Conformation and Orientation of Met-enkephalin Analogues in a Lyotropic Liquid Crystal Studied by the Magic-Angle- and Near-Magic-Angle-Spinning Two-Dimensional Methodology in Nuclear Magnetic Resonance: Relationships between Activities and Membrane-Associated Structures

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Abstract: The preferred orientation and conformation of biologically active [D-Ala^2]- and inactive [L-Ala^2]Met-enkephalins have been studied by liquid-crystal NMR spectroscopy employing magic-angle- and near-magic-angle-spinning (MAS/NMAS) two-dimensional methodology. The solvent employed was a CsPFO (cesium perfluorooctanoate) liquid crystal so that less proton signals interfere with the spectrum under the MAS/NMAS conditions. The conformation and orientation of [D-Ala^2]- and [L-Ala^2]Met-enkephalins were determined in this anisotropic environment from the analysis of the ^{1}H−^{1}H direct couplings obtained under the NMAS condition and of the ^{1}H−^{1}H ROE factors obtained under the MAS condition. These data were analyzed by the minimization based on pseudoenergy analysis. The results showed that the preferred orientations and the motional restrictions of the tyrosine residues of Met-enkephalin analogues are perpendicular to each other, although the preferred conformations are extended ones for both compounds. Also it is revealed from the NMR analysis that the tyrosine residue is much embedded in the liquid-crystal aggregates for the inactive analogue in contrast to the active one. The interaction between the membrane and the tyrosine residue is pointed out to relate to the activity of enkephalin.

Introduction

Enkephalin is an endogenous neuropeptide with morphine-like activities and is composed of Met-enkephalin (Tyr^1-Gly^2-Gly^3-Phe^4-Met^5) and Leu-enkephalin (Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5).[1] To date, many conformational studies have been performed to understand the biological activities of this compound, but only revealed that the molecule can take diverse forms in the solid state[2−4] as well as in solution.[5] Under the circumstances, the conformational and orientational analyses in the membrane environment are very crucial to understand the biological function. This may be understood from the facts that the specific interactions between the opioid peptides and lipid membranes play an important role in the peptide's function, catalyzing the peptide−receptor interaction for the initial step of enkephalin function.[6]

When the amino acid sequence of enkephalin is substituted with D-Ala in the 2 position (Tyr^1-D-Ala^2-Gly^3-Phe^4-Met^5), it is shown that the biological activity of the analogue is preserved or enhanced relative to the native one.[7] However, if it is replaced by L-Ala, the molecule becomes inactive. Although the conformation and orientation of the analogue seem to give a clue for the structure−activity relationships, it was only predicted from an empirical calculation of conformational energy that [D-Ala^2]Met-enkephalin as well as Met-enkephalin tends to form a β turn conformation.[8] Much less is known about the membrane-associated conformation and orientation of [D-Ala^2]-Met-enkephalin due to the lack of an experimental method applicable for such a system.

High-resolution NMR has become a distinctive approach in determining the membrane-associated conformation of molecules, where a micellar or small unilamellar vesicle solution is used as a model system of a membrane. The membrane-associated conformations of Leu-enkephalin and its analogues ([D-Ala^2]Leu-enkephalin, [D-Ala^2]Leu-enkephalinamide, and [L-Ala^2]Leu-enkephalin) have been elucidated by Millon *et al.* using the vesicle of the mixed membrane of perdeuterated phosphatidylcholine and phosphatidylserine.[9] The authors concluded that the activity of the peptide may be related to the conformational changes associated with the interaction between the molecule and the negatively charged membrane. In the case of Met-enkephalin, it seems to form a folded "β turn" predominantly in SDS (sodium dodecyl sulfate) and lysophosphatidylcholine micelles.[10,11] In all such systems, however, solute molecules reorient rapidly in every direction and the

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anisotropic nature of direct couplings disappears, giving no information on the orientation of solute molecules.

On the other hand, liquid-crystal NMR spectroscopy has been used to investigate the orientation as well as the structure of solutes in the anisotropic environment.[12−14] In conformational studies, the liquid crystal has some advantages as a model for biological membranes compared with other systems such as micellar or unilamellar vesicle solutions. The liquid crystal itself orients to exhibit direct couplings under the external magnetic field, and also direct couplings are observed for the molecules dissolved in this medium, which give precise information on molecular geometry. The direct couplings can be determined from the NMR spectral analysis, but the NMR spectrum becomes very complex when direct couplings come into appearance. This limits the applicability of the liquid-crystal NMR within only small molecules in its conventional version.[15,16] However, molecules oriented in liquid crystals quite resemble in their situation those dissolved in the biological membrane.[17] Hence, it will be quite important and interesting if the membrane-associated conformation and orientation can be studied through the use of direct couplings obtained in liquid crystals without any serious restrictions.

