spin-spin analysis and cross-relaxation rates agreed with each other (±0.2 Å) and with the crystal structure distances. This proved that the dipolar formalism was applicable to complex natural products and hence proton relaxation parameters can be effectively used to determine the conformation of natural products. The seeming discrepancies between the H30-H31 distance determination from relaxation rate were attributed to experimental and theoretical complications due to non-first-order coupling of H31 to H32. The framework of saxitoxin, as distinct from the side chain, is rigid and the correlation time for overall rotation is $\tau_c = 8.3 \times 10^{-11}$ s.

Internal rotation of the side chain was evaluated from averaged $^3J_{28,33}$ and $^3J_{28,34}$ as well as the observed relaxation parameters $\sigma_{28,33}$ and $\sigma_{28,34}$. Rotamer populations, derived from the scalar coupling constants, and used with similar equations, predicted the average distances $d_{28,33}$ and $d_{28,34}$ from the averaged σ values.

Thus, combined use of the proton relaxation parameters and scalar coupling constants not only completely defined the total absolute stereochemistry of saxitoxin in solution but also gave details of the overall and internal rotations of the molecule. The speed, accuracy, and low concentrations of noncrystalline materials required indicate extensive future use of dipolar coupling in the area of natural-product stereochemistry.

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Proton Spin–Lattice Relaxation Studies of [D-Ala²-Met⁵]Enkephalin

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Abstract: Application of selective and nonselective proton relaxation rate measurements to molecules outside the $\omega_0^2\tau_c^2 \ll 1$ limit is explored using [D-Ala²-Met⁵]enkephalin. Monoselective, biselective, and nonselective measurements yielded cross-relaxation rates, σ, and F ratios; from these, it was deduced that enkephalin has a relatively rigid backbone, internal motion of the Ala², Phe⁴, and Met⁵ side chains, a small reorientation of the Tyr¹ aromatic ring, and proximity of the Ala² and Met⁵ methyl groups. These data support but do not prove the existence of the β-turn conformations. All proton relaxation is dominated by dipolar mechanisms.

Introduction

This study presents novel approaches to the use of mono-, bi-, and nonselective spin-lattice relaxation times for peptides with correlation times outside the extreme narrowing limit.

In the previous papers in this series, we reported proton spin-lattice relaxation rate studies of saxitoxin,[2] whose crystal structure was known,[3,4] and isoleucine.[5,6] These established the mechanisms of proton relaxation and demonstrated the measurement of correlation times and distances for interproton vectors, but the question of larger molecules which do not satisfy the extreme narrowing condition still remained; large natural products and biopolymers in general require spectrometers of high frequency and have long correlation times.

Because of topical interest in enkephalins as a new class of endogenous neurotransmitter peptides[7] and to test the eventual possibility of applying these methods to larger polypeptides and proteins, we report studies of [D-Ala²-Met⁵]enkephalin.

NMR studies of zwitterionic and cationic enkephalins have appeared.[8-12] ^{13}C T_1 data have been interpreted in terms of motion[11,12] and proton spectral parameters, other than relaxation times, have been used to propose various conformations for the zwitterion.[10] The proton relaxation studies of

