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Solution Conformation of [D-Pen^2,D-Pen^5]enkephalin in Water: A NMR and Molecular Dynamics Study

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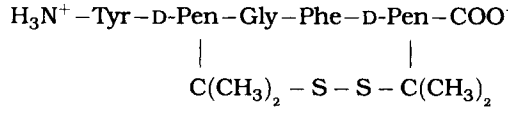
The solution conformation of [D-Pen^2,D-Pen^5]enkephalin (DPDPE), a highly potent δ-selective opioid agonist, was examined by means of NMR, molecular mechanics and molecular dynamics methods. The structural information in the solvent water was obtained employing one- and two-dimensional methods of ^{1}H and ^{13}C-NMR spectroscopy. Based on the distance geometry technique using the ROE data as input, 400 conformers were obtained and considered in the structure analysis. Alternatively, about 2000 conformers were stochastically generated and related to the NMR data after energy minimization. The structure analysis provides one conformer in agreement with all NMR data, which belongs to the lowest energy conformation group. This structure may serve as a reference conformer for DPDPE analogues synthesized with the aim of activity increase.

Keywords: DPDPE; enkephalins; NMR structure analysis; molecular dynamics simulations

INTRODUCTION

[D-Pen^2,D-Pen^5]enkephalin (DPDPE), a conformationally restricted cyclic pentapeptide with the sequence



has been found to be one of the most selective and potent neurotransmitters known for the δ-opioid receptor [1]. The conformation of DPDPE, which plays an important role for the understanding of the interaction between the peptide and the receptor, has been the subject of several investigations including NMR, molecular mechanics and molecular dynamics studies [2–6]. Unfortunately, these studies are contradictory and provide different models for the solution conformation of DPDPE. Working on the conformational investigation of a series of peptides, where amino acids of DPDPE are replaced by non-natural amino acids, we tried to gain further insight into the conformational behaviour of DPDPE as a reference for the conformations of DPDPE analogues.

MATERIALS AND METHODS

NMR Spectroscopy

For the NMR investigations on DPDPE a sample of the peptide from the Bachem company was used. The NMR measurements were performed on a Varian UNITY400 and Bruker AMX500 spectrometer at 299 K and 285 K, respectively. A 5 mM sample of the peptide in H_2O/D_2O (9 : 1) was examined. All spectra were recorded using the Redfield method in the acquisition dimension. TPPI was used for the indirect dimension in the 2D spectra. All 2D spectra were processed using a squared cosine bell as window function in both dimensions. FID size in F1 was doubled by zero filling; no zero filling was done in

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F2. Since phase-sensitive processing is impossible in the F2 dimension of the HMBC, magnitude calculation was used without changing the window function. To suppress the water signal the presaturation technique was used.

Signal assignment in the ^{1}H-NMR spectrum was achieved by using DQF-COSY and ROESY spectra [7]. The problem of the ambiguous assignment of the diastereotopic γ methyl groups (D-Pen^2, D-Pen^5) and α protons (Gly^3) was solved based on the generated conformers (see below). In the ^{13}C-NMR spectrum signals were assigned utilizing HMQC and HMBC NMR techniques [7]. To obtain information on the interatomic distances the standard pulse sequence for ROESY [8] using a mixture time of 150 ms was applied with a minor modification. Instead of a continuous wave spin lock, a pulsed spin lock calculated for a spin lock field strength of 4 kHz was used. The ROEs were offset corrected and calibrated using the distance between the α protons in the Gly^3 residue. The vicinal coupling constants $^3J_{NH,C_\alpha H}$ were determined from the 1D ^{1}H-NMR spectrum. In the case of the D-Pen^5 amide proton which is overlapped by the aromatic protons of Phe^4, the coupling constant was extracted from a row of a TOCSY spectrum with *z*-filter [9] using a spin lock field strength of 10 kHz. The amide proton temperature gradients were determined from 1D ^{1}H-NMR spectra recorded in the range 280–303 K with exception of D-Pen^5 where the gradient was determined from DQF-COSY spectra recorded at the same temperatures.

