INTRAMOLECULAR MOTION IN PEPTIDES DETERMINED BY ^{13}C NMR: A SPIN-LATTICE RELAXATION TIME-STUDY ON MSH-RELEASE-INHIBITING FACTOR*

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1. Introduction

Measurement of spin-lattice relaxation times (T_1) [1, 2] of individual carbon nuclei provides information about intramolecular motions as well as correlation times for over-all tumbling of molecules in solution. Such information should extend and complement data gained from X-ray crystallographic studies by allowing observation of molecules in motion and by providing a monitor for solvent effects on overall molecular conformations. An added advantage is that intermolecular solute–solute effects are reduced in solution.

Spin-lattice relaxation time measurements have been used to determine the nature of intramolecular motions in a number of systems of biological interest such as cholesterol [3], sucrose [3], adenosine 5′-monophosphate [3], lecithin [4], ribonuclease [5], thyrotropin-releasing hormone [6, 7] and poly-L-lysine [7, 8]. These studies have been greatly aided by recent theoretical investigations [3, 9–13] as well as by research on small organic systems [14–21]. We have undertaken T_1 measurements on Pro–Leu–Gly–NH2 (MSH-R-IF) (fig. 1), a peptide which was suggested to be the natural factor responsible for the inhibition of the release of melanocyte-stimulating hormone from the pituitary [22, 23]; the factor is released from oxytocin by enzymes present in the hypothalamus [22, 24, 25]. On the basis of 300 MHz ^1H NMR studies in dimethyl sulfoxide and preliminary X-ray crystallographic investigations it was proposed that the preferred conformation of Pro–Leu–Gly–NH_2 consists of a 10-membered β-turn, which is closed by a hydrogen bond between the *trans* carboxamide proton of the glycinamide moiety and the C=O group of proline [26].

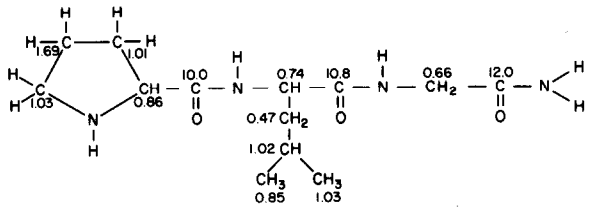

Fig. 1. Structure of MSH-R-IF with T_1 values (in seconds) for carbon atoms obtained in D_2O.

We have found MSH-R-IF to be a conformationally flexible molecule in which the Leu side chain is capable of segmental motion and the Pro ring undergoes enhanced rotation about the αCH–CO bond and rapid interconversion between ringpuckered confor-

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mations. MSH-R-IF behaves as a more compact molecule than its dimethylamide analog which cannot form a β-turn. Both these molecules undergo solvent-induced perturbations which may be a consequence of conformational rearrangements of the peptide backbone.

2. Materials and methods

NMR spectra of ^{13}C in natural abundance were obtained at 25.16 MHz on a Varian XL-100-15 spectrometer in the pulsed Fourier transform mode with complete proton noise decoupling. Spin-lattice relaxation times (T_1) were measured with an accuracy of 15% using the inversion–recovery method of Freeman and Hill [27]. Peptide samples were run at 37°C in 12 mm tubes in D_2O or deuterated dimethylsulfoxide ($(CD_3)_2SO$) at concentrations of 50 mg/ml with tetramethylsilane as external standard. Crystalline Pro–Leu–Gly–$NH_2 \cdot 1/2\ H_2O$ [28] was the same as used in earlier biological [22] and conformational investigations [26]. The Pro–Leu–Gly–N $(CH_3)_2$ was secured by Dr. K.U.M. Prasad by treatment of 2.0 g of ethyl carbobenzoxy-L-prolyl-L-leucylglycinate [29] with 10 ml of anhydrous dimethylamine in 50 ml of methanol for 24 hr at 4°C in a sealed pressure bottle to give *N*-carbobenzoxy-Pro–Leu–Gly–N $(CH_3)_2$ [(yield: 91%; m.p. 129–130°C, $[\alpha]_D^{25}$ −85°C (*c*2, ethanol). *Anal.* Calculated for $C_{23}H_{34}O_5N_4$: C, 61.9; H, 7.67; N, 12.5. *Found:* C, 62.0; H, 7.70; N, 12.4] which was subjected to hydrogenolysis (10% Pd/C) in 25 ml methanol and chromatographed on a silica gel column to give the oily and hydroscopic free base in 79% yield. The base was characterized by amino acid analysis and reconversion to the carbobenzoxy derivative.

