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NMR ROTATING FRAME RELAXATION STUDIES OF INTRAMOLECULAR MOTION IN PEPTIDES. TYROSINE RING MOTION IN METHIONINE-ENKEPHALIN

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SUMMARY: Proton, rotating frame, nuclear magnetic spin-lattice relaxation measurements have been performed in aqueous solution on the unlabelled tyrosyl ring nuclei in [1-($\alpha,\beta\beta,\delta\delta$-$^2H_5$)-L-Tyr,5-L-Met]-enkephalin. In the temperature range measured, 7°C to 30°C, the chemical shift difference between the two epsilon protons is modulated by motions of the main chain and the ring with an average rate of the order of $\sim$ 400 sec^{-1} This work confirms an earlier suggestion by us that the ring of the tyrosyl residue in Methionine-enkephalin is quite rigidly fixed with respect to the peptide main chain compared with overall molecular tumbling.

We recently suggested (1) that ^1H and ^{13}C nuclear magnetic resonance (NMR) relaxation in the rotating frame should be a useful tool for studying rates of intramolecular motion in peptides. There has been recent renewed theoretical and experimental interest in this problem (2,3,4) particularly with regard to aromatic ring motions.

This paper reports quantitative experimental results on intramolecular motions of the tyrosyl residue in the peptide NH_3^+-L-Tyr-Gly-Gly-L-Phe-L-Met-OH (Methionine-enkephalin) in aqueous solution. This part of the molecule is of particular interest because of its requirement for binding activity to the opiate receptor(s) (5) and the analogy between the tyrosyl residue in the potentially flexing peptide and the rigid tyramine ring system in the classical opiates (6). Based upon the circumstantial evidence furnished us by conventional, laboratory frame, spin-lattice relaxation in Methionine-enkephalin, we had previously sug-

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gested (7) that the tyrosyl ring was rather rigidly fixed with respect to the rest of the peptide molecule in a coordinate system undergoing overall tumbling with the molecule.

As discussed originally (1) the application of rotating frame methods to peptides requires that extraneous motion-modulated dipolar couplings be removed from the nuclear system under study. Given peptide synthetic methods this is most easily achieved by appropriate specific isotopic substitution. In the present case the tyrosyl residue of the peptide under study was in the form of [$\alpha,\beta\beta,\delta\delta$-$^2H_5$]-L-Tyrosine. Hence, only the epsilon protons remained.

EXPERIMENTAL: The peptide was synthesized and purified as previously described (7). The labelled tyrosine was a gift from Professors V.J. Hruby and A.O. Spatola. The material used for the NMR experiments travelled as a single component upon thin layer chromatography in two solvent systems as it co-migrated with previously prepared, unlabelled Methionine-enkephalin. NMR samples were made up in 5mm tubes as follows. 16.1 mg of [1-($\alpha,\beta\beta,\delta\delta$-$^2H_5$)-L-Tyr,5-L-Met]-enkephalin was dissolved in 0.7 ml ^2H_2O (99.7 atom percent; Merck, Sharp and Dohme). A sample spotted on indicator paper showed a pH (apparent) near 2.2. The 40 mM solution was degassed through 6 freeze-pump-thaw cycles and transferred to the NMR sample tube under a nitrogen atmosphere.

Our basic ^1H 100 MHz JEOL PFT-100 crossed-coil spectrometer has been described elsewhere (8). The detection portion of the instrument is now equipped with home-made quadrature detection apparatus.

A complete description of the circuitry to perform rotating frame relaxation experiments on our modified spectrometer is provided in a companion publication (9). Low RF spin-locking fields are obtained through on-resonance spin-locking which in turn requires quadrature phase detection. The apparatus makes use of a phase alternating pulse sequence (10). The nuclear magnetism is therefore aligned parallel and antiparallel to the rotating RF field in sequential pulses. Spin-locking is achieved through the use of a two-channel RF multiplexer preceding the RF transmitter, one channel of which contains a variable RF attenuator and phase shifter.

