A Carbon-13 Nuclear Magnetic Resonance Study of the Molecular Dynamics of Methionine-enkephalin and α-Endorphin in Aqueous Solution†

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ABSTRACT: Nuclear magnetic resonance (NMR) of ^{13}C is used to probe dynamical properties of [2-[2-^{13}C]glycine]-, [3-[2-^{13}C]glycine]methionine-enkephalin, and α-endorphin. Spin–lattice relaxation times (T_1) are reported at 20 MHz for the enriched enkephalin samples as functions of pH, temperature, and concentration. Relaxation times are also reported for enkephalin and α-endorphin containing ^{13}C in natural abundance. The relaxation times are used to monitor the relative flexibility of the carbon atoms in the peptides. The results imply that in enkephalin the [3-glycine] residue is more restricted than the [2-glycine] residue at low peptide concentrations. However, this difference in flexibility decreases upon increasing peptide concentration. We found optimal correlation times for the overall molecular reorientation of the peptide backbone at 32 °C and 100 mg/mL to be $\tau_x = 3.2 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, and $\tau_z = 9.9 \times 10^{-11}$ s. These values are conformation independent. The rates of internal motion of the side chains are of the same order of magnitude and are also conformation independent. The activation energy for the overall rotational process is found to be 20–27 kJ/mol. Our data do not indicate discontinuous molecular aggregation in the concentration range of 2–100 mg/mL of peptide. The binding properties of enkephalin with egg lecithin and phosphatidylserine were investigated. The T_1 results for α-endorphin indicate that the peptide is a flexible, possibly random-coil structure. The enkephalin segment of α-endorphin differs little in motional properties from the rest of the peptide. This is consistent with the relative biological activities reported for α-endorphin and enkephalin.

Carbon-13 (^{13}C) spin–lattice relaxation times (T_1) have been used to elucidate the overall as well as the segmental motional characteristics of peptide hormones. Studies on glycine-containing hormones and hormone analogues such as luliberin (LH-RH) (Deslauriers et al., 1977a), oxytocin (Deslauriers et al., 1974; Walter et al., 1974), and [3-proline,4-glycine]-oxytocin (Deslauriers et al., 1977b) have shown that the presence of a glycyl residue increases the local flexibility of the peptide backbone at the glycyl residue; this flexibility may be of significance when the peptide interacts with its receptor ["induced-fit" mechanism (Anteunis et al., 1977; Lewis et al., 1971)].

A new class of biologically active peptides has been discovered recently. Hughes and co-workers (Hughes et al., 1975) have demonstrated the presence of two pentapeptides, methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu), in extracts of pig brain. These peptides act as opiate agonists (Bradbury et al., 1976) in their interactions with morphine receptors, and they probably act as neurotransmitters or neuromodulators in pain pathways to the brain (Kosterlitz and Hughes, 1976a,b). Methionine-enkephalin is thought to be the enzyme cleavage product of β-lipotropin, a 91-residue peptide synthesized by the pituitary (Li and Chung, 1976a). Larger peptides, now known under the generic name endorphins, are also cleavage residues, and their biological activity as opiate agonists has been tested in various laboratories (Li and Chung, 1976b; Lazarus et al., 1976). Among these peptides is α-endorphin, a 16-residue molecule identical to the subunit [61–76] of β-lipotropin. It has been shown to possess an opioid activity about 60% of that of methionine-enkephalin in various bioassays (Rónai et al., 1977). β-Endorphin (subunit [61–91] of β-lipotropin), on the other hand, is about eight times more potent than methionine-enkephalin and possesses much longer lasting analgesic properties (Loh et al., 1976). In the latter case, it has been suggested (Hollósi et al., 1977; Austen et al., 1977) that the peptide could be folded, with the tyrosine end responsible for the activity buried in the folded structure. Presumably the folding protects the peptide from the enzymes of the surrounding medium.

In order to understand the structure–activity relations in the enkephalins and endorphins, and to relate the structure of the enkephalins to those of opiates, a number of physicochemical studies have been undertaken. Nuclear magnetic resonance (NMR) of ^1H (Bleich et al., 1977; Jones et al., 1977; Roques et al., 1976; Garbay-Jaureguiberry et al., 1976) and ^{13}C (Combrisson et al., 1976) have been used to delineate the overall conformational properties of the enkephalins. In solution, the enkephalins appear to prefer a folded conformation, although there is some controversy about the exact nature of the folded structure (Jones et al., 1977).

Published ^{13}C NMR T_1 values on enkephalin suggest that the two glycine residues are more flexible than the phenylalanine residue. In order to elucidate this aspect further, we have synthesized [2-[2-^{13}C]glycine]methionine-enkephalin and [3-[2-^{13}C]glycine]methionine-enkephalin. Specific enrichment permits the measurement of accurate T_1 values for the [2-glycine] and [3-glycine] α carbons which show partial overlap in the 20 MHz ^{13}C spectra. Accurate T_1 values allow comparison with those obtained for the [4-phenylalanine] and [1-tyrosine] residues. Furthermore, the use of ^{13}C-enriched material permits studies at lower concentrations (over two orders of magnitude) and thus detection of effects due to molecular aggregation. The enriched samples are more suitable

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to monitor possible changes in the overall and/or internal dynamics of the peptide upon interaction with membrane-like model systems of the opiate receptor.

Herein we report the ^{13}C T_1 values of the ^{13}C-enriched glycine residues of enkephalin in aqueous solution as functions of both temperature and concentration. We have correlated our observed T_1 data for the ^{13}C-enriched glycine residues with the macroscopic viscosities of the solutions. In addition, we have compared our data on the ^{13}C-enriched material with those obtained for the unenriched peptide at 100 mg/mL, in solutions of various pH. We have calculated optimized parameters both for rates of overall molecular tumbling and for internal motion of each of the residues in enkephalin. We have also measured the T_1 values of all the residues in α-endorphin for comparison with those of enkephalin, in the hope of identifying possible dynamical origins of the activity of the former. We report preliminary attempts to monitor the interaction of the ^{13}C-enriched enkephalins with lecithin and phosphatidylserine.

Materials and Methods

Synthesis of [2-[2-^{13}C]Glycine]methionine-enkephalin. Glycine ^{13}C enriched ([2-^{13}C], 90%) was obtained from Merck, Sharp and Dohme (Canada). The methyl ester hydrochloride of [2-^{13}C]glycine was prepared by standard methods using thionyl chloride (Brenner and Huber, 1953). *t*-Boc[2-^{13}C]Gly was prepared from [2-^{13}C]glycine and di-*tert*-butyl dicarbonate (Moroder et al., 1976).

[2-[2-^{13}C]Glycine]methionine-enkephalin was prepared by standard solution methodology. *t*-Boc-Tyr-[2-^{13}C]Gly-hydrazide was prepared from *t*-Boc-Tyr and [2-^{13}C]Gly methyl ester using dicyclohexylcarbodiimide (DCC) followed by hydrazinolysis of the ester.

t-Boc-Gly-Phe-Met methyl ester was prepared from *t*-Boc-Gly and Phe-Met methyl ester using DCC. The protected pentapeptide was obtained by condensing *t*-Boc-Tyr-[2-^{13}C]Gly azide with Gly-Phe-Met methyl ester (Honzl and Rudinger, 1961). The free peptide was obtained by saponification followed by deprotection with F_3AcOH. The peptide was purified by partition chromatography on Sephadex G-25 using the system *n*-BuOH, HOAc, H_2O (4:1:5).

