Conformational Diversity of [D-Pen^2,D-Pen^5]Enkephalin as Studied by Magic-Angle Spinning Liquid-Crystal NMR Spectroscopy and Multiconformational Analysis

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Abstract: The conformation of biologically active [D-Pen^2,D-Pen^5]enkephalin (DPDPE) has been studied by liquid-crystal NMR spectroscopy employing magic-angle spinning (MAS) two-dimensional methodology and by computer-assisted multiconformational analysis. The NMR structural parameters of DPDPE were obtained in a CsPFO (cesium perfluorooctanoate) liquid-crystal. The NMR structural information was acquired in the anisotropic environment based upon the vicinal coupling constant of $^3J(HNC_\alpha H)$ and the $^1H-^1H$ ROE factors obtained under the MAS condition. These data were submitted to multiconformational analysis based on the ECEPP/2 potential energy function and the Metropolis Monte Carlo simulation. As a result of the energy calculation, four conformers are obtained for DPDPE which are considered to exist in the anisotropic environment. The major contribution is determined among these probable conformers in the liquid-crystal medium. Moreover, it is suggested from the NMR multiconformational analysis that the D-Pen^5 residue, the C-terminal "address" segment, is responsible for the conformation of the "message" segment (N-terminal tetrapeptide sequence). The conformational flexibility is discussed for the spatial arrangement of the Tyr^1 and Phe^4 aromatic rings in the message segment of the bioactive conformation in relation to the activity of DPDPE.

Introduction

The endogenous peptide enkephalin is composed of Leu-enkephalin (Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5) and Met-enkephalin (Tyr^1-Gly^2-Gly^3-Phe^4-Met^5)[1] and possesses biological activities similar to those of morphine. But, the receptor subtype selectivity is different between enkephalin and morphine: the former prefers the δ-receptor, while the later binds to the μ-receptor preferentially.[2] To date, many studies have been performed to reveal the structure−activity relationships of enkephalins. On the basis of these studies, it has been suggested that the structure of enkephalin can be divided into two segments, the "message" segment (N-terminal tetrapeptide sequence) and the remaining C-terminal "address" segment.[3] The N-terminal message segment is required for the recognition of the opioid receptor. The message segment is composed of the spacer residue (Gly^2-Gly^3) and two pharmacophore residues, Tyr^1 and Phe^4, in which the amine and phenolic groups of Tyr^1 and the aromatic ring of Phe^4 are included.[4] The C-terminal address segment is considered to stabilize a specific "bioactive conformation" among various conformations accessible to the N-terminal message segment.

The conformational features that determine the receptor selectivity of enkephalin have been investigated widely on the basis of the X-ray crystal,[5,6] the NMR solution,[7,8] and computer-simulated[9] structures. Those studies have revealed a conformational diversity of the molecule in the crystal state as well as in solution. In an attempt to confirm the bioactive conformation of enkephalin, the solution conformation was investigated for the cyclic analogue of [D-Pen^2,D-Pen^5]enkephalin, DPDPE (Tyr^1-D-Pen^2-Gly^3-Phe^4-D-Pen^5), which is one of the most potent and δ-receptor-selective analogues.[10,11] Since DPDPE possesses a 14-membered structure with a highly constrained conformation, a limited number of conformations are allowed and NMR studies have succeeded to a certain extent in determining its solution conformation. Topochemical approaches have also progressed in recent years.[12] From the results, it has been deduced that the relative proximity and the topographical relationship between the two pharmacophore residues in the Tyr^1 and Phe^4 are crucial for the receptor binding affinity.

On the other hand, it has been proposed that the specific interaction of opioid peptides with lipid bilayer membranes plays an important role in the receptor selectivity.[13−15] According to

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this idea, it is the membrane-associated conformation that is essential for the molecular recognition of opioid receptor and is related to biological activities. Recent developments in NMR spectroscopy have made it possible to study molecular conformations in micelles or unilamellar vesicles which can be adopted as a model system for biological membranes. By using this methodology, the membrane-associated conformations of DPDPE have been studied by Matsunaga et al. using vesicles composed of perdeuterated phosphatidylcholine,[16] and the authors have proposed a membrane-associated conformation of DPDPE as well as a mode of interaction of DPDPE with the membrane medium. Our present work intends to further promote the investigation of the membrane-associated nature of DPDPE and the elucidation of the bioactive conformation of this molecule.