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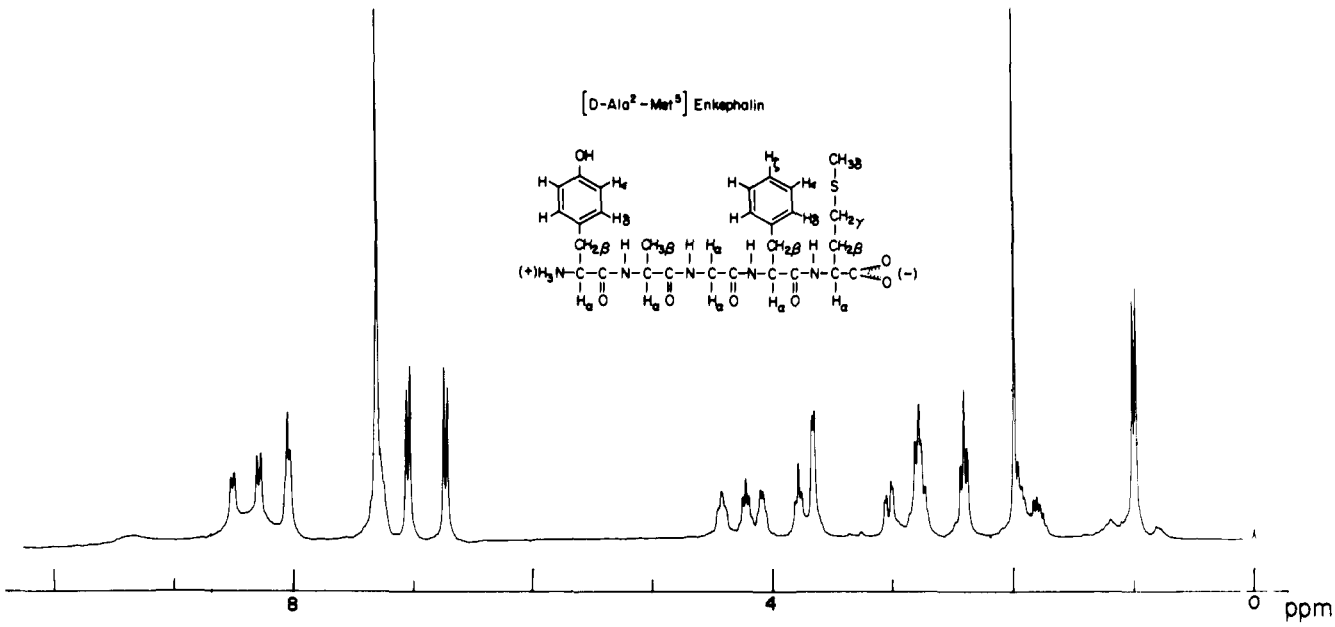

Figure 1. The proton magnetic resonance spectrum of zwitterionic [D-Ala²-Met⁵]enkephalin. The concentration was 10 mM in Me_2SO-d_6 and the temperature = 26 °C.

enkephalin[11] have relied entirely upon nonselective spin-lattice relaxation rates which, on their own, are of limited use and complicated to interpret.

Experimental Section

Pure[D-Ala²-Met⁵]enkephalin was supplied by Dr. V. Garsky (Wyeth Laboratories). The samples were prepared by dissolving 1.8 mg of the peptide in 0.25 mL of Me_2SO-d_6. Samples were thoroughly degassed and care was taken to avoid complications from other paramagnetic impurities such as metal ions. Small amounts of EDTA were added to some samples to further test for paramagnetic impurities. Spectra were obtained with a Bruker WH-270 equipped with a Nicolet 1180 computer. To measure the relaxation rates, a $(180°\text{-}\tau\text{-}90°\text{-}T)_n$ pulse sequence was performed using 20 different τ values, which were chosen short enough to describe the initial behavior of the recovery curve of the fastest relaxing protons. In the semilogarithmic plot of the $(A_\infty - A_\tau)/2A_\infty$ quantities vs. τ, the fitting of the experimental data to the best straight line calculated via a least-squares analysis indicates a 98% confidence for the reported relaxation rates, when A_τ values relative to $\tau < T_1$ were taken into account for the relaxation rate calculations. The nonselective 180° pulse was typically 20 μs and the selective one, provided by the decoupler channel, was 10 ms. The temperature was controlled to ±1 °C by the Bruker temperature unit.

Results and Discussion

The ^1H NMR spectrum of [D-Ala²-Met⁵]enkephalin (**1**), shown in Figure 1, is similar to those reported for zwitterionic [Met⁵]enkephalin.[8,9,12] The assignment of all the peaks in the spectrum was carried out by homonuclear decouplings and comparison with previous studies.[8,11] Two ^{13}C T_1 studies of [Met⁵]enkephalin, an analogue of **1**, exist,[11,12] but only a preliminary discussion of ^1H T_1 values is now available.[11] In fact, even if ^{13}C T_1 measurements present many problems because of the low ^{13}C natural abundance, they are much easier to interpret in terms of motion.[13-15]

For protons it is well known that the spin-lattice relaxation rate of the nucleus, i, interacting with the neighboring, j, is described by

$$R^i = \sum_{m,j\neq i} {R_m}^{ij} \qquad (1)$$

where m are the possible relaxation mechanisms such as spin rotation (SR), chemical shift anisotropy (CSA), intermolecular (XDD) and intramolecular (IDD) dipole-dipole interaction, and scalar coupling (SC). Furthermore, in any conventional nonselective measurement (NS) we have to take into account cross-relaxation contributions arising from XDD, SC, and IDD, and to describe a nonselective relaxation rate, eq 1 has to be modified:

$$R^i(\text{NS}) = R^i + \sum_{j\neq i} \sigma^{ij} \qquad (2)$$

where the σ^{ij} terms are the cross-relaxation parameters for the IDD interaction of nuclei i and j.

