Solution Conformation of Enkephalin. A Nuclear Magnetic Resonance Study of ^{13}C-Enriched Carbonyl Carbons in [Leu^5]-enkephalin†

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ABSTRACT: By using ^{13}C enrichment in [Leu^5]-enkephalin, it has been possible to improve the assignment of carbonyl resonances in the nuclear magnetic resonance spectrum and to remove some of the ambiguities in the derived ϕ and χ dihedral angles, thereby providing information about the conformation of this molecule in solution. The combined use of ^{13}C and ^{1}H nuclear magnetic resonance experiments leads to the conclusion that [Leu^5]-enkephalin contains a type I β bend at residues Gly^3-Phe^4 in dimethyl-d_6 sulfoxide (Me_2SO-d_6) solution. Furthermore, the side chains of Tyr^1, Phe^4, and Leu^5 exist predominantly in one conformation (tg$^-$) in this solvent. A comparison is made between the conformation found in Me_2SO-d_6 and those determined by X-ray diffraction and conformational energy calculations.

Because enkephalin, a noncyclic peptide (Tyr-Gly-Gly-Phe-Leu),[1] binds to the same receptor site as rigid opiate agonists, there has been considerable interest, both experimental and theoretical, in the structure–activity relationships and the conformation of this endogenous analgesic peptide in solution. The conformation of [Leu^5]- and [Met^5]-enkephalins has been investigated previously by using a variety of methods—NMR (Bleich et al., 1976, 1977; Combrisson et al., 1976; Garbay-Jaureguiberry et al., 1976, 1977; Khaled et al., 1977; Jones et al., 1976, 1977; Deslauriers et al., 1979; Tancrède et al., 1978), X-ray diffraction (Smith & Griffin, 1978), conformational energy calculations (Isogai et al., 1977; De Coen et al., 1977; Momany, 1977; Balodis et al., 1978; Loew & Burt, 1978), CD (Khaled et al., 1977), Raman (Han et al., 1979). In general, these studies agree that enkephalin has a well-defined compact conformation in the crystal and in solution but differ in the nature of the conformation, specifically in the location and type of bend in the backbone, the extent of motional freedom of the side chains, and even in the assignments of some of the nuclear magnetic resonances (from which the conformation in solution is deduced).

In this paper, we address some of these discrepancies using carbon-13 NMR as the primary tool for studying the conformation of this peptide. As with ^{1}H NMR spectroscopy, ^{13}C shieldings and couplings are sensitive to minor structural changes. The differences in chemical shift exhibited by carbon in different environments are generally larger than those for hydrogens, and ^{13}C–^{13}C and ^{13}C–^{1}H couplings provide information about the intervening dihedral angles (Wehrli & Wirthlin, 1976). However, this approach to the study of peptide conformation has not been explored extensively because (when reliance is placed on the natural abundance of ^{13}C) spectral assignments, especially for carbonyl carbons, are sometimes ambiguous (and hence chemical shifts cannot be related to structural changes), and couplings are not readily observed. Thus, ^{13}C enrichments are required to enhance the ^{13}C signals for easy detection. Since enkephalin is a small molecule, such enrichment is easily accomplished by direct synthetic procedures.

In order to be able to make unequivocable assignments of the carbonyl resonances to the correct individual carbons, and to examine the sensitivity of ^{13}C chemical shifts to changes in solvent, state of ionization, and conformational states, several [Leu^5]-enkephalins were synthesized, viz., those in which the carbonyl groups were enriched in ^{13}C in (1) Gly^2, (2) Gly^3, (3) Phe^4, (4) Leu^5, and (5) all five residues, respectively. Direct methods of synthesis, enabling purifications of intermediates, were employed.

Once the first four compounds are used to identify the C′ resonances, the $^3J_{^{13}C'-^{13}C'}$ coupling constants of the fifth one (the completely enriched one) yield sets of values of the dihedral angles ϕ, even though the relation between ^{13}C′–^{13}C′ coupling constants and dihedral angles (Bystrov et al., 1977) has not been tested experimentally as extensively as that for C$^\alpha$H–NH hydrogen–hydrogen couplings (Bystrov et al., 1973). Nevertheless, it is clear (Bystrov et al., 1977) that carbons that are trans to each other have large coupling constants (>3 Hz) and those that are gauche to each other have small coupling constants (≤1 Hz). However, it is not yet possible to obtain very precise values of ϕ from ^{13}C′–^{13}C′ coupling constants. Additional information about ϕ is obtainable from ^3J values for conformationally dependent ^{13}C′–C$^\alpha$–N–^{1}H and ^{13}C′–N–C$^\alpha$–^{1}H carbon–hydrogen spin interactions (Bystrov et al., 1975). Finally, the more extensively used relation (Bystrov et al., 1973) for ^3J hydrogen couplings (C$^\alpha$H–NH) can be employed to establish the values of ϕ as well as possible. These conformationally sensitive three-bond carbon–carbon and carbon–hydrogen coupling constants, to be used here to determine the conformation of enkephalin in solution, are illustrated in Figure 1.

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[1] The nomenclature used in this paper follows the recommendations of the IUPAC–IUB Commission on symbols for amino acid derivatives and peptides [(1975) *Biochemistry 14*, 449; (1972) *J. Biol. Chem. 247*, 977], for naming synthetic modifications of natural peptides [(1967) *J. Biol. Chem. 242*, 555], and for describing polypeptide conformation [(1970) *Biochemistry 9*, 3471]. Isotopic substitution is indicated by designating the isotope, and the part of the molecule to which the label applies, in brackets preceding the name; e.g., [*Gly-1*-^{13}C]^2[Leu^5]-enkephalin indicates that a ^{13}C label occurs in the glycyl 1 or C′ carbon of the second residue from the amino terminus of enkephalin with leucine as the fifth residue. Other abbreviations used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Me_2SO-d_6, deuterated dimethyl sulfoxide; Me_4Si, tetramethylsilane; T_1, spin–lattice relaxation time; δ, chemical shift; CD, circular dichroism; IR, infrared; cP, centipose; Boc, *tert*-butyloxycarbonyl; DCC, *N,N′*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; OBzl, benzyloxy; HONSu, *N*-hydroxysuccinimide; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; TsOH, *p*-toluenesulfonic acid; TLC, thin-layer chromatography.

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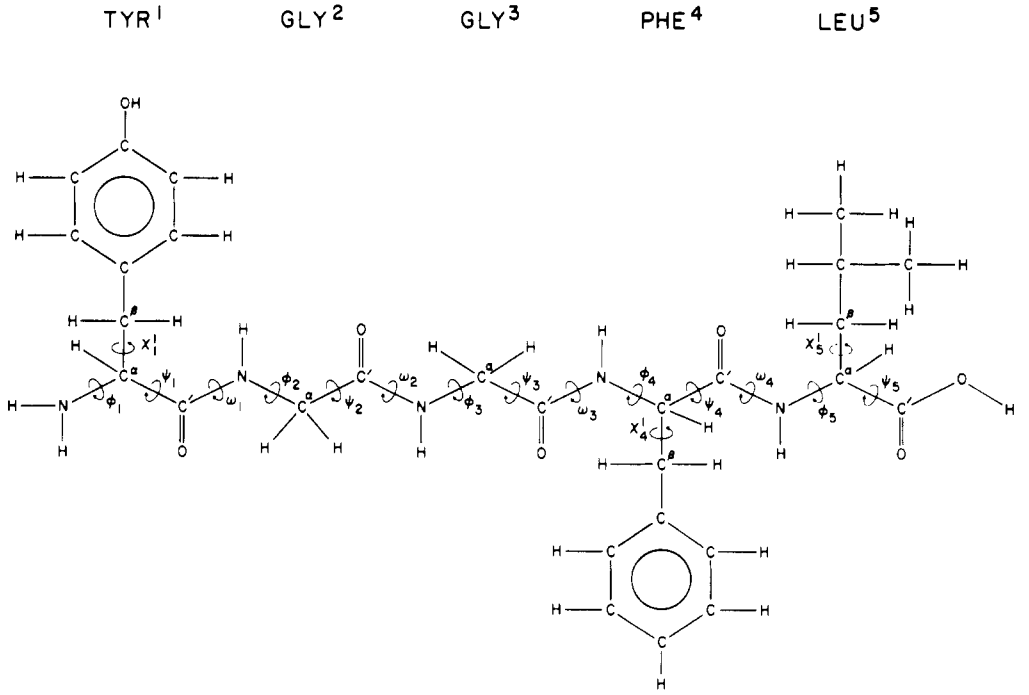

FIGURE 1: The three-bond coupling constants and related dihedral angles in [Leu^5]-enkephalin. The dihedral angle ϕ is related to the $^3J_{C'-NH}$, $^3J_{C'-C'}$, and $^3J_{C'-C^\alpha H}$ coupling constants. The dihedral angle χ^1 is related to the $^3J_{C'-C^\beta H}$ coupling constant.