To simplify such complex spectra and widen the applicability of liquid-crystal NMR spectroscopy, the authors have proposed a comprehensive technique where the nuclear Overhauser effect (NOE) in the rotating frame (ROE) is measured by means of MAS (magic-angle sample spinning) and 2D techniques, and analyzed by the pseudoenergy method.[18−20] The MAS technique has been applied to liquid crystals and also to artificial vesicles,[21−23] but it really loses the important information of direct couplings. Information on the anisotropic orientation can be recovered in the MAS mode without serious complication in the spectral pattern by setting the angle of sample rotation slightly different from the exact magic angle.[18,19,24] Such a method of sample rotation, which may be called near-magic-angle spinning (NMAS), reduces the number of direct couplings obtained from the experiment compared with those obtained from the experiment with a static sample, since small direct couplings are scaled down to much smaller values and masked within the line widths. The authors have proposed a composite method where ROEs measured under the MAS condition and direct couplings observed under the NMAS condition are used together in the pseudoenergy calculation. By this method, the authors determined the preferred orientations and conformations of Met-enkephalin[20] and Leu-enkephalin.[18]

The solvent used in our study is the cesium perfluorooctanoate (CsPFO) lyotropic liquid crystal. It is known that mixtures of fluorocarbon and hydrocarbon surfactants are nonideal and show various unique physicochemical properties, since the fluoromethylene chain has a larger exclusion volume than the methylene chain.[25] So a more general system to mimic the biomembrane environment may be provided by the potassium laurate or the DMPC/CHAPSO (dimyristoylphosphatidylcholine/3-((cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonate)[26] liquid-crystal system. However, the CsPFO liquid-crystal system offers some advantages over the other systems. When a liquid-crystal sample is spinning about the magic angle, solvent peaks which have disappeared due to a strong intramolecular coupling network reappear in the MAS/NMAS NMR spectra. The CsPFO liquid crystal can be devoid of nuclei under observation. Moreover, it forms a bilayer-like disk-shaped micelle which is stable near pH 7.[27] Therefore, it can represent a preliminary step toward more biomemrane-like systems, and we decided to use this solvent to investigate the conformations and orientations of enkephalins in detail.

In the present study, we have extended our NMR studies that combined the $^1H-^1H$ direct couplings observed under near-magic-angle spinning (NMAS) and the rotating frame nuclear Overhauser effects (ROE) observed under the MAS condition to yield the orientation and conformation of molecules in liquid crystals. We have determined and discussed the preferred conformation and orientation of [D-Ala^2]- and [L-Ala^2]Met-enkephalins in the CsPFO liquid crystal by the composite method.

Experimental Section

Preparation of the NMR Sample. [D-Ala^2]- and [L-Ala^2]Met-enkephalins (acetate form) were purchased from Sigma Chemical Co. and used without further purification. The liquid-crystal component, cesium perfluorooctanoate (CsPFO), was prepared as described by Boden *et al.*[27] Cesium hydroxide was added to an aqueous solution of perfluorooctanoic acid (Tokyo Kasei Organic Chemicals) until neutralized. The solution was dried and then redissolved to 40% by weight in water (80 wt % H_2O/20 wt % D_2O). [D-Ala^2]- and [L-Ala^2]Met-enkephalins were added to 1.4 wt %. The sample was confirmed to be a nematic phase 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 phase-sensitive proton ROESY/MAS spectra of [D-Ala^2]Met-enkephalin were recorded on a Varian UNITY-500 NMR spectrometer operating at 500 MHz using a "nanoprobe" in which a sample spun about the magic angle with a speed of 3.5 kHz. The ROESY/MAS spectra of [L-Ala^2]Met-enkephalin were recorded on a Varian VXR-200 spectrometer at a frequency of 200.0 MHz using a solid CP/MAS probe in which a sample was spun with a speed of 2.3 kHz. The strength of the spin-lock field was 3.0 kHz on a Varian UNITY-500 and 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 192 and 2048 points, respectively. Zero filling was applied to 2K in both F_1 and F_2 dimensions before Fourier transformation. 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.

The MAS and NMAS 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

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temperature of air flow. Prior to each 2D experiment, the spinning axis was carefully referenced to the exact magic angle by monitoring a ^{2}H signal of deuterium oxide, and then it was adjusted to a desired angle by a screw attached to the bottom of the probe. The angle was calibrated by using a sample of CH_2Cl_2 dissolved in a nematic liquid crystal. The FID data of 2048 points in the t_2 dimension were collected for 0.1−0.2 s repeatedly with an interval of 1.0 s to give the data in the t_1 dimension. The direct and indirect couplings were measured from homo-2*DJ* spectra. The 2D FID data were multiplied by a sifted sine-bell window function in both t_1 and t_2 dimensions.

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.

