Determination of the Three-Dimensional Structure of Crystalline Leu-Enkephalin Dihydrate Based on Six Sets of Accurately Determined Interatomic Distances from ^{13}C-REDOR NMR and the Conformation-Dependent ^{13}C Chemical Shifts

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We have determined the three-dimensional structure of [^{13}C,^{15}N]-labeled Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) dihydrate (crystallized from aqueous methanol) on the basis of six sets of accurately determined ^{13}C···^{15}N interatomic distances by rotational echo double resonance (REDOR) and some additional constraints from ^{13}C chemical shifts. This compound has not yet been refined by X-ray diffraction. Six kinds of [^{13}C,^{15}N]-doubly-labeled samples, in which the doubly-labeled positions are four-bonds apart (four samples) and five-bonds apart (two samples), were chemically synthesized. These labeled peptides (100%) and an isotopically diluted one with unlabeled samples (60% or 30%) were crystallized from aqueous methanol solution. ^{13}C or ^{15}N chemical shifts were carefully evaluated prior to and after every REDOR experiment in order to check that the crystalline polymorphs under consideration were not modified either by loss of or by freezing of motion of solvent molecules in the crystals. Accurate and precise interatomic distances (±0.10 Å) were obtained from REDOR factors of infinite dilution, which were extrapolated from the data of 100% and 30% isotopically diluted samples to eliminate dipolar contributions from the labeled nuclei of neighboring molecules in the crystals. These distance data were converted to a possible set of local torsion angles (ϕ_i and ψ_i) in a peptide unit of the respective amino acid residue of interest using standard bond lengths and angles in a sequential manner. It turned out that a unique set of the torsion angles corresponding to the most appropriate three-dimensional structure was determined with reference to some additional constraints from the conformation-dependent displacements of ^{13}C chemical shifts of certain peptide units. The three-dimensional structure thus obtained was finally subject to a calculation for energy minimization in order to ensure that the conformation obtained was at least at one of local minima. Finally, the biological consequence of the peptide structure thus determined is discussed.

Introduction

Enkephalin is an endogenous morphine-like substance and a mixture of two pentapeptides, Tyr-Gly-Gly-Phe-Met-OH (Met-enkephalin) or Tyr-Gly-Gly-Phe-Leu-OH (Leu-enkephalin).[1] The sequence of the first four amino acids is commonly found in the N-terminus region of endorphin and is essential for its binding to δ-, μ-, or κ-receptors.[2] Enkephalins are very flexible in aqueous media and are in equilibrium among several preferred conformations.[3] It is, therefore, difficult to gain insight into "the active conformation" of these substances, which is required for them to exhibit their physiological function from solution data. Cyclic analogues with restrained conformations[4] have been studied by both solution NMR and X-ray diffraction in the solution and crystalline state, respectively. In the crystalline state a number of crystalline polymorphs have been revealed by X-ray diffraction studies that depend on the solvent composition for crystallization of individual crystalline polymorphs. The resulting structures can be classified into the two types, as a whole, either the β-bend[5] or extended[6] forms, although detailed conformations in the backbone and side chains are different among a variety of polymorphs. In particular, it has been suggested that the β-bend form binds to the μ-receptor while the extended form binds to the δ-receptor.[7] However, this does not directly imply that these structures correspond in a simple way to the active conformation because such a form could be realized when these molecules are bound to their respective receptor molecules which are located in the hydrophobic environment of the cell membrane. It is, therefore, very important to develop a means to analyze the three-dimensional

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structure of biologically active peptides bound to the receptor molecules. For this purpose, enkephalin could be an ideal substrate if such a receptor-bound complex were isolated. In practice, however, it is very difficult to do so at present because only a small amount of such receptor molecules is currently available.

Alternatively, it has also been demonstrated that the secondary structure of peptides can be determined by measurements of either interatomic distances from partial recoupling of the dipolar interaction[8,9] or torsion angles.[10] In particular, determination of interatomic distances by REDOR (rotational echo double resonance) is most promising for this purpose, because the most straightforward data analysis results in very precise and accurate data comparable to those obtained by X-ray diffraction using a reasonable computation time,[8] provided that every plausible source of error for the determination of the interatomic distances inherent in the REDOR experiments could be eliminated in advance: contributions from the intermolecular dipolar interaction from labeled nuclei, contributions from natural abundant isotopes, the effect of a finite π pulse, etc.[11,12] Garbow and co-workers[13] were the first to measure three interatomic distances of a systematically labeled tripeptide Pro-Leu-Gly-NH_2 to yield the β-turn II structure, although they did not arrive at a unique structure from experimental constraints alone. Subsequently, Naito et al. systematically applied this technique to elucidate the three-dimensional structures of *N*-acetyl-Pro-Gly-Phe[11,12] from experimental constraints alone because molecular dynamics simulation was not always useful for crystalline peptides.[12,16]