Synthesis of [3-[2-^{13}C]Glycine]methionine-enkephalin. This peptide was synthesized as previously described for the [2-[2-^{13}C]glycine]peptide, except that *t*-Boc-Tyr-Gly azide was coupled with [2-^{13}C]Gly-Phe-Met methyl ester that was obtained from F_3AcOH deprotection of Boc-[2-^{13}C]Gly-Phe-Met methyl ester. Deprotection and purification were similar to the previous case. All peptides had correct elemental and amino acid analyses.

Lipids. Phosphatidylserine from Serdary Research Laboratories, London, Canada, was stored in chloroform at −15 °C. Egg lecithin from Lipid Products Co., South Nutfield, England, was stored in chloroform–methanol. Solvents were evaporated by gently bubbling dry nitrogen through the mixture immediately prior to use. Lipid dispersions were prepared by sonicating the lipid–water–enkephalin mixture in a bath type sonicator to opalescence for 0.5–1.0 h, depending on the concentration. Solutions were maintained under N_2 to avoid oxidation of the lipids.

NMR Measurements. Samples were dissolved in D_2O; pH values are meter readings uncorrected for the deuterium isotope effect (Glascoe and Long, 1960). Solution pH was adjusted using NaOH or HCl diluted in D_2O.

^{13}C NMR spectra were obtained using Varian CFT-20 and XL-100 and Bruker HX-270 NMR spectrometers operating at 20, 25, and 68 MHz, respectively. All spectra were recorded with broad-band proton-noise decoupling. Chemical shifts are reported in parts per million downfield from external tetramethylsilane and are accurate to ±0.05 ppm. Sample temperatures were measured before and after T_1 determination by replacing the sample with a tube containing water and a thermocouple junction and are estimated to be accurate to ±1°. T_1 data were obtained using the inversion–recovery sequence (Freeman and Hill, 1971; Vold et al., 1968) or the fast inversion–recovery technique (FIRFT) (Canet et al., 1975). T_1 values were calculated from semilogarithmic plots. The 90° pulse widths were 25 μs at 68 and 20 MHz, and 14 μs at 25 MHz. From 1000 to 2000 scans/spectrum were accumulated at 68 MHz; the number of scans/spectrum for the ^{13}C-enriched material varied with sample concentration. At 68 and 20 MHz a minimum of 15 values of the time τ between the 180° and 90° pulses was used to determine T_1, including at least three values corresponding to a fully relaxed spectrum. At 25 MHz only ten values could be used, including two for the fully relaxed spectrum. Measurements performed on the ^{13}C-enriched material showed a reproducibility in T_1 of 10% or better.

Viscosity Measurements. Viscosity measurements were carried out on 0.6-mL samples of pure H_2O and D_2O as well as on the samples used in the T_1 studies, using an Ostwald (flow) microviscometer (Tanford, 1967).

Computational Techniques. The conformations of enkephalin chosen for least-squares fitting of observed T_1 values to optimal rotational diffusion constants had either a fully extended backbone or a compact conformation containing a β bend (Venkatachalam, 1968), with the [3-glycine] and [4-phenylalanine] residues in the corners of the turn. The latter conformation was generated by use of the stereoalphabet strategy (Ralston and de Coen, 1974), and the coordinates were then computed for this structure. The moments of inertia for each of the conformations were calculated from the Cartesian coordinates of all the atoms using the computer program XYZ (QCPE 178, modified by W. M. Murphy). Graphical representations of the conformations were drawn from the coordinates relative to the principal axes of the moment of inertia tensor using the ORTEP thermal-ellipsoid plot program for crystal structure illustrations (Johnson, 1965). Optimal diffusion constants for anisotropic rigid-body reorientation of enkephalin in the two conformations were computed by least-squares fitting to the observed T_1 values, as described by Somorjai and Deslauriers (1976). Rates of internal motion about individual C–C bonds in the side chains and terminal residues were estimated using the computer program described in Deslauriers and Somorjai (1976).

Results

Rates of Overall Molecular Reorientation and Internal Motion in Enkephalin. Figure 1a shows the structure of methionine enkephalin along with the ^{13}C T_1 values obtained for a 100 mg/mL sample containing ^{13}C in natural abundance. In order to estimate the rates of both overall tumbling of enkephalin, and its internal rotational motions, we have fitted the observed T_1 data to both folded and extended conformations. This procedure helps determine the sensitivity of the calculated rotational diffusion constants (and correlation times) to the overall molecular shape. The folded conformation which was chosen contains a β turn (Venkatachalam, 1968), the corners of which are formed by the [3-glycine] and [4-phenylalanine] residues of enkephalin. The conformation compatible with the ^1H NMR data (Jones et al., 1976) is shown in Figure 1a. A folded conformation has also been found by X-ray diffraction for the peptide in the solid state (Smith and Griffin, 1977).

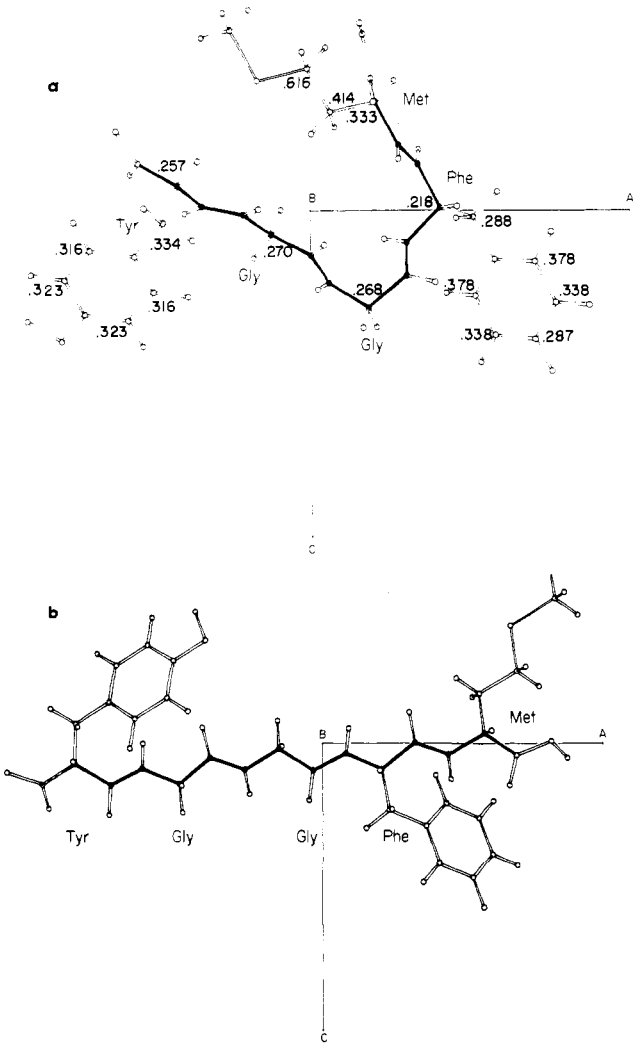

FIGURE 1: ORTEP illustration of methionine-enkephalin in folded (a) and fully extended (b) conformations. In both cases the molecule is viewed along the *B* axis. The *A*, *B*, and *C* axes are the principal axes of the moment of inertia tensor. The numbers in (a) represent the NT_1 values (in seconds) observed at 20 MHz and 32 °C for a solution of 98.27 mg/mL D_2O of the pentapeptide at natural abundance, pH 3.5.

