CONFORMATIONAL STUDIES OF TACHYKININ PEPTIDES USING NMR SPECTROSCOPY

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1. Summary

Conformational analysis of two tachykinin family peptides: Scyliorhinin I (ScyI) and Scyliorhinin II (ScyII) was carried out by 1D- and 2D-NMR (DQF-COSY, TOCSY, HMQC, HMBC, NOESY and ROESY) and molecular dynamics calculation methods in water and DMSO. Scyliorhinin I is a equipotent agonist of NK-1 and NK-2 tachykinin receptors and Scyliorhinin II is a selective agonist of NK-3 tachykinin receptor. In DMSO, two groups of conformations (major and minor) were obtained for both peptides based on the experimental data. The conformations proposed for ScyI represent a folded structure, which show certain similarities to the structures reported for other NK-1 and NK-2 tachykinin agonists. In water ScyII displays a flexible, extended structure, whereas in DMSO the structure is more compact and in the fragment from centre to the C-terminus several β-turns may be present.

2. Introduction

In 1986, Conlon et al. [1] isolated from the intestine of the common dogfish (*Scyliorhinus caniculus*) two peptides named Scyliorhinin I (ScyI) and Scyliorhinin II (ScyII). The first peptide appeared to be the naturally occurring tachykinin family peptide displaying equipotent high affinity [2, 3] and agonistic potency [4] towards both NK-1 and NK-2 tachykinin receptors. ScyII, on the other hand, is a selective agonist of NK-3 tachykinin receptor [3] and in addition contains one disulfide bridge, which is a unique structural element among all naturally occurring tachykinins. The amino

acid sequences of the peptides investigated are as follows:

ScyI: AKFDKFYGLM-NH2;

ScyII: SPSDSKCPDGPDCFYGLM-NH2

In this paper we report the conformational studies of both peptides. The results obtained will be further discussed in relation to the 3D structures published for other tachykinins.

3. Materials and Methods

Peptides synthesis: Both peptides were synthesised by the conventional solid-phase method using the Boc chemistry. Finally purification was carried out on a semipreparative reversed-phase C18 HPLC column.

NMR experiment: The sample concentrations are in a range from 5 to 10 mM. The ^{1}H, ^{13}C NMR experiments were performed on a Varian Unity 500 Plus spectrometer, operating at 500 MHz for proton and 125.7 MHz for carbon resonance frequency. All spectra were recorded at 303 K except for the temperature coefficients of the chemical shift which were measured for the amide proton resonances throughout the temperature range 295–318 K.

NMR signal assignment: The assignment of the proton chemical shifts of ScyI and ScyII were made using DQF-COSY, TOCSY, NOESY, and ROESY experiments. For ScyII in water, ^{1}H and ^{13}C HMQC and HMBC spectra were used additionally.

Vicinal coupling constants and torsion angles calculations: The $^{3}J_{HN\alpha}$ coupling constants were extracted mainly from the 1D proton spectrum at 295 K after resolution enhancement recorded at 500 MHz. The coupling constants of the overlapped amide protons were measured from complementary DQF-COSY. The possible torsion angles ϕ were calculated from relevant $^{3}J_{HN\alpha}$ values using the Karplus-type equation reported in the literature [5,6].

Temperature dependence of the chemical shift of the NH proton: Temperature coefficients $\Delta\delta/\Delta T$ were calculated from the 1D spectra. The following temperatures: 295 K, 303 K, 308 K, 313 K and 318 K were used.

Proton-proton distance calculations: Except for ScyII in water where the ROESY spectrum recorded at a mixing time of 300 ms was used, the interproton distances were calculated from the NOESY spectra recorded at mixing times of 400 ms using the procedure described in the literature [7].

Energy minimisation and MD simulated annealing: The initial molecular structures of ScyI and ScyII were generated by the BIOPOLYMER module of the SYBYL package. The starting conformations were set to the extended form. Then energy minimisation was performed using the Kollman All-Atom force field.