To prove the value of this procedure, it is natural to try to extend this approach to elucidate the three-dimensional structure of a biologically active pentapeptide such as enkephalin.[1] The secondary structure of Leu-enkephalin dihydrate, in particular, was initially demonstrated by an X-ray diffraction study as a β-bend form corresponding to a morphine-like secondary form.[5e] This form was later withdrawn because further refinement of this form by X-ray diffraction was unsuccessful.[5f] This is probably due to the complexity of polymorphs which are very easily converted from one form to the other,[14,15] depending on the number of solvent molecules remaining in the crystal. Solid-state NMR spectroscopy for this purpose has an advantage over X-ray diffraction because conversion of polymorphs can be easily monitored during the course of experiments. For this purpose, we synthesized six kinds of [^{13}C,^{15}N]-labeled Leu-enkephalins by means of a solid-phase peptide synthesizer, followed by isotopic dilution (60% or 30%) with unlabeled enkephalin to eliminate dipolar contributions from neighboring labeled nuclei[11,12] and crystallized them from aqueous methanol to yield dihydrate. Undoubtedly it is essential to utilize additional experimental constraints in order to arrive at a unique three-dimensional structure, otherwise many redundantly labeled samples are required. We emphasize here that ^{13}C chemical shifts for the C_α, C_β, and carbonyl carbons for a given amino acid residue are very sensitive to variations in the local secondary structural unit, as seen in the compiled data from a number of model polypeptides with known secondary structure,[17−19] and can be used as additional constraints. It is also demonstrated that these polymorphs are readily distinguishable from their ^{13}C NMR spectra recorded in the solid state.[14,15]

In this paper we were able to construct the three-dimensional structure of Leu-enkephalin on the basis of the interatomic distances determined by REDOR experiments. The revealed structure is a sort of β-bend form but quite different from that originally proposed.

Experimental Section

The isotopically labeled amino acid [1-^{13}C]Tyr, [1-^{13}C]Gly, [2-^{13}C]Gly, [^{15}N]Gly, [1-^{13}C]Phe, [^{15}N]Phe, and [^{15}N]Leu were purchased from CIL, Inc. Fmoc-OSu was purchased from Peptide Institute Inc. (Osaka, Japan) and used to synthesize Fmoc isotopically labeled amino acids.[20] [1-^{13}C]Tyr-Gly-[^{15}N]-Gly-Phe-Leu (**I**) was obtained by liquid-phase synthesis based on Boc chemistry to avoid consumption of large amounts of the labeled Gly and Tyr in solid-phase synthesis. The other ^{13}C,^{15}N doubly-labeled Leu-enkephalins were synthesized by using an ABI 431 A peptide synthesizer based on Fmoc chemistry: Tyr-[1-^{13}C]Gly-Gly-[^{15}N]Phe-Leu (**II**), Tyr-Gly-[1-^{13}C]Gly-Phe-[^{15}N]Leu (**III**), Tyr-[^{15}N]Gly-[1-^{13}C]Gly-Phe-Leu (**IV**), Tyr-Gly-[^{15}N]Gly-[1-^{13}C]Phe-Leu (**V**), Tyr-[^{15}N]Gly-[2-^{13}C]Gly-Phe-Leu (**VI**). They were cleaved from resin, purified by a reversed-phase HPLC using 0.05% TFA−H_2O and 0.05% TFA−acetonitrile on a Bondasphere C-18 column, and finally lyophilized. Thirteen crystalline preparations (100%, 60%, and 30% isotopically diluted samples using unlabeled peptide) were obtained by recrystallization from aqueous methanol (1:1) at 4 °C.

^{13}C NMR spectra were recorded on a Chemagnetics CMX-400 spectrometer with a triple-resonance probe for a 5 mm o.d. zirconia rotor. ^{1}H, ^{13}C, and ^{15}N resonance frequencies were 400.16, 100.64, and 40.55 MHz, respectively. The XY4 pulse sequence for irradiation of ^{15}N was used to compensate for the flip-angle error, off-resonance effect, and fluctuation of H_1 field.[8b] The length of the π pulses for ^{13}C and ^{15}N were 10.8 and 11.8 μs, respectively. A ^{1}H decoupling field of 70 kHz was used in order to make the ^{13}C spin−spin relaxation time (T_2) as long as possible. Crystalline samples of 30 mg each were placed in the central portion of a 5 mm o.d. rotor (6 mm width) to keep the homogeneity of radio field.[12] Crystalline peptides containing the mother liquid were tightly sealed by gluing the Teflon caps to the rotor to prevent evaporation of the mother liquid during the magic-angle spinning under dried compressed air.[14,15] All NMR spectra were recorded at 0 °C to reduce dielectric loss. The numbers of data acquisition were varied from 64 to 12 800 to obtain a reasonable signal-to-noise ratio by taking into account ^{13}C T_2 values of individual peaks. The rotor spinning speed was controlled to 4000 ± 3 Hz. REDOR and full-echo NMR spectra were recorded at various N_cT_r to include more than four points until $\Delta S/S_0$ values were 0.3 at least, respectively, where Nc and Tr are the number of rotor cycle and rotor period, respectively. The normalized REDOR difference (REDOR factor) was defined as $\Delta S/S_0$ = (full echo − REDOR)/(full echo) to remove the reduction of the signal due to the transverse relaxation. The signal intensities were analyzed as the sum of the center and spinning sideband intensities to take into account of all orientations of the powder sample.[12]

Results

Prior to and after every REDOR experiment, we confirmed whether the crystalline polymorphs of the samples used in the experiment were changed as viewed by ^{13}C and ^{15}N chemical shifts with reference to the sample of natural abundance[15] as summarized in Table 1. This is to evaluate the possible evaporation of solvent molecules from crystalline samples through a pinhole at the top of the Teflon cap, since a stream of compressed dried air results in a substantial conformational change unless sample rotors are tightly sealed.[14,15] It is found that the carbonyl ^{13}C chemical shifts of Gly_2 (167.2 ppm) and