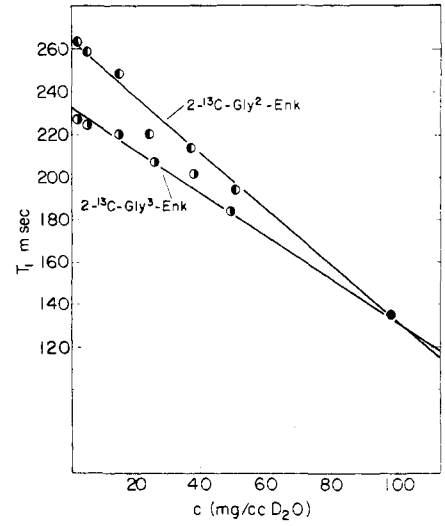

FIGURE 2: T_1 values for [2-[2-^{13}C]glycine]enkephalin and [3-[2-^{13}C]-glycine]enkephalin as a function of concentration at 32 °C. The full circle at c = 100 mg/mL represents the T_1 value for both the [2-glycine] and [3-glycine] residues obtained from a sample of methionine-enkephalin containing ^{13}C in natural abundance.

Figure 1b shows the fully extended conformation of enkephalin. This provides the maximum anisotropy for the inertia tensor and potentially the greatest possible anisotropic overall molecular motion for the monomer. Table I shows the optimal values of the rotational correlation times (τ) obtained for rotation about the principal axes (Figure 1) of the moment of inertia tensor. Table I, column 3, contains the optimal parameters obtained when using all the atoms of enkephalin in a folded conformation. These parameters are $\tau_x = 1.9 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, and $\tau_z = 1.1 \times 10^{-10}$ s rad^{-1}. Column 4 shows the optimal rotational diffusion constants from a least-squares fit to the fully extended conformation to be $\tau_x = 1.8 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, and $\tau_z = 1.0 \times 10^{-10}$ s rad^{-1}. Eliminating the CH_3 groups, which are known to rotate rapidly, does not affect these parameters. Column 5 contains the results of fitting the T_1 values for the backbone carbons only of folded enkephalin, yielding $\tau_x = 3.2 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, and $\tau_z = 9.9 \times 10^{-11}$ s rad^{-1}. Column 6, in which the backbone carbon T_1 values were used with a fully extended backbone conformation, gives optimal values of $\tau_x = 1.1 \times 10^{-10}$, $\tau_y = 3.3 \times 10^{-10}$, and $\tau_z = 2.2 \times 10^{-10}$ s rad^{-1}. The results of these calculations are independent of physically reasonable starting values chosen for τ_x, τ_y, and τ_z. Table I also shows the observed T_1 values for enkephalin (column 2) as well as the values calculated for the proton-bearing carbons using the different sets of optimal values τ_x, τ_y, and τ_z. Table II shows the calculated optimal rates of rotation about the individual C–C bonds in the side chains (τ_{int}) which will reproduce the observed T_1 values, given the previously computed optimal rates of overall tumbling. The latter were those obtained from fitting of observed T_1 values to the backbone carbons only. Because the computer program used to calculate the rates of internal rotation assumes that the overall motion of the molecule has at least cylindrical symmetry (ellipsoid of revolution), it was necessary to average two of the correlation times (and corresponding diffusion constants) obtained from Table I; these average values are given in Table II.

NT_1 Values of Glycyl Residues—Comparison with Other Residues. The T_1 data on enkephalin at 100 mg/mL (Table I) indicate that the NT_1 values (where N is the number of hydrogens directly bonded to the carbon) of the glycine residues are larger than those of the phenylalanine residue (Combrisson et al., 1976, and the present work). The specifically enriched enkephalins allow us to investigate the properties of the two glycyl residues separately, at low concentrations, and to compare the data with those for the other residues in the peptide. The purpose of measuring T_1's as functions of both sample concentration and temperature was to separate contributions from overall molecular reorientation and segmental motion within the peptide backbone. Figure 2 shows the effect of varying sample concentration on the T_1 values observed for methionine-enkephalin in D_2O at 32 °C. Enkephalin is thought to aggregate at high concentrations (Khaled et al., 1977); therefore, we sought a discontinuity in the concentration dependence of T_1. There is no break in the curve between concentrations of 2 and 100 mg/mL for either of the enriched enkephalins. The data for these compounds extrapolate to the values observed for methionine enkephalin at 100 mg/mL. In order to determine whether pH-induced conformational changes in enkephalin could be detected by T_1 measurements, we measured the T_1 values of the enriched samples between pH 1.5 and 10.0, at 32 °C for a peptide concentration of 5 mg/mL. The data are shown in Table III. The T_1 values for each sample varied less than 5% over the pH range investigated.

TABLE I: Least-Squares Fit of the T_1 Values for Enkephalin in the Extended (E) and Folded (F) Conformations.

Atom	Exptl T_1 (s)[a]	Calcd T_1^F (s), all atoms $\tau_x = 1.9 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, $\tau_z = 1.1 \times 10^{-10}$ (s rad^{-1})	Calcd T_1^E (s), all atoms $\tau_x = 1.8 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, $\tau_z = 1.0 \times 10^{-10}$ (s rad^{-1})	Calcd T_1^F, backbone $\tau_x = 3.2 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, $\tau_z = 0.99 \times 10^{-10}$ (s rad^{-1})	Calcd T_1^E, backbone $\tau_x = 1.1 \times 10^{-10}$, $\tau_y = 3.3 \times 10^{-10}$, $\tau_z = 2.2 \times 10^{-10}$ (s rad^{-1})
Tyr $C_\alpha H$	0 257	0.311	0.270	0.284	0.254
Tyr $C_\beta H_2$	0.167	0.156	0.152	0.146	0.116
Tyr $C_\delta H$	0.316	0.313	0.321	0.282	0.228
Tyr $C_\epsilon H$	0.323	0.345	0.334	0.360	0.288
Tyr $C_\eta H$	0.316	0.345	0.334	0.360	0.288
Tyr $C_\theta H$	0.323	0.314	0.321	0.282	0.228
Gly $C_\alpha H_2$	0.135	0.154	0.148	0.137	0.134
Gly $C_\alpha H_2$	0.134	0.147	0.148	0.127	0.135
Phe $C_\alpha H$	0.218	0.268	0.271	0.219	0.254
Phe $C_\beta H_2$	0.144	0.148	0.152	0.129	0.121
Phe $C_\delta H$	0.378	0.309	0.324	0.271	0.236
Phe $C_\epsilon H$	0.338	0.301	0.334	0.280	0.283
Phe $C_\xi H$	0.378	0.301	0.334	0.280	0.283
Phe $C_\eta H$	0.338	0.309	0.324	0.271	0.236
Phe $C_\theta H$	0.287	0.323	0.352	0.290	0.197
Met $C_\alpha H$	0.333	0.323	0.270	0.303	0.254
Met $C_\beta H_2$	0.207	0.149	0.152	0.131	0.116
Met $C_\gamma H_2$	0.308	0.149	0.152	0.131	0.116
Met S-CH_3	1.41	0.101	0.104	0.092	0.083

[a] Obtained at a concentration of 98.3 mg/mL D_2O, T = 32 °C.