Molecular Mechanics and Dynamics Calculations

The theoretical investigations including structure generation, molecular mechanics and part of the dynamics calculations were performed employing the QUANTA4.1 molecular modelling package [10], which is based on the CHARMm23.1 force field.

Firstly, based on the distance geometry technique [11], structures were directly generated using the ROE data as input. In order to mimic the water environment, the calculations were carried out using a dielectric constant of ε=80. Because of the lack of an unambiguous stereochemical assignment of the γ methyl groups in the D-Pen^2 and D-Pen^5 residues, all possible assignments of the γ methyl groups were considered in the structure generation resulting in four different distance geometry runs. The distance constraints conformational search yielded 400 structures, which were subsequently energy-minimized.

That conformation which was found to be in agreement with the NMR data was finally subjected to two molecular dynamics simulations. At first, a 150 ps trajectory was calculated in time steps of 1 fs after heating and equilibration periods of 10 ps considering the solvent water implicitly by using a dielectric constant of ε=80. In order to verify our structure at a still higher level, a second molecular dynamics run with the same lengths of the heating and equilibration periods as before and 100 ps simulation time was performed by embedding the solute into 1000 water molecules within a 30 Å cubic box employing periodic boundary conditions. This second trajectory was calculated by means of the CHARMM23f5 program [12], but maintaining the force field parameters of QUANTA/CHARMm23.1 for comparison. In all MD simulations the SHAKE algorithm for the X–H bonds was applied.

Alternatively to the determination of the DPDPE conformation on the basis of the distance constraints, 2000 conformers were stochastically generated and geometry optimized using the random sampling and peptide flip algorithms of the QUANTA molecular modelling package [10] for structure and energy comparison.

RESULTS AND DISCUSSION

NMR Data

The ^{1}H and ^{13}C chemical shift data of DPDPE are given in Table 1. Investigations on the conformational behaviour of peptides using NMR spectroscopy include the determination of the vicinal coupling constants, temperature gradients of the amide protons and nuclear Overhauser effects (NOEs). These parameters provide information about the peptide backbone angles, intramolecular hydrogen bonds and interproton distances, respectively [13,14].

Table 2 shows the vicinal coupling constants $^3J_{NH,C_\alpha H}$ determined for DPDPE. The corresponding peptide backbone angles ϕ were calculated using the BYSTROV parameters for the angular dependence of the coupling constants [15].

The evaluation of the amide proton temperature gradients in DPDPE (Table 2) reveals that the amide proton of the D-Pen^5 residue has a very small value compared with the other amide protons. Thus, it could be concluded that this proton is shielded from the solvent and involved in an intramolecular hydrogen bond. This interpretation is supported by similar values for the temperature gradients of DPDPE observed also in the solvent DMSO.

Table 1 ^{1}H and ^{13}C Chemical Shifts for DPDPE in Water[a]

	Tyr[1]	D-Pen[2]	Gly[3]	Phe[4]	D-Pen[5]
NH		8.27	8.55	8.53	7.35
H_α	4.44	4.18	(pro-R)4.42	4.53	4.30
			(pro-S)3.61		
H_β	3.23			3.12	
$H_{\beta'}$	3.08			3.21	
$H_{\gamma,pro\text{-}R}$		1.57			1.35
$H_{\gamma,pro\text{-}S}$		0.86			1.28
$H_{ar,ortho}$	6.91			7.31	
$H_{ar,meta}$	7.21			7.41	
$H_{ar,para}$				7.37	
C_α	55.3	62.1	43.1	57.9	64.2
C_β	37.2	51.2		37.2	53.9
$C_{\gamma,pro\text{-}R}$		28.2			27.6
$C_{\gamma,pro\text{-}S}$		27.1			27.9
$C_{2,6}$	131.7			130.0	
$C_{3,5}$	117.2			129.8	
C_4	156.2			128.3	
C_1	126.6			136.9	
C_{CO}	170.5	171.1	172.0	173.3	175.0

[a]In p.p.m. calibrated using an independent sample of TSS in water.