TABLE 1: ^{13}C and ^{15}N Chemical Shifts of [^{13}C,^{15}N]-Doubly-Labeled Enkephalin Dihydrate (ppm)[a]

sample	^{13}C,^{15}N chemical shifts[b]	Tyr	Gly	Gly	Phe	Leu
I	C═O	167.8				
	NH			81.8, 86.6		
II	C═O		170.7			
	NH				92.8, 94.2	
III	C═O			167.0		
	NH					103.6
IV	C═O			167.2		
	NH		82.6, 83.4			
V	C═O				170.3, 170.7	
	NH			82.4, 86.9		
VI	Cα			42.8, 44.9		
	NH		82.0, 83.7			
naturally abundant sample	C═O	168.1	171.2	167.2	170.2	
	Cα			42.6, 44.0		
	NH		82.1, 83.5	82.1, 86.4	93.3	104.0, 102.9

[a] ^{15}N NMR signals of Gly2, Gly3, Phe, and Leu are observed as doublet signals (see ref 15). [b] ^{13}C and ^{15}N chemical shifts are referred to TMS and $^{15}NH_4NO_3$, respectively.

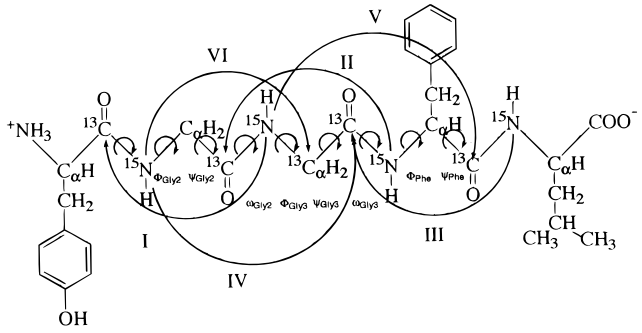

Figure 1. Schematic representation between the sites of a variety of [^{13}C,^{15}N]-doubly-labeled Leu-enkephalin dihydrates and their related torsion angles. The locations of the ^{13}C and ^{15}N nuclei in each sample (designated by the roman number) are indicated by the head and tail of the curved arrow, respectively.

Phe (170.2 ppm) are characteristic of a typical β-sheet form for the respective residues.[17−19] These data were used as additional constraints to select possible sets of torsion angles from the conformation map as deduced from the following REDOR experiments.

We have determined interatomic distances of six kinds of ^{13}C,^{15}N-doubly labeled samples, **I**−**VI**: **I**−**III** and **VI** are for four- bonds apart and **IV** and **V** are for five-bonds apart (Figure 1). Figure 2 shows an example of ^{13}C REDOR (top) and full-echo (bottom) NMR spectra for **I** of isotopically undiluted (100%) (panel A) and diluted (30%) samples (panel B). Many minor signals arising from the naturally abundant nuclei are prominent from the 30% isotopically diluted sample, because their ^{13}C T_2 values are longer than those of the carbonyl nuclei of the Tyr residue. The extent of the reduced peak intensity for the 30% labeled sample was found to be smaller than that of the 100% labeled one (Figure 2B) due to reduced dipolar recouplings. This reduction of the peak intensity was obtained after subtracting the contribution from the naturally abundant nuclei. Such a reduction in the peak intensity with the same N_cT_r was found for all samples, owing to intermolecular dipolar interactions from labeled nuclei from a neighboring molecule.[11,12] Figure 3 exhibits the normalized echo difference $\Delta S/S_0$ values plotted against its concentrations, used to extrapolate the REDOR factor to infinite dilution free from such dipolar contributions from the neighboring molecules.[11,12] We repeated a series of similar experiments for a variety of N_cT_r values of more than four points. Figure 4 compares the REDOR factors $\Delta S/S_0$ vs N_cT_r with (bottom) and without (top) infinite dilution.

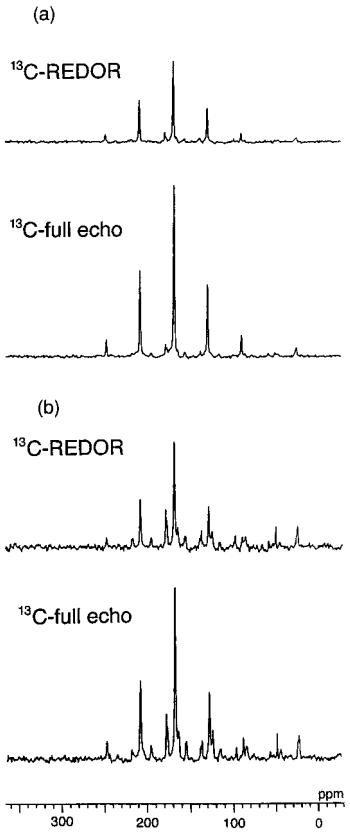

Figure 2. ^{13}C-REDOR and ^{13}C full-echo spectra of **I** in the dihydrate crystal under the conditions of (A) undiluted and (B) diluted to 30% with unlabeled sample. $N_cT_r = 12$ ms.

The resulting interatomic distances 4.72 (top) and 4.20 Å (bottom) for **I** were obtained by fitting a theoretical curve obtained by considering the finite length of a π pulse[12] to the experimental points, as summarized in Table 2.