TABLE II: Least-Squares Fit of the T_1 Values with Inclusion of Internal Rotation.

Carbon	Exptl T_1 (s)	Extended: Calcd T_1 (s)[a]	Extended: τ_{int} (s rad^{-1})	Folded: Calcd T_1 (s)[b]	Folded: τ_{int} (s rad^{-1})
Tyr $C_\alpha H$	0.257	0.254		0.284	
Tyr $C_\beta H_2$	0.167	0.169	1.8×10^{-10}	0.168	3.1×10^{-10}
Gly $C_\alpha H_2$	0.135	0.134		0.137	
Gly $C_\alpha H_2$	0.134	0.135		0.127	
Phe $C_\alpha H$	0.218	0.254		0.219	
Phe $C_\beta H_2$	0.144	0.143	3.5×10^{-10}	0.147	8.5×10^{-10}
Phe $C_\theta H$	0.287	0.290	5.0×10^{-11}	0.289	5.5×10^{-11}
Met $C_\alpha H$	0.333	0.254		0.303	
Met $C_\beta H_2$	0.207	0.206	1.0×10^{-10}	0.208	1.25×10^{-10}
Met $C_\gamma H_2$	0.308	0.309	0.75×10^{-11}	0.307	0.77×10^{-11}

[a] Calculated using $\tau_z = 2.2 \times 10^{-10}$, $\tau_x = \tau_y = 2.2 \times 10^{-10}$ s rad^{-1}. [b] Calculated using $\tau_z = 9.9 \times 10^{-11}$, $\tau_x = \tau_y = 2.4 \times 10^{-10}$ s rad^{-1}.

TABLE III: The pH Dependence of T_1 for $^{13}C_\alpha$ in [2-Glycine]- and [3-Glycine]enkephalin.

Peptide[a]	pH	T_1 (s)
[2-[2-^{13}C]Gly]enk	1.5	0.226
	3.5	0.225
	10.0	0.235
[3-[2-^{13}C]Gly]enk	1.5	0.256
	3.5	0.250
	10.0	0.261

[a] For both peptides, the concentration was 4.95 mg/mL D_2O. The temperature was 305 K.

Effects of Temperature and Viscosity. In order to evaluate the contribution to T_1 of overall molecular reorientation (τ_{mol}) and internal reorientation (τ_{int}), we sought to perturb them differentially. By varying the temperature of the sample it was expected that the overall reorientation, as monitored principally at the [3-glycine] residue, would be more perturbed than the excess internal rotation, as monitored at the [2-glycine] residue. Furthermore, it is of interest to determine whether any variations in observed T_1 values and corresponding effective correlation times (τ_{eff}) could be simply related to changes in macroscopic sample solution viscosity.

Figure 3a,b shows the variation of $1/T_1$ vs. temperature for [2-[2-^{13}C]glycine]methionine-enkephalin for various sample concentrations. As expected, a general increase in T_1 values is observed as the temperature is increased. The viscosities of all samples were determined as functions of temperature and sample concentration. In all cases, a linear relation is observed between τ_{eff} and the viscosity (η) of the solution, as expected if the Stokes–Einstein relation (Tanford, 1967) is valid:

$$\tau = \frac{V_m \eta}{kT}$$

where τ is the correlation time for molecular motion, V_m the molecular volume, η the sample viscosity, k the Boltzmann constant, and T the absolute temperature. A linear relation between $1/T_1$ and η/T is also observed. Figure 4a,b shows plots of τ_{eff} vs. η for sample concentrations of 2 and 38 mg/mL. In all cases studied, the [3-glycine] residue was more influenced by η than was the [2-glycine] residue.

In view of the linearity of the τ_{eff} vs. η curves, it was of interest to compare the observed τ_{eff} values with those *calculated* for a particle the size of enkephalin in a solution of known viscosity. In order to calculate the value of τ_{eff} from the Stokes–Einstein relation, it is necessary to know the volume of the particle. A minimum molecular volume can be determined by the "Method of Atomic Increments" (Edward, 1970), which involves summing the van der Waals volumes of all the atoms in the molecule. The calculated minimum molecular volume for enkephalin is found to be ca. 540 Å^3. Table IV shows the τ values calculated for overall motion of a particle the size of enkephalin for the different solution viscosities studied. These data can be compared with the experimental results on the [2-glycine] and [3-glycine] residues. The calculated values generally lie between those observed for the

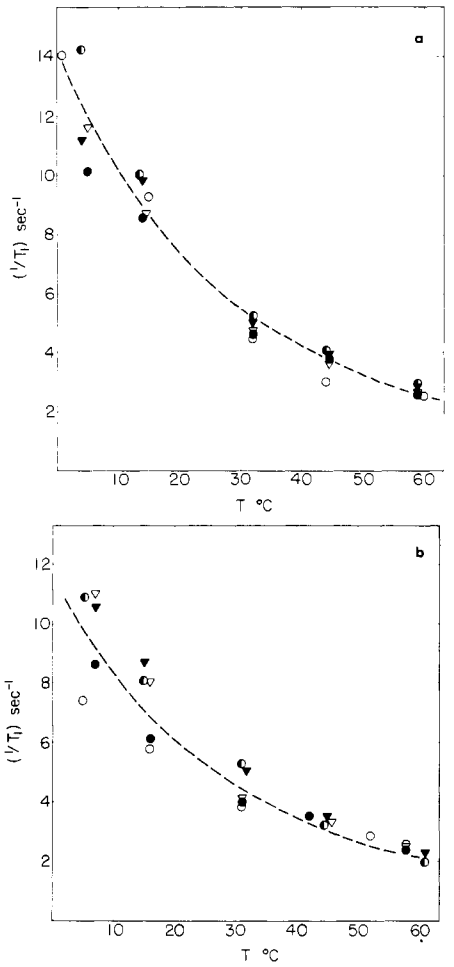

FIGURE 3: (a) $1/T_1$ values for the [2-glycine] residue of methionine-enkephalin as a function of temperature for concentrations of enkephalin of (○) 1.95, (●) 4.30, (▽) 14.79, (▼) 37.88, and (◐) 50.53 mg/mL D_2O. (b) $1/T_1$ values for the [3-glycine] residue of methionine-enkephalin, as a function of temperature for concentrations of enkephalin of (○) 1.95, (●) 4.99, (▽) 11.63, (▼) 37.76, and (◐) 44.63 mg/mL D_2O.