For the purpose of our study, magic-angle-spinning (MAS) liquid-crystal NMR spectroscopy is suited, which has been developed by the authors as a new version of NMR spectroscopy using a liquid-crystal as the medium of the model membrane.[17−20] The MAS liquid-crystal NMR spectroscopy can yield preferred conformations even for complicated molecules and is very well suited for studies of molecules of biological interest. The new method is rather comprehensive and is based on measurement of the nuclear Overhauser effect NOE in the rotating frame (ROE) by means of 2D techniques for the liquid-crystal sample under the MAS condition, the data being analyzed by the pseudoenergy method. The method has been applied to Leu-, Met-, and [D-Ala²]Met-enkephalins which are biologically active and [L-Ala²]Met-enkephalin which is an inactive. The results showed that there is a characteristic difference in the orientation of Tyr¹ and Phe⁴ residues between the active and inactive Met-enkephalin analogues,[20] giving a clue to the receptor recognition of the enkephalin.

Although the method described is a sophisticated one, it is still possible that the conformation of enkephalin determined above is an average over an ensemble of diverse conformations. This situation may be said to be common to structural analysis based on conventional NMR spectroscopy in solution. Therefore, it is necessary to further analyze the conformational diversity of molecules in the liquid-crystal to obtain the information about the structure−activity relationships. After recent advancement in computer-assisted techniques of conformational analysis, a number of authors have reported about the methodology to treat the conformational diversity of molecules in solution, and different algorithms have been presented such as the MEDUSA by Ernst et al.,[21] the ensemble-averaging (EA) protocol by Cuniasse et al.,[22] and the Monte Carlo procedures by Nikiforovich[23] or Meirovitch et al.[24] These approaches are expected to provide useful knowledge for the study of the conformational diversity and structure−activity relationships of the opioid peptide treated here.

In the present study, we have extended our NMR studies that combine MAS liquid-crystal NMR spectroscopy and the computer-assisted multiconformational analysis. We have determined and discussed the conformational diversity of cyclic enkephalin analogue DPDPE in the CsPFO liquid-crystal in relation to the bioactive conformation of the message and address segments.

Experimental Section

Preparation of the NMR Sample. DPDPE was purchased from Sigma Chemical Co. and used without further purification. The liquid-crystal component, cesium perfluorooctanoate (CsPFO), was prepared as described previously.[20] The liquid-crystal solution was dissolved to 40 wt % in water (80 wt % H_2O/20 wt % D_2O). DPDPE was added to 1.4 wt %. The nematic phase of the sample aligned spontaneously to orient its normal axis parallel to the external magnetic field, and it was confirmed at 25 °C by measuring the ^2H NMR spectra. The ^2H NMR spectra were recorded on a Varian VXR-200 NMR spectrometer at a frequency of 30.7 MHz.

NMR Measurements. The MAS NMR spectra of the nematic sample were measured on a Varian VXR-200 spectrometer using a solid CP/MAS probe. The sample temperature was maintained at 25 °C by controlling the temperature of air flow. Prior to each 2D experiments, the spinning axis was carefully referenced to the exact magic angle by monitoring a ^2H signal from deuterium oxide in the liquid-crystal sample.

The phase-sensitive proton ROESY/MAS spectra of DPDPE were recorded on a Varian VXR-200 spectrometer at the frequency of 200.0 MHz using a solid CP/MAS probe in which a sample was spun with a rate of 2.3 kHz. The strength of the spin-lock field was 2.0 kHz on a Varian VXR-200 spectrometer. These spectra were measured at 25 °C. Data sets with F_1 and F_2 axes were taken at 256 and 2048 points, respectively. Zero filling was applied to 2K in the F_1 dimension, and Fourier transformation was performed with 2K points for both dimensions. The data were multiplied by a shifted sine-bell window function in the F_1 dimension and by an unshifted Gaussian window function in the F_2 dimension.

Chemical shifts were referenced to the water signal at 4.6 ppm, and then it was suppressed by a presaturation pulse. The proton resonances were assigned by COSY/MAS and ROESY/MAS experiments taking into account the assignments reported in the literature previously.[10,11]

Energy Calculations. All energy calculations for DPDPE were performed using the ECEPP/2 potential field[25−27] with a solvation free energy term[28] which takes into account solvation effects implicitly. The solvation free energy was evaluated as follows:

$$E_{\mathrm{sol}} = \sum_{\mathrm{atoms}\ j} \sigma_j A_j \qquad (1)$$

where σ_j is the solvation parameter of atom j and A_j is its solvent-accessible surface area. The conformation of DPDPE was modeled by the 12 backbone dihedral angles ϕ, ψ, and ω and the 11 side-chain dihedral angles χ. Electrostatic interactions were taken into account under the dielectric constant of $\epsilon = 10$,[29] which is considered to reflect the value of the membrane/water interface.