Likewise, the function relating the dihedral angle χ^1 to the three-bond $C^\alpha H-C^\beta H$ hydrogen–hydrogen coupling constant is a multiple-valued one (Kopple et al., 1973), leading to ambiguities in the values of χ^1, including the impossibility of distinguishing between the two C^β hydrogens and between g^+ and g^- states (Jones et al., 1976). Also, a given $C^\alpha H-C^\beta H$ coupling constant could correspond either to a discrete value of ϕ or to an averaged value for a distribution over a population of rotamers. Some of this ambiguity can be resolved by using the $^3J_{^{13}C'-C^\beta H}$ carbon–hydrogen coupling constants in conjunction with the $^3J_{C^\alpha H-C^\beta H}$ hydrogen–hydrogen values (see Figure 1).

Besides making possible the use of such chemical shift and coupling data to determine conformation, ^{13}C enrichment also facilitates the measurement of spin–lattice relaxation times, T_1, at low concentrations, without NOE enhancement, especially for nonprotonated carbons. The values of T_1 of the carbonyl carbons can provide information about relaxation mechanisms, internal segmental motion, molecular reorientation and tumbling, intermolecular interactions, and even hydrogen bonding or ion-pair formation. In enkephalin, a compound of intermediate size, polarity, and symmetry, intramolecular dipolar relaxation of the nonprotonated carbonyl carbons by nearby hydrogens would be expected to contribute to the relaxation. Such a dipole–dipole relaxation mechanism has a $1/r^6_{C\cdots H}$ dependence. Thus, if an intramolecular hydrogen bond exists in the molecule, the resulting short C′···H distance will be reflected in the value of T_1 (Maciel & Savitsky, 1964; Jackman & Trewella, 1976; Llinás et al., 1977). A hydrogen bond can also affect T_1 by limiting the segmental mobility of the molecule. On the other hand, if a molecule of the size of enkephalin has a very rigid conformation, then the dipole–dipole relaxation mechanism may no longer dominate over the molecular-rotation mechanism. Thus, an additional tool used here to elucidate the conformation of enkephalin in solution is the behavior of the spin–lattice relaxation times of the carbonyl carbons.

The primary solvent used in this study of the neutral form of enkephalin was Me_2SO-d_6. This solvent was chosen over H_2O to reduce the degree of ionization of the α-amino and α-carboxyl end groups.

Experimental Section

Materials

All of the amino acids (except glycine) were of the L configuration. The individual 1-^{13}C-enriched amino acids (94% enrichment) were custom synthesized by KOR Isotopes, Inc., Cambridge, MA 02142. Other amino acids were purchased from Aldrich Chemical Co. Commercial [Leu^5]-enkephalin with natural-abundance isotopic content, was purchased from Bachem Chemical Co; chromatography on DEAE-Sephadex indicated that the commercial peptide had ~30% impurity.

Boc azide, hydrazine hydrate, isopentyl nitrite, TFA, and TsOH were obtained from Aldrich Chemical Co. HOBt, DCC, HONSu, *N*-ethylmorpholine, $SOCl_2$, and benzyl chloroformate, also from Aldrich Chemical Co., were purified before use. Solvents and inorganic salts were reagent grade or better and were used without further purification. DEAE-Sephadex was obtained from Pharmacia Chemical Co.

Deuterated solvents and reagents used in NMR experiments (100% Me_2SO-d_6, >99.5% CD_3OD, 100% D_2O, 99.8% D_2O, DCl, and NaOD) were obtained from Aldrich Chemical Co.

Methods

The purity of the amino acid derivatives, intermediates, and peptides was checked routinely by TLC on Merck silica gel plates, F 254, 0.25 mm, by using the following solvent systems: (a) chloroform–methanol, 9:1; (b) chloroform–methanol–acetic acid, 95:20:3; and (c) 1-butanol–acetic acid–water, 4:1:1.

Methods used for the detection of materials by TLC were ultraviolet light, ninhydrin reagent for free amino groups, chlorine/potassium iodide–starch reagent for NH groups, and Barton reagent for hydrazides.

Melting points (uncorrected) were determined with a Thomas-Hoover melting point apparatus. Specific rotations were measured with a Perkin-Elmer 141 polarimeter. NMR spectra were obtained for all intermediates and final products

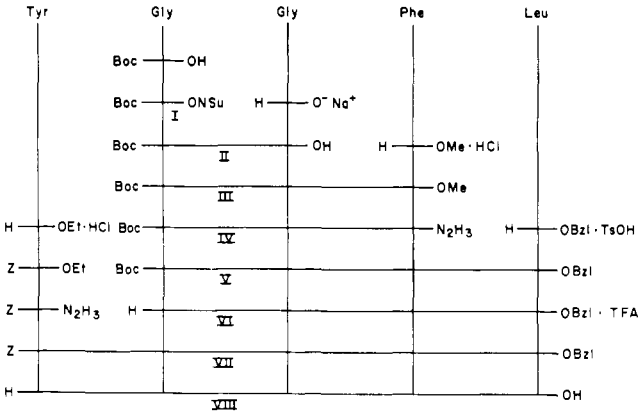

FIGURE 2: Schematic representation of the synthesis of [Leu^5]-enkephalin.

with a Varian EM-390 spectrometer and found to be in full agreement with proposed covalent structures. Amino acid analyses were carried out with a Technicon TSM-1 autoanalyzer on samples that had been hydrolyzed in 6 N HCl for 24 h at 105 °C in deoxygenated, sealed ampules. To check for racemization, an enzymatic digestion was carried out with aminopeptidase M in 0.10 M Tris-HCl buffer (pH 8.5) at 37 °C for about 24 h. The amino acid compositions of these enzymatic hydrolyzates were also checked on the TSM-1 autoanalyzer. Microanalyses for C, H, N composition were carried out by Galbraith Laboratories.

Synthetic Methods. A new synthesis of [Leu^5]-enkephalin was developed, and the following six compounds were all synthesized by classical solution methods according to the general scheme shown in Figure 2: [Leu^5]-enkephalin, [[*Gly-1*-^{13}C]^2,Leu^5]-enkephalin, [[*Gly-1*-^{13}C]^3,Leu^5]-enkephalin, [[*Phe-1*-^{13}C]^4,Leu^5]-enkephalin, [*Leu-1*-^{13}C]^5-enkephalin and [[*Tyr-1*-^{13}C]^1, [*Gly-1*-^{13}C]^2, [*Gly-1*-^{13}C]^3, [*Phe-1*-^{13}C]^4, [*Leu-1*-^{13}C]^5]-enkephalin. This synthetic scheme was chosen to utilize the expensive ^{13}C enriched materials most efficiently.

Boc-Gly-ONSu (I) (Anderson et al., 1964) and the sodium salt of glycine were coupled to give Boc-Gly-Gly-OH (II). The protected dipeptide was treated with Phe-OMe and DCC by using the modification of König & Geiger (1970) to give Boc-Gly-Gly-Phe-OMe (III) (Voelter et al., 1976) in 85% yield. III was converted to its hydrazide IV in the usual manner and coupled with Leu-OBzl·TsOH according to the method of Honzl & Rudinger (1961) to give a 73% yield of the fully protected tetrapeptide Boc-Gly-Gly-Phe-Leu-OBzl (V). After the removal of the Boc group by trifluoroacetic acid, the tetrapeptide VI was acylated with Z-Tyr-N_3 prepared in situ from Z-Tyr-N_2H_3 (Bergmann & Fruton, 1937; Wünch & Jentsch, 1964) to give the fully protected pentapeptide VII in 80–90% yield. A minor product (detected on TLC) was also present; it could not be separated efficiently at this stage. Upon hydrogenation of crude VII, pure [Leu^5]-enkephalin was isolated by chromatography on DEAE-Sephadex as described by Bower et al. (1976) The overall yield from V to the final product VIII was 67%.

Boc-Gly-Gly-Phe-N_2H_3 (IV). Boc-Gly-Gly-Phe-OMe (2.02 g, 5.1 mmol) (Voelter et al., 1976) was dissolved in 10 mL of methanol and 2 mL of 85% hydrazine hydrate was added. The reaction mixture was left at room temperature for 6 h. The solvent was evaporated under reduced pressure and the residue dried over potassium hydroxide at 0.1 mm pressure overnight. The resulting foam was recrystallized from ethanol–ether to give 1.78 g, 88% yield, of IV: mp 117–118 °C; $[\alpha]^{22}_D$ +2.14° (*c* 5, MeOH); R_f 0.32 (b). Anal. Calcd for $C_{18}H_{28}N_5O_5$: C, 54.81; H, 7.16; N, 17.75. Found: C, 54.87; H, 7.15; N, 17.93: Amino acid anal. (6 N HCl, 105 °C, 24 h): Gly, 1.96; Phe, 1.04.