Analysis of Direct Coupling. The direct coupling can be expressed as follows:[15]

$$D_{ij} = -\frac{h\gamma_i\gamma_j}{4\pi^2 r_{ij}^3}\left\langle\frac{3\cos^2\theta_{ij}-1}{2}\right\rangle \quad (1)$$

where γ is the gyromagnetic ratio of a proton, r_{ij} is the distance between protons i and j, θ_{ij} is the angle between the $i-j$ vector and the applied magnetic field, and the angular bracket denotes an ensemble average.

The angular averaging of direct couplings arising from internal motions can be expressed as

$$\begin{aligned}\frac{\langle 3\cos^2\theta_{ij}-1\rangle}{2} = {} & (\cos\alpha_x^{ij})(S_{yy}-S_{zz}) + (\cos^2\alpha_y^{ij})(S_{yy}) + \\ & (\cos^2\alpha_z^{ij})(S_{zz}) + (2\cos\alpha_x^{ij})(\cos\alpha_y^{ij})(S_{xy}) + \\ & (2\cos\alpha_x^{ij})(\cos\alpha_z^{ij})(S_{xz}) + (2\cos\alpha_y^{ij})(\cos\alpha_z^{ij})(S_{yz}) \quad (2)\end{aligned}$$

where α_x^{ij}, α_y^{ij}, and α_z^{ij} are the angles between the $i-j$ vector and an arbitrary molecular frame (x, y, z) fixed in a subsystem of a liquid crystal where the molecule is dissolved, and S_{pq} (p, $q = x$, y, z) is an element of an order matrix. The order matrix defined in the molecular frame is related to the principal order matrix defined in the principal axis system (x', y', z'), the latter being the diagonalized form of the former. The principal order parameters ($S_{x'x'}$, $S_{y'y'}$, $S_{z'z'}$) determine the orientation of the principal axis system (PAS) in the external magnetic field. The direction of the major axis of orientation is chosen along the z' axis in the present study.

If the motion is assumed to be symmetric about the z' axis in PAS, an independent variable in the principal order parameters is only $S_{z'z'}$ because of the relation of $S_{x'x'} = S_{y'y'} = -S_{z'z'}/2$.[15] The order matrix in a molecular frame, $\mathbf{S}_{mf}$, is related to the principal order matrix $\mathbf{S}_{PAS}$ by an orthogonal transformation, $\mathbf{R}$:

$$\mathbf{S}_{mf} = \mathbf{R}\mathbf{S}_{PAS}\mathbf{R}^{\ddagger} \quad (3)$$

where the elements of $\mathbf{R}$ characterize the Euler angles (α, β, and γ) necessary for the rotation of the principal axis system onto the molecular frame.[20] The Euler prescription from an x', y', z' axis system to an x, y, z axis system is as follows: (1) rotate about the z' axis by an angle α and transform to an intermediate frame, x^{i1}, y^{i1}, z^{i1} ($z^{i1} = z'$), (2) rotate about the y^{i1} axis by an angle β to transform to a second intermediate frame, x^{i2}, y^{i2}, z^{i2} ($y^{i2} = y^{i1}$), and (3) rotate about the z^{i2} axis to complete the transformation to x, y, z ($z = z^{i2}$).

Conformation and Orientation Search. Introduction of the direct couplings into the conformational search can be achieved by considering a pseudoenergy composed of the direct coupling data[28] and a distance pseudoenergy.[20] In the actual course of the conformational search, 336 initial conformations were generated randomly and the ROE distance constraints were included by describing the pseudoenergy in terms of the dihedral angle, and the energy was minimized with the chain-growth procedure,[29] affording the improved dihedral angles. In this procedure, the backbone dihedral angles, ω, were fixed at 180° and the pseudoatom was placed according to the Wüthrich rule.[30] Then 1000 initial sets of order parameters were generated randomly for each of these conformers, and the pseudoenergy composed of the direct coupling data, where independent variables are S_{yy}, S_{zz}, S_{xy}, S_{xz}, and S_{yz}, was minimized against the experimental direct couplings. In this manner a set of internal variables (S_{yy}, S_{zz}, S_{xy}, S_{xz}, and S_{yz}) was determined for each conformer which gave the minimum energy value. In the calculation of the direct couplings of tyrosine and phenylalanine, a flip-flop motion (rotation of 180°) was assumed for aromatic rings.[4]

In the approach under the axially symmetric motion model, it is unnecessary to rotate the PAS by an angle α (*i.e.*, $\alpha = 0°$), since the motion is assumed to be axially symmetric about the z' axis. Therefore, independent parameters are reduced to $S_{z'z'}$, β, and γ. The best fit parameters were obtained by calculation in manner similar to the fitting by five order parameters (S_{yy}, S_{zz}, S_{xy}, S_{xz}, S_{yz}) described above. In this procedure, the molecular frame (x, y, z) was initially set to the principal axis system of the inertia of the molecule. The major axis of inertia was chosen to correspond to the z axis. Then, 10 000 sets of values were generated randomly and optimized for $S_{z'z'}$, β (in the range of −180° to +180°), and γ (in the range of 0−180°).