The recent use of mono- (SE) and biselective (BS) techniques simplified ^1H relaxation rate studies; R^i, in the initial rate approximation,[16] is obtained by a monoselective measurement, and the σ^{ij} are calculated from SE and BS measurements.[17] Evaluation of stereochemistry and internal motion of small molecules such as monosaccharides,[16,17] amino acids,[18] neurotoxins,[2] and nucleotides[19] by these techniques is firmly established because their correlation times satisfy the relation $\omega_0^2\tau_c^2 \ll 1$; in this case, theory states that $R^i(\text{NS})/R_0{}^i(\text{SE}) = 1.5$[20] if only XDD and IDD mechanisms dominate the relaxation pathway of the nucleus, i. With low-concentration samples, it is reasonable to assume that the XDD mechanism is not efficient; the actual relevance of IDD is directly related to $R^i(\text{NS})/R_0{}^i(\text{SE})$ values. From SE and BS measurements, correlation times[5] and interproton distances[2] can be defined because, in general,

$$\sigma^{ij} = \hbar^2\gamma^4(r_{ij})^{-6} f({\tau_c}^{ij}) \qquad (3)$$

and knowing r_{ij} (i.e., geminal protons), $f({\tau_c}^{ij})$ can be estimated or vice versa. With larger molecules the 1.5 criterion is inapplicable because σ^{ij} and R^i depend differently on $\omega_0^2\tau_c^2$ and because the F ratio, $R^i(\text{NS})/R_0{}^i(\text{SE})$, exhibits a behavior similar to that of the NOE, decreasing from 1.5 to 0 at increasing values of τ_c.

Even if it is reasonable to assume that the IDD mechanism dominates the proton relaxation pathway in biopolymers as well as in simpler systems, the problem of contamination of the samples from dissolved oxygen, paramagnetic impurities in substances or solvents, and concentration effects can still exist. In thoroughly degassed solutions, with small amounts of metal ion chelating agents such as EDTA, operating at low concentrations, it has been clearly demonstrated that the IDD is the dominant mechanism.[26,27]

Table I. ^{1}H NMR Parameters for [D-Ala²-Met⁵]Enkephalin (12 mM in Me_2SO-d_6 at 26 °C)

		δ, ppm[a]	R^i(NS)[b]	$R_0{}^i$(SE)[b]	F
Tyr¹	NH	8.41			
	H_α	3.83	2.83	3.26	0.87
	H_β	*c*			
	H_δ	7.07	0.94	0.90	1.04
	H_ϵ	6.81	1.31	1.21	1.08
	OH	9.39			
Ala²	NH	8.56	4.00	5.08	0.79
	H_α	4.27	1.23	1.26	0.98
	H_β	1.03	3.36	2.67	1.26
Gly³	NH	8.11	*c*		
	H_α	3.69	3.55	3.78	0.94
Phe⁴	NH	8.33	3.33	3.26	1.02
	H_α	4.47	1.65	1.66	0.99
	H_{β_1}	3.07	*d*	*d*	*d*
	H_{β_2}	*c*			
	$H_{\delta-\zeta}$	7.35	0.98	0.87	1.12
Met⁵	NH	8.09	*c*		
	H_α	4.13	1.32	1.28	1.03
	H_{β_1}	1.83	*c*		
	H_{β_2}	1.96	3.93	3.67	1.07
	H_γ	2.46	2.74	2.53	1.08
	H_ϵ	2.03	0.76	0.66	1.15

[a] Calculated using Me_4Si as internal standard. [b] The estimated experimental error of ±1% yields a ±5% error in the F parameters. [c] The overlap with other peaks introduced errors into their measurement. [d] The relaxation behavior of such a strongly coupled spin system will be analyzed in a future paper.