Boc-Gly-Gly-Phe-Leu-OBzl (V). To a stirred solution of IV (1.18 g, 3 mmol) in 14 mL of dimethylformamide at −20 °C were added 2.46 mL of 3.66 N hydrogen chloride in dimethoxyethane and 0.54 mL of isopentyl nitrite. After 15 min, the temperature was lowered to −30 °C. *N*-Ethylmorpholine (1.15 mL, 9 mmol) was added, followed by a solution of Leu-OBzl·TsOH (1.18 g, 3 mmol) (Zervas et al., 1957) and *N*-ethylmorpholine (0.38 mL, 3 mmol) in 5 mL of dimethylformamide. After being stirred at 4 °C for 2 days, the reaction mixture was diluted with ethyl acetate (50 mL) and washed with 1 N hydrochloric acid, 5% sodium bicarbonate solution and saturated sodium chloride solution. The organic layer was dried with sodium sulfate and concentrated to dryness in vacuo. The residue was recrystallized from ethanol–ether to give 1.28 g, 73% yield, of V: mp 162.5–163.5 °C; $[\alpha]^{22}_D$ −18.1° (*c* 2, MeOH); R_f 0.75 (b). Anal. Calcd for $C_{31}H_{42}N_4O_7$: C, 63.90; H, 7.26; N, 9.62. Found: C, 63.92; H, 7.14; N, 9.82. Amino acid anal. (6 N HCl, 105 °C, 24 h): Gly, 1.93; Leu, 1.05; Phe, 1.00. Treatment with chymotrypsin in 0.1 M Tris-HCl buffer (pH 8.5) at room temperature cleaved the tetrapeptide at the Phe-Leu bond in 20 min.

H-Gly-Gly-Phe-Leu-OBzl·TFA (VI). V (278 mg, 0.48 mmol) was dissolved in 2.5 mL of anhydrous trifluoroacetic acid at 4 °C for 15 min. The excess reagent was removed in vacuo and the residual oil dried over potassium hydroxide at 1 mm pressure to constant weight (4 days). The product appeared as a foam, with quantitative yield, and was used without further purification.

Z-Tyr-Gly-Gly-Phe-Leu-OBzl (VII). Z-Tyr-N_2H_3 (160 mg, 0.49 mmol) (Bergmann & Fruton, 1937; Wünch & Jentsch, 1964) was dissolved in 0.8 mL of dimethylformamide. The solution was kept at −20 °C with stirring. A methanolic hydrogen chloride solution (0.51 mL, 2.84 N) was added, followed by 0.083 mL of isopentyl nitrite. After being stirred at −20 °C for 15 min, the reaction mixture was cooled to −30 °C. *N*-Ethylmorpholine (0.19 mL) was added, followed by a solution of VI (0.48 mmol) and *N*-ethylmorpholine (0.064 mL) in 2.5 mL of dimethylformamide. After being stirred at 4 °C for 2 days, the reaction mixture was worked up as described for V. The crude product appeared as a thick oil. The oil was dissolved in a small amount of ethanol and precipitated with ether to give 339 mg of VII, R_f 0.38 (a), 0.73 (b), contaminated by a minor product, R_f 0.18 (a), 0.66 (b). Upon repeated crystallization, VII showed a melting point of 159–162 °C but still not totally free of the minor product. Therefore, VII was used directly for the next step after the first treatment with ethanol and ether.

[Leu^5]-enkephalin (VIII). VII (339 mg) was hydrogenated in 10 mL of ethanol and 0.4 mL of acetic acid over 10% palladium/carbon; after 1.5 h, TLC showed that the reaction was completed. The catalyst was removed by filtration and washed thoroughly with hot ethanol. The combined filtrate was concentrated to dryness in vacuo and the residue (276 mg) chromatographed on a DEAE-Sephadex (acetate form) column with a pyridine/acetic acid buffer (pH 6) (Bower et al., 1976) to give 177 mg, 67% yield from V, of pure [Leu^5]-enkephalin: mp 206–207 °C [lit. (Bower et al., 1976) mp 206 °C; (Voelter et al., 1976) mp 206–208 °C]; R_f 0.49 (c). Amino acid anal. (6 N HCl, 105 °C, 24 h): Gly, 1.95; Leu, 1.04; Tyr, 1.00; Phe, 1.00. The aminopeptidase M digest of enkephalin gave Gly 1.93, Leu 1.01, Tyr 1.02, and Phe 1.05,

Table I: Assignments of C′ Chemical Shifts in Enkephalin at 32 °C

	chemical shift (ppm)[a]											
	Me_2SO-d_6		Me_2SO-d_6 (DCl),	1:1 Me_2SO-d_6/D_2O		D_2O				D_2O		
residue	this work	lit.[b]	this work[c]	this work	lit.[d]	this work, pD 7.7	this work, pD 6.0	lit.,[e] pH 3.6	lit.,[f] pH 4.78	this work, pD 1.2	lit.,[f] pH 0.87	CD_3OD, this work
Tyr[1]	172.88	170.9	169.04	171.17	171.9	174.06	170.76	173.78	169.9	170.32[g]	169.4[g] 165.5	172.05
Gly[2]	168.92	166.8	169.04	171.50	170.9	171.94	171.71	172.20	171.0	171.60	171.0	171.46
Gly[3]	168.61	167.0	169.04	171.17	170.8	171.32	171.32	171.83	170.7	171.16	170.7	171.06
Phe[4]	170.75	168.8	171.66	172.61	170.6	172.58	172.64	171.01	172.1	173.20	172.8	173.16
Leu[5]	174.36	172.2	174.28	178.78	178.2	180.00	180.01	176.34 (Met)	178.0 (Met)	176.17	174.8 (Met)	179.09

[a] Downfield from Me_4Si. The peptide concentration was 0.018 M in our experiments. [b] Values for 0.018 M, estimated from Figure 1 of Khaled et al. (1977). [c] Values in this column were determined by adding an equivalent amount of DCl to the solution. [d] From Garbay-Jaureguiberry et al. (1977). [e] Values from Deslauriers et al. (1978) for [Met[5]]-enkephalin. [f] Values estimated from Figures 5A and 5B of Khaled et al. (1977), which pertain to [Met[5]]-enkephalin at pH 4.78 and 0.87, respectively. [g] Only peak was observed in our work (see Figure 4) even after the solution was kept at pD 1.2 for ~24 h (with no apparent hydrolysis).

indicating that no racemization had taken place during synthesis.

Before the material was used for NMR measurements, all the following criteria for purity were met: (a) the amino acid analysis of the acid and enzymatic hydrolyzates yielded correct molar ratios; (b) the C, H, N analyses agreed with the theoretical values; (c) only one spot was detected with TLC in the different solvent systems with the various detection methods, and (d) all traces of the volatile buffer were removed (no pyridinium acetate was detected by NMR at the δ 1.91 peak).

NMR. The ^{13}C NMR spectra were recorded at 20 MHz and 23.4 MHz with a Varian CFT-20 spectrometer (5- and 8-mm tubes) and with a Brüker HX-90 spectrometer (5- and 10-mm tubes), respectively. Both spectrometers were operated in the Fourier transform mode and were equipped with variable temperature probes. Internal Me_4Si (at a concentration comparable to that of the peptide) was used as a reference except for aqueous solutions where internal dioxane at δ 67.40 was used. In all samples used for relaxation studies, dissolved oxygen was removed by purging for 15 min with nitrogen. Measurement of the spin–lattice relaxation times, T_1, were made by using the inversion–recovery Fourier transform method of Freeman & Hill (1971) with delay times between the 180°–τ–90° sequences of at least five times the longest observed relaxation time (Levy & Peat, 1975; Harris & Newman, 1976) and several values of τ, where τ is a variable delay time. Values of T_1 were determined by a least-squares fit of a semilogarithmic plot of M_τ vs. τ to the best straight line, where M_τ is the value of the magnetization (i.e., peak area) for a given value of τ. In some cases, the intercept deviated slightly from $2M_0$ because of the inability to set pulse widths at fractional values of microseconds on the CFT-20 spectrometer. The only ^{13}C resonances discussed here are the ones corresponding to carbonyl carbons.

Proton NMR spectra were obtained on a 250-MHz spectrometer (Carnegie-Mellon) and on a 90-MHz Varian EM-390 and a Brüker HX-90 spectrometer. For measurements at 250 MHz and on the EM-390, the lock signal was provided by the proton signal of Me_4Si. For measurements on the HX-90, the deuterated solvent provided the deuterium lock signal. Chemical shifts are reported in ppm downfield from internal Me_4Si. In all samples used for NOE or relaxation studies, dissolved oxygen was removed by purging with nitrogen for 15 min.

Adjustments of pD in aqueous solutions were made with dilute DCl or NaOD solutions (0.1–1.0 N) and measured with a pH meter equipped with an Ingold electrode. The values of pH obtained from the pH meter were corrected to values of pD by using the equation of Glasoe & Long (1960), pD = pH + 0.4.