RF field strength was calibrated by disconnecting the excitation RF pulse and using the spin-locking RF pulse as an excitation pulse. Corrections were made whenever the spectral line used for calibration was not on resonance (11). The smallest spin-locking field (100 rad/sec) was chosen to be a value above which contributions from inhomogeneities of the static magnetic field were negligible, while a maximum spin-locking field of $\sim 10^4$ rad/sec was used to avoid probe damage. As discussed in (9), the performance of the equipment was tested on cyclohexane with results comparable to those of previous workers. The sample had to be kept spinning in order to eliminate temperature gradients within the sample. Changing the spinning rate from 10 to 60 Hz did not produce any change in rotating frame relaxation times and gross RF inhomogeneities could therefore be disregarded. Earlier experiments were performed with 0.6 ml of sample in ordinary 5 mm high resolution NMR tubes. Later experiments with cylindrical micro-tubes (Wilmad 508-CP) supported the earlier data but reduced the scatter in the relaxation plots. The sample temperature was controlled by air pre-cooled in a copper coil immersed in a Forma-Temp bath. The temperatures were determined by the resonance

separation in a sample of methanol using the Varian calibration chart (12).

RESULTS: The normal ^1H NMR spectrum of the labelled peptide showed only a single narrow peak at δ = 6.88 ppm in the tyrosyl aromatic region. This corresponds to the upfield resonance in the normal AA'BB' spectrum of the unlabelled peptide (7).

Relaxation data was fitted via a non-linear least squares procedure to a single exponential decay. Each decay curve consisted of at least 10 points. The parameters A and τ, relevant to the rotating frame experiments (1), were derived by another non-linear least squares fit to,

$$\frac{1}{T_{1\rho}} - \frac{1}{T_1} = \frac{A\tau}{1+\omega^2\tau^2} \qquad (1)$$

where ω is the magnitude of the spin-locking RF field. Each fit was to at least 6 field strengths. The errors quoted in all derived parameters are 95% confidence limits (that is, approximately two standard deviations).

The A and τ values obtained from this work are discussed below and are given in Table Ia. Apparent activation energies obtained from Arrhenius plots

TABLE Ia

Derived parameters from rotating-frame spin-lattice, and laboratory frame spin-lattice, relaxation experiments on NH_3^+-[$\alpha,\beta\beta,\delta\delta$-$^2H_5$]-L-Tyr-Gly-Gly-L-Phe-L-Met-OH in ^2H_2O at 100 MHz.

Temperature (^oC)	Rotating Frame*	Laboratory Frame†
7	$A = 275 \pm 48$ sec^{-2} $\tau = 2.1 \pm 0.7 \cdot 10^{-3}$ sec	$T_1 = 1.81 \pm 0.13$ sec
10	$A = 203 \pm 75$ $\tau = 2.5 \pm 1.6 \cdot 10^{-3}$	2.03 ± 0.06
12	$A = 198 \pm 33$ $\tau = 3.9 \pm 2.3 \cdot 10^{-3}$	1.85 ± 0.12
16.5	$A = 77 \pm 47$ $\tau = 2.7 \pm 2.8 \cdot 10^{-3}$	2.30 ± 0.09
30	$A = 60 \pm 6$ $\tau = 5.1 \pm 1.5 \cdot 10^{-3}$	3.10 ± 0.16