Multiconformational Analysis. The computer-assisted multiconformational analysis was performed mainly according to the Monte Carlo procedures of Nikiforovich et al.[23] after some minor modifications. At first, the four local potential energy minima were reached from 1500 initial conformations which were generated randomly and minimized

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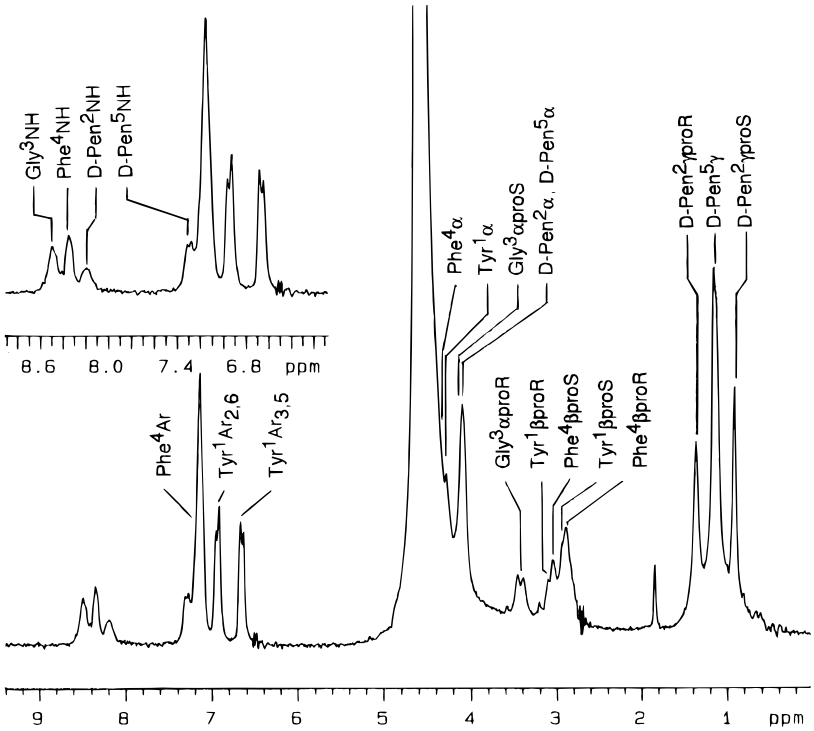

Figure 1. MAS NMR spectra of DPDPE dissolved in the CsPFO liquid-crystal measured at 25 °C and at 200 MHz. The sample spinning rate is 2.3 kHz. Assignment of the signals is also included.

by the Monte Carlo minimization (MCM) method proposed by Li and Sheraga.[30] Then the Monte Carlo simulation method was applied to each energy-minimized conformer to obtain a statistical sample for the calculation of the statistical weight value for the NMR structural parameter as mentioned below. The Markov chain was started from one of the minima. The energy was calculated for the starting conformation and named "old energy", E^{old}. Then, a trial conformation was obtained by changing each dihedral angle by a random value between $-1°$ and $+1°$ from the starting value. Here, all the dihedral angles were changed at the same time. The energy, which was named "new energy", E^{new}, was calculated for the trial conformation. This value of E^{new} was compared with E^{old} and decided to be accepted or rejected according to the usual Metropolis criterion (temperature $T =$ 298 K was utilized). In this way, we generated a Markov chain of 40 000 steps for each starting structure. That is, a statistical sample contains 40 000 conformations.

Selection of Statistical Weights in the Multiconformational Analysis. A statistical weight value, w_i ($i = 1-4$), is assigned to each of the four conformers, which denotes the relative probability of its existence. This set of the conformer statistical weights, $\{w_i\}$, was selected in the following manner. The four random values were generated within the range of 0 and 1 which satisfy $\sum w_i = 1$ and $w_i > 0$. Each w_i value was assigned to each of the four conformers, creating a set of $\{w_i\}$. Thus the generated set of $\{w_i\}$ was checked one by one to ascertain if it satisfied all the conditions expressed as follows:

$$\frac{|\sum_{i=1}^{N} w_i \langle A^{calc}\rangle_{ik} - \langle A^{exp}\rangle_k|}{(\sum_{i=1}^{N} (w_i D_{ik}^{calc})^2 + (D_k^{exp})^2)^{1/2}} < t_k \qquad (2)$$