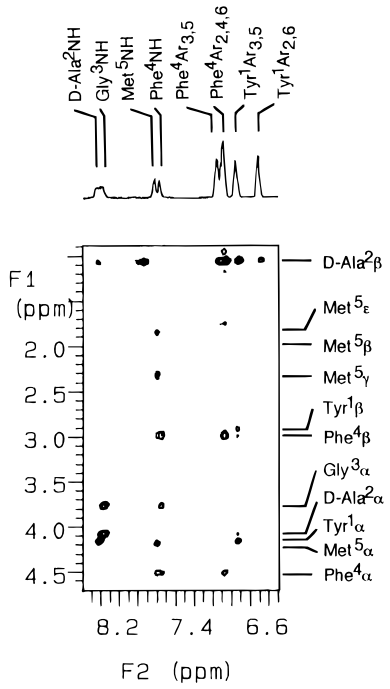

Figure 1. Partial ROESY/MAS spectra of [D-Ala^2]Met-enkephalin dissolved in the CsPFO liquid crystal at 25 °C (500 MHz). The mixing time is 100 ms. The signal appearing at ($F_1 = 1.1$ ppm, $F_2 = 7.9$ ppm) is the spinning side band of D-Ala$^2\beta$ protons.

Results

ROESY/MAS Experiment. Figure 1 shows the partial ROESY/MAS spectra of [D-Ala^2]Met-enkephalin dissolved in the CsPFO liquid crystal measured under the MAS condition. Figure 2 shows the profile of some important ROEs of [D-Ala^2]- and [L-Ala^2]Met-enkephalins among a total of 42 and 33 ROEs, respectively, obtained from ROESY/MAS experiments.

The enhanced resolution observed under the MAS condition clearly shows the merit of MAS for the liquid-crystal sample. The improved resolution facilitates the observation of long-range ROEs as well. In the present study, long-range ROE effects were observed in abundance as shown in Figure 1, and especially numerous interresidue ROEs were clearly detected. Therefore, the conformation of [D-Ala^2]Met-enkephalin in the CsPFO liquid crystal is considered to be in rather high rigidity.

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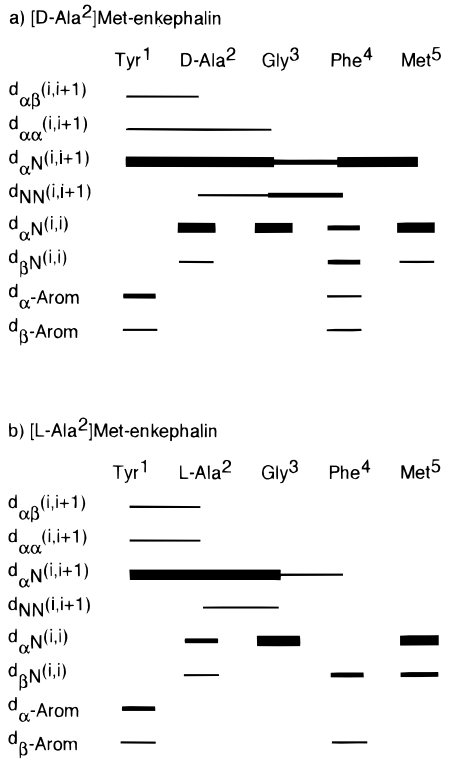

Figure 2. Profile of some important ROEs of (a) [D-Ala^2]- and (b) [L-Ala^2]Met-enkephalins dissolved in the CsPFO liquid crystal.

The combined use of NOESY and MAS techniques has been reported by a few researchers adopting the artificial membrane as a solvent.[22,31] However, NOEs are sometimes hard to be observed for molecules with intermediate size because of their improper correlation time which gives rise to vanishing cross-relaxation.[32,33] The ROESY experiment can avoid such a problem,[34] since positive enhancements are always expected for molecules with any size. Furthermore, the problem of spin diffusion is reduced in the ROESY experiment, and hence ROESY combined with the MAS technique (ROESY/MAS) will be an effective technique for the structural study in liquid crystals.

Direct Couplings Observed under the NMAS Condition. Direct couplings masked under the MAS condition can be restored and adjusted to moderate size in terms of the NMAS technique: the direct couplings are scaled to an extent of $(3\cos^2\theta - 1)/2$ from the original couplings observed in a static sample where θ means an angle between the axes of the sample spinning and the external magnetic field. Under such a NMAS condition, a homo-2*DJ* (homo 2*DJ*/NMAS) experiment is suitable for the measurement of ^{1}H$-^1$H direct couplings since the splitting ($\Delta\nu$) observed in the homo-2DJ/NMAS spectra corresponds to the sum of scalar (J) and direct (D) couplings:[24]

$$\Delta\nu = (3\cos^2\theta - 1)(D + J) \qquad (4)$$

Because the anisotropy in the scalar coupling constant J is negligible, its value is practically the same as the isotropic one. Therefore, it can be obtained from the homo-2*DJ* spectra of the same sample measured under the MAS condition (homo 2*DJ*/MAS spectra).