The solvent was implicitly accounted for by using a distance-dependent dielectric constant. The total conformational energy was the sum of the electrostatic energy E_{ES}, non-bonded energy E_{NB}, (including hydrogen bond energy, E_{HB}), and torsional energy E_{TOR}. The relative energy of the *i*th local minimum is expressed as $\Delta E_i = E_i - E_0$, where the zero subscript refers to the energy of the lowest-energy conformations. The SYBYL-standard geometry, partial atomic charges, and other force field parameters were used for all amino acid residues. Then the interproton distances derived from NOEs (for ScyI and ScyII in DMSO) and ROEs (for ScyII in water), respectively, were used as distance constraints in the energy minimisation with the AMBER force field. According to the NMR data, the geometry of the peptide bonds, was fixed to *cis* or *trans*. Also the chirality of all C_α (except for the Gly residue) was fixed to L. For further calculations the lowest energy conformers were selected, and after removing the NMR constrains, the energy minimisation step was again performed using the AMBER force field not including solvatation. All computations were carried out on an SGI POWER CHALLENGE workstation using SYBYL (version 6.2) software (TRIPOS, Inc.).

4. Results

In the TOCSY and DQF-COSY spectra, both peptides displayed two distinct sets of residual proton resonances in the amide and a proton regions. Except for ScyII in water, where only few amino acid residues displayed two sets of resonances, this enabled to perform an independent conformational analysis of the major and minor species. Based on the data obtained from the analysis of the NMR spectra (location of hydrogen bonds, torsion angles, configuration of peptide bonds, and interproton distances) probable solution structures of ScyI and ScyII were determined. Careful analysis of the NOESY and ROESY spectra revealed the presence of several *cis* peptide bond: Ala1-Lys2 and Gly8-Leu9 for the ScyI major species; Asp4-Lys5 and Lys5–Phe6 for the ScyI minor species; Pro11–Asp12 for ScyII in water; Cys7–Pro8, Gly10–Pro11 and Val15–Gly16 for ScyII in DMSO major species; Pro2–Ser3, Pro11–Asp12, Val15–Gly16 for the ScyII minor species and enabled the number of interproton distances to be determined: 98, 69, 59, 151, 62, respectively. The *cis* peptide bonds were identified by the presence of cross peaks $H_{ai} - H_{ai+1}$ and the lack of the $H_{ai} - H_{ai+1}$ in the NOESY spectra, and by the proton exchange peaks n the ROESY spectra. The results obtained for ScyI indicate that this peptide has a folded structure in DMSO. The lowest energy conformer of the major species (Fig.1a) reveals that two β-turns along the peptide backbone are present in the fragment Ala1–Tyr7.

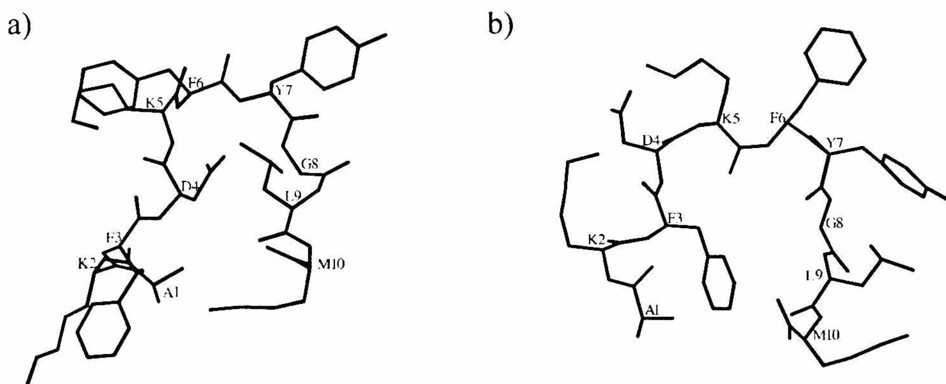

Figure 1. *Three-dimensional structures of the lowest energy conformers of the major (a) and minor (b) species of ScyI in DMSO-d_6.*