We repeated up to 70 measurements (six samples, two or three concentrations, and 4−5 N_cT_r values) of similar experiments for the isotopically undiluted and diluted samples **II**−**VI**. Data for the REDOR factors for infinitely diluted samples **II**−**VI** are summarized in Figures A−E in the Supporting Information. The extrapolated interatomic distances thus obtained are summarized in Table 2 together with those obtained for undiluted samples.

TABLE 2: Interatomic Distances for the $^{13}C,^{15}N$-Doubly-Labeled Enkephalin Dihydrate

	sample					
distances (Å)	**I**	**II**	**III**	**IV**	**V**	**VI**
observed	4.72 ± 0.10	3.79 ± 0.10	4.80 ± 0.10	5.10 ± 0.10	5.60 ± 0.10	4.55 ± 0.10
	$(4.20 \pm 0.05)^a$	$(3.61 \pm 0.05)^a$	$(4.53 \pm 0.05)^a$	$(4.90 \pm 0.05)^a$	$(5.15 \pm 0.05)^a$	$(4.53 \pm 0.05)^a$
calculated	4.684	3.747	4.745	5.139	5.627	4.664

[a] Interatomic distance determined from undiluted doubly-labeled sample.

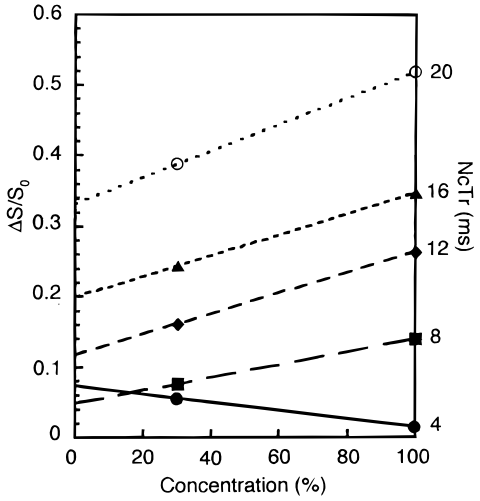

Figure 3. Plot of the REDOR factor (normalized echo difference), $\Delta S/S_0$, versus the concentration of an isotopically labeled sample of **I**.

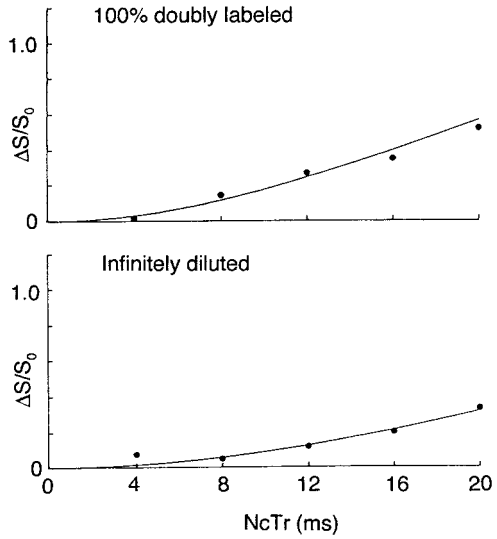

Figure 4. Plot of the REDOR (normalized echo difference),$\Delta S/S_0$ (top, without dilution; bottom, extraporated to infinite dilution) versus N_cT_r values. The observed $^{13}C\cdots^{15}N$ distances are thus determined as 4.72 (top) and 4.20 Å (bottom).

Discussion

Determination of Local Torsion Angles of the Peptide Units Based on Distance and Chemical Shifts Constraints. The interatomic distances for **I**−**III** are related to the torsion angles (ϕ and ψ) of the respective amino residues, (ϕ_{Gly_2}, ψ_{Gly_2}), (ϕ_{Gly_3}, ψ_{Gly_3}), (ϕ_{Phe}, ψ_{Phe}), as illustrated in Figure 1. The distances for **IV** and **V** are also related to (ψ_{Gly_2},ω_{Gly_2}, ϕ_{Gly_3}) and (ψ_{Gly_3}, ω_{Gly_3}, ϕ_{Phe}), respectively. Further, the distance of **VI** depends on the torsion angles ψ and ω, namely (ψ_{Gly_2}, ω_{Gly_2}). We then attempted to convert these interatomic distances to the local torsion angles of constituent amino acid residues as a step to achieve the three-dimensional structure of Leu-enkephalin dihydrate. For this purpose, it is very useful to draw conformation maps to give the C−N interatomic distances as a function of the respective torsion angles (Figures 5−7). In particular, the conformation maps for amino acid residues four-bonds apartwere drawn as a function of the ϕ and ψ or ϕ and ω angles with increments of 1° from −180° to 180° by the program CONF4 written in FORTRAN language,[12] using the standard bond lengths and angles. The conformation maps for amino acid residues five-bonds apart were also calculated by CONF5 in a similar manner, in which it connects the *i*th residue to (i + 1)th residue with other types of the torsion angles. In addition, we utilized ^{13}C chemical shift data as an additional constraint