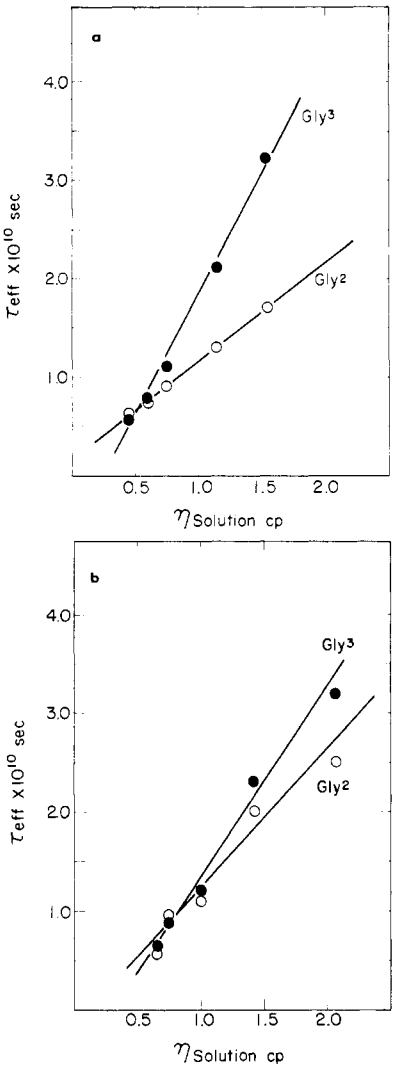

FIGURE 4: τ_{eff} values for the [2-glycine] and [3-glycine] residue of methionine-enkephalin as a function of the viscosity (η) of the solution. (a) The concentration is 1.95 mg/mL D_2O for [2-glycine]- and [3-glycine]-enkephalin. (b) The concentration is 37.88 and 37.76 mg/mL D_2O for [2-glycine]- and [3-glycine]enkephalin, respectively.

[2-glycine] and [3-glycine] residues, the fit being much better than the expected order of magnitude agreement for τ, given the assumptions required for the calculations. Using the experimental values for τ_{eff}, T, and η, the molecular volume can also be calculated from the slope of $\tau_{eff}T$ vs. η. In this latter case, a molecular volume of 586 Å^3 is found, a value very similar to that obtained from the atomic increments method.

Calculation of τ_{int} for [2-Glycine]. Knowing τ_{eff} for the [3-glycine] and [2-glycine] residues, it is possible to estimate τ_{int} for the [2-glycine] residue, if τ_{eff} for the [3-glycine] residue is assumed to be most representative of τ_{mol}. This is not necessarily the case if the residue in position 3 has the tendency for additional internal flexibility, a characteristic of glycine residues. Thus, the [4-phenylalanine] residue may be more representative of the overall molecular reorientation. However, we have not yet been able to obtain ^{13}C-enriched phenylalanine-containing material. The results of these calculations, using the molecular coordinates of both the folded and extended conformations of enkephalin, are given in Table V. It is clear from Table V that τ_{int} increases with increasing concentration of enkephalin.

Activation Energies for Molecular Reorientation. Activation energies for rotational processes can be determined from T_1 or τ values using the Arrhenius expression:

$$X = X^0 \exp(E_a/RT)$$

where X is $1/T_1$ or τ, E_a the activation energy, T the absolute temperature, and R the gas constant. Figure 5 shows a typical plot of log $(1/T_1)$ vs. $1/T$ for the [3-glycine] residue of a sample of enkephalin at a concentration of ca. 45 mg/mL. Table VI lists the values of E_a determined from similar plots for the [2-glycine] and [3-glycine] residues of enkephalin at various concentrations. With the exception of the [2-glycine] residue at a concentration of 1.95 mg/mL, all the E_a values range between 20 and 26 kJ/mol, with an estimated error of approximately 20–25%, assuming an experimental accuracy of about 10% for T_1. The differences between the values in Table VI are therefore not significant.

Enkephalin–Lipid Mixtures. Morphine is known to bind stereospecifically with lipids such as cerebroside sulfates and phosphatidylserine (Abood and Hoss, 1975a,b; Loh et al., 1974; Abood et al., 1977). These lipids are known to be part of the opiate pharmacophore, and the enkephalins, being competitors for the opiate receptors in vivo, might also bind to them. To determine if such an interaction could be monitored by changes in either chemical shifts (δ), line widths ($\Delta\nu$), or spin–lattice relaxation times (T_1), samples were prepared

TABLE IV: Effective (Experimental) and Calculated Correlation Times for C_α of Glycyl Residues in ^{13}C-Enriched Enkephalins.

c (mg/mL)[a]	Temp (°C)	η[b] (cP)	[2-[2-^{13}C]-Gly]enk τ_{eff} ($\times 10^{+10}$)[c]	[3-[2-^{13}C]-Gly]enk τ_{eff} ($\times 10^{+10}$)[c]	Calcd τ ($\times 10^{+10}$)[d]
2	5	1.81	1.7	3.2	2.5
	15	1.29	1.3	2.1	1.8
	34	0.88	0.89	1.1	1.1
	45	0.57	0.73	0.78	0.79
	60	0.52	0.59	0.55	0.59
5	5	1.75	2.1	2.5	2.6
	15	1.32	1.4	1.8	1.8
	34	0.90	0.89	1.1	1.1
	45	0.69	0.72	0.79	0.85
	60	0.52	0.58	0.59	0.60
15	5	1.74	2.5	2.7	2.7
	16	1.29	1.9	2.1	1.7
	34	0.86	0.96	1.1	1.3
	46	0.68	0.75	0.81	1.1
	54	0.59	0.57	0.55	0.74
42	5	1.89	2.5	3.2	2.92
	16	1.49	2.0	2.3	1.94
	34	0.95	1.1	1.2	1.29
	46	0.74	0.98	0.87	0.92
	54	0.55	0.57	0.65	0.66
50	5	2.45	2.6	3.8	3.5
	16	1.76	1.9	2.4	2.2
	34	1.07	1.1	1.3	1.6
	46	0.80	0.71	1.1	1.3
	58	0.62	0.46	0.60	0.76

[a] The concentrations given in this table are approximate, being different for the [2-[2-^{13}C]glycine] and [3-[2-^{13}C]glycine]enkephalin. The actual concentrations are for [2-glycine]enkephalin 1.95, 4.30, 14.79, 37.88 and 50.53 mg/mL D_2O, and for [3-glycine]enkephalin 1.95, 4.99, 14.81, 37.76, and 44.63 mg/mL D_2O. [b] The viscosity (in cP) was measured in an Ostwald microviscometer; 0.6 mL of the sample was used. [c] The effective, or experimental, correlation time in s rad^{-1} calculated from the experimental T_1 values of Table VI. [d] Calculated from the Stokes-Einstein relation, in s rad^{-1}.