where $\langle A^{exp}\rangle$ is the mean value of the measured NMR structural parameter such as J or ROE and $\langle D^{exp}\rangle$ is its standard deviation, $\langle A^{calc}\rangle$ and $\langle D^{calc}\rangle$ are the calculated mean value and standard deviation of the structural parameter, respectively, which are evaluated from a statistical sample in the Monte Carlo simulation as mentioned above, i and k are indexes related to the number of conformers and the number of measured parameters, respectively, and t_k is the Student's t value. In the present study, the value of t_k was set equal to 1.96, which corresponds to the 95% level of the confidence interval. The procedure was continued until the number of selected sets of $\{w_i\}$ was equal to 100 000.

In the actual course of multiconformational analysis, pseudoatoms were set according to the Wüthrich rule.[31] The vicinal coupling constant of $^3J(HNC_\alpha H)$ was calculated according to the Karplus-type equation.[32] All of the computer calculations were performed by using the FORTRAN program written by the authors. All calculations were performed on SGI IRIS Indy R4000SC and FUJITSU S-4/IP workstations.

Results

The ROESY/MAS Experiment. Figure 1 illustrates the 1D MAS NMR spectra of DPDPE dissolved in the CsPFO liquid-crystal. Figure 2 shows the partial ROESY/MAS spectra. From the ROESY spectra, the corresponding interproton distances were derived successfully. Table 1 lists the interproton distances which were defined semiquantitatively as the lower and upper limits: strong = 2.0−2.7 Å, medium = 2.7−3.3 Å, and weak = 3.3−4.0 Å.[18] Assuming that $ROE^{upp} = \langle ROE\rangle^{exp} + D^{exp}$ and $ROE^{low} = \langle ROE\rangle^{exp} - D^{exp}$, it was possible to estimate the values of $\langle ROE\rangle^{exp}$'s and D^{exp}'s for use in the inequalities of eq 2.[23]

In Table 1, the $^3J(HNC\alpha H)$ coupling constant of the D-Pen^5 residue is also listed, which was obtained by analyzing the 1D MAS spectra. The standard deviation of 0.5 Hz was arbitrarily set for the $^3J(HNC_\alpha H)$ by considering the digital resolution of the 1D MAS spectra. In the present MAS experiments, liquid-crystal NMR spectra were obtained at high resolution, which made the spectral analysis easy and comparable to that of the other linear enkephalins reported previously.[17,19,20] Nevertheless, the splitting from $^3J(HNC_\alpha H)$ was not observed clearly in the

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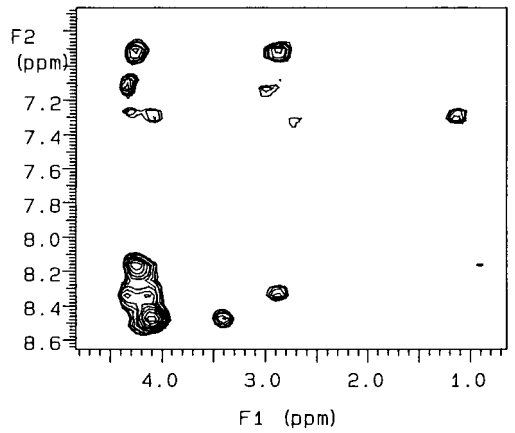

Figure 2. Partial ROESY/MAS spectra of DPDPE dissolved in the CsPFO liquid-crystal measured at 25 °C and at 200 MHz. The mixing time is 100 ms. The sample spinning rate is 2.5 kHz.

Table 1. NMR Data for DPDPE Observed in the CsPFO Liquid Crystal

vector	^3J(HNC$_\alpha$H), Hz	vector	ROE
D-Pen^{5}αH−D-Pen^{5}NH	6.8 (0.5)	Tyr^{1}αH−Tyr^1βH$_{proS}$	+
		Tyr^{1}αH−Tyr^{1}ArH$_{2,6}$	+++
		Tyr^{1}αH−D-Pen^{2}NH	+++
		D-Pen^2γH$_{proS}$−D-Pen^{2}NH	+
		Gly^{3}αH$_{proS}$−Gly^{3}NH	+++
		Gly^{3}αH$_{proR}$−Gly^{3}NH	++
		Gly^{3}αH$_{proS}$−Phe^{4}NH	++
		Phe^{4}αH−Phe^4βH$_{proS}$	++
		Phe^{4}αH−Phe^{4}ArH$_{2,6}$	+++
		Phe^4βH$_{proR}$−Phe^{4}NH	++
		Phe^{4}αH−D-Pen^{5}NH	+
		Phe^{4}NH−D-Pen^{5}NH	++
		D-Pen^{5}αH−D-Pen^{5}NH	+
		D-Pen^5γH−D-Pen^{5}NH	++