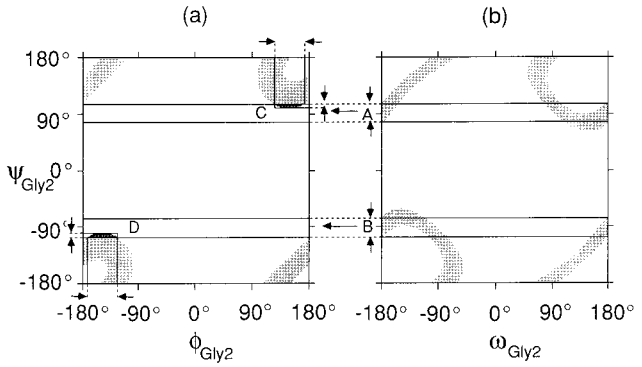

Figure 5. Systematic procedure to determine the torsion angles (ϕ_{Gly_2} and χ_{Gly_2}) (shaded area) from the conformation map for (a) **I** based on an interatomic distance of 4.72 ± 0.10 Å and (b) **VI** based on an interatomic distance of 4.55 ± 0.10 Å. The widths of the respective strips were defined by the corresponding two arrows.

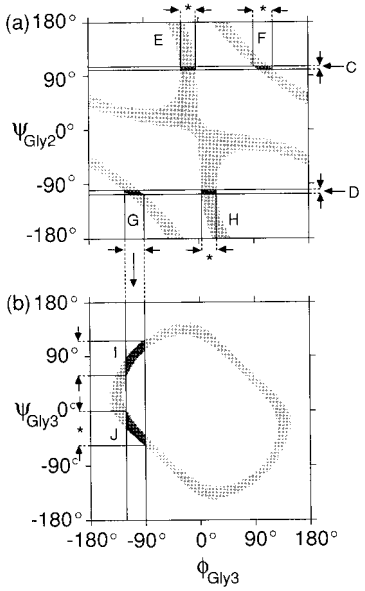

Figure 6. Systematic procedure to determine the torsion angles (ϕ_{Gly_3} and χ_{Gly_3}) (shaded area) from the conformation map for (a) **IV** based on a distance of 5.10 ± 0.10 Å and (b) **II** based on a distance of 3.79 $\pm$ 0.10 Å. The asterisked regions, G, E, and F, are ruled out by an additional constraint of the conformation-dependent ^{13}C chemical shifts. The widths of the respective strips were defined by the corresponding two arrows.

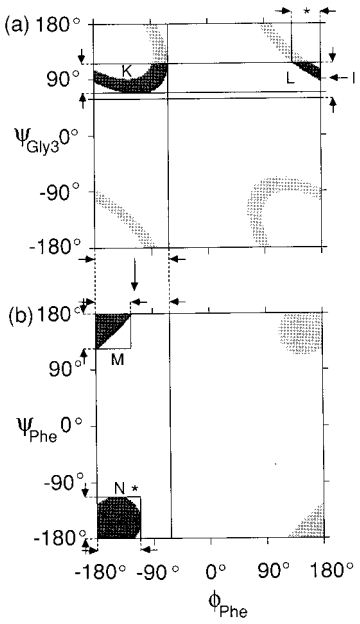

Figure 7. Systematic procedure to determine the torsion angles (ϕ_{Phe} and χ_{Phe}) (shaded area) from the conformation map for (a) **V** based on a distance of 5.60 ± 0.10 Å and (b) **III** based on a distance of 4.80 ± 0.10 Å. The asterisked regions, K and M, were ruled out by an additional constraint of the conformation-dependent ^{13}C chemical shifts. The widths of the respective strips were defined by the corresponding two arrows.

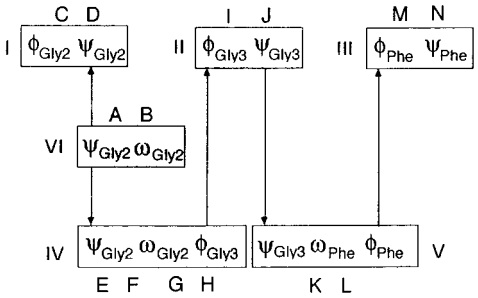

Figure 8. Block diagram to show how respective torsion angles are uniquely determined by the combined examinations of the conformation maps based on the distances of four-bonds apart (**I**, C and D; **II**, I and J; **III**, M and N; **VI**, A and B) and five-bonds apart (**IV**, E, F, G, H; V, K and L).

for selection of a set of possible torsion angles.[17−19] In Figure 8 we summarize a block diagram showing how a unique set of torsion angles for each amino acid residue is systematically determined by combination of the conformation maps in a sequential manner.