3. Results and discussion

If all carbon atoms of a given molecule possess an identical degree of mobility, and the molecule is tumbling isotropically, the spin-lattice relaxation time (T_1) for each carbon should be inversely proportional to the number of hydrogens directly attached to it [2, 3]. Thus, T_1 data give information about the dynamic states of molecules in different solutions. T_1 values along with the assignments of the ^{13}C resonances have been determined for Pro–Leu–Gly–NH_2 and its dimethylamide derivative in D_2O and $(CD_3)_2SO$ (table 1).

Table 1

^{13}C chemical shifts and spin-lattice relaxation times in Pro–Leu–Gly–NH_2 and its dimethyl derivative in D_2O and DMSO-d_6.

		Pro–Leu–Gly–NH_2		Pro–Leu–Gly–$N(CH_3)_2$	
D_2O		Chemical shift (in ppm)	T_1 value (in sec)	Chemical shift (in ppm)	T_1 value (in sec)
Pro	α	61.07	0.86	61.12	0.65
	β	31.63	1.01	31.66	0.62
	γ	26.48	1.69	26.12	0.82
	δ	47.62	1.03	47.63	0.60
	C=O	178.99	10.0	178.33	11.0
Leu	α	53.63	0.74	53.38	0.52
	β	40.81	0.47	41.14	0.42
	γ	25.53	1.02	25.55	0.61
	δ	23.27	0.85	23.32	0.66
	δ'	21.86	1.03	21.84	0.69
	C=O	176.52	10.8	175.97	11.2
Gly	α	43.25	0.66	42.03	0.48
	C=O	175.26	12.2	170.86	12.2
$N-CH_3$					1.5
$N-CH_3$					1.2
DMSO-d_6					
Pro	α	61.69	0.26	61.65	0.82
	β	31.91	0.33	32.03	0.54
	γ	27.31	0.47	27.31	0.68
	δ	48.20	0.33	48.24	0.54
	C=O	175.87	5.3	175.39	4.3†
Leu	α	52.17	0.29	51.87	0.45
	β	*	*	*	*
	γ	25.89	0.72	25.88	0.73
	δ	24.59	0.60	24.64	0.67
	δ'	23.24	0.63	23.28	0.79
	C=O	173.69	3.3	173.50	4.3†
Gly	α	43.39	*	43.26	0.36
	C=O	172.34	4.7	169.46	6.7
$N-CH_3$				36.59	≃ 4.
$N-CH_3$				37.22	≃ 2.

† Both resonances superimposed, only an average value is reported.

* Hidden under solvent peak.

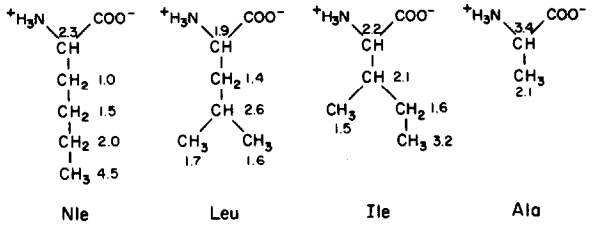

Fig. 2. T_1 values of carbons of DL-norleucine (20 mg/ml), L-leucine (25 mg/ml), L-isoleucine (45 mg/ml) and L-alanine (100 mg/ml) obtained in D_2O.

T_1 values were also measured for side-chain carbons of certain amino acids (fig. 2).