amide proton region except for the splitting of D-Pen^{5}NH-D-Pen^{5}C$_\alpha$H. These observed and unobserved splittings were probably caused by the smaller ^3J(HNC$_\alpha$H) coupling constants in the present case compared to the cases of other linear enkephalins, suggesting a different conformation in the main chain of DPDPE. This difference is also supported by the fact that the amide protons in D-Pen^2 and D-Pen^5 were shifted to higher field compared to the chemical shifts of the corresponding amide protons in other enkephalins (Figure 1).

Selection of the Conformations of DPDPE with Local Energy Minima. The energy minimization was performed by using the MCM method as mentioned in the Experimental Section. As a result, four conformers with local energetic minima were obtained successfully from 1500 initial conformers generated randomly. Table 2 lists the dihedral angles for these four conformers. In this procedure, the 1500 conformers were clustered at first using an root mean square criterion for the deviations of the dihedral angles[33] with a threshold of 10°. From this criterion, the 1500 conformers were devided into 10 clusters. The 10 conformers, each of which has the minimum energy in each of the 10 clusters, were chosen, and the root-mean-square distances (rmsd) were calculated among the 10 conformers. Finally, the four conformers were selected from the 10 conformers with a threshold of 1.0 Å for the rmsd criterion.

In this case, our objective is to obtain a complete set of energy-minimized conformers which are significantly different. For this purpose, we have used the ECEPP/2 force field with the solvation energy term and the MCM method in which a large set of initial random conformations is subjected to the local minimization in the potential energy and to the Metropolis test for acceptance or rejection of the minimized conformation. According to this procedure, we could find the four probable conformers successfully. While the MCM method does not lead to a rigorous Boltzmann sampling, it is known to be more efficient than other methods in generating low-energy conformations.[34] In the present study, although we have attempted other methods such as the chain-growth procedure and the modified version of it,[35] the MCM method seemed to be very efficient in finding a set of energy-minimized conformers which are different significantly in the structural parameter.

Table 2. Dihedral Angles for the Four Energy-Minimized Conformers

		model			
		1	**2**	**3**	**4**
Tyr^1	ψ	138	164	143	143
	ω	−178	179	−178	−178
	χ^1	178	58	179	179
	χ^2	−105	−94	−103	−105
	χ^6	180	180	180	−179
D-Pen^2	ϕ	88	83	79	80
	ψ	20	33	31	29
	ω	170	168	165	175
	χ^1	−65	−62	−61	−63
	$\chi^{2,1}$	46	45	42	41
	$\chi^{2,2}$	79	71	72	70
Gly^3	ϕ	−42	−66	−63	−67
	ψ	−69	−115	−115	−102
	ω	−175	−173	−171	−171
Phe^4	ϕ	−114	−103	−103	−105
	ψ	−60	4	0	−5
	ω	177	177	179	−177
	χ^1	168	58	−175	−57
	χ^2	−120	−99	−100	−81
D-Pen^5	ϕ	66	138	139	137
	χ^1	−56	−85	−83	−85
	$\chi^{2,1}$	22	67	70	67
	$\chi^{2,2}$	77	59	60	60
	∠CSSC	96	116	115	116
energy (kcal/mol)		−63	−9.5	−10.3	−12.5

Metropolis Monte Carlo simulation. Each energy-minimized conformer as obtained above was submitted to a Metropolis Monte Carlo simulation to calculate the NMR structural parameters $\langle J^{calc}\rangle$ and $\langle ROE^{calc}\rangle$ and their standard deviations, $\langle D^{calc}\rangle$, for use in eq 2. In this procedure, the $\langle ROE^{calc}\rangle$ was calculated corresponding to the interproton distance. The Metropolis Monte Carlo simulations were performed with 40 000 steps for each conformation by selecting for each dihedral angle a random value within the range of [−1°,1°] around the original value, as mentioned in Experimental Section. As a result, we could calculate the NMR structural parameters for the four probable conformations successfully. It might be possible to generate a larger Markov chain to better simulate the NMR structural parameters. However, too large a scale of the sampling may lead the molecule to reach to another local minimum conformation that is already included in the simulation.