*$1/T_{1\rho} - 1/T_1 = \frac{A\tau}{1+\omega_1^2\tau^2}$

†$Mz(t) = M_0(1-2e^{-t/T_1})$

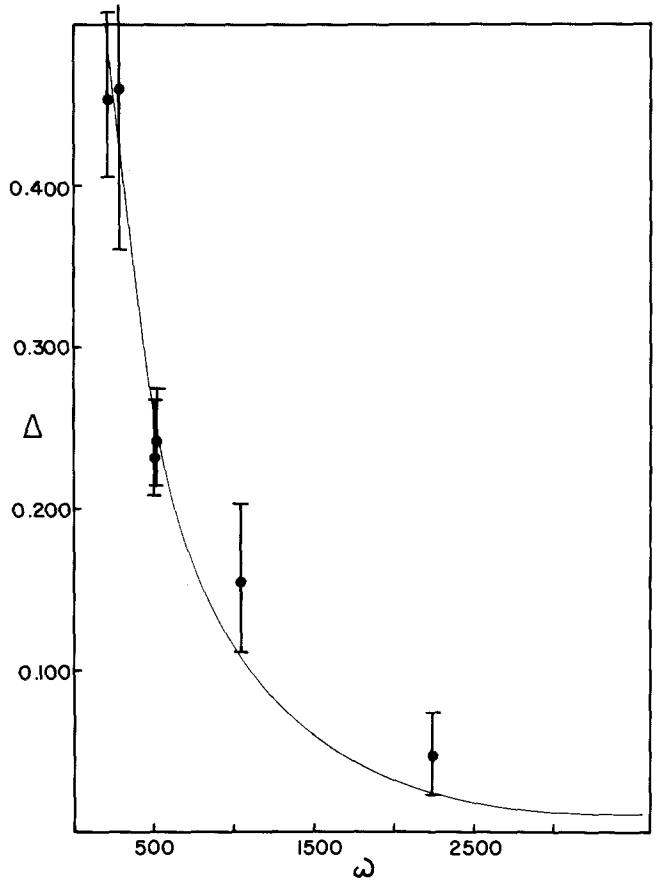

Figure 1 Typical data plot of $\Delta = 1/T_{1\rho} - 1/T_1$ (in sec) vs. ω (in rad/sec) showing propagated energy bars and reproducibility of points. This figure is for data taken at 7°C. Smooth curve is theoretical curve for the non-linear least squares determined parameters A = 275 sec^{-2}, $\tau = 2.1 \cdot 10^{-3}$ sec.

of this data are: T_1, -4.02 ± 1.2 kcal/mole; $A^{\frac{1}{2}}$, 5.5 ± 2.8 kcal/mole; τ, -2.7 ± 2.4 kcal/mole. Figure 1 shows a typical plot of equation (1) with propagated error bars and the computer generated theoretical curve. The data contained in Table Ib gives the populations of tyrosyl side chain rotamers derived from classical analysis (13) of the alpha-beta coupling constants for the normal peptide from spectra taken at 360 MHz, under the same physico-chemical conditions, as given in a previous publication (14).

DISCUSSION: In the rotating frame experiment applied to the present case the A parameter of equation (1) is proportional to the square of the maximum chemical

TABLE Ib

Tyrosyl side chain rotamer (χ^1) populations based on previous data (14). Met-enkephalin in ^2H_2O, 30°C.

Coupling Constants and Shifts:

δ_A = 3.16 ppm
δ_B = 3.16
J_{AX} = 7.5 Hz
J_{BX} = 7.5 Hz

Populations:

g^+ = 0.54 ($\chi^1 = -60^o$)
t = 0.45 ($\chi^1 = 180^o$)
g^- = 0.008 ($\chi^1 = +60^o$)

shift difference between epsilon protons in various conformers to which the ring has access, and τ is the average exchange time which modulates this difference. For two conformeric environments, $A = \Delta\nu^2/4$ where $\Delta\nu$ is the difference in radial frequencies between the two proton shifts. An A value of, for example, 275 sec^{-2} which we find at 7°C corresponds to a chemical shift between epsilon protons in the tyrosyl ring of 0.066 ppm. The basic problem raised by our data is therefore: what motions of the tyrosyl side chain modulate the chemical shift difference between the epsilon protons at an average rate of $1/\tau \sim 400$ sec^{-1} at 7°C with a low temperature dependence for $1/\tau$ and a high one for the chemical shift difference?

For both peptides and proteins (15,16) steric effects result in a negative correlation between the side chain torsional angles χ^1 and χ^2 for aromatic residues. That is χ^2 is found to be always at $\chi^2 = \sim 90^o$ independent of the rotameric state of χ^1. Theoretical work on Methionine-enkephalin (17) shows that a global energy minimum exists in which the tyrosyl side chain is in the conformation χ^1, $\chi^2 = g^+$, 82^o and is there stabilized by a hydrogen bond involving the tyrosyl hydroxyl group. Our population analysis shows that this cannot be the only conformer in solution. In fact, g^+ and t are equally populated under our conditions.