Information from R^i(NS)/$R_0{}^i$(SE) Ratios (Called F Ratios). A nonselective ^{1}H T_1 study of **1** was performed. To reduce experimental and interpretational errors as much as possible only the relaxation rates of well-resolved, first-order coupled protons were evaluated (Table I). On the basis of these relaxation parameters alone it is difficult to get structural or dynamic information for an unknown molecule because each value can be affected by different unknown cross-relaxation contributions.[5] According to ^{13}C data NT_1's for residues 2–5 of enkephalin have progessively faster motions proceeding from backbone along the side chain. Using these NT_1 values and the log $\omega_0\tau_R$ vs. log $\omega_0\tau_c$ plots of Werbelow,[21] it can be predicted for enkephalin-like molecules that the NOE (and hence F values) for backbone protons should be relatively insensitive to side-chain motions. Therefore, the curve shown in Figure 2 was used to calculate the correlation time which modulates the relaxation process of backbone protons. The fact that all measured $F^{H\alpha}$ and F^{NH}, save F^{NH} of Ala², have the same value (0.94 ± 0.07) argues that the F values are dominated by τ_c for the overall motion and that these τ_c values are characteristic of backbone motions. Hence F values are, like NOEs, useful parameters for studying peptides whose motions are outside the extreme narrowing conditions. The lower F^{NH} of Ala² could arise from an effective slower local motion or from a nondipolar contribution to the spin-lattice relaxation of this amide proton. Using Figure 2, for **1** a value of τ_c (7.0 ± 0.5) × 10^{-10} s was derived, in satisfactory agreement with that reported in a ^{13}C T_1 study,[12] $\tau_c{}^{C^\alpha H} = 3 \times 10^{-10}$ s, considering the higher temperature and less viscous solvent of the ^{13}C measurement.

The side-chain protons generally have higher values of F, showing that faster correlation times dominate the inter-side-chain interactions. The methyl protons of Met⁵ and Ala² exhibit the highest F ratio values. Since their experimental selective relaxation rates still contain relevant cross-relaxation contributions arising from the intra-methyl-proton interactions, even larger ratios for these have to be expected. From a temperature-dependence study of the two methyl groups (in the range of 20–50 °C), positive activation energies were found

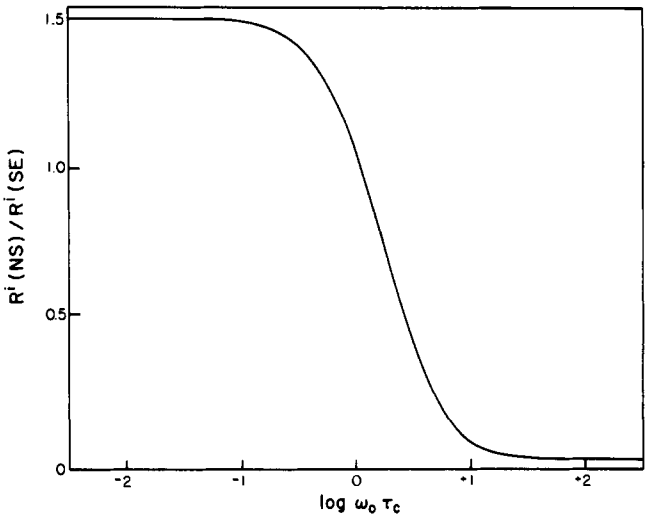

Figure 2. A plot of the ratio $F = R^i(NS)/R^i(SE)$ vs. log $\omega_0\tau_c$. The terms and use of this diagram are explained in the text.

for the relaxation process. The Met⁵-CH_3 and Ala²-CH_3 have positive E_A = 0.9 and 2.8 kcal/mol, respectively; therefore relatively fast reorientations and no spin-rotation contribution to the relaxation pathway are predicted for these protons[29] in the experimental conditions of the present study. The further complication of cross-correlation contributions for such A_3 spin systems[30] was avoided because initial slopes were used to calculate the relaxation rates.[31] Thus, it is reasonable to assume for the two methyl groups that F = 1.5, as expected for protons completely relaxing through the IDD mechanism and in the $\omega_0{}^2\tau_c{}^2 \ll 1$ dynamical region, if any cross-relaxation contribution was removed from the selective relaxation rate. Dividing the R^{CH_3}(NS) by 1.5, the obtained rate, $R^{CH_3}_{calcd}$(SE), should not contain σ_{CH_3} terms; therefore, from the difference $R^{CH_3}_{exptl} - R^{CH_3}_{calcd}$, the quantity $2\sigma_{CH_3}$ can be evaluated, where the coefficient 2 takes into account the interaction of each nucleus of the methyl group with its two neighboring protons. Because the interproton distance for these interactions is known, from the calculated σ's, the effective correlation times can be obtained. These were found to be 10^{-11} and 2.8×10^{-11} s for Met⁵ and Ala² internuclear methyl vectors, respectively. The agreement with a previous ^{13}C T_1 study on a similar peptide[22] is excellent for the Met⁵-CH_3; in fact, from the reported T_1 = 1.37 s, the methyl protons of the methionine residue exhibit a $\tau_c = 1.1 \times 10^{-11}$ s. Furthermore, the three times slower correlation time of Ala²-CH_3 is consistent with the different activation energies found for the two CH_3's and with their different relaxation times.