As an example of the results of our simulation, we illustrate in Figure 3 the population distribution of several dihedral angles obtained during the simulation of conformer **1** which is considered to be the most populated one in the liquid-crystal solvent as mentioned below. As is apparent from Figure 3, the dihedral angles in the Tyr^1 residue show a typical profile of a concentrated distribution. All the dihedral angles of the D-Pen^2

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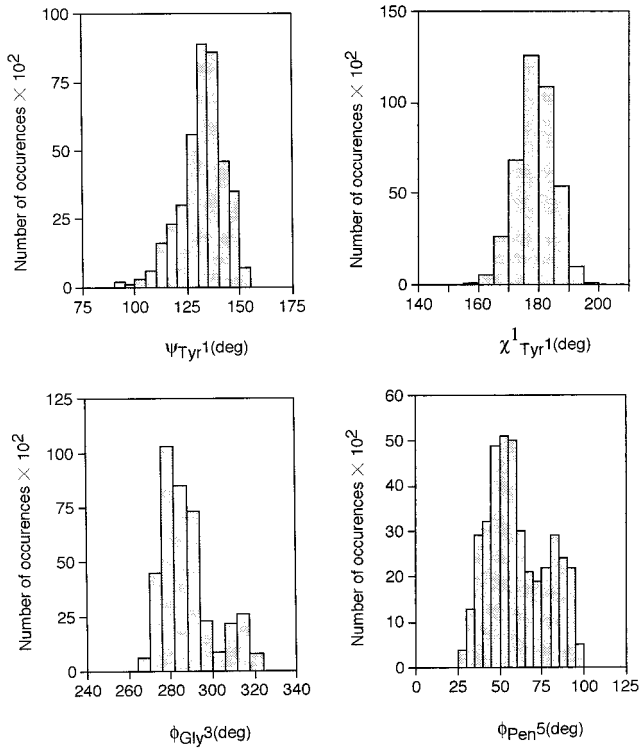

Figure 3. Population profile of the dihedral angles in conformer **1** of DPDPE obtained from a statistical sample containing 40 000 conformations.

Table 3. Energies and Statistical Weights for the Conformers **1**−**4** Obtained from the Multiconformational Analysis

i	E_i (kcal)[a]	E_i^{av} (kcal)[b]	w_i^{low}	w_i^{upp}	w_i^{av}
1	−6.32	1.03	0.26	0.71	0.40
2	−9.46	0.52	0.00	0.43	0.15
3	−10.33	2.80	0.00	0.62	0.19
4	−12.42	−1.82	0.00	0.70	0.26

[a] The value is adopted from the original energy for each conformer. [b] The value is the averaged energy of each statistical sample which contains 40 000 conformations.

and Phe[4] residues showed the same distribution profiles as those of the Tyr[1] residue. On the other hand, the angles of Gly[3]ϕ and D-Pen[5]ϕ showed a population of "random coil" type which is distributed within the range 60−80°.[24] This is probably due to the steric flexibility in these residues. It is worth mentioning that the D-Pen[5] residue is situated in the C-terminal address segment which is considered to be responsible for the stability of the conformation of the message segment.[4,14]

Determination of Statistical Weight Value in the Multiconformational Analysis. In the above treatment, we obtained four conformations as the energy-minimized ones and their NMR structural parameters, $\langle J^{calc}\rangle$ and $\langle ROE^{calc}\rangle$, were calculated upon the basis of the Monte Carlo simulations. Then the calculation of statistical weight values was performed using eq 2 for the four probable conformers as referenced to the experimental NMR structural data. Table 3 lists the energies and statistical weight values thus obtained for the four conformers. The stereoscopic view is shown in Figure 4 for conformers **1** and **4**.

It is worth noting that the highest values are observed for conformer **1** in the statistical weight values of $w^{av}{}_i$, $w^{low}{}_i$, and $w^{upp}{}_i$ (Table 3), which mean the average, lower limit, and upper limit of weight values, respectively. Especially, the $w^{low}{}_i$ of conformer **1** takes nonzero value (0.26) while those of other