Figures 5a and b show the possible area of the torsion angles, (ϕ_{Gly_2}, ψ_{Gly_2}) and (ψ_{Gly_2}, ω_{Gly_2}), drawn as shaded area(s) from the experimentally determined interatomic distances of 4.72 ± 0.10 and 4.55 ± 0.10 Å for **I** and **VI**, respectively. The two candidates A and B were selected for ψ_{Gly_2} angles as 91.5° ± 14.5° and −91.5° ± 14.5° by assuming the torsion angle of ω_{Gly_2} as 180°(Figure 5b). Subsequently, the two possible shaded areas for a set of (ϕ_{Gly_2}, ψ_{Gly_2}) of **I** were chosen as the cross-section of the shaded area in Figure 5a with the two horizontal strips from the blacked areas A and B of ψ_{Gly_2} angles C (149° ± 22°, 103° ± 3°), D (−149° ± 22°, −103° ± 3°). The two horizontal strips from ψ_{Gly_2} arising from the blacked areas C and D thus determined were connected with the conformation map for IV drawn for the interatomic distances 5.10 ± 0.10 Å (Figure 6a). Four possible cross-sections for (ψ_{Gly_2}, ϕ_{Gly_3}) were thus determined: E (103° ± 3°, −17° ± 12°), F (103° ± 3°, 106.5° ± 15.5°), G (−103° ± 3°, −106.5° ± 15.5°), H (−103° ± 3°, 17 ± 12°). One can eliminate any possibility of conformations E, F, and H as viewed from the carbonyl ^{13}C chemical shifts of Gly_3 (167.3 ppm) as β-sheet form (ϕ,χ) = (−129 ± 20°, 124 ± 20°). Then there remains only the blacked area G (−103 ± 3°, −106.5 ± 15.5°). This in turn selects exclusively the area of D (ψ_{Gly_2}) for the Gly_2 residue. The vertical strip from G (ϕ_{Gly_3} = −106.5° ± 15.5°) was then combined with the conformation map for **II** obtained from a distance of 3.79 ± 0.10 Å (Figure 6b). The resulting two possible blacked areas were determined by the cross-section (ϕ_{Gly_3}, ψ_{Gly_3}): I (−106.5° ± 15.5°, 87° ± 29°) and J (−106.5° ± 15.5°, −28° ± 28°). The latter set was ruled out on the basis of the constraint of ^{13}C carbonyl chemical shifts of Gly_3 as β-sheet form, and the resulting unique set of the torsion angle is I (−106.5° ± 15.5°, 87° ± 29°).

The two possible values for the ϕ_{Phe} angle were determined by the cross-section of the conformation for **V** from 5.60 ± 0.10 Å with the horizontal strip from ψ_{Gly_3}, 87° ± 29°, to yield the blacked area with the torsion angles (ψ_{Gly_3}, ϕ_{Phe}): K (92.5° ± 23.5°, −122° ± 58°), L (102° ± 14°, 159° ± 21°). We have chosen the former on the basis of the ^{13}C chemical shifts of Phe as β-sheet. Finally, the obtained blacked area K (92.5° ± 23.5°, −122° ± 58°) was applied to the next conformation map **III** as drawn for a distance of 4.80 ± 0.10 Å, shown in Figure 7b. Subsequently, two possible values for ψ_{Phe} were determined as the cross-section with the vertical strip ϕ_{Phe}, −122 ± 58°, then the resulting set of the torsion angles (ϕ_{Phe}, ψ_{Phe}) (M (−152° ± 27.5°, 152° ± 28°), N (−146° ± 34°, −146.5° ± 33.5°)) for the conformation map for **III**. Moreover, the selection for the possible area of the torsion angle based on an additional constraint of the chemical shifts of the C═O carbon for Phe as β-sheet was applied to these candidates, and the resulting set was the blacked area with the torsion angle M (−152° ± 27.5°, 152° ± 28°). The final unique sets of torsion angles are summarized in Table 3.

The three-dimensional structure of Leu-enkephalin obtained from the sets of the torsion angles from the REDOR experiments is illustrated in Figure 9. This structure was then subjected to energy minimization using the KGNMD program from the MOTECC package,[21] as summarized in Table 3. The torsion angles obtained are within the range of those obtained by the REDOR experiments. The maximum deviation of the interatomic distance is found to be +0.11 Å in the case of **VI** for four-bonds apart (Table 2).

Undoubtedly, the accuracy in the interatomic distances could seriously deteriorate when a dipolar pair under consideration is involved in any kind of molecular motion. It should be taken into account that molecular motion, if any, would make the measured interatomic distance longer.[12] In general, it is advisable to freeze such motion by measurements of the interatomic distances at low temperature. It is plausible that the freezing motion of the solvent molecules tends to induce conformational changes of enkephalin at low temperature.[15] A convenient and reliable way to check this possibility is to measure a series of relaxation parameters sensitive to different types of motions with a different time scale.[15] We pointed out that no such motion is involved for the present crystalline polymorph as far as the backbone motion is concerned as measured by T_1, $T_{1\rho}$, T_{CH}, T_2.[15]

General Procedure To Determine the Three-Dimensional Structure from the Interatomic Distances. One of the major objectives of this study is to develop a general procedure to determine the unique three-dimensional structure of biologically

TABLE 3: Comparison of the Observed Torsion Angles Based on Interatomic Distances and Chemical Shift Data with Those Calculated by the Energy Minimization (deg)

torsion angle	Gly_2 (ϕ)	Gly_2 (ψ)	Gly_3 (ϕ)	Gly_3 (ψ)	Phe (ϕ)	Phe (ψ)
experimental	-149 ± 22	-103 ± 3	-106.5 ± 15.5	92.5 ± 23.5	-152.5 ± 27.5	152 ± 28.0
calculated	-150.35	-104.74	-105.0	90.6	-152.84	155.4

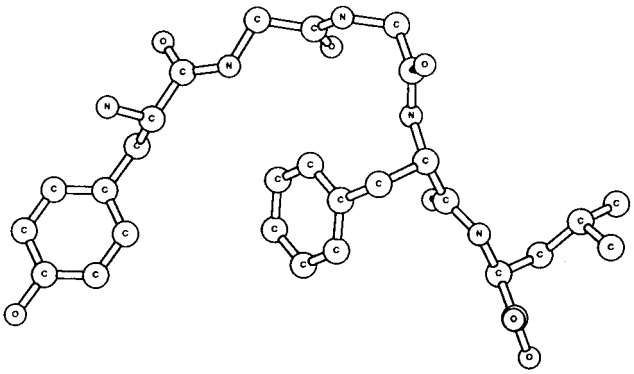

Figure 9. Three-dimensional structure of Leu-enkephalin dihydrate determined uniquely by the successive application of the conformation maps as well as additional constraints of the conformation-dependent ^{13}C chemical shifts. This structure was not changed by an energy minimization procedure, starting from the experimentally determined structure.