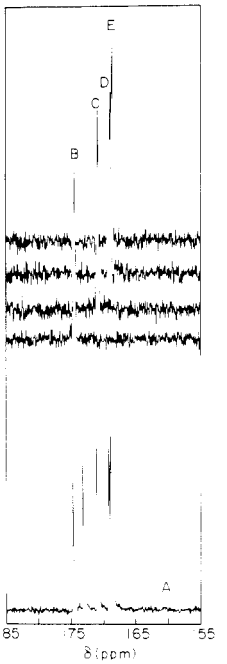

FIGURE 3: Spectra illustrating carbonyl-^{13}C, 20-MHz assignments of [Leu[5]]-enkephalin in Me_2SO-d_6 from compound enriched at the following carbonyl group: (A) all five; (B) Leu[5]; (C) Phe[4]; (D) Gly[2]; (E) Gly[3]. The peak in spectrum A (that lies between those of spectra B and C) is assigned to the Tyr[1] carbonyl (temperature, 32 °C).

In some ^1H NMR experiments, difference spectra were taken to separate resonances more clearly. For example, the C^α protons of glycine were separable by irradiation of the Gly[2] NH resonance and subtraction from the nonirradiated spectrum. This gave a difference spectrum consisting solely of Gly[2] components (coupled and uncoupled) since the Gly[3] portion canceled out.

Results

The C′ carbonyl resonances were assigned by using the separate selectively ^{13}C enriched enkephalins. Figure 3 illustrates how the resonances are distinguishable in Me_2SO-d_6; similar separations of peaks were observed in D_2O. The chemical shifts of the C′ resonances are shown in Table I, together with data from the literature. The absolute values of the chemical shifts differ between this work and the lit-

Table II: Solvent Perturbation of C′ Carbons of [Leu^5]-enkephalin at 32 °C

residue	Me_2SO-CD_3OD (ppm)	Me_2SO-Me_2SO(DCl) (ppm)	Me_2SO-D_2O (pD 7.7) (ppm)	Me_2SO-D_2O (pD 6.0) (ppm)	Me_2SO-D_2O (pD 1.2) (ppm)	Me_2SO-1:1 mixture (ppm)	D_2O (pD 7.7)-D_2O (pD 1.2) (ppm)	D_2O (pD 6.0)-D_2O (pD 1.2) (ppm)	D_2O (pD 7.7)-D_2O (pD 6.0) (ppm)
Tyr^1	+0.83[a]	+3.84	−1.18	+2.12	+2.56	+1.71	+3.74	+0.44	+3.30
Gly^2	−2.54	−0.12	−3.02	−2.79	−2.68	−2.58	+0.34	+0.11	+0.23
Gly^3	−2.45	−0.43	−2.71	−2.71	−2.55	−2.56	+0.16	+0.16	0
Phe^4	−2.41	−1.09	−1.83	−1.89	−2.45	−1.86	−0.62	−0.56	−0.06
Leu^5	−4.73	+0.08	−5.64	−5.65	−1.81	−4.42	+3.83	+3.84	−0.01

[a] Positive and negative values indicate upfield and downfield shifts, respectively, in going from the first to the second solvent.

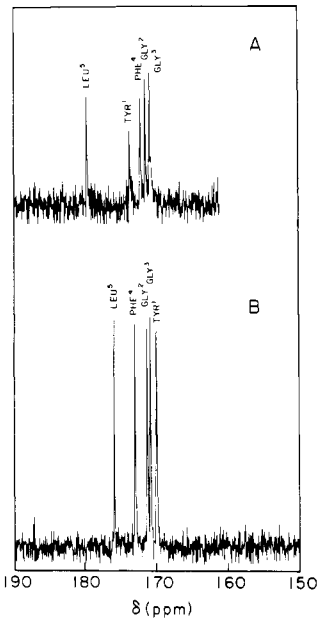

FIGURE 4: Comparison of ^{13}C, 20-MHz spectra of the carbonyl regions of [Leu^5]-enkephalin (5×10^{-3} M in D_2O) at two different pDs. (A) pD ~7.7, unadjusted; (B) pD ~1.2, adjusted by adding dilute DCl (temperature, 32 °C).

erature because of differences in temperature and references. In any given solvent (except D_2O at pD 1.2 and 6.0) our relative order of the chemical shifts differs from those reported in the literature.

As can be seen in Table I, the ^{13}C chemical shifts depend on the nature of the solvent. In Table II, the differences in chemical shifts in the solvent pairs of Table I are listed. These shifts may reflect different conformations and hence different exposures of the carbonyl carbons in the two solvents (Urry & Long, 1976).

In Me_2SO-d_6, the addition of sufficient DCl to titrate enkephalin altered all C′ shifts except those of Gly^2 and Leu^5. Tyr^1 is shifted to higher field and both Phe^4 and Gly^3 are shifted to lower field. This suggests that both Tyr-NH_2 and Leu-COOH are in their neutral (uncharged) forms in Me_2SO-d_6; addition of DCl protonates the α-amino group (the α-carboxyl group having already been protonated before addition of DCl), and this protonation influences the ^{13}C shifts of all the carbonyl carbons except those of Gly^2 and Leu^5.

An upfield shift, upon the addition of DCl, would be expected for Tyr^1, if its α-amino group became protonated (Dwek, 1973). However, inductive effects from protonation of the α-amino group would not account for the downfield shifts of Phe^4 and Gly^3, because they are so far removed (along the backbone) from the α-amino group. Thus, a conformational change is probably responsible for the downfield shifts of Phe^4 and Gly^3. If the Leu^5 NH were hydrogen bonded in Me_2SO-d_6 (as in a Gly^3-Phe^4 β bend; see below), disruption of this hydrogen bond upon addition of DCl would cause a downfield shift of the Phe^4 carbonyl resonance. [The resonance of a carbonyl carbon in a CO–NH group is shifted downfield and upfield if its CO and NH moieties, respectively, become hydrogen bonded (Llinás et al., 1977). Thus, the *rupture* of a hydrogen-bonded NH would shift the carbonyl carbon resonance downfield.] However, it is not clear why protonation of the α-amino group induces the conformational change (affecting also the Gly^3, but not the Gly^2 or Leu^5, carbonyls), unless the chloride counterion perturbs the conformation (in which the α-amino and α-carboxyl end groups are interacting with each other) by possible ion-pair formation.

In D_2O–Me_2SO (1:1 mixture), where the α-NH_2 and α-COOH groups are probably charged, the C′ resonance of Tyr^1 is upfield from that in Me_2SO, and the resonance of Leu^5 is downfield from that in Me_2SO (see Table II), as expected for such states of ionization (Dwek, 1973). However, the large downfield shifts of the Gly^2, Gly^3, and Phe^4 carbonyls indicate that conformational changes accompany the addition of D_2O (and charging of the α-amino and α-carboxyl groups), since protonation of α-amino or deprotonation of α-carboxyl groups, by itself, would not shift the resonances (more than one residue removed from the site of charging) appreciably.

Further conformational changes probably occur on going from D_2O–Me_2SO (1:1 mixture) to 100% D_2O (pD 7.7) because Tyr^1 and Leu^5 undergo large downfield shifts. At pD 7.7 in D_2O, essentially all of the structure that was present in Me_2SO is disrupted, and addition of DCl causes little further change in conformation. The only major observed effects of acidification (in D_2O) are an upfield shift of Tyr^1 (in going from pD 7.7 to 6.0), due to protonation of its α-amino group (in this pD region near its pK_a). Further acidification from pD 6.0 to 1.2 shifts Leu^5 upfield due to protonation of the α-carboxyl group. The behavior in D_2O is different from that in Me_2SO. In Me_2SO, protonation of the α-amino group of Tyr^1 shifted the Phe^4 resonance downfield; in D_2O, the protonation leaves the Phe^4 chemical shift unaffected. Our use of ^{13}C-enriched compounds enabled these changes of chemical shift to be followed and all carbon resonances to be assigned unambiguously (see Figure 3 for spectral identifications in Me_2SO-d_6; similar spectra were obtained in D_2O–Me_2SO mixtures and in D_2O).

By selective proton or carbon decoupling in the spectra of the ^{13}C-enriched compounds, it was possible to make the assignments of all of the proton resonances shown in Table III. The corresponding assignments of Garbay-Jaureguiberry et al. (1976, 1977) are similar.

The temperature dependences of the chemical shifts of the ^{13}C carbonyl carbons of enkephalin in Me_2SO-d_6 are shown in Figure 5. The slopes of all curves, except that of Tyr, are negative and no crossovers exist (except for Tyr at high temperatures) to confuse the assignments.