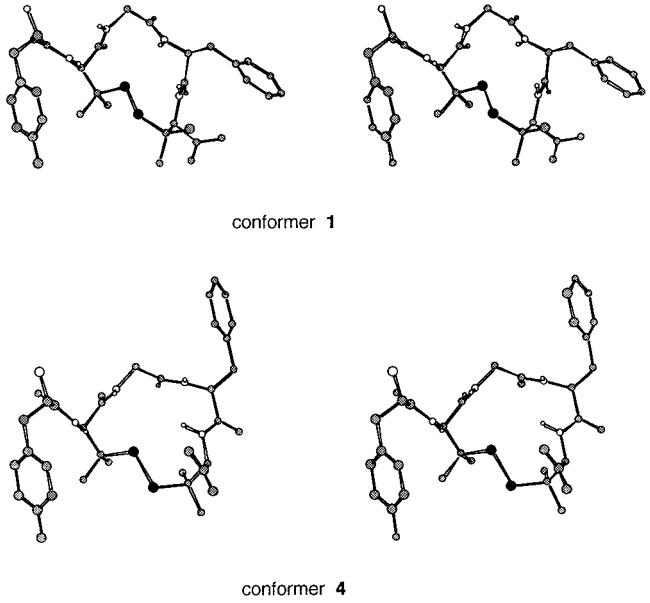

Figure 4. Stereoscopic view of conformers **1** and **4** of DPDPE.

conformers show nearly zero values. That is, conformer **1** is considered to be present significantly in the liquid-crystal medium although it is the most unstable conformation in aqueous solution. This may be caused by the presence of a specific conformational effect from the oriented liquid-crystal medium. Therefore, the enhanced population of the conformer **1** is considered to be obtained probably as a result of simulating experimental NMR data measured in the liquid-crystal.

In this way, we can pick up conformer **1** as the most populated one in the CsPFO liquid-crystal medium. But, since no single conformer can fully satisfy the observed NMR structural parameters, it is sure that the other conformers contribute more or less as a equilibrium mixture. Thus, the conformational diversity of DPDPE in the liquid-crystal medium is described as follows: the molecule presents mainly as an equilibrium mixture of the major conformer **1** and the minor ones **2**−**4**.

Discussion

The approach performed in the present study expands our methodology of membrane-associated structural determination, which combines the novel liquid-crystal NMR spectroscopy under the magic-angle spinning and a computer-assisted simulation technique. By using this method of analysis, the membrane-associated conformational diversity is treated for a relatively small peptide, DPDPE, and the bioactive conformation of it is deduced.

It should be noted that we have studied the conformational diversity which takes into account the solvation effect by using the solvation energy function for the aqueous solution. Although it is desirable to use a solvation energy function specific for the liquid-crystal medium, any such function is not yet reported except for some preliminary studies.[29] Therefore, in the present study, we have decided to use the solvation energy function for the aqueous solution in building up an initial model of the conformation of DPDPE. We have performed a multiconformational analysis to discuss the real conformation in liquid-crystal medium referencing to the experimental NMR data observed in this medium. The result showed that the model of conformation of DPDPE in aqueous solution does not straightforwardly reflect that in liquid-crystal medium: the most stable conformer in aqueous solution, conformer **4**, fails by itself to

reproduce exactly the experimental NMR structural parameters observed in the liquid-crystal medium and the most populated conformer is the conformer **1** in the liquid-crystal, whereas it is conformer **4** in the aqueous phase.

The present analysis of the membrane-associated conformational diversity of DPDPE shows that this molecule is present mainly as an equilibrium mixture of the major conformer **1** and the minor ones **2**−**4**. All four conformers seem to have a specific spatial arrangement of the Tyr[1] and Phe[4] aromatic rings which is slightly different from that found in the other linear enkephalins dissolved in the CsPFO liquid crystal.[17,19,20] That is, the Tyr[1] aromatic ring in DPDPE orients toward the same direction as the other side chains including the Phe[4] aromatic ring when judged with reference to the plane formed by peptide backbone, while that in the linear enkephalins orients toward the opposite direction from the other side chains. Interestingly, Matsunaga et al. have investigated the membrane-associated conformation of cyclic DPDPE and acyclic DPDPE which is the reduced analogue of DPDPE by using the phospholipid vesicles as a model membrane. They have reported that the most stable conformation of acyclic DPDPE changes between aqueous solution and vesicles, whereas that of cyclic DPDPE is not considered to change significantly between these two media. Their result showed a difference in the spatial arrangement of the Tyr[1] and Phe[4] aromatic rings between cyclic DPDPE and acyclic DPDPE, which is similar to our result. That is, according to their results, the Tyr[1] and Phe[4] side chains of acyclic DPDPE are well separated on either side of the plane formed by peptide backbone while the conformation of cyclic DPDPE favors the close interaction of the Tyr[1] and Phe[4] aromatic rings. Such difference was considered to be brought about by the different mode of interaction between the N-terminal residues Tyr[1] and D-Pen[2] and the membrane. Therefore, the specific spatial arrangement of the Tyr[1] and Phe[4] aromatic rings observed in the present study may also be caused by the different mode of interaction between the molecule and the liquid-crystal aggregates, which is related to the relatively large hydrophobicity at the 2 position of DPDPE.