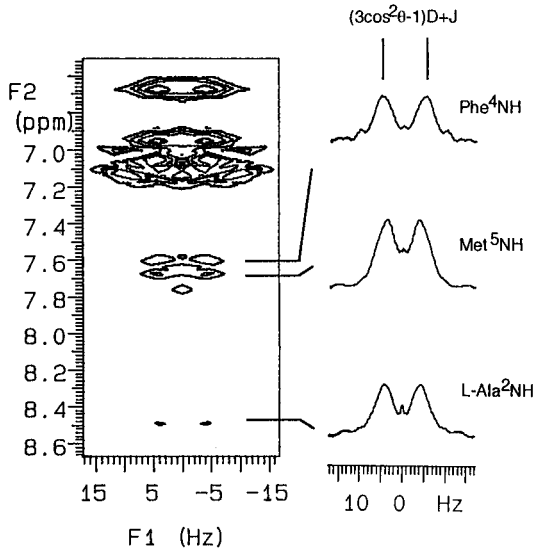

Figure 3. Partial homo-2*DJ*/NMAS ($\theta = 52.7°$) spectra of [L-Ala^2]-Met-enkephalin dissolved in the CsPFO liquid crystal at 25 °C (200 MHz).

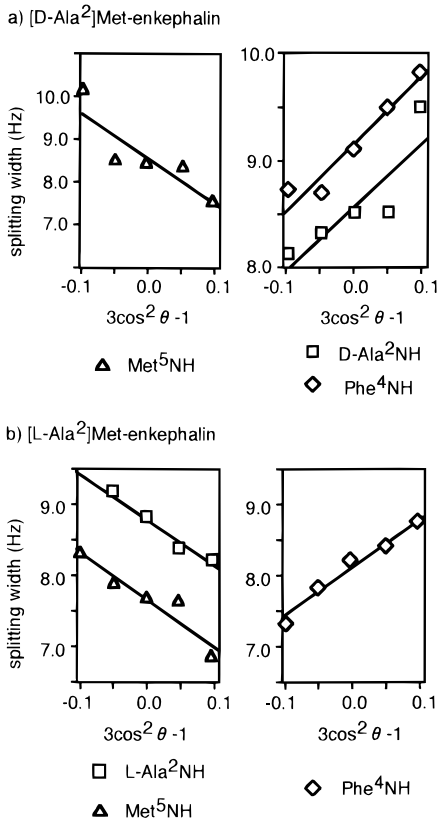

Figure 4. Dependence of the splitting width upon the angle (θ) between the axes of sample spinning and the external magnetic field.

Figure 3 illustrates the partial spectra of homo-2*DJ*/NMAS measured under $-2°$ deviation from the exact magic angle ($\theta = 52.7°$). The splitting pattern is clearly resolved for each proton in Figure 3. If the splitting $\Delta\nu$ obtained from Figure 3 is plotted against $3\cos^2\theta - 1$, the direct coupling can be obtained as a slope of linear relation according to eq 4. Figure 4 shows such a plot for amide protons of [D-Ala^2]- and [L-Ala^2]-Met-enkephalins, and the direct couplings thus obtained are listed in Table 1. The partner proton(s) of these splittings was assigned in the simulation calculation from geometric considerations together with the absolute signs. It is noteworthy that the slope in the plot of $\Delta\nu$ against $3\cos^2\theta - 1$ (Figure 4)

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Table 1. Observed and Calculated Dipolar Couplings

vector	[D-Ala²]Met-enkephalin obsd[a]	[D-Ala²]Met-enkephalin calcd 1[b]	[D-Ala²]Met-enkephalin calcd 2[c]	[L-Ala²]Met-enkephalin obsd[a]	[L-Ala²]Met-enkephalin calcd 1[b]	[L-Ala²]Met-enkephalin calcd 2[c]
$Tyr^1Ar_2-Tyr^1Ar_3$ } $Tyr^1Ar_5-Tyr^1Ar_6$ }	−9.6 (2.3)	−11.9	−11.8	4.3 (1.7)	2.6	6.8
$Ala^2NH-Ala^2\alpha H$	5.9 (2.6)	3.3	1.3	−6.5 (1.7)	−8.2	−2.8
$Phe^4Ar_2-Phe^4Ar_3$ } $Phe^4Ar_5-Phe^4Ar_6$ }	7.1 (1.4)	5.7	5.0	7.8 (1.1)	8.9	4.4
$Phe^4NH-Phe^4\alpha H$	6.3 (1.4)	6.3	6.3	6.7 (2.2)	6.7	6.7
$Met^5NH-Met^5\alpha H$	−9.6 (4.0)	−5.6	−15.0	−6.7 (2.2)	−8.9	0.3

[a] The standard deviations are given in parentheses. [b] The values are fitted by five order parameters. [c] The values are fitted by three parameters ($S_{z'z'}$, β, and γ).

