complex media. Therefore, this information cannot be used as an accurate indicator of possible hydrogen bonds located within the three-dimensional structure. The analysis of the temperature coefficients shows that none of them is close enough to zero to indicate the presence of stable hydrogen bonds or complete shielding from the solvent. C-terminal amide protons of Trp and the N-terminal proton of pGlu were not visible in the spectra. This is due to fast H-D exchange with the solvent. Using the proton-proton distances evaluated from the NOE experiment, a possible three-dimensional structure of Emp-AKH was constructed. The starting conformer was subjected to Molecular Dynamics at 1,000 K to overcome all possible energy barriers. This step was followed by Restrained Molecular Dynamics Energy Minimisation procedures allowing the structure to "settle down" in its energetic minimum. As a result, 25 final structures were generated and single point energy calculation was performed on all of them. The analysis of these results allowed to elucidate the lowest energy conformer, shown in Fig. 2. Using the hydrogen bond acceptance rules, the placing of such bonds was calculated and it became clear that a hydrogen bond between HN–Val and C=O of Phe is present.

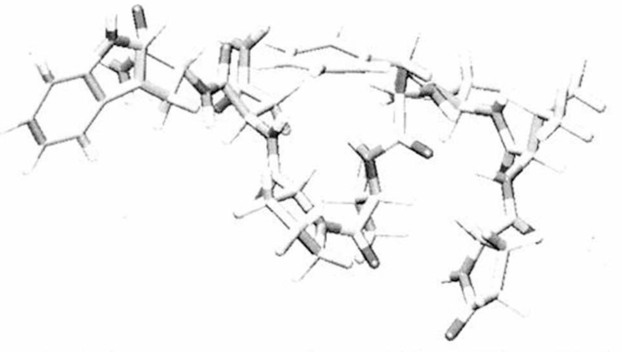

Fig. 2. Lowest energy conformer of Emp-AKH peptide in SDS solution obtained using Restrained Molecular Dynamics Simulation.

DISCUSSION

There is general consensus that small peptides in aqueous solutions may adopt a variety of different conformations, which can be elucidated by the use of different physical conditions such as solvent and/or temperature changes. It has been shown that in the presence of excessive SDS concentration, i.e. higher than 4 mM, SDS forms micelles that can be used as mimics of negatively charged bilayers; these can also provide an anisotropic environment similar to those of lipid vesicles (Zhong & Johnson, 1992).

The use of low temperature (283 K) and SDS micelles, which are good membrane mimics, allowed for "freezing" of the conformer in its energy minimum and thus for complete assignment of 2D NMR spectra of the Emp-AKH peptide. Because there is a strong possibility that Emp-AKH may interact with a receptor located in a membrane environment when exerting its action, the use of SDS micelles as a "solvent" has merit.

The resonances of all protons, with the exception of C-terminal amide protons and N-terminal HN–pGlu group, have been assigned. The absence of the aforementioned resonances indicates that these protons are not deeply buried in the molecule and are easily accessible to the solvents. The formation of pyroglutamate from glutamate, which is an intramolecular cyclisation between the amino and the f-gamma-carboxy group, results in the cancellation of the hydrophilic properties of the N-terminal peptide "tail". Hence the first residue does not take a part in hydrogen bonding and therefore the stretch of residues 1 to 5 can adopt an extended structure.

By analysing the structure obtained by the use of Restrained Molecular Dynamics we could assign positions of hydrogen bonds. It was observed that a hydrogen bond is present between the C=O group of Phe and HN–Val. Since the peptide is very flexible in aqueous solution this bond is definitely responsible for stabilisation of the turn observed in the backbone chain, as well as for the overall shape of the peptide.

The superposition of backbone chains of the lowest energy conformers of Emp-AKH shows a characteristic conformation in the presence of SDS micelles and possibly in its physiological environment (Ohno et al., 1993) with pGlu and Trp–NH2 groups hanging freely and thus being easily accessible for the solvent.

Our previous NMR study on the conformation of Emp-AKH, performed in DMSO-d6 solution, indicated the presence of a beta-turn consisting of residues 5 to 8 and a beta-sheet consisting of residues 1 to 5. Unfortunately, the results obtained in DMSO solution may have not reflected what occurs in the physiological environment because of solvating effects of this solvent.

Recently, another study proposed a different secondary structure for the AKH/RPCH peptides using circular dichroism spectroscopy (Cusinato et al., 1998). In an aqueous solution at low temperature a P II extended conformation becomes apparent. However, in the presence of 0.6% SDS the majority of the peptides adopt a beta-turn.

Another molecular modelling study on Emp-AKH using molecular and Langevin dynamics has been reported recently (Zubrzycki, 1988). The results presented in that report, as well as the results obtained in the present study, were compared with left-handed P II structure calculated using the HyperChem modelling programme. The NMR results of the present study are consistent with those obtained by Langevin dynamics and aqueous solutions simulation. Results of the theoretical study (Zubrzycki, 1988) performed for a simulated in vacuo and membrane mimicking environment have shown that the first five residues created a left-handed helix that was slightly tighter than the P II conformation. Results obtained for simulation performed in aqueous solution and using Langevin dynamics led to conformers whose "tails" did fit better to the P II conformation.

Previous studies on structure prediction in which Chou and Fasman algorithms were used (Zubrzycki & Gäde, 1994) indicated the existence of a beta-structure formed by the group of four amino acids involved in the beta-turn. The analysis of ϕ and ψ dihedral angles of the NMR structure obtained in the present study shows that, although there is an extended structure present which is covered by residues 1–5, it cannot be classified as a β-structure or a PII conformation. The same applies to the actual turn region. It cannot be classified as possessing any defined turn. Since the differences between beta-structure, PII conformation and experimentally and theoretically obtained models are quite significant, we may speculate that hydrophobic residues are responsible for the hormone-receptor interactions which is, however, only feasible if the first five residues create some sort of extended structure – something between a beta-structure or a P II conformation which allows for such interactions. This theory is strengthened by the presence of two strongly hydrophobic residues, Phe and Trp, in the sequence of Emp-AKH whose side chains create a hydrophobic template.

The results presented here are supported by an earlier study (Sato et al., 1993). In this work the first generation HTH analog of Hypertrehalosemic hormone (HTH), isolated from a tropical cockroach, incorporating a beta-turn was synthesised and its biological activity was measured. It was shown that only the isomer approximating a type II beta-turn exhibits biological activity.

The possibility of interaction with the extrinsic part of the receptor should also be considered and, in this case, the structure of a "tail" consisting of five residues should not influence hormone-receptor interaction. In our opinion the presented data help understanding of the selectivity of adipokinetic hormone receptors. The model presented here is in favour of hydrophobic interactions between the peptide and its membrane receptor. It can be assumed that receptor selectivity is based mainly on topographical and hydrophobic similarities between molecular conformation of peptide hormones.

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