The first one, a type IV β-turn was found at the N-terminus. The second one, a type III' β-turn, was detected in the centre of the molecule with Lys5 and Phe6 in positions i+1 and i+2, respectively. The structure is stabilised by several intramolecular hydrogen bonds (Figure 1a). These data are in good accordance with the proton temperature coefficients obtained and the values of $^3JHN_\alpha$, which indicate some of bent points formed in the backbone, especially in the region of Phe3–Leu9. More uniform values of the $^3JHN_\alpha$ coupling constants for the minor species of ScyI adopts a more extended conformation in this case. The analysis of 10 lowest energy trajectory structures reveals that the minor species is an equilibrium mixture of conformers with 1 or 2 turns present in the fragment from the centre to the C-terminus, depending on the energy distribution. The lowest-energy conformer of the minor species (Figure 1b) is characterised by the presence of two *cis* peptide bonds Asp4–Lys5 and Lys5–Phe6, which form a turning point for the bent fragment 3-7. In addition a type VI β-turn in the fragment 2-5 was found (with a *cis* peptide bond in position i+2). The results obtained indicate that the ScyII structure in water is less defined than in DMSO. In the first solvent the calculated lowest energy conformer (Figure 2) shows that the N-terminal fragment represent a flexible, extended structure. The bent structure is in fact located in the fragment 7–13 between two Cys residues. This finding is also supported by the $^3JHN_\alpha$ coupling constants which except those obtained for Gly10 (3.1 Hz) are in the range of 5.5–7.9 Hz. Significantly more ordered structure was found for ScyII in DMSO. Both lowest energy conformers of the major and minor species are stabilised by several β-turns. In the case of the major species (Figure 3a) four are type IV β-turns present in the fragments: Lys6–Asp9, Cys7–Gly10, Asp9–Asp12, Gly10–Cys13 and Asp12–Val15. The others found in the regions Pro2–Ser5 and Pro8–sPro11 are type I' and VI β-turns, respectively.

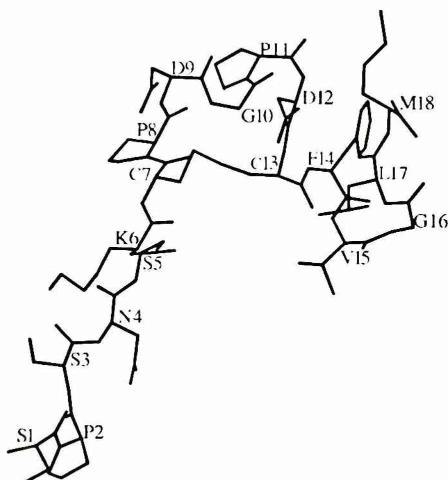

Figure 2. *Three-dimensional structure of the lowest energy conformer of ScyII in water.*

The structure is in good agreement with the low values of the temperature coefficients of Ser5 (-2.67), Phe14 (-2.67), Val15 (-2.67) and Met18 (-2.00) indicating the presence of stable hydrogen bonds which stabilise the ordered structure of ScyII around these residues. For the 3D structure of the minor species of ScyII in DMSO, the temperature coefficient study and especially the analysis of the calculated structure of the lowest energy conformer (Fig. 3b) indicate that the fragment from the centre to the C-terminus is relatively more rigid than the N-terminal one, since six β-turns were found in this region (Lys6–Asp9 (type III), Asp9–Asp12 (type VI), Gly10–Cys13 (type IV), Pro11–Phe14 (type II'), Phe14–Leu17 (type I) and Val15–Met18 (type I)) compared with one β-turn in the N-terminal region (Ser1–Asn4 (type I)).