Table 2 Amide Proton Temperature Gradients, Coupling Constants $^{3}J_{NH,C_\alpha H}$ and Backbone Angles ϕ[a]

Residue	Grad.[b]	Backbone angle ϕ[c]				$^{3}J_{NH,C_\alpha H}$[d]	$^{3}J_{NH,C_\alpha H}$[e]	$^{3}J_{NH,C_\alpha H}$[f]
D-Pen[2]	−9.0	−80	−40	86	154	7.7	8.1	9.5
Gly[3]	−8.8	−121	−67	67	121	4.2/8.8	$\sum$13.8[g]	$\sum$13.9[g]
Phe[4]	−6.6	−163	−77	28	92	6.2	7.6	7.5
D-Pen[5]	−1.1	−60	−60	92	148	8.7	7.9	8.1

[a]The angle notation refers to [16].
[b]Amide proton temperature gradient, in p.p.b./K.
[c]In degrees, calculated from the NMR coupling constants.
[d]In Hz, determined by NMR.
[e]In Hz, recalculated from a MD trajectory with implicit solvent description.
[f]In Hz, recalculated from a MD trajectory with explicit solvent description.
[g]For Gly[3] the KARPLUS equation only provides the sum of both coupling constants $^{3}J_{NH,C_\alpha H}$ and $^{3}J_{NH,C_\alpha H'}$.

From the ROESY spectrum of DPDPE, 17 distance restraints were obtained to be used in the structure determination (Table 3). These distances were allowed to vary by ±0.5 Å with the exception of those distances including pseudoatoms necessary for the description of methyl groups. In these cases, the upper distance limit was raised by another 0.9 Å.

Conformational Analysis

Among the 400 structures generated by the distance geometry technique with following geometry optimization, we found one conformer in rather good agreement with the NMR data. All other structures differ more or less from the NMR data and cannot be considered as structure alternatives.

Detailed inspection of the obtained conformer shows a γ-turn around the Phe[4] residue, which is stabilized by an intramolecular hydrogen bond between the amide proton of the D-Pen[5] residue and the carbonyl group of the Gly[3] residue (Figure 1). All other amide protons are pointing outwards, which is in full agreement with the amide proton temperature gradients determined by the NMR measurements (Table 2).

The comparison of the distances determined from the ROE data with the corresponding distance values from both MD trajectories shows (Table 3) that all

Table 3 Comparison between ROE Derived and Calculated Distances[a]

ROE between				r_{ROE}[b]	r_{MIN}[c]	r_{MD}[d,e]	r_{MD}[e,f]
Tyr^1	H_α	Pen^2	H_N	2.15	2.16	2.48	2.14
Pen^2	H_N	Pen^2	H_α	3.00	2.95	2.91	2.93
Pen^2	H_N	Pen^2	$H_{\gamma S}$	2.50	3.95	3.31	3.68
Pen^2	H_α	Pen^2	$H_{\gamma S}$	2.10	2.97	2.95	2.93
Pen^2	H_α	Pen^2	$H_{\gamma R}$	2.20	3.11	3.07	3.13
Pen^2	H_α	Gly^3	H_N	1.95	2.24	2.20	2.25
Pen^2	$H_{\gamma R}$	Gly^3	H_N	2.20	2.78	2.83	2.85
Pen^2	$H_{\gamma R}$	Pen^5	$H_{\gamma S}$	2.00	3.11	3.30	3.34
Gly^3	H_N	Gly^3	$H_{\alpha S}$	2.40	2.86	2.88	2.88
Gly^3	$H_{\alpha S}$	Phe^4	H_N	3.00	3.48	3.49	3.50
Gly^3	$H_{\alpha R}$	Phe^4	H_N	2.30	2.22	2.44	2.52
Phe^4	H_N	Phe^4	H_α	2.45	2.20	2.19	2.20
Pen^5	H_N	Pen^5	H_α	2.55	2.93	2.89	2.91
Pen^5	H_N	Pen^5	$H_{\gamma S}$	2.85	4.11	3.43	3.39
Pen^5	H_N	Pen^5	$H_{\gamma R}$	4.00	4.63	4.51	4.49
Pen^5	H_α	Pen^5	$H_{\gamma S}$	2.00	2.90	2.91	2.93
Pen^5	H_α	Pen^5	$H_{\gamma R}$	2.10	3.22	3.15	3.14