Table III: ^1H-^1H Chemical Shifts and Coupling Constants of [Leu^5]-enkephalin in Me_2SO-d_6 at 22 °C[a]

residue	chemical shifts (ppm downfield from Me_4Si)				coupling constants, J (Hz)				
	NH	$C^\alpha H$	$C^\beta H_2$	others[b]	$^3J_{NH-C^\alpha H}$	$^3J_{C^\alpha H-C^\beta H}$	$^2J_{\alpha\alpha}$	$^2J_{\beta\beta}$	others[b]
Tyr^1	*c*	3.60	$C^\beta H_A$ 2.64 $C^\beta H_B$ 2.90	ϕ_o 6.72 ϕ_m 7.02 ϕ_{OH} ~9.2[d]	*c*	$C^\beta H_A$ 8.5 $C^\beta H_B$ 4.7		13.5	ϕ_{om} 8.4
Gly^2	8.51	3.71			6.5 4.0		17.8		
Gly^3	7.97	3.68			5.9 4.4		17.8		
Phe^4	8.17	4.48	$C^\beta H_A$ 2.74 $C^\beta H_B$ 3.07	ϕ 7.25 7.27	8.4	$C^\beta H_A$ 9.2 $C^\beta H_B$ 3.4		13.8	*c*
Leu^5	8.02	4.12	$C^\beta H_A$ 1.53 $C^\beta H_B$ 1.53	$C^\gamma H$ 1.66 CH_3 0.88	7.9	$C^\beta H_A$ 12.4 $C^\beta H_B$ 1.0		13.5	$C^\beta H_A$-$C^\gamma H$ 7.4 $C^\beta H_B$-$C^\gamma H$ 6.5 $C^\gamma H$-CH_3 5.5

[a] The peptide and Me_4Si concentrations were 0.018 M. [b] ϕ_o, ϕ_m, and ϕ_{OH} are the ortho, meta, and hydroxyl protons of the phenolic group, and ϕ designates the unidentified protons of the phenyl ring. [c] Not observed. [d] This peak was very broad and was observed only in resolution-enhanced spectra. Its appearance at 9.2 ppm indicates that the Me_2SO-d_6 was dry because, if H_2O were present, the OH protons (at ~9 ppm) would exchange rapidly with H_2O protons (at ~4 ppm), and hence the OH peak would appear at ~4 ppm.

Table IV: ^{13}C Coupling Constants of [Leu^5]-enkephalin in Me_2SO-d_6 at 32 °C

residue	$^3J_{^{13}C-^{13}C}$	$^3J_{^{13}C-C^\alpha H}$	$^3J_{^{13}C-NH}$	$^3J_{^{13}C-C^\beta H}$	$^2J_{^{13}C-C^\alpha H}$	$^2J_{^{13}C-NH}$
Tyr^1	0.9	*a*	*a*	2.7 1.0	*a*	*a*
Gly^2	0.9[b] 1.0	*a*	*a*		4.3	2.6
Gly^3	1.0[b] 1.0	1.7[c]	*a*		3.8	3.9
Phe^4	1.0[b] 1.0	1.7	1.5	3.1[d]	6.2	*a*
Leu^5	1.0		0.8	2.6 2.6	4.8	

[a] Indeterminate because of overlaps or exchange broadening of proton resonances. [b] The first value in each pair is the coupling to the preceding C′ (toward the N terminus), and the second value is to the following C′. [c] Although only one $^3J_{^{13}C-C^\alpha H}$ coupling constant was observed, it is likely that the Gly^2 C′ line width is sufficiently broad to obscure the presence of two similar coupling constants, which would arise because of the nonequivalence of thr protons of Gly^3 (see Table III); $^3J_{^{13}C-C^\alpha H}$ is much less sensitive to small variations in the dihedral angle ϕ in the range of −60° to −80° than is $^3J_{NH-C^\alpha H}$. [d] Only one or averaged coupling constant; i.e., the resonance for each β proton displays only eight lines whereas each Tyr or Leu β proton has 15 lines in resolution enhanced spectra. This doubling of lines disappears in aqueous solutions, indicating that preferred side-chain conformers exist in Me_2SO but not in water. Conceivably, the doubling of the lines for Leu could arise from the two possible values for ϕ_{Leu} (±60 °C; see Table V) and similarly for Tyr if it is close to Leu.

In contrast to results reported by Khaled et al. (1977), we found no dependence of the chemical shift on peptide concentration (in Me_2SO-d_6) in the concentration range of 0.025 to 0.0010 M. Our results agree with those of Tancrède et al. (1978) who found no evidence for aggregation from the concentration dependence of the T_1's for the α carbons (in D_2O) and with those of Khaled et al. (1977) who found no concentration dependence of the circular dichroism in water. All chemical shifts were reproducible to within ±0.05 ppm, a limit dictated by instrumental characteristics (spectral width, SW = 4000, and data points, DP = 8000) and thermal fluctuations (±1 °C). Because of the limited amount of ^{13}C-enriched material available, we did not examine the concentration dependence above 0.025 M. Since the concentration dependence reported by Khaled et al. (1977) was observed both by NMR and circular dichroism (in Me_2SO), it may have arisen from the presence of impurities in the sample (since they used a Bachem sample, and we found ~30% impurity in our Bachem samples).

Some three- and two-bond proton–proton coupling constants are given in Table III, and various coupling constants involving ^{13}C carbonyl carbons are given in Table IV. Those of Table III are similar to the values reported by Garbay-Jaureguiberry et al. (1976). No use was made of the values of the ^2J's because they do not depend on conformation.

The values of the ^{13}C spin–lattice relaxation times T_1 of all of the carbonyl carbons of [Leu^5]-enkephalin in Me_2SO-d_6 were identical within the experimental error of ±0.15 s. At 32 °C, the following values of T_1 (in seconds) were obtained: Tyr^1, 1.59; Gly^2, 1.54; Gly^3, 1.49; Phe^4, 1.54; and Leu^5, 1.49.

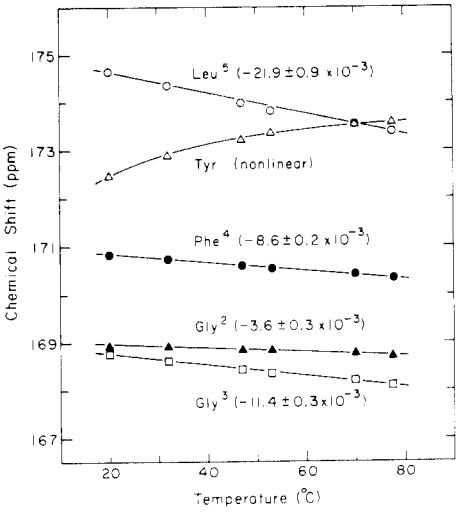

FIGURE 5: Effect of temperature on the [^{13}C]carbonyl carbon chemical shifts of [Leu^5]-enkephalin in Me_2SO-d_6. The slopes (in ppm per deg) are given in parentheses. The peptide concentration is 0.018 M. The curves shown are for the compound enriched at all five C′ positions. However, individually labeled compounds showed the same temperature dependence.

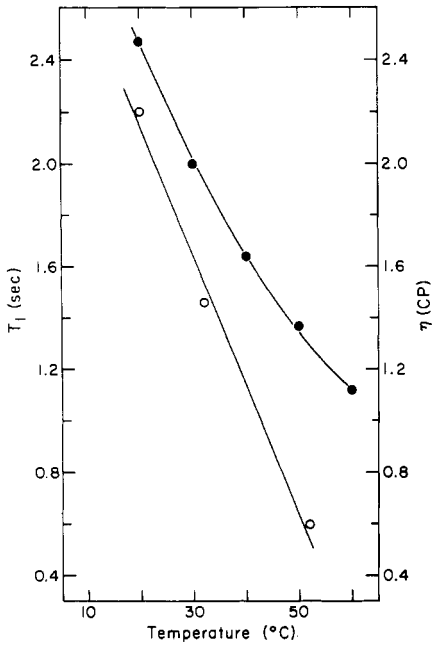

FIGURE 6: Temperature dependence of the Leu^5 C′ spin–lattice relaxation time (T_1) in Me_2SO-d_6. (○) T_1 of Leu^5 C′ in seconds. (●) η of Me_2SO in centipoise (cP) (Schläfer & Schaffernicht, 1960). Assuming isotropic rotational diffusion, the activation energy E_a for rotational reorientation [calculated from the formula $E_a = |RT^2 \, d \ln T_1/dT|$ (Lenk, 1977)] is ~9 kcal/mol; this is somewhat greater than the value of 4.8–6.2 kcal/mol reported by Tancrède et al. (1978).

These were independent of concentration in the range examined (≤0.025 M). Since the ^{13}C resonances of Tyr and Leu are well separated, it was possible to examine the temperature dependence of their T_1 values. That for Leu^5 is shown in Figure 6, together with the temperature dependence of the viscosity of Me_2SO. Similar results were obtained for Tyr (not shown in Figure 6).

In certain cases, intramolecular NOEs in the proton NMR spectra have been useful in providing conformational information about backbone and side-chain dihedral angles (Leach et al., 1977; Rae et al., 1977; Von Dreele et al., 1978). However, no NOEs could be observed for enkephalin in Me_2SO-d_6, suggesting that NH and C$^\alpha$H protons are not very close to each other.