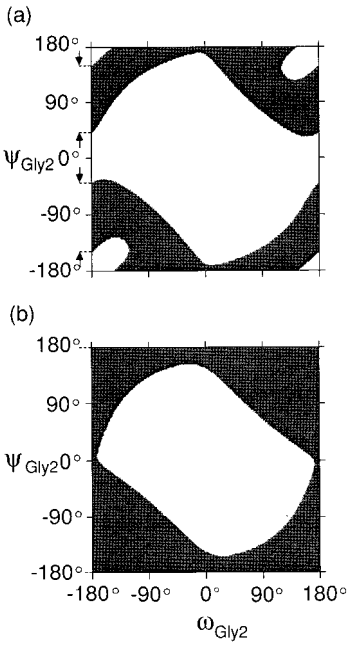

Figure 10. Conformational maps for **VI** based on the interatomic distance of 4.55 Å (see Figure 5b) with increased experimental errors: (a) ± 0.20 Å and (b) ± 0.41 Å.

important molecules by a method other than the diffraction method, which is applicable to membrane- or receptor-bound ligand molecules. It is expected on the basis of the aforementioned procedure that the accuracy of the distances is crucially important in order to select a unique set of torsion angles which are used to arrive at the three-dimensional structure based on the interatomic distances. It is emphasized that the present approach is based on the interatomic distance with an accuracy of ± 0.10 Å. It is worthwhile to examine what happens when the error range is relaxed to involve experimental error larger than the ± 0.10 Å inherent to a number of experimental procedures. The conformation maps for sample **VI**, for instance, were extended to accommodate experimental data with an accuracy of ± 0.30 and ± 0.41 Å, as shown in Figure 10. Obviously, it is very difficult to determine a unique set of torsion angles because all of the shaded area is allowed. Therefore, it is essential to utilize a method such as REDOR which is capable of determining the interatomic distances with an accuracy of ± 0.10 Å for the distances either four- or five-bonds apart. In fact, it is not recommended to use distances more than five-bonds apart because the accuracy of ± 0.05 Å cannot always be retained in a distance longer than 5 Å.

We proposed here a systematic procedure to determine the three-dimensional structure of Leu-enkephalin based on six kinds of accurately determined distances of a systematically [^{13}C,^{15}N]-labeled sample using some additional constraints for the conformation-dependent ^{13}C chemical shifts.[17−19] In general, it can be pointed out that the minimum number of such distances is $2(N-2)$ for a peptide consisting of N amino acid residues as a sum of $(N-2)$ for four-bonds apart, which depends on (ϕ_i and ψ_i), $(N-3)$ for five-bonds apart, which depend on (χ_i, ω_i, and ϕ_{i+1}), and 1 for four-bonds apart, which depends on (χ_i and ω_i) angles. The successive application of the distances four-bonds apart which are related to ϕ_i and ψ_i angles was previously proposed by Garbow and co-workers.[13] In the present paper, however, we extended this approach to involve additional sets of distances including five-bonds apart (see Figures 1 and 8) and another four-bonds apart distance, which is related to χ_i and ω_i angles as additional constraints. This means one can significantly reduce the number of candidates from the conformation map. Nevertheless, it appears that at least four areas can remain, in general, as plausible candidates for the conformation in each residue. The total number of such plausible conformations is $\Pi_2{}^{N-1}k_i$,where k_i is the number of candidates of the ith residue, although k_i depends on the accuracy and combination of the interatomic distances. It is, therefore, emphasized that introduction of additional constraints other than the interatomic distances is essential to minimize a pertinent set of torsion angles. It is tempting to rely on a computation procedure, such as molecular dynamics simulation as in solution NMR, to minimize such a possibility and arrive at the three-dimensional structure of peptides from a minimum amount of experimental data. We demonstrated, however, that such an approach does not always usefully account for the conformational calculations of the solid samples because the overall structure is mainly determined by intermolecular interactions rather than by the intramolecular interactions.[16]

Instead, we used an empirical approach to select the local conformation of the amino acid residues under consideration utilizing the conformation-dependent ^{13}C chemical shifts as the parameters with transferability from the accumulated database,[17−19,22,23] although the availability of such data is limited to five kinds of conformations (α_I-helix, β-sheet, α_{II}-helix, collagen-type helix, and Silk I) for Ala residues and the first two forms for other amino acid residues, in general. In fact, these data have been applied, so far, to reveal the conformational features of fibrous proteins such as collagen and silk fibroin[17−19] and also membrane proteins such as bacteriorhodopsin.[22] It is emphasized that these kinds of constraints are especially useful to rule out irrelevant area in the conformation map, although current sets in the base are not always sufficient.