[a]In Å.
[b]ROE derived distances.
[c]Distances of the CHARMm minimized structure.
[d]Distances averaged over a MD trajectory with implicit solvent description.
[e]Distance averaging is based on the relation

$$\langle d_{ij} \rangle = \left[\frac{1}{N} \sum_{k=1}^{N} (d_{ij})^{-3} \right]^{-\frac{1}{3}}$$

[f]Distances averaged over a MD trajectory with explicit solvent description.

distances agree within ±0.5 Å (8 out of 17 distances) or within −0.5 Å for the lower and +1.4 Å for the upper limit in the case of pseudoatoms representing the methyl groups (9 out of 17 distances). Comparing with the MD data, it was considered that the shorter distances contribute more to the ROE intensities than the larger ones by application of a weighting procedure suggested in [17].

The torsion angles for the suggested DPDPE conformer arising from the distance constraints calculations and those averaged from the two MD trajectories are given in Table 4. The backbone angles ϕ of the proposed conformer agree with the corresponding angles determined *via* the NMR coupling constants within 20° (Tables 2 and 4). The $^3J_{NH,C_\alpha H}$ coupling constants recalculated by means of the ϕ angles from both dynamics simulations are also given in Table 2 and provide maximum angle deviations of 20° for the corresponding torsion angles.

The averaged dihedral angles and their rms values demonstrate that the peptide ring system exhibits considerable rigidity. Only ψ_{Phe^4}, ϕ_{Pen^5} and, of course, the rotation angles of the outer parts of the ring (Tyr^1) indicate more flexible regions. High flexibility can be assumed for the side chains of the amino acids Tyr^1 and Phe^4. Thus, we renounce giving explicit values for the torsion angles χ_1 and χ_2. Changes of the disulphide bond orientation described by other authors [4] were not observed in our simulations.

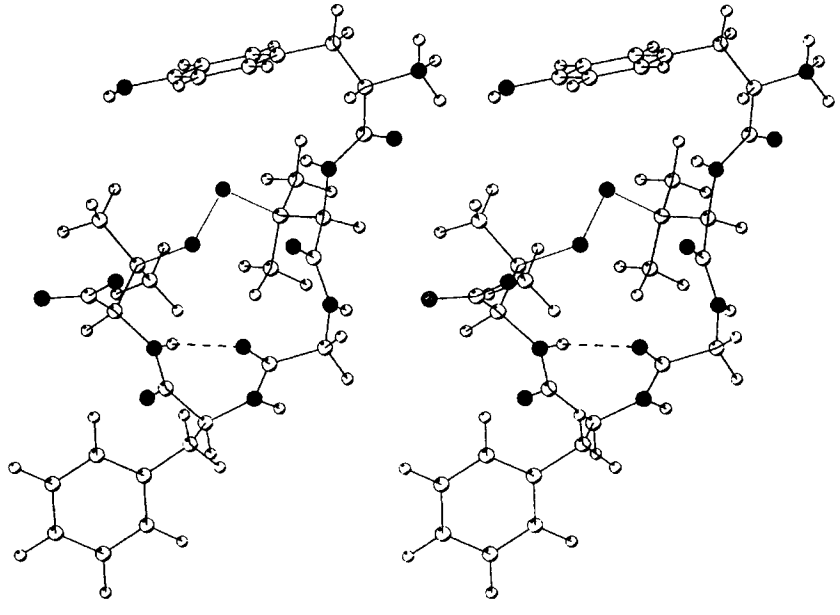

Figure 1 Stereoview of the DPDPE conformer.