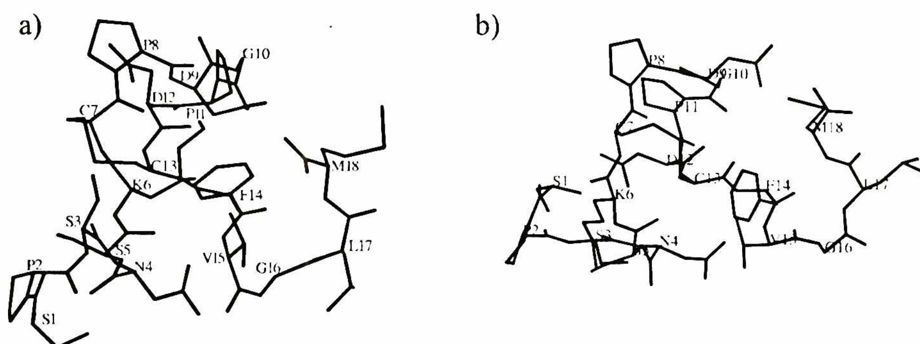

Figure 3. *Three-dimensional structures of the lowest energy conformers of the major (a) and minor (b) species of ScyII in DMSO-d_6.*

5. Discussion

The results presented above indicate the presence of several *cis* peptide bonds in the peptides investigated, which is usually not a common feature. The explanation could be that under the conditions of the experiments the structures of both peptides are flexible. This is also supported by the presence of two groups of the NMR signals. In this respect, the NMR data reflect a population-weighted average and the conformations discussed cannot be interpreted in terms of static structures but should rather be called the "NMR structures" of these peptides. In fact, this is a problem common for conformational studies of peptides. Keeping the above in mind we would like to stress that our results have provided evidence for the folded structure of ScyI in DMSO. The conformations obtained, although different from those published for selective agonists of the NK-1 and NK-2 tachykinin receptors also revealed certain similarities, in particular with the proposed bioactive conformation of the selective NK-2 agonist [β-Ala8]NKB(4-10) [8]. On the other hand, the conformational flexibility of the fragment Leu-Met-NH_2 is believed to be required for the selectivity of the ligands towards the NK-1 tachykinin receptor [9]. Similar to other tachykinins, ScyII in water has no structural elements that could be connected with its activity. Recent conformational studies [10] of a selective NK-3 tachykinin agonist [pGlu6, NMePhe8, Aib9]SP_{6-11} revealed that in the DMSO solutions this SP analogue forms a type IV β-turn with Phe7 and NMePhe8 in the centre, followed by a helical segment extending to the C–terminus. According to the authors, the stable helical segment plays an important role for the recognition in the NK-3 tachykinin receptor. This secondary structure element is not present in conformations proposed for ScyII in this solvent. Recent pharmacological studies of our ScyII analogues modified in position 16 (equivalent to position 9 in SP) indicate that, in contrast to the published results [10], the introduction of the Aib residue in this position produce an analogue with weak antagonistic activity towards the NK-3 tachykinin receptor, whereas the substitution of Gly16 with a helix breaker — Sar retains the agonistic activity (Menarini Ricerche Spa, unpublished results). These results give a good starting point for further conformational investigations of the ScyII analogues.

6. Conclussions

The NMR data of peptides studied reflect a population-weighted average and the discussed conformations can not be interpreted in terms of static structures and should be rather called "the NMR structures" of these peptides.

Our results have provided evidence for folded structure of ScyI in DMSO. The obtained conformations, although different from these published for selective agonists of NK-1 and NK-2 tachykinin receptors, revealed certain similarities, especially with the proposed bioactive conformation of selective NK-2 agonist [β-Ala8]NKB(4-10). On the other hand, the conformational flexibility of the fragment Leu-Met-NH_2 is believed to be required for the selectivity of the ligands towards NK-1 tachykinin receptor.

Similar to the other tachykinins, ScyII in water does not have any structural elements, which could be connected with its activity. In contradiction to the published results for other NK-3 agonists, our studies on ScyII did not give any evidence for the presence of a helical segment on the C-terminus.

Acknowlegements

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