Discussion

Tables I and II demonstrate the sensitivity of the ^{13}C chemical shifts to changes in solvent and pH. While the ^{13}C chemical shifts reflect interactions with the solvent, they also are influenced by solvent- or pH-induced changes in conformation. For example, the C′ chemical shift of Phe^4 in Me_2SO is different upon protonation of the Tyr^1 NH_2 group even though the Gly residues in between are not affected significantly. Thus, the altered chemical shift of C′ of Phe^4 probably reflects a conformational change or an interaction between the ends of the molecule. [The large and opposite temperature dependences of the Tyr^1 and Leu^5 carbonyl chemical shifts in Me_2SO (Figure 5) also indicate some interaction of the end residues or change in state of ionization.] Such a pD-induced perturbation was also observed for the C$^\alpha$H protons of Phe^4 and Met^5 in [Met^5]-enkephalin in Me_2SO (Roques et al., 1976; Jones et al., 1977; Anteunis et al., 1977). Since changes in pH can alter chemical shifts for several reasons (such as inductive effects arising from changes in state of ionization, changes in conformation, etc.), the use of the pH dependence of the chemical shift to identify resonances (Christl & Roberts, 1972; Combrission et al., 1976) is not always reliable. Some of these effects may be the origin of the different ^{13}C assignments found in the literature (see Table I) and provided the motivation for our use of specifically enriched ^{13}C compounds to assign the resonances.

While it is reasonable to expect that the dependence of the ^{13}C chemical shift on the nature of the solvent (see Table II) can provide information about the relative exposure of a group to the solvent, compared with its tendency to be hydrogen-bonded internally (the so-called solvent perturbation technique) (Urry & Long, 1976), the situation is more complicated here since the conformation apparently differs in different solvents. For example, the transfer from Me_2SO-d_6 to the 1:1 mixture of Me_2SO/D_2O causes an upfield shift of the C′ resonance of Tyr^1, while the transfer from Me_2SO-d_6 to D_2O (pH 7) causes a downfield shift. [Ionization of the C-terminal COOH group would lead to a downfield shift of the Leu C′ and an upfield one for the Tyr C′ if a conformational change were not involved (Dwek, 1973).] Although Garbay-Jaureguiberry et al. (1976) and Jones et al. (1976) found the conformation of enkephalin to be similar in Me_2SO-d_6 and D_2O, these data would indicate that at least the environment of Tyr is different.

Since solvent may affect the conformation, it is of interest to examine the temperature dependence of CO and NH shifts in a particular solvent (viz. Me_2SO-d_6) to obtain information about relative solvent shielding or hydrogen bonding. For example, Kopple et al. (1969a,b) demonstrated that intramolecular hydrogen bonded or buried NH groups exhibit smaller temperature coefficients than those exposed to solvent. Ohnishi & Urry (1969) demonstrated that hydrogen-bonded NH and CO groups both have chemical shifts that differ from similar groups that are exposed to solvent. Solvent shielded or hydrogen-bonded NH and CO moieties have decreased temperature coefficients (assuming the conformation remains unchanged). Thus, even though the NH resonances of [Leu^5]-enkephalin (Garbay-Jaureguiberry et al., 1977) do not have temperature coefficients as small as those of [Met^5]-enkephalin (Jones et al., 1976), some solvent shielding is indicated by the small temperature coefficients (Garbay-Jaureguiberry et al., 1977). The NH proton temperature coefficients (in ppm/deg) in Me_2SO-d_6, reported by Garbay-Jaureguiberry et al. (1977), are Tyr^1, not observed; Gly^2, -9×10^{-3}, Gly^3, -4.1×10^{-3}; Phe^4, -6.7×10^{-3}; and Leu^5, -3.3×10^{-3}. From the graph in Figure 5, the temperature coefficients for the carbonyl carbons are Tyr^1, of opposite slope, compared with the others, and nonlinear; Gly^2, -3.6×10^{-3}; Gly^3, -11.4×10^{-3}; Phe^4, -8.6×10^{-3}; and Leu^5, -21.9×10^{-3}. If the lowest NH temperature coefficient is paired with the lowest CO coefficient, this might imply the existence of a hydrogen bond between the Leu^5 NH and the Gly^2 CO. Such a bond would imply the existence of a β bend centered on Gly^3-Phe^4.

The fact that the ^{13}C chemical shift of Gly^2 CO in Me_2SO-d_6 is downfield with respect to that of Gly^3 CO indicates that the Gly^2 CO is more deshielded, possibly by hydrogen bonding. This observation would support the conclusion suggested from the temperature coefficients. The fact that the lower values of the temperature coefficient of Leu^5 NH and Gly^2 CO are not zero could be attributed to nonlinearity (hence weakness) of the proposed hydrogen bond. In the proposed β bend at Gly^3-Phe^4 [which, as will be shown below, is considered to be of type I (Lewis et al., 1973)], Gly^2 CO and Leu^5 NH are hydrogen bonded, and Gly^3 NH, Gly^3 CO, and Phe^4 CO point *outward* from the bend, and Phe^4 NH points slightly inward (and is probably shielded by the Phe^4 ring), as illustrated in Figure 7. The reduced temperature

Table V: Dihedral Angles Consistent with ^3J Coupling Constants in Me_2SO-d_6 at 32 °C

residue	ϕ (deg) from $^3J_{^{13}C-^{13}C}$	ϕ (deg) from $^3J_{^{13}C-NH}$	ϕ (deg) from $^3J_{^{13}C-C^{\alpha}H}$	ϕ (deg) from $^3J_{NH-C^{\alpha}H}$	χ (deg) from $^3J_{^{13}C-C^{\beta}H}$	χ (deg) from $^3J_{C^{\alpha}H-C^{\beta}H}$	rotamer populations[a] from $^3J_{^{13}C-C^{\beta}H}$	rotamer populations[a] from $^3J_{C^{\alpha}H-C^{\beta}H}$
Tyr[1]				[b]	30[c] to 60			0.2 g^-g^+
					−30 to 60	20,[c] −95		0.8 tg
					−90, +90	120,[d] 140[d]	tg^- [e]	
					+120,[d] −120[c]	**−50, −170**		
Gly[2]	**±60**	[b]	[b]	**90, −175**				
	+120, −120			**30, −70**				
Gly[3]	+60, **−60**	[b]	0, 180	90, −175				
	±120		**−60 to −80**	25, **−65**				
Phe[4]	+60, **−60**	**−60 to −80**	**−60 to −80**	**−85**, 45	−60, 60[c]	145, 25	tg^- [e]	
	±120			−155, 75	±120[c]	±10, ±110[c]		0.1 g^-g^+
						−60, 60[c]		**0.9 tg**
Leu[5]	**±60**	**±60**, ±120	[b]	**−80, 40**	**−60**, 60[c]	**−60**, 60[c]	tg^- [e]	**1.0 tg**
	±120			−160, **80**	±120			0 g^-g^+

[a] Preferred rotamer populations about C^{α}–C^{β} bond, calculated using the treatment of Pachler (1964). See text and Figure 8 for explanation of gg and tg symbols. [b] Indeterminable because of line broadening or overlaps. [c] Eliminated because it does not agree with the Pachler-type analysis; i.e., this is not a tg^- conformation. [d] Not selected because the Ph and CO are eclipsed in this conformation, making it one of high energy. [e] 100% tg^-, within an uncertainty of ±10%, because of the uncertainty in the relation between χ and coupling constant.

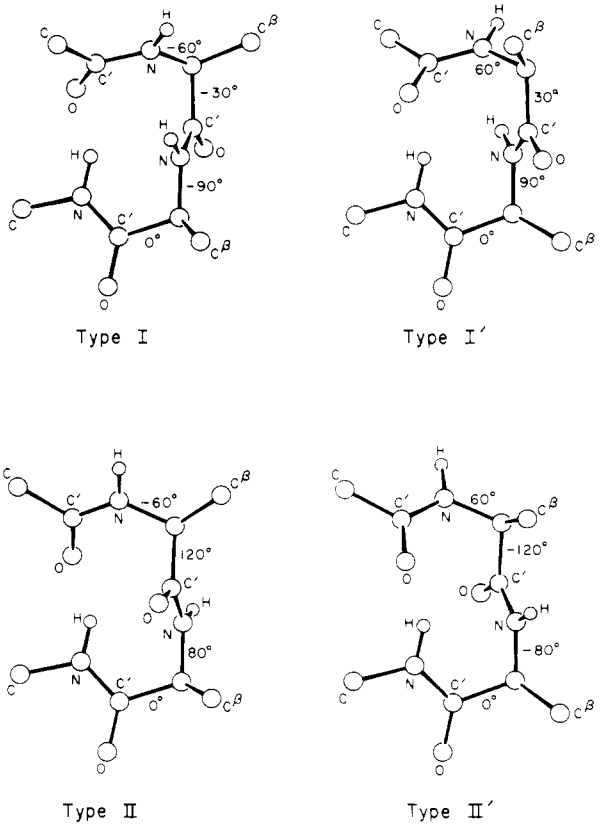

FIGURE 7: Several types of β bend, with relevant dihedral angles indicated (Lewis et al., 1973; Zimmerman & Scheraga, 1977).