Table 4 Dihedral Angles[a] for the DPDPE Conformer Fulfilling the NMR Data Compared with Conformers Proposed by other Authors

Resid.	Angle	This study					Alternative proposals			
		Min.[b]	MD[c]	r.m.s.	MD[d]	r.m.s.	[4]	[3]	[2]	[2]
Tyr[1]	ψ	141	45	90	126	29	164	163	149	153
	ω	−176	−179	9	−175	9	−173	−177	−175	−175
D-Pen[2]	ϕ	139	98	27	119	23	111	149	135	140
	ψ	−151	−147	17	−148	27	14	−153	−143	−113
	ω	179	177	7	176	6	173	−175	−177	−162
	χ_1	−51	−54	9	−48	10	−180	−78		
	χ_2	−75	−70	9	−68	10	143	178		
Gly[3]	ϕ	70	81	17	81	15	−98	78	78	118
	ψ	88	63	26	57	22	−18	−111	−72	−17
	ω	176	176	8	175	8	177	−164	−170	−178
Phe[4]	ϕ	80	75	15	75	16	−72	−85	−67	−149
	ψ	−70	−17	47	−8	40	−46	38	−53	−53
	ω	−176	−178	8	−179	9	−175	172	−169	178
D-Pen[5]	ϕ	144	109	35	104	30	83	61	127	102
	χ_1	−47	−53	9	−55	10	−70	−87		
	χ_2	174	162	16	159	17	119	60		
	CS–SC	−109	−109	6	−110	6	−110	110		

[a]In degrees; the angle notation refers to [16].
[b]CHARMm minimized structure.
[c]Average structure from a MD simulation with implicit solvent description.
[d]Average structure from a MD simulation with explicit solvent description.

The two MD trajectories on the DPDPE conformer indicate that the hydrogen bond of the γ-turn found in the minimized structure is maintained at about 30% when the solvent water was simulated by a global dielectricum and to 23% when explicitly considering the water environment.

In view of the relatively great number of ROE distances to methyl group protons (9 out of 17 distances), which have to be simulated as pseudoatoms, thus automatically leading to more distance uncertainty, the proposed structure rather well reflects all NMR data (ROE distances, coupling constants, hydrogen bonds).

Based on this structure, it was possible to solve the problem of the stereo-chemical assignment of the diastereotopic γ methyl groups of the D-Pen[2] and D-Pen[5] residues and the α protons of Gly[3]. For the D-Pen[2] residue the upfield shifted methyl signal was assigned to the *pro*-S methyl group, because this proton is lying in the shielding region of the Tyr[1] aromatic ring. Using the observed ROE between the lowfield shifted methyl signal, i.e. the *pro*-R methyl group, of the D-Pen[2] residue and the upfield shifted methyl signal of D-Pen[5], the latter methyl group could be assigned to the *pro*-S configuration. For the Gly[3] residue, the strong splitting of the proton resonances arises from the chemical anisotropy effects of the adjacent carbonyl groups. Only the *pro*-S proton is within the shielding region of the carbonyl groups of both the D-Pen[2] and Gly[3] residues shifting that signal to higher field when compared with the *pro*-R proton.

It seemed to be interesting to look for the suggested conformer among the 2000 structures generated by the random sampling and peptide flip algorithms. Considering all conformers within a range of 40 kJ mol referred to the lowest energy structure, we find practically the same conformer about 20 kJ mol more unstable than the most stable structure generated. Thus, the conformer derived from the NMR data belongs to the lowest energy conformation group.

Finally, it may be necessary to compare our structure with the various proposals by other authors, since these models do not agree with our data (Table 4) [2–4]. For a final decision, all these alternatives were constructed based on the available dihedral angles, but none of the structures was able to fulfil our NMR data. Even after energy minimization and molecular dynamics calculations these structures did not agree with our experimental data. It should be emphasized that our conformational

analysis for the solvent water is based on far more ROE-derived distance restraints than those of other authors [2–4].

CONCLUSIONS

In this paper, we performed a conformational analysis of DPDPE using NMR data obtained in water in connection with molecular mechanics and dynamics calculations. The proposed solution conformation is in good agreement with all NMR data and belongs to the lowest energy conformation group. Therefore, it may be justified to consider this conformer as a reference structure for the discussion of the conformation of DPDPE analogues.

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