The motion of the aromatic rings of Tyr¹ and Phe⁴ was tested by a similar approach. Each of the H^δ and H^ϵ doublets of the tyrosine ring has different relaxation rates because of the interaction of the H^δ with the nearby β protons. The two lines of each doublet exhibit a significantly different behavior in the nonselective experiment and an even more noticeable one in the selective case. As previously discussed,[23] such an AA′BB′ spin system, where the para and meta couplings are small enough, can be approximated to an AB system; the outer line of each doublet has greater J mixing and, therefore, a greater cross-relaxation contribution than the inner lines. Experimentally, they exhibit greater multiexponential behavior. Despite this, it was still possible to obtain non-, mono-, and biselective relaxation rates for each doublet, following the recovery of the inner lines from partially relaxed spectra. Because the two H^δ and H^ϵ in the ring have meta and ortho distances of 4.05 and 2.44 Å, respectively,[24] no detectable meta cross relaxation can occur at least for correlation times faster than 10^{-8} s; for any range of molecular motion it is always less

than 1% of that arising from the H^{δ}-H^{ϵ} ortho interaction. In this way, in a monoselective experiment on such doublets, any ortho cross relaxation is removed. This was tested on *N*-acetyltyrosine ethyl ester, in which both *F* values for the H^{δ} and H^{ϵ} were found to be 1.5:[25] the actual $\sigma_{H\delta-H\epsilon}$ was evaluated by the difference between the relaxation rates obtained from both a double and a monoselective measurement. From the small experimental $\sigma = 0.05$ and the interproton distance 2.44 Å, $\tau_c = 5.8 \times 10^{-10}$ s for the H^{δ}-H^{ϵ} vector. This value is only slightly faster than the one found for backbone protons showing that the aromatic ring is not freely rotating along the C^{δ}-C^{ϵ} axis of the tyrosine side chain.

A selective experiment on the aromatic region of Phe[4] does not remove all the cross relaxation arising from the ortho interaction because of the degeneracy of these five protons. The experimental *F* value for this complex peak is considerably lowered as just discussed for the methyl group. Even if the correlation times for the interproton vectors in the Phe ring are difficult to evaluate, a relevant internal reorientation can be assumed from the *F* ratio >1.1 and from the slow nonselective and selective relaxation rates.

Information from a Combination of Mono- and Biselective Relaxation Rates. In addition to using *F* ratios, it is possible to study conformational dynamics by proton-proton NOEs[26,27] and by mono- and biselective spin-lattice experiments. These two complementary techniques yield the cross-relaxation parameters σ between pairs of protons; this has been done for gramicidin S,[26] tyrocidine A,[28] alloisoleucine,[5] and saxitoxin.[2] However, such techniques when applied to **1** in Me_2SO-d_6 yields σ values close to zero. The latter confirms the conclusion from *F* ratios concerning correlation times.

Although NOEs between backbone protons were zero, a detectable positive NOE between the CH_3 groups of Ala[2] and Met[5] was found. Not only does this confirm different correlation times for backbone and side-chain motions and a fast correlation time for the latter, but it indicates a significant peptide conformation which permits CH_3—CH_3 interactions. Such a conformation, the β turn, has been proposed.[7,9]

Conclusions

The [D-Ala[2]-Met[5]]enkephalin was studied by nonselective and selective ^1H spin-lattice measurements and by proton-proton NOEs. A relatively rigid backbone structure, internal motion of the Ala[2], Phe[4], and Met[5] side chains, a small reorientation of the Tyr[1] aromatic ring, and proximity of Ala[2] and Met[5] side chains were deduced from our data. All these features fit the β_1 turn previously proposed[8,10] for the conformation of the opioid peptide and a similarity of the first residue with the rigid morphine ring. Although at 270 MHz this method gives zero cross-relaxation rates for backbone protons, it should be possible by changing the experimental condition (i.e., temperature, viscosity, or frequency of the instrument) to evaluate interproton distances as shown here. In general, this kind of approach complements ^{13}C T_1 studies; it gives simultaneous evaluation of structural interproton distances and motional correlation times in relatively dilute solutions. ^{13}C T_1 experiments require a much more concentrated sample and so ^1H T_1 studies can be more representative of in vivo environments.

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