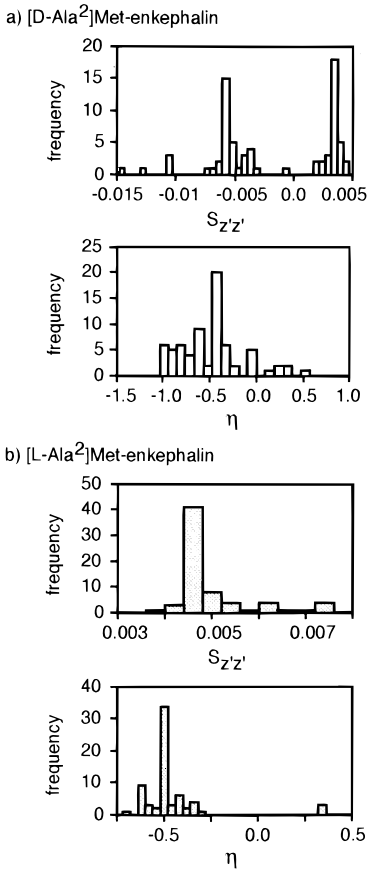

Figure 5. Histogram of the distribution of frequency for $S_{z'z'}$ and η.

reflects the relative sign of the *D* and *J* couplings. Particularly, it is interesting that the direct couplings in aromatic ring of Tyr^1 and Ala^2NH-$Ala^2C_\alpha H$ observed for [D-Ala²]- and [L-Ala²]Met-enkephalins exhibit the inverse sign of *D* compared with each other, where the pertinent *J* couplings are known to be positive. This observation suggests that the orientations of Tyr^1 and Ala^2 residues differ from each other in the CsPFO liquid-crystal aggregates.

Determination of Preferred Conformation and Full Order Parameters. By means of the pseudoenergy minimization procedure, we could obtain 71 and 68 solutions of [D-Ala²]- and [L-Ala²]Met-enkephalins, respectively, which would give convergency with the experimental results. The average root-mean-square distances (rmsd) for the conformers of [D-Ala²] and [L-Ala²]Met-enkephalins were 0.71 and 0.95 Å, respectively. The rmsd value was evaluated by the method of MacLachlan.[35]

Figure 5 shows the histogram of the distribution of $S_{z'z'}$ and η obtained from the above 71 and 68 solutions, η being the parameter describing asymmetric motion and defined as $(S_{x'x'} - S_{y'y'})/S_{z'z'}$. While the histogram of η of [D-Ala²]Met-enkephalin shows a reasonable distribution profile around a single expected value, two contributions are possible in the plot of $S_{z'z'}$: one being around $S_{z'z'} = -0.006$ and another being $S_{z'z'} = 0.002-0.005$. Table 2 lists the corresponding two possible sets of order parameters (orientations 1 and 2). The principal values of the order parameters are listed in Table 3. The calculated direct couplings are listed in Table 1 together with the experimental values. In the case of [L-Ala²]Met-enkephalin, we could determine the orientation in the CsPFO liquid crystal successfully, since the histograms for both $S_{z'z'}$ and η show a reasonable distribution (Figure 5). Tables 1−3 also list the fitting results of [L-Ala²]Met-enkephalin.

Table 2. Order Parameters

	[D-Ala2]Met-enkephalin orientation 1	[D-Ala2]Met-enkephalin orientation 2	[L-Ala²]Met-enkephalin
S_{xx}	0.002 437	−0.001 233	−0.002 285
S_{yy}	−0.000 410	−0.000 545	−0.000 325
S_{zz}	−0.002 027	−0.001 778	0.002 610
S_{xy}	0.001 680	−0.000 112	−0.001 109
S_{xz}	0.000 055	0.001 742	−0.000 514
S_{yz}	0.004 560	−0.001 970	−0.003 014

Table 3. Principal Axes Order Parameters

	[D-Ala²]Met-enkephalin orientation 1	[D-Ala²]Met-enkephalin orientation 2	[L-Ala²]Met-enkephalin
$S_{x'x'}$	0.0044	−0.0025	−0.0035
$S_{y'y'}$	0.0016	−0.0010	−0.0010
$S_{z'z'}$	−0.0060	0.0035	0.0045