TABLE V: τ_{int} for the [2-Glycine] Residue of Enkephalin.

c_{enk} (mg/mL D_2O)	Exptl T_1, Gly[2] (s)	Extended[a] Fitted T_1 (s)	Extended[a] Fitted τ (s rad^{-1})	Folded[b] Fitted T_1 (s)	Folded[b] Fitted τ (s rad^{-1})
1.95	0.263	0.260	4.5×10^{-11}	0.262	6.8×10^{-11}
4.30	0.258	0.256	4.7×10^{-11}	0.258	7.0×10^{-11}
14.79	0.248	0.249	5.0×10^{-11}	0.249	7.8×10^{-11}

[a] Calculated using an overall molecular reorientation characterized by $\tau_z = 2.2 \times 10^{-10}$ s rad^{-1} and $\tau_x = \tau_y = 2.2 \times 10^{-10}$ s rad^{-1}, which is an average of the values for these parameters given in Table I. [b] Calculated using $\tau_z = 9.9 \times 10^{-11}$ s rad^{-1} and $\tau_x = \tau_y = 2.4 \times 10^{-10}$ s rad^{-1}.

by sonicating an aqueous lipid-peptide mixture until the solution cleared (this usually required 0.5–1.0 h). The results are shown in Table VII. No effect on any of the parameters measured (δ, $\Delta\nu$, T_1) was detected. This could result from a slow exchange between bound (undetectable) and free species or from an interaction between the lipids and only a very small fraction of the enkephalin sample. We measured the macroscopic viscosity of the lipid-containing solutions and found a good correlation between this and the observed T_1 values of the dissolved enkephalins.[1]

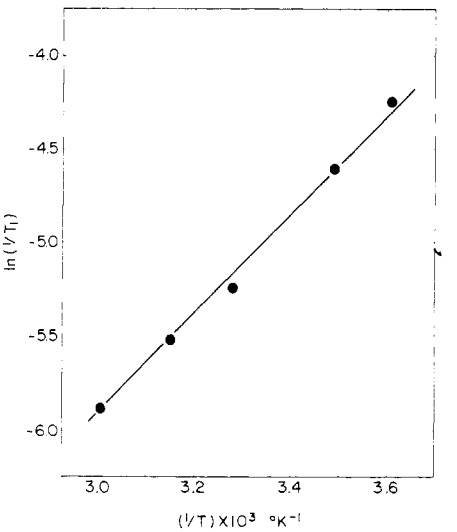

FIGURE 5: ln $(1/T_1)$ as a function of $(1/T)$, a typical Arrhenius plot. The concentration of [3-glycine]enkephalin was 44.63 mg/mL D_2O, pH 3.5.

TABLE VI: Activation Energies for Rotational Motion of Enkephalin.

c[a] (mg/mL D_2O)	T (K) [2-[2-^{13}C] Gly]	T (K) [3-[2-^{13}C] Gly]	Exptl T_1 (s) [2-[2-^{13}C] Gly]	Exptl T_1 (s) [3-[2-^{13}C] Gly]	E_a (kJ)[b] [2-[2-^{13}C] Gly]	E_a (kJ)[b] [3-[2-^{13}C] Gly]
2	278	274	0.135	0.071	14.4	25.4
	289	288	0.174	0.108		
	305	305	0.263	0.227		
	325	317	0.352	0.334		
	332	333	0.386	0.402		
5	280	278	0.116	0.095	19.7	21.0
	289	287	0.163	0.118		
	305	305	0.258	0.225		
	315	318	0.284	0.262		
	332	332	0.408	0.390		
15	280	278	0.091	0.086	21.5	21.3
	289	288	0.125	0.117		
	305	305	0.248	0.220		
	317	318	0.306	0.266		
	332	332	0.412	0.390		
42	280	277	0.095	0.089	22.4	20.7
	288	287	0.115	0.102		
	306	305	0.202	0.202		
	318	318	0.291	0.258		
	335	332	0.427	0.358		
50	279	277	0.092	0.070	25.7	21.9
	288	387	0.123	0.100		
	305	305	0.187	0.190		
	318	318	0.311	0.249		
	335	332	0.517	0.355		

[a] See footnote *a* of Table IV. [b] Calculated from the slope of an Arrhenius plot. The estimated error in E_a is ±20–25% assuming the T_1 values show a reproducibility of ±10%.

T_1 Values of α-Endorphin. α-Endorphin has been shown to have 60% of the activity of the enkephalins themselves.

[1] Note Added in Proof: ^{13}C NMR studies of mixtures of both [2-[2-^{13}C]glycine]- and [3-[2-^{13}C]glycine]methionine-enkephalin with phosphatidylserine have shown a great sensitivity of T_1 to the pH of the mixture. Maximum interaction between enkephalin and phosphatidylserine occurs under acidic conditions. A full report is in progress.

TABLE VII: Experimental Data on the Lipid–Enkephalin Binary System.

Lipid[b]	c_{lipid}/c_{enk}[a]	T_1 (s)	pH	δ (ppm)	Line width (Hz)
EL	10	0.229	3.5	n.o.[d]	n.o.
	20	0.236	3.5	n.o.	n.o.
PS[c]	4	0.198	6.8	43.47	6.5
	4	0.237	11.8	43.47	4.9
	4	0.258	11.8	43.45	5.8
	11	0.215	7.4	43.46	6.3
	11	0.242	12.0	43.46	5.5
	11	0.248	12.0	43.43	6.1
	85	0.220	6.8	43.42	5.9
	85	0.238	11.9	43.44	5.6

[a] For the egg lecithin–enkephalin mixtures, the concentration of the enkephalin was held constant at 2.83 mg/mL D_2O. For the phosphatidylserine–enkephalin mixtures, the enkephalin concentration was held at 3.00 mg/mL D_2O for $(c_{lipid}/c_{enk}) = 4$ and 11 and was equal to 0.75 mg/mL D_2O for the last ratio given. In all cases, the enkephalin used was [2-[2-^{13}C]Gly]enkephalin and the temperature was 32 °C. In the absence of lipid, $T_1 = 0.25$ s for the concentration indicated. [b] EL, egg lecithin; PS, phosphatidylserine. [c] See footnote 1. [d] n.o., not observed.

Considering the molecular weight, the ^{13}C NMR spectrum of α-endorphin has unusually narrow resonances at 25 MHz; at 68 MHz most carbon resonances can be distinguished. The ^{13}C chemical shifts of most of the resonances can be assigned by comparing the observed chemical shifts with those calculated for a random-coil polypeptide. A number of these assignments were confirmed by obtaining ^{13}C chemical shifts under acidic, neutral, and basic conditions at 68 MHz. The effects of titration of individual residues are confined to the residues being titrated, thus allowing definitive assignments (Deslauriers and Smith, 1976).

Lowering the pH of the solution from 5.0 to 2.0 results in upfield shifts of the α and β carbons of the C-terminal threonine residue. Furthermore, the glutamic acid α-, β- and γ-carbon resonances are perturbed due to titration of the side chain. Raising the pH from 5.0 to 8.0 resulted in downfield shifts of the tyrosine α- and β-carbon resonances. Assignments of the individual glycine residues were made by comparison with the ^{13}C-enriched enkephalins, the lower-field glycyl α-carbon resonance being assigned to glycine-3.

To compare the reorientational motion in α-endorphin and the enkephalins, T_1 measurements (25 MHz) were performed on ^{13}C at natural abundance for 100 mg of α-endorphin/1.1 mL of D_2O at 32 °C. However, with peptides the size of α-endorphin, T_1 values may not be accurate monitors of molecular flexibility if relaxation occurs outside the "extreme narrowing" range ($\omega_0^2\tau^2 \geq 1$) (Lyerla and Levy, 1974). To test this possibility, we determined the nuclear Overhauser enhancements (NOE) (Noggle and Schirmer, 1971) for the carbons in α-endorphin using the gated-gyrocode technique (Feeney et al., 1970; Freeman and Hill, 1971b) in order to obtain proton noise-decoupled spectra with and without Overhauser enhancement. Full NOE values were obtained for all resonances, indicating that the relaxation occurs within the motional narrowing limit and allowing a direct comparison of the T_1 values of α-endorphin and the enkephalins.