3.1. *T_1 values for carbon atoms of the peptide backbone of MSH-R-IF in D_2O*

If the backbone carbons are assumed to be equally rigid in MSH-R-IF and the molecule is tumbling isotropically, a T_1 value of 0.40 sec would be expected for the CH_2 group of Gly. The observed value of 0.66 sec (table 1) suggests that the CH_2 group of Gly undergoes a slightly greater degree of motion than do the αCH groups of Pro and Leu (see section 4 and table 2). For the Pro, Leu and Gly carbonyl carbons we found T_1 values of 10.0, 10.8 and 12.2 sec using a cycle time of 30 sec. Again, the Gly residue shows the longest relaxation time.

3.2. *T_1 values for side-chain carbon atoms of MSH-R-IF and selected amino acids in D_2O*

The β and γ carbons of Leu in MSH-R-IF and its dimethyl derivative undergo increasing degrees of segmental motion [2–4, 15, 17], as judged by the lengthening of T_1 values in going from Cα to Cγ (see section 4). The CH_3 groups of Leu are most interesting. If all relaxation occurred via dipole–dipole interactions, CH_3 groups incapable of rapid internal rotation would relax three times as fast as CH groups which were equally immobilized [3]; however, if the CH_3 groups are rotating rapidly, they should take three times as long as a CH group to relax. In fact, the T_1 values are intermediate to these limits, implying a restricted rate of rotation due to steric hindrance.

In order to see whether restricted rotation was intrinsic to the amino acids containing CH_3 groups, we compared the relaxation times in D_2O of Nle, Leu and Ile, all of which have identical molecular weights, with those of Ala and Leu in MSH-R-IF (fig. 2). As noted with Leu in MSH-R-IF we see that segmental motion does occur along the side chain of Nle, as judged by the increase in T_1 values for carbons along the chain. The relaxation time of the methyl group is greater than 4.5 sec (the cycle time used was 15.0 sec, which is only $3T_1$ of the CH_3 group, therefore the value of 4.5 sec may be slightly underestimated). In Ile, the relaxation times of the two CH_3 groups differ from each other by a factor of two, indicating that the CH_3 in the longer segment has roughly twice the mobility. Some segmental motion is indicated by the T_1 of the Ile Cγ (1.6), as well as for Cγ of Nle (1.5 sec). The Cα and Cβ of Ile appear to have equal mobility, with T_1's of 2.2 and 2.1 sec, respectively (this again parallels the results with Nle). The δCH_3 of Ile is not rotating freely, as indicated by its T_1 value of 3.2 sec (for free rapid rotation, T_1 would be approximately $3T_1$(αCH), 6.6 sec). As a result of steric crowding, the motion of the two δCH_3 of Leu (T_1 of 1.6 and 1.7 sec, respectively) is apparently even more restricted. Interestingly, the βCH_3 group of Ala is also less mobile than might be expected. In summary, a certain degree of restricted rotation is inherent to the amino acids, although further conformational constraints may be imposed upon their incorporation into a peptide structure.

The ring carbons of Pro in MSH-R-IF gave some unexpected T_1 values. If one considered the Pro ring as being rigid [30] and not undergoing any preferential mode of tumbling with respect to the rest of the molecule, one would expect T_1 values for the β, γ and δCH_2 groups to be similar and half that of the αCH. However, the T_1 values measured were 0.86 sec (Cα), 1.01 sec (Cβ), 1.69 sec (Cγ) and 1.03 sec (Cδ). The CH group of Pro relaxes in a time comparable to that of the CH group of Leu in the peptide backbone. The longer relaxation time of Cγ compared to Cβ and Cδ can be explained by a flipping of this carbon into and out of the plane of the ring formed by the other four atoms (see section 4). The ^1H NMR analysis of poly-L-Pro has indicated that several conformations were in rapid equilibrium; two Cγ puckered ring conformations (*exo* and *endo* relative to the C=O moiety) were the predominant solution conformations [31].