Table 4. Fitting Results of the Axially Symmetric Model of Motion

param	[D-Ala²]Met-enkephalin	[L-Ala²]Met-enkephalin
$S_{z'z'}$	−0.0071	0.0033
β	11.7°	94.2°
γ	41.4°	95.2°

Comparison of the Full Order Matrix with the Result of the Axially Symmetric Model. In order to determine which orientation of [D-Ala²]Met-enkephalin is significant, a model calculation was performed further under the assumption of the simple model of axially symmetric motion. As a result, we were able to obtain the converged values of $S_{z'z'}$, β, and γ as shown in Table 4. Table 1 lists the calculated direct couplings from a best fit solution. It is seen that the $S_{z'z'}$ value of [D-Ala²]-Met-enkephalin is negative and of the same order compared with the value reached above in the fitting using five order parameters. Therefore, the contribution around $S_{z'z'} = -0.006$ is probably significant in Figure 5. This means that the orientation 1 listed in Table 3 is dominant for [D-Ala²]Met-enkephalin in the liquid crystal. The fitting results of [L-Ala²]-Met-enkephalin are also listed in Tables 1 and 4. The $S_{z'z'}$ value

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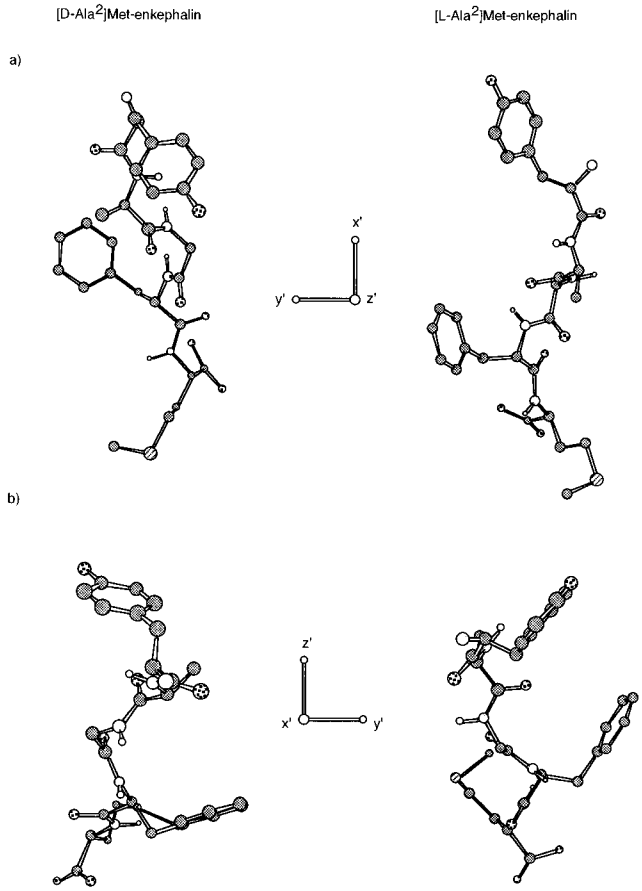

Figure 6. Preferred orientation and structure of Met-enkephalin analogues dissolved in the CsPFO liquid crystal viewed from the (a) z' and (b) x' axes. The principal order frame (x', y', and z') are also included (orientaion 1 in Table 3 is adopted for [D-Ala^2]Met-enkephalin).

corresponds well with the value obtained from the full order matrix fitting listed in Table 3, indicating the validity of the method of the analysis.

Discussion

Preferred Conformation of Met-enkephalin Analogues. Figure 6 shows a best fit solution obtained from the above fitting calculations. Here, we sought a single average structure, and no special model was assumed for multiple stable conformations and orientations. However, the motions of membrane-associated molecules may be so complex that selecting an appropriate model will be difficult. Recently, methodology treating conformational and/or orientational diversity was proposed.[36−38] In our study, if more information is obtained from such an experiment as that utilizing ^{13}C NMR, the possibility of conformational and orientational diversity may be discussed in detail. This is a subject of our future study of extension.

It is seen that extended forms are predominant for both [D-Ala^2]- and [L-Ala^2]Met-enkephalins dissolved in the CsPFO liquid crystal as supported by continuous strong ROE signals of $d_{\alpha N}(i, i+1)$ for $i = 1-3$ (Figure 2), which differs from the result of Met-enkephalin taking a folded form predominantly.[20] Moreover, according to empirical calculations of conformational energy, it was reported that D-Ala^2-Gly^3 as well as Gly^2-Gly^3 sequences tend to form a β turn.[8,39,40] So the stabilization of the extended form is possibly related to the interaction between the molecule and the CsPFO liquid crystal.