At 25 MHz the average value of T_1 for the α carbons of the optically active residues in the peptide backbone was 0.14 s. No gradation in T_1 values was observed between the α carbons of the central portion of the peptide and the terminal residues. The T_1 values for the enkephalin sequence in α-endorphin are similar to T_1 values for the remainder of the endorphin sequence. The ortho and meta carbons of the aromatic rings of the tyrosine and phenylalanine residues all have comparable T_1 values, averaging 0.25 s. Thus, the T_1 values of the backbone carbons in α-endorphin decreased by ca. 40% with respect to those of enkephalin itself, whereas those of the aromatic residues decreased only ca. 25%; the latter is not unexpected if the aromatic rings have significant internal rotation. The internal rotation appears to be relatively independent of the overall reorientation in a random-coil peptide. The glycyl resonances of α-endorphin, which overlap at 25 MHz, show NT_1 values (0.24 s) significantly longer than those of the optically active residues.

In order to obtain more accurate T_1 values for α-endorphin, studies were undertaken at 68 MHz where no overlap occurs for the resonances of the glycine residues or of the central and terminal threonine residues. Due to the limited size of our experimental sample (100 mg) and the larger sample volume required at 68 MHz, the data were obtained at a concentration of 100 mg/1.7 mL, thus not allowing direct comparison between T_1 data obtained at 25 and 68 MHz. Figure 6 shows the NT_1 values observed at 68 MHz, 27 °C, and pH 5.0 for α-endorphin. The average T_1 value for the backbone carbons, excluding the glycine residue, is 0.22 s. The [3-glycine] and [6-threonine] residues have longer NT_1 values than the surrounding residues. As at 25 MHz, the aromatic rings of the tyrosine and phenylalanine residues have similar T_1 values. Segmental motion is apparent in the methionine and lysine residue side chains, as judged by the gradation in NT_1 values. The glutamine side chain is less restricted than the side chain of the glutamic acid residue, possibly due to interaction of the latter with the solvent. The CH_3 groups generally have NT_1 values which are five times longer than those of the carbons to which they are attached, indicating great rotational freedom. Internal motion occurs within the pyrrolidine ring of proline, as seen for this residue in other peptides (Deslauriers and Smith, 1977), particularly at the β and γ carbons. The δ-carbon, however, is more restricted than the former two. The hydroxyl-bearing threonine and serine side chains are hindered, possibly due to interaction of the hydroxyl groups with the solvent. Increasing the temperature to 35 °C leads to an increase in the average T_1 values of the α carbons in the peptide backbone (0.26 s, excluding the glycine residues). The C terminus of the peptide shows a larger increase in T_1 values than does the N terminus. The general behavior of the side chains is similar to that observed at 27 °C, although the threonine and serine residues show more side-chain flexibility at the higher temperature.

Discussion

The studies presented here were undertaken with the purpose of understanding the dynamical properties of enkephalin and α-endorphin and relating these to their biological potencies with the ultimate goal of studying interactions with membranelike systems.

Our studies of the T_1 values of the ^{13}C-enriched enkephalins have shown that the [3-glycine] residue is motionally more restricted than the [2-glycine] residue. Furthermore, as the concentration of the solution increases, the mobility of the [2-glycine] residue decreases relative to that of the [3-glycine] residue. This decrease in mobility could be due either to the formation of a specific backbone conformation or to molecular aggregation. Enkephalin has been reported to aggregate at high concentration (Khaled et al., 1977). However, we find no discontinuity in T_1 as a function of peptide concentration in D_2O, implying that *if* aggregation occurs it does so gradually

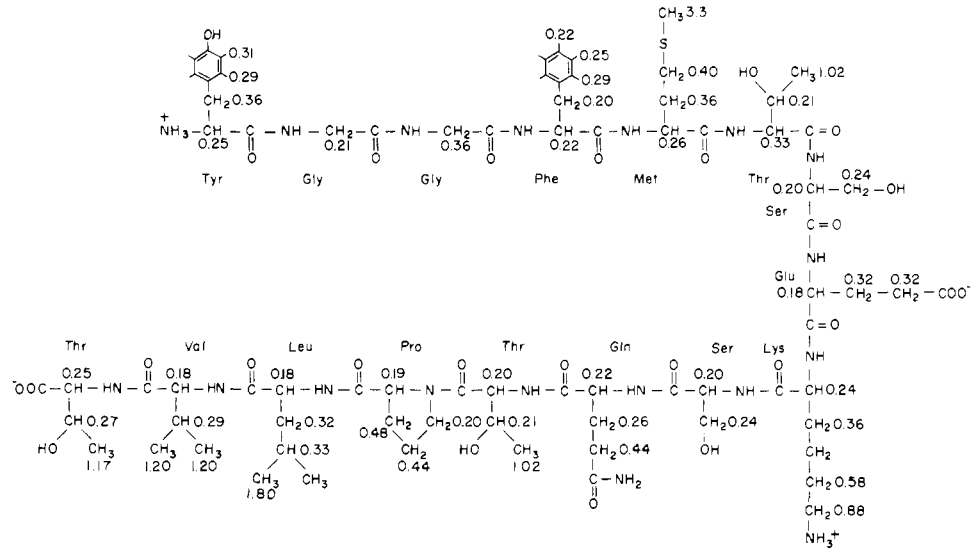

FIGURE 6: The amino acid sequence of α-endorphin. The numbers represent the NT_1 values observed at 68 MHz and 27 °C for a solution of 100 mg/1.7 mL D_2O, pH 5.0.

over the concentration range of 2–100 mg/mL and that no clear distinction can be made between monomer and aggregate under these conditions. In addition, excellent agreement has been obtained between the observed correlation times for overall molecular reorientation and those calculated from the Stokes–Einstein relation, under all conditions, leading us to believe that molecular aggregation is not significant in determining the ^{13}C T_1 values in enkephalin.

The approximate nature of the Stokes–Einstein relation cannot be overemphasized; however, due to the direct relation between V_m and τ in the Stokes–Einstein equation, we believe that if the effective volume of the particle were changing as a function of concentration we would not observe such excellent agreement between calculated and observed τ values at all peptide concentrations.

The T_1 data obtained from the natural abundance ^{13}C resonances in enkephalin at a concentration of 100 mg/mL were used to calculate the rates of overall and internal reorientation in enkephalin. We have found the overall molecular motion of enkephalin to be only slightly anisotropic ($\tau_x = 3.2 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, $\tau_z = 9.9 \times 10^{-11}$ s rad^{-1}). The range of values obtained does not depend on the choice of molecular conformation (β turn or fully extended). This demonstrates that in potentially flexible molecules of this size it is difficult to detect anisotropic overall molecular motion by performing a least-squares fit of observed T_1 data to various rigid molecular conformations. Similar conclusions were obtained in the case of angiotensin-II (Deslauriers et al., 1977c). The rates of internal rotation about the side chains are of the same order of magnitude as the rates of overall reorientation. The values of the rates of internal reorientation about the C–C bonds in the side chains do not depend strongly on the overall molecular conformation. Thus, as has been found for angiotensin-II, rotational motion in individual side chains can be detected and estimated without precise knowledge of the overall molecular shape.