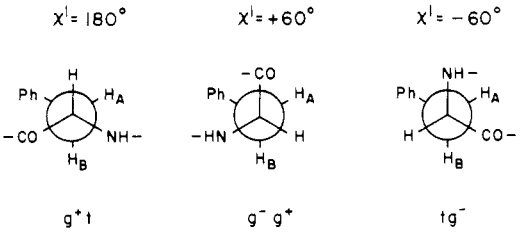

FIGURE 8: Conformations about the C^{α}–C^{β} bond of L-Phe[4]. The rotamer symbols refer to the relative positions between H^{α} and H_A and H_B, respectively (see text).

coefficient of Phe[4] NH (relative to that of Gly[2]) suggests that such shielding might occur in the type-I β-bend conformation (in contrast to the behavior in an extended conformation).

Whereas the temperature coefficients suggest the existence of a β bend at Gly[3]-Phe[4] in Me_2SO-d_6 solution, a Gly[2]-Gly[3] bend was proposed on the basis of X-ray diffraction studies (Smith & Griffin, 1978) and postulated on the basis of an application of empirical rules for predicting backbone structure (Bradbury et al., 1976). Such a bend would imply the existence of a hydrogen bond between Tyr[1] CO and Phe[4] NH. However, the temperature coefficients of this CO and NH chemical shift do not support this. Also, the nonlinear increasingly downfield chemical shift of the Tyr[1] carbonyl carbon with increasing temperature is not compatible with hydrogen bonding; i.e., hydrogen bonding causes a downfield shift (due to deshielding of the CO by the nearby proton), so that the rupture of the hydrogen bond as the temperature increases should cause an upfield shift (Llinas et al., 1977).

The proton chemical shifts and coupling constants for [Leu[5]]-enkephalin in Me_2SO-d_6 in Table III agree with those of Garbay-Jaureguiberry et al. (1976, 1977) with some minor exceptions that can be accounted for by differences in temperature and reference systems (i.e., use of internal vs. external Me_4Si). These coupling constants lead to many possible values of the corresponding dihedral angles, ϕ and χ (Bystrov et al., 1973; Kopple et al., 1973). These are given in Table V. However, the number of possibilities can be reduced by considering the ^3J carbon–proton and carbon–carbon coupling constants of Table IV (Bystrov et al., 1975, 1977). The values of ϕ and χ, consistent with the ^3J coupling constants involving ^{13}C, are given in Table V. The acceptable dihedral angles indicated in boldfaced type in Table V are those that are consistent with *all* of the coupling constants of Tables III and IV. In particular, ϕ of both Gly[3] and Phe[4] is restricted to the neighborhood of ~−70°. This value of ϕ for *both* Gly[3] and Phe[4] is consistent with a type I bend at Gly[3]-Phe[4] (see Figure 7) and rules out the type I′, II and II′ β bends (Lewis et al., 1973; Zimmerman & Scheraga, 1977).

Before considering the conformations of the side chains of Tyr[1], Phe[4], and Leu[5], it is necessary to recall that the standard convention for polypeptide nomenclature [(1970) *Biochemistry* 9, 3471] bases the definition of χ^1 on the N–C^{α}–C^{β}–C^{γ} moiety; this is the convention adopted in Figure 8 which applies to Phe[4] as an example. Usually, the terms t, g^+, and g^- (trans, gauche plus, and gauche minus) refer to values of χ^1 in the neighborhood (i.e., within 20–30°) of 180°, +60°, and −60°, re-

spectively [Flory, 1969, with the convention of (1970) *Biochemistry 9*, 3471]. However, since we are interested here in the positions of both H_A and H_B, we shall use a double notation, e.g., g^+t, where g^+ refers to H_A and t to H_B (see Figure 8 and also Jones et al., 1976), and this notation applies to the H^α–C^α–C^β–H_A and H^α–C^α–C^β–H_B moieties, with the retention of the numerical values of χ^1 given in Figure 8. The method of Pachler (1964), applied to the $C^\alpha H$–$C^\beta H$ coupling constants, can provide information about the relative amounts of g and t conformers but cannot distinguish between g^+ and g^-. However, we can do so by use of the ^3J coupling constants involving ^{13}C.

Applying the method of Pachler (1964) to the Tyr[1] proton $C^\alpha H$–$C^\beta H$ coupling constants, we find 20% g^-g^+ and 80% tg (or gt) for the rotamer populations around the C^α–C^β bond. However, of the two tg (or gt) rotamers, only the g^+t conformation has one $C^\beta H$ proton (viz., H_A) trans to the carbonyl carbon of the same residue. Such a ^{13}C–$C^\beta H$ trans coupling constant is large ($\gtrsim$8 Hz), according to Wherli & Wirthlin (1976) and Bystrov et al. (1977), whereas coupling constants for gauche protons to ^{13}C are small (1 or 2 Hz). Even if the three possible staggered conformations about the C^α–C^β bond were equally probable, or if rotation about this bond were completely free, the trans ^{13}C–$C^\beta H$ contribution would yield an average coupling constant of ~4.3 Hz (Hansen et al., 1975). Therefore, since the proton coupling data ($^3J_{C^\alpha H\text{-}C^\beta H}$ = 8.5 and 4.7 Hz) indicate a predominance of tg (or gt) rotamers, the ^{13}C coupling data ($^3J_{^{13}C\text{-}C^\beta H}$ = 1.0 and 2.7 Hz) are most consistent with the predominantly tg^- rotamer.

In general, from the tentative Bystrov-type curve for $^3J_{^{13}C\text{-}C^\beta H}$ (Wehrli & Wirthlin, 1976), ^3J = 2.7 corresponds to a band of dihedral angles near ±(30 to 60)° or near ±120°, whereas ^3J = 1.0 corresponds to dihedral angles around ±90°. Because a dihedral angle χ^1 of +120° is a high-energy conformation (because the carbonyl group and phenyl group are eclipsed), this can be eliminated from consideration, leaving −60° or −90° (+60° and +90° are not consistent with tg^-). Similarly, the Leu[5] proton coupling data ($^3J_{C^\alpha H\text{-}C^\beta H}$ = 12.4 and 1.0 Hz) indicate a predominance of tg (or gt) rotamers, and the $^3J_{^{13}C\text{-}C^\beta H}$ coupling constants (2.6 Hz) distinguish this as the tg^- rotamer.

Although the Phe[4] $^3J_{^{13}C\text{-}C^\beta H}$ coupling constant of 3.1 Hz is slightly larger than those observed for Tyr and Leu, the side chain of Phe[4] also exists predominantly as a tg^- rotamer; this is also consistent with a Pachler-type analysis of $^3J_{C^\alpha H\text{-}C^\beta H}$ = 3.4 and 9.2 Hz. The possibility exists that there is a small population of g^+t and g^-g^+ rotamers which does not increase the average value of $^3J_{^{13}C\text{-}C^\beta H}$ significantly and is still consistent with the values of $^3J_{C^\alpha H\text{-}C^\beta H}$ in Table III.

On the basis of the above analysis, the side chains of Tyr[1], Phe[4], and Leu[5] are seen to exist predominantly in the tg^- conformation, at least as far as χ^1 is concerned.

These ^{13}C NMR results demonstrate the usefulness of ^{13}C enrichment, not only in the proper assignment of resonances but also in the determination of the values of χ^1 and in showing that all these residues with side chains in [Leu[5]]-enkephalin have rotational isomers about their C^α–C^β bonds that are not equally probable. On the contrary, the side chains have strongly preferred conformations in Me_2SO-d_6. This conclusion is in partial agreement with that of Garbay-Jaureguiberry et al. (1977) and with those for [Met[5]]-enkephalin of Bleich et al. (1976, 1977); both groups suggested that the side chain of Tyr[1] is restricted in its rotational motion, but (in contrast to the analysis reported here) both they and Jones et al. (1976, 1977) concluded that there are significant populations of *several* rotamers about the C^α–C^β bonds of Phe[4] and Leu[5].

Whether the predisposition of these side chains for preferred values of χ^1 facilitates recognition by the receptor cannot be answered until the receptor can be isolated and bound to enkephalin. Nevertheless, since, as pointed out by Loew & Burt (1978), the tyramine moiety of their calculated conformation with χ^1 near −90° resembles the corresponding portion of the morphine molecule, our experimental conformation with χ^1 of Tyr[1] near −60° would do likewise; i.e., no conformational change from that of the free enkephalin molecule would be required to achieve a resemblance to this portion of the morphine molecule.