In Figure 1, it is seen that the resonances of Gly^{3}NH and Met^{5}NH protons show downfield shifts compared with the results of Met-enkephalin. This observation suggests that the substitution with D-Ala^2 causes a change in environment surrounding the Gly^{3}NH and Met^{5}NH protons. The Gly^{3}NH proton is exposed by the enhanced hydrophobic interaction between the substituent in the 2 position of [D-Ala^2]Met-enkephalin and the liquid-crystal medium. Consequently, the conformation may be changed to an extended form, and the Met^{5}NH proton becomes exposed. The absolute $S_{z'z'}$ value of [D-Ala^2]- and [L-Ala^2]Met-enkephalins (0.006 and 0.005, respectively) is about 6 times as large as that of Met-enkephalin. This result is also attributed to the enhanced hydrophobic interaction between the molecule and the CsPFO liquid crystal.

Comparison of the Orientation between [D-Ala^2]- and [L-Ala^2]Met-enkephalins. The most preferred orientation of [D-Ala^2]Met-enkephalin is one in which the z' axis, for which the order parameter ($S_{z'z'}$) is negative, is orthogonal to the normal axis of the CsPFO aggregates which is parallel to the external magnetic field.[27] The z' axis is almost parallel to the principal axis of the inertia tensor of the molecule as seen from the Euler angle in Table 4 ($\beta = 11.7°$). Therefore, [D-Ala^2]Met-enkephalin orients preferably so that the principal axis of the inertia tensor is orthogonal to the normal axis of the liquid-crystal aggregates. On the contrary, the z' axis of [L-Ala^2]Met-enkephalin is parallel to the normal axis of the liquid crystal, since $S_{z'z'}$ shows a positive value, opposite that of [D-Ala^2]Met-enkephalin. However, the Euler angle, β, indicates that the principal axis of the inertia tensor of the molecule is orthogonal to the z' axis, *i.e.*, the normal axis of the liquid crystal. Therefore, the located states of the two compounds are considered to be similar to each other in the liquid crystal: that is, both the active and inactive compounds are situated with their principal axes of inertia directed almost perpendicular to the normal axis of the liquid-crystal aggregates. An important difference is in their dynamic profiles; that is, the D-Ala^2 analogue is moving (self-rotating) preferentially around the z' axis whereas the L-Ala^2 analogue is around an axis perpendicular to the z' axis. This difference may be closely related to the difference in the side-chain orientation described below.

There is a remarkable difference in the side-chain orientation of the Tyr^1 residue between [D-Ala^2]- and [L-Ala^2]Met-enkephalins as clearly seen in Figure 6. The Tyr^1 side chains in these two enkephalins orient orthogonal to each other along the z' axis (Figure 6b). Moreover, those side chains point to opposite sides with regard to the x' axis which is the most motionally restricted axis (Figure 6b). Therefore, the difference in the orientation and the motional restricted nature is mainly based upon the Tyr^1 side chain. The Tyr^1 aromatic ring of [D-Ala^2]-Met-enkephalin is remote from all other side chains, while those other side chains orient toward similar directions about the backbone structure projected on the $x'-z'$ plane. This result indicates that the interaction between the Tyr^1 residue and the liquid crystal is weak, similar to those of Leu- and Met-enkephalins.[18,20] Therefore, the weak interaction is thought to be important for their activity.

As for the interaction of Tyr^1 residues with the artificial vesicle membrane, there seems to be a common difference between active [D-Ala^2]- and inactive [L-Ala^2]Leu-enkephalin.[9]

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Although the active molecules prefer a folded form in the case of the Leu-enkephalin analogue and an extended form in the case of the Met-enkephalin analogue, the locations of the Tyr^1 residues are similar to each other. The Tyr^1 residue is known to play a crucial role for the enkephalin activity.[41,42] In this way, the mode of interaction of the Tyr^1 residue with the membrane may be related to the activity of enkephalin in a wider sense including Met- and Leu-enkephalins as well.

Conclusion

Two-dimensional NMR spectroscopy has been applied to [D-Ala^2]Met-enkephalin dissolved in a CsPFO liquid crystal. MAS and NMAS conditions were shown to be useful for successful determination of ROE factors and direct coupling constants. These experimental values were analyzed by the use of a target function minimization algorithm. It was derived that Met-enkephalin analogues (active [D-Ala^2]Met-enkephalin and inactive [L-Ala^2]Met-enkephalin) are in extended forms in the liquid crystal. Principal values of the order matrix showed that the motions of these molecules are restricted about the x' axes of the principal order frame for both compounds in the liquid crystal, but the modes of rotational motions within the liquid crystal are quite different from each other when viewed from the principal axes of inertia. Particularly, the orientations and motions of Tyr^1 residues are different characteristically.

The methods developed in the present study can be applied to any molecule whose NMR spectra are first order in pattern under the MAS and NMAS conditions, providing the information on conformation and orientation in the liquid-crystal medium. Thus, the traditional liquid-crystal NMR may be extraordinarily widened in its applicability by the improved version used in the present study, and applied interestingly to study the conformation and orientation of biologically active molecules in model membranes.

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