The above result is also supported by the MAS liquid-crystal NMR experiments as seen in Figure 1: the D-Pen[2] and D-Pen[5] amide protons were shifted to higher field compared with the other amide protons, indicating an apolar environment for the D-Pen[2] and D-Pen[5] amide protons. This means that the hydrophobic D-Pen[2] and D-Pen[5] residues interact with the liquid-crystal aggregates much more strongly than the other side chains do. Especially the D-Pen[2] amide proton is shifted to higher field remarkably as compared with the chemical shifts of amide protons at the 2 position in the other enkephalins.[17,19,20] The mode of interaction of DPDPE with the liquid-crystal aggregates presumably differs from the other enkephalins mainly because of the difference in the hydrophobicity in the side chains at the 2 position. Therefore, the spatial arrangement between the Tyr[1] and Phe[4] aromatic rings of DPDPE is different from that of the other enkephalins. However, it has been proposed that the δ receptor selectivity of DPDPE is related to the adverse steric interaction between D-Pen[2] methyl group and the δ receptor binding site,[36] and it was shown in the case of the cyclic analogues of enkephalin that it is essential to leave the Tyr[1] residue out of the cycle in order to preserve activity.[37] Such a situation of the Tyr[1] residue may be necessary to compensate for the unfavorable hydrophobicity at the 2 position and to yield the bioactive conformation in the case of the cyclic analogues of enkephalin.

A bioactive conformation in the two pharmacophore residues, Tyr[1] and Phe[4] in the message segment, has been proposed from the solution NMR study and from the topochemical approach with opioid peptides such as DPDPE and morphiceptin.[4,10−12,38] According to the proposed bioactive conformation, it is required that $\psi \cong 140°$ with the side chain in trans conformation ($\chi^1 \cong 180.0°$) in the Tyr[1] residue and that $\phi \cong -120°$ with the side chain in gauche (−) conformation ($\chi^1 \cong -60.0°$) in the Phe[4] residue. Among the four conformers obtained in the present study, only conformer **4**, which is the most stable one in aqueous solution and possesses a second highest population in liquid-crystal medium (Table 3), satisfies this requirement for the bioactive conformation. Moreover, the Tyr[1] and Phe[4] aromatic rings in the conformer **4** are likely to take a similar spatial arrangement to those in the other linear enkephalins.[17,19,20] As seen from Table 2 and Figure 4, the conformer **4** is similar to conformer **1** except for the angle χ_1 in the Phe[4] side chain in the message segment ($\chi_1 = -57°$ for **4** but 168° for **1**) although the angle ϕ in the D-Pen[5] residue in the address segment differs largely from that in the conformer **1** ($\phi = 137°$ for **4** but 66° for **1**). Therefore, the DPDPE molecule may change its conformation from the one in conformer **1** to another in conformer **4** in the message segment to show the biological activities. This change is probably caused by the conformational flexibility in the C-terminal address segment of the D-Pen[5] residue (Figure 3).

Conclusions

The MAS liquid-crystal NMR spectroscopy has been applied to [D-Pen[2],D-Pen[5]]enkephalin(DPDPE) dissolved in the CsPFO liquid-crystal, and the MAS condition was shown to be useful for the determination of ROE factors and $^3J(HNC_\alpha H)$ couplings. These experimental values were subjected to computer-assisted multiconformational analysis, and the conformational diversity was analyzed successfully for the DPDPE molecule dissolved in the liquid-crystal medium. As a result, a major component was determined together with the probable second component in the anisotropic environment. It follows from comparison of the probable conformers of DPDPE with the conformation of the other enkephalins that the orientation of the Tyr[1] and Phe[4] residues is important for its activity. This orientation between the Tyr[1] and Phe[4] aromatic rings may be brought about by the interaction between the C-terminal address segment and the liquid-crystal aggregates in the anisotropic environment.

The methods developed in the present study can be applied widely to biologically active molecules, providing information on conformational diversity in the liquid-crystal medium. The liquid-crystal NMR method may be said to be extraordinarily widened in its applicability by the improvements due to magic-angle-spinning and multiconformational analysis, thereby offering novel information on the conformation of biologically active molecules in model membranes.

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