Significance of Determined Conformation of Leu-Enkephalin Dihydrate. As pointed out, no three-dimensional

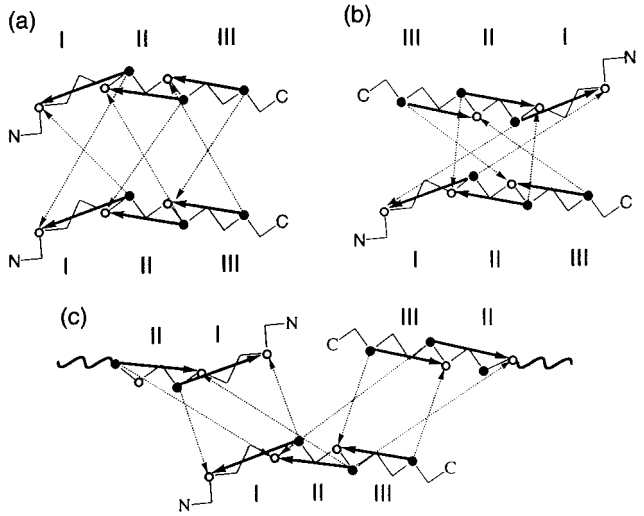

Figure 11. Three kinds of models for putative intermolecular packing as illustrated in the cartoons: (a) interaction through parallel β-sheet form, (b) through an antiparallel β-sheet, and (c) through interaction of three molecules. Solid and dotted arrows correspond with the dipolar interactions between intramolecular and intermolecular $^{13}C\cdots^{15}N$ pairs, respectively.

structure of Leu-enkephalin dihydrate is available from X-ray diffraction studies,[5f] probably due to the complexity of the phase-transition/evaporation of the solvents in the crystal. Our three-dimensional structure is quite different from that previously reported.[5] The resulting torsion angles of the present bend structure in the central region thus obtained are not the same as those of any β-turn type I and II and γ-turn conformation or of previously reported cyclic analogues.[4] It is interesting that the observed partial torsion angles, from the ϕ_{Gly_2} to χ_{Phe} angle of this peptide, are very similar to those of two polymorphs of the previously reported extended conformation[6b] within the range $\pm 10°$.

It appears that no direct intramolecular hydrogen bond is found in this crystal, as illustrated in Figure 9, despite the slightly bent structure at the central region, probably because this structure could be stabilized by intermolecular hydrogen bonds with neighboring molecules or solvent molecules such as water or methanol. In fact, we noticed the presence of considerable dipolar contributions from the labeled nuclei of neigboring molecules as demonstrated by the differential distances between diluted and undiluted conditions, as shown in Table 2. In particular, the dilution effect of **I** and **III** is larger than that of **II**. The following three models can be envisioned to account for the presence of such a dilution effect. The intermolecular dipolar contribution should be the same if the putative intermolecular hydrogen bonds is formed as a parallel β-sheet form, as illustrated in Figure 11a. Here, the solid and dotted arrows correspond to the dipolar interactions between intramolecular and intermolecular $^{13}C\cdots^{15}N$ pairs, respectively. When the peptides form as an antiparallel β-sheet (Figure 11b), the dilution effct of **II** is larger than those of **I** and **III**. Further, if a β-sheet is formed, as shown in Figure 11c, the dilution effect of **I** and **III** is expected to be larger than that of **II**, consistent with our present finding. Figure 11c illustrates the molecular packing taking into account the pseudo-point symmetry because a single NMR line is generally observed for each labeled nuclei. Therefore, we suggest that a β-sheet structure is formed as one peptide interacts with two peptides to form an antiparallel β-sheet. It is further possible to locate the position of the labeled nuclei of the neighboring molecules by taking the second dipolar interaction into account as a three-spin system for REDOR analysis, as demonstrated in our previous work.[11] Detailed analysis of the manner of this intermolecular packing is beyond the scope of this work and will be published separately.

In addition, the secondary structure of Leu-enkephalin or its analogue has been characterized by solution NMR techniques including cases in micelle,[24] liquid crystalline nematic solvent,[25] and liposome.[26] However, no consistent structural data are available from them, including the present approach. It is, therefore, very important to extend this approach to study the three-dimensional structure of enkephalin bound to lipid bilayers or receptor molecules as a means of revealing its active conformation. For this purpose, the present approach could provide the most reliable way because we rely on experimental constraints alone, free from any assumption in relation to molecular dynamics calculations such as its crystalline form or knowledge of the secondary structure of the receptor molecule itself. In this respect, it is pointed out that ^{13}C chemical shifts of this peptide taking a variety of polymorphs are very useful to probe how the present conformation of this peptide is retained under different conditions.

Conclusion

This is the first paper to reveal the complete three-dimensional structure of pentapeptide, enkephalin dihydrate, based on six sets of systematically determined interatomic distances and conformation-dependent ^{13}C chemical shifts as the experimental constraints. The latter constraint is especially useful for sorting out inappropriate conformations among a number of possible candidates. The final selected conformation was not well reproduced by a molecular dynamics calculation but a simple procedure of energy minimization. This approach can be undoubtedly applied to clarify the three-dimensional structures of biologically active peptides which are bound to membrane lipids or receptor molecules. It turns out that the latter approach is very useful to rule out inappropriate candidates.

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Supporting Information Available: Figures A−E illustrating plots of the REDOR factor (normalized echo difference), $\Delta S/S_0$, versus the concentration of the isotopically labeled sample for **II**−**VI**, respectively (5 pages). Ordering information is given on any current musthead page.

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