No dependence of T_1 values on pH has been observed for either ^{13}C-enriched peptide between pH 1.5 and 10.0. Thus, any conformational change which might occur as a function of pH is not accompanied by significant changes in the rates of overall molecular reorientation or internal reorientation of the [2-glycine] or [3-glycine] residues. The lack of influence of pH on the T_1 values further implies that at a concentration of 4.95 mg/mL no change in the state of aggregation of the peptide occurs between pH 1.5 and 10.0.

We have observed a linear relation between the value of τ_{eff} and η/T for both ^{13}C-enriched peptides at all the concentrations and temperatures investigated. This implies that the T_1 values of the enkephalins are sensitive to the rate of overall molecular reorientation of the peptide. This motion slows down with increasing solution viscosity. The [3-glycine] residue always shows a greater dependence on η than does the [2-glycine] residue in the ^{13}C-enriched enkephalins. This is due to the lesser contribution of internal motion to the T_1 of the former, as has been observed previously for proline in solutions of varying viscosity (Deslauriers and Smith, 1977).

By studying the temperature dependence of T_1 values of the ^{13}C-enriched glycyl residues in enkephalin, we can obtain effective activation energies for the rotational reorientation of the glycyl residues. Between peptide concentrations of 5 and 45 mg/mL the values of E_a range between 20 and 26 kJ/mol. These values are essentially constant for the [3-glycine] residue in enkephalin over this concentration range. The error on the values of E_a is estimated to be ±20–25%, assuming a reproducibility in T_1 measurements of ±10%. The E_a values are in the range of those determined for the α carbons in luliberin (Deslauriers et al., 1977a) at a concentration of ca. 100 mg/mL (20–27 kJ/mol).

The study of α-endorphin was undertaken in order to determine whether this peptide possessed any conformational properties different from those of the enkephalins. α-Endorphin shows a well-resolved ^{13}C NMR spectrum; NOE measurements yielded a full enhancement, indicating that the effective correlation times for the carbons in this peptide are relatively short ($<10^{-10}$ s). The good correspondence between the calculated and the observed spectrum of α-endorphin implies that α-endorphin exists in a flexible, possibly random, conformation. We found no difference in the T_1 behavior of the enkephalin segment and the remaining peptide sequence in α-endorphin. Incorporation of the pentapeptide sequence of enkephalin into α-endorphin produces a larger effect on the backbone carbons of the enkephalin sequence than it does on the aromatic residues. This indicates that the internal rotation of the aromatic residues contributes appreciably to their T_1 values and that this rotation is affected relatively slightly by incorporation of the aromatic residues into a larger, random

conformation peptide. The observation that the tyrosyl and phenylalanyl aromatic rings have equal T_1 values does not necessarily imply that the tyrosine residue is relatively restricted in mobility as a consequence of particular conformational features of the enkephalins. Studies on angiotensin-II (Deslauriers et al., 1977c) and on [4-phenylalanine, 8-tyrosine]-angiotensin-II (Deslauriers et al., 1975a) have shown that the T_1 behaviors of the aromatic rings of tyrosine and phenylalanine are dependent upon *both* the nature of the ring (i.e., hydroxylated or not) *and* the position the residue occupies in the peptide. The tyrosyl residue, bearing a hydroxyl group, has been shown to reorient more slowly than a phenylalanyl residue occupying the same position in a peptide (Deslauriers et al., 1975a). Thus, the equality of T_1 values in the tyrosyl and phenylalanyl residues is a compound effect. Having a tyrosyl residue in an N-terminal position would generally enhance the mobility of this residue with respect to a nonterminal residue; however, the presence of the hydroxyl group in tyrosine, which is known to hydrogen bond to solvent (Deslauriers et al., 1975b), restricts the motion of the ring with respect to that of a phenylalanyl residue. We believe that the T_1 behavior of the tyrosyl residue in enkephalin and α-endorphin reflects the intrinsic properties of a terminal tyrosyl residue rather than any particular conformational feature of the peptide.

Conclusion

In these studies we have attempted to probe some of the dynamic properties of the enkephalins and α-endorphin. We have shown that the [3-glycine] residue in enkephalin is motionally more restricted than the [2-glycine] residue at low peptide concentrations. Upon increasing peptide concentration, the T_1 values of both glycyl residues decrease, the decrease for the [2-glycine] residue being the more pronounced. This can be viewed as a restriction of the segmental motion in the peptide with increasing concentration. We do not believe that molecular aggregation occurs discontinuously in the concentration range of 2–100 mg/mL of peptide in view of the good correspondence between calculated and observed τ values over all the concentrations and temperatures studied. We have found the optimal correlation times to describe the overall molecular reorientation of the peptide backbone to be $\tau_x = 3.2 \times 10^{-10}$, $\tau_y = 1.7 \times 10^{-10}$, and $\tau_z = 9.9 \times 10^{-11}$ s rad^{-1}. These values are conformation independent. The rates of internal motion of the side chains are of the same order of magnitude as those for overall motion and are also conformation independent. No pH dependence of T_1 values was noted, again indicating that no change in the dynamical properties or the state of aggregation of the peptide takes place. The effective correlation time of the peptide depends linearly on solvent viscosity, the [3-glycine] residue having a larger dependence than the [2-glycine] residue. The latter confirms that the overall molecular reorientation of enkephalin is better monitored at the [3-glycine] residue. Activation energies of 20–27 kJ/mol were determined for the rotational process; these values are constant for the [3-glycine] residue of enkephalin at all sample concentrations and may reflect the activation energy for overall molecular reorientation. They constitute further evidence against aggregation at the concentrations studied.

Our studies on α-endorphin reveal a flexible, possibly random-coil structure for this peptide. The enkephalin portion differs little in motional properties from the remaining peptide segments in the molecule. The peptide backbone seems more restricted than the side chains upon incorporation of the enkephalin segment of the peptide into α-endorphin. These data may help explain the biological activity of α-endorphin (60% of enkephalin) in the sense that the enkephalin segment of the peptide does not appear to be buried or masked by the remaining peptide segment of α-endorphin. The fact that no enhancement of activity is noted in this peptide over that of enkephalin may imply that the random nature of the remaining peptide segment does not in any way favor increased formation of the biologically active conformation of enkephalin. In this context it would be of interest to compare these results with similar measurements on β-endorphin which shows increased activity when compared to the enkephalins; in this case the nature of the peptide chain may provoke an increase in the population of the biologically active conformation of the enkephalin segment.

Opiates are reported to interact stereospecifically with phosphatidylserine and cerebrosides. By analogy, we expected similar interactions with the enkephalins. Our attempts at monitoring such interactions have so far been unsuccessful.[1] Further attempts should be made using a ^{13}C-enriched tyrosine in enkephalin, since this residue is thought to be the primary locus of interaction with biological receptors (Horn and Rodgers, 1976). Another potentially useful approach involves spectroscopic monitoring of the lipids upon interaction with the enkephalins.

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