Taking into account previous work and the results obtained here the following experimental facts must be explained. The tyrosine aromatic spin system is AA'BB'

under the conditions used. The alpha-beta tyrosyl spin system analysis shows that g^+ and t conformers are equally populated independent of temperature. The latter analysis also shows that the two tyrosyl beta protons have the same chemical shift as compared to a different solvent system where they may be as much as 0.14 ppm different (unpublished results using data taken during work on reference 14). Our computer experiments and theoretical considerations show that the χ^2 angle is fixed at $\sim 90^0$ (or 270^0) independent of χ^1 for aromatic ring containing residues. Rotating frame relaxation experiments show that there is a temperature dependent chemical shift difference between the two epsilon protons on the tyrosine ring and that this difference is being modulated by a motional process with a characteristic average rate of ~ 400 sec^{-1} whose temperature dependence has not been determined with good accuracy.

We believe that the simplest explanation of our results is that we are observing a combination of rate process k and at least one of the processes ℓ and m in,

$$\begin{array}{ccc} g^+, \sim 90^0 & & t, \sim 90^0 \\ \ell \downarrow\uparrow & \overset{k}{\rightleftharpoons} & \downarrow\uparrow m \\ g^+, \sim 270^0 & & t, \sim 270^0 \end{array}$$

Our reasoning is as follows. The diminished chemical shift between the tyrosyl beta protons in the present case, where we have observed a difference of up to 0.14 ppm, means that the rate process $g^+ \rightleftharpoons t$ must have a constant > 90 sec^{-1}. If ring flipping were a very slow process we would expect to observe a chemical shift difference between tyrosyl ring protons of the same order as at 7°C (4) but, being in the slow exchange limit, would be temperature independent. This is not our case. We are therefore forced to the conclusion that the change in chemical shift parameter is due to ring flip averaging which is taking place on the same time scale as the χ^1 conformational transition but with a greater activation energy. Hence, as the temperature rises the flipping rate, which must at 7°C be taking place with 600 $sec^{-1} \lesssim 1/\tau \lesssim 1000$ sec^{-1}, increases to where at 30°C we cannot observe the averaged shift difference in our experiment and $T_1 = T_{1\rho}$. At this point we cannot tell whether we are observing this averaging as an outcome of both

processes ℓ and m or whether those two have very different rate constants.

The derived apparent activation energy for T_1 is the same as that for the activation energy of viscous flow for ^2H_2O (18) as is generally observed for an overall molecular rotational diffusion process (19). In view of the data scatter for the activation energy of our rotating frame derived lifetime τ, very little can be said. If our explanation of the results is accepted the activation energy of the chemical shift difference between ring protons is a measure of ring flipping activation energy. Barriers of this magnitude have been predicted for this process on theoretical grounds for tyrosyl residues in flexible geometry situations and correspond to flipping rates of the magnitude required (2).

The overall rates of tumbling of Methionine-enkephalin have been investigated by us for the global minimum conformation (17) using our rotational diffusion coefficient optimization technique for peptides (20). Rotation is assymetric with an average rotational diffusion coefficient $\langle R \rangle = 1.1 \cdot 10^{10}$ sec^{-1}. Our present work indicates that the tyrosine ring in this molecule is being carried back and forth between g^+ and t χ^1 states at a rate of $\sim$400 sec^{-1}. At the same time ring flipping is being carried out at 7°C with a rate comparable to this but becoming more rapid at 30°C. Thus, comparison between these various rates shows clearly that on the time scale of diffusion controlled reactions the tyrosine ring system is quite rigidly fixed with respect to the rest of the molecule. We are in the process of increasing the accuracy of our analysis by the use of other specifically labelled enkephalins. In addition we are improving our data scatter by certain instrumental improvements.

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