The values of T_1 of the carbonyl carbons at 32 °C are remarkably similar in Me_2SO-d_6, as were those of the C^α carbons in Me_2SO–D_2O mixtures or in D_2O (Garbay-Jaureguiberry et al., 1977; Tancrède et al., 1978). The values of T_1 reported here for enkephalin in Me_2SO display less variation than did those of Garbay-Jaureguiberry et al. (1977) for the C′ carbons in 1:1 Me_2SO–D_2O solution. Since we have shown that the conformation changes in going from Me_2SO to 1:1 Me_2SO–D_2O solution, the variation in carbonyl T_1 values from residue to residue found by Garbay-Jaureguiberry et al. (1977) can be accounted for by a contribution from segmental motion (at the end residues) to relaxation in 1:1 Me_2SO–D_2O. Such segmental motion does not contribute significantly to the relaxation in Me_2SO and, hence, the values of T_1 of the inner and end residues are similar. Furthermore, our values of T_1 for C′ carbons are similar to those of Garbay-Jaureguiberry et al. (1977) for the Leu CH_3 group. These observations indicate that the molecule undergoes an overall tumbling rather than segmental motion. This is also indicated by the similar temperature dependence for T_1 of Leu[5] and η of Me_2SO-d_6 (see Figure 6). The two curves diverge only at high temperature, where the terminal Leu residue may begin to exhibit segmental motion, besides that arising from overall molecular tumbling. In addition, the decrease in T_1 with increasing temperature is that expected for rotation of nuclear spins, i.e., overall tumbling (Brown et al., 1963; Hubbard, 1963; Atkins, 1967), in contrast to the reverse relationship (increase in T_1) for dipolar relaxation or scalar relaxation (Wehrli & Wirthlin, 1976); such a reverse relationship was observed for the behavior of the values of T_1 for the α carbons of enkephalin, where dipolar relaxation involved the α hydrogen (Tancrède et al., 1978). The small differences in the values of T_1 from residue to residue suggest the conclusion that rotational diffusion appears to be isotropic, i.e., that the molecule is spherical rather than extended.

In comparison with the few C′ relaxation studies which have been reported, [Leu[5]]-enkephalin behaves more like the cyclic peptide gramicidin S (Allerhand & Komoroski, 1973) than the more flexible acyclic peptides thyrotropic releasing factor (TRF) and melanocyte stimulating hormone release inhibiting factor (MSF) (Deslauriers et al., 1973, 1974). The values of T_1 of the carbonyl carbons of gramicidin S in methanol vary from 1.5 to 1.8 s. Even though the molecular weight of gramicidin S is approximately twice that of [Leu[5]]-enkephalin, the viscosity of methanol (0.45 cP at 43 °C; *CRC Handbook*, 1973) is only about one-fourth that of Me_2SO-d_6 (1.92 cP at 32 °C). Thus, since

$$\tau_c = \frac{V_M \eta}{kT} \qquad (1)$$

(Bovey, 1969), where τ_c is the effective correlation time, V_M is the molecular volume, η is the viscosity of the medium, k is the Boltzmann constant, and T is the absolute temperature,

τ_c for the motionally constrained gramicidin S is similar to that for the acyclic enkephalin. On the other hand, even though the acyclic compounds TRF and MSF have smaller molecular weight and volume than enkephalin, they exist as mixtures of several (unaggregated) rotamers which undergo rapid segmental motion, which increases their values of T_1. For example, values of T_1 for TRF in D_2O range from 7 to 16 s, and those for MSF in Me_2SO-d_6 range from 3 to 5 s. Finally, the value of T_1 of [Leu^5]-enkephalin corresponds to a reasonable value of τ_c; using the crystal density of 1.13 g/cm^3 (Smith & Griffin, 1978), a value of τ_c of about 3.8×10^{-10} s is calculated for enkephalin (in Me_2SO-d_6) from eq 1. In their studies with $^{13}C^{\alpha}$-enriched enkephalin, Tancrède et al. (1978) measured the overall molecular reorientation correlation time, τ_c, and found it to be of this magnitude; they interpreted this value and its linear dependence on concentration to indicate the absence of aggregation. Since we found no dependence of the chemical shift on concentration, we assume that our measured values of T_1 pertain to the unaggregated molecule.

If any of the carbonyls were hydrogen bonded, thereby introducing dipolar relaxation from the neighboring hydrogen, their effect on T_1 might not be detected because the rate of dipolar relaxation from neighboring protons is too slow to compete with the spin-rotation mechanism (i.e., overall tumbling of the molecule) because the hydrogen is two bonds removed from the carbonyl carbon (Jackman & Trewella, 1976; Llinàs et al., 1977; Maciel & Savitsky, 1964). From the relaxation behavior of the C′ carbons, it was concluded that [Leu^5]-enkephalin tumbles as a fairly rigid conformation with relatively little segmental motion. If any of the carbonyls are hydrogen bonded, the contribution of such bonds to the relaxation would not be detected; i.e., the behavior of the values of T_1 is not inconsistent with the possible existence of such a hydrogen bond.

From the values of ϕ for Gly^3 and Phe^4, we have concluded that the β bend is of type I, rather than I′, II, or II′. If [Leu^5]-enkephalin is as rigid as the measurements of T_1 indicate, then NOE measurements for pairs of protons in close spatial proximity should also distinguish, qualitatively, between these different types of β bends (Leach et al., 1977). The closest distances between pairs of protons are 3.0, 3.0, 2.2, and 2.2 Å for the Gly^3 C$^{\alpha}$H–Phe^4 NH interproton distance in types I, I′, II, and II′ bends, respectively. Since we could not observe *any* NOE, this observation supports the conclusion that the β bend in Me_2SO-d_6 is of type I.

On the basis of the various types of results presented here, it is suggested that [Leu^5]-enkephalin exhibits a preferred conformation in Me_2SO-d_6 solution—most likely involving a type I β bend at Gly^3-Phe^4. This conformation is stabilized by a hydrogen bond between Leu^5 NH and Gly^2 CO as well as by an interaction between the α-carboxyl and α-amino end groups. The side chains appear to be relatively immobilized in Me_2SO-d_6 solution. Such immobilization might enhance the binding to opiate receptors.

This proposed type I β bend conformation at Gly^3-Phe^4 is one of a family of conformations with relatively low values of the conformational energy, based on calculations carried out for the neutral enkephalin molecule in the absence of solvent (Isogai et al., 1977). The only conformations with significantly lower calculated energies are those in which the Tyr^1 OH is involved in a hydrogen bond. However, interactions with the solvent, not included in the calculations of Isogai et al. (1977), could easily weaken the phenolic hydrogen bond. Indeed, the data of Table II demonstrate the influence of changes in solvent and/or pH on the conformation in solution. Such possible effects of solvent are also indicated by calculations of Némethy et al. (1978) and Hodes et al. (1979), who considered the effects of hydration on the conformations of terminally blocked dipeptides and found that there are fewer internal hydrogen bonds in the low-energy conformations in the presence of water. The predominant conformations of the side chains may also be influenced by interactions with the solvent. For example, for the type I β bend conformation of enkephalin considered here (ϕ_1, ψ_1, ϕ_2, ψ_2, ϕ_3, ψ_3, ϕ_4, ψ_4, ϕ_5, ψ_5 = −81°, 137°, −103°, 162°, −64°, 45°, −71°, 40°, −159°, 138°, respectively, with all ω's = 180°), the computed minimum energies for the side chain of Phe^4, in the absence of solvent, are 6.11, 5.18, and 12.93 kcal/mol for (χ^1,χ^2) = (−62°, 110°), (178°, 75°), and (82°, 99°), respectively (G. Nemethy, private communication). This calculation indicates that the g$^+$t side-chain conformation should be favored slightly over the tg$^-$ conformation found here. However, Hodes et al. (1979) found that, for terminally blocked dipeptides, the differences in energy between various side-chain conformations are generally smaller when the effects of hydration are included in the calculations. Thus, the results obtained here appear to be in general (although not perfect) agreement with previous calculations (Isogai et al., 1977). Only when solvation effects (Hodes et al., 1979) are introduced explicitly into the calculations on enkephalin can direct comparisons be made between the calculated conformation and the one reported here.

Conclusion

These combined ^1H and ^{13}C NMR studies indicate that [Leu^5]-enkephalin exists as a well-defined compact conformation (rather than as an ensemble of conformations) in Me_2SO-d_6 solution. It is suggested that an intramolecular hydrogen bond between the Leu^5 NH and CO of Gly^2 occurs in a type I β bend centered at Gly^3-Phe^4. Furthermore, all three residues with side chains show considerable conformational rigidity in the tg$^-$ conformation. Although such a conformation is one of relatively high conformational energy (calculated without taking solvent effects into account), the introduction of solvent effects could lead to a relatively low energy for the type I β bend. The role of solvent is also indicated by the fact that a different type of β bend conformation (type I′) is observed in the crystal structure, where *inter*molecular interactions play a role in the crystal similar to that of the solvent in solution. The type I β bend conformation of enkephalin in Me_2SO-d_6 positions the critical tyramine moiety in an orientation analogous to that existing in the rigid framework of morphine, which may account for its ability to simulate the analgesic properties of opiates.

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