It was of interest to compare chemical shifts and T_1 values of MSH-R-IF with those of Pro–Leu–Gly–$N(CH_3)_2$ (table 1). The latter peptide is unable to form a β-turn with a hydrogen bond between the *trans*

carboxamide proton of the Gly residue and the carbonyl oxygen of Pro as was proposed for MSH-R-IF [26]. Although MSH-R-IF and its dimethyl analog behave in D_2O in a similar manner with respect to *relative* T_1 values of the various segments of the molecules, the T_1 values of MSH-R-IF are, on the average, 1.5 times greater than those of the methenyl, methylene and methyl carbons in Pro–Leu–Gly–$N(CH_3)_2$. Thus, MSH-R-IF tumbles more quickly in solution and behaves as a more compact molecule than its dimethyl analog. This is consistent with the proposed β-turn model [26], but neither proves nor disproves it.

3.3. *Solvent effects on chemical shifts and T_1 values*

Upon changing solvent from D_2O to $(CD_3)_2SO$, the carbonyl carbons shift upfield. All other carbon resonances shift downfield, with the exception of the Cα of Leu (table 1) which moves upfield 1.5 ppm. This shift may reflect steric compression of the Cα of Leu due to a conformational change in MSH-R-IF and Pro–Leu–Gly–$N(CH_3)_2$ caused by the solvent.

The relaxation times of MSH-R-IF differ in D_2O and $(CD_3)_2SO$. The largest differences occur in the Pro residue, where a decrease by a factor of three is seen for all carbons bearing hydrogen atoms. The carbons in the side chain of Leu undergo a decrease of 1.5 in T_1 value. The carbonyl carbons undergo a reduction in T_1 of two or three. The large differences in T_1 cannot be ascribed to a decreased rate of rotational diffusion due to a change in macroscopic viscosity since the viscosities of dimethyl sulfoxide and D_2O are comparable [32, 33]. The T_1 values of the methenyl, methylene and methyl carbons of Pro–Leu–Gly–$N(CH_3)_2$ in D_2O and $(CD_3)_2SO$ are similar, but the ratios of T_1 for the carbonyl carbons in the two solvents are between 2 and 3, indicating specific solvent interactions. The above data imply an aggregation of MSH-R-IF in $(CD_3)_2SO$, with a significant increase in the rigidity of the Pro residue. However, this aggregation is not the basis for the conformational change observed with MSH-R-IF, since the 1.5 ppm upfield shift of the Cα of Leu during the solvent transition was not only observed with Pro–Leu–Gly–NH_2 but also with Pro–Leu–Gly–$N(CH_3)_2$, which apparently does not aggregate.

4. T_1 values and correlation times

The T_1 values of most carbon atoms are dominated by dipole–dipole relaxation from the contiguous hydrogen(s) [2, 15]. It is possible to estimate from the T_1 values a correlation time, τ, related to the rate of reorientation of the C–H bonds relative to the applied magnetic field. In the limit of rapid isotropic reorientation

$$\frac{1}{T_1} = N\hbar^2\gamma_C^2\gamma_H^2 r_{CH}^{-6}\tau,$$

where N is the number of hydrogens at a distance r from the carbon nucleus of magnetogyric ratio γ_C, and $\hbar$ is Planck's constant/2π. When internal motions are present, two or more correlation times must be introduced, and quantitative interpretation becomes difficult. Therefore, as a first approximation one can use the relationship above to estimate τ for the backbone α-carbon of Leu, and consider deviations from this value for other carbon atoms as indicators of their relative mobilities, table 2. The shorter the correlation time, the greater is the mobility of the carbon atom. We must emphasize the approximate nature of these calculations; they are presented for illustrative purposes only. At present we prefer to discuss the

Table 2
Approximate correlation times for reorientation of individual ^{13}C atoms of Pro–Leu–Gly–NH_2 in D_2O*.

	Atom	τ ($\times 10^{-11}$) sec
Pro	α	5.4
	β	2.3
	γ	1.4
	δ	2.3
Leu	α	6.3
	β	5.0
	γ	4.6
	δ	1.8
	δ	1.5
Gly	α	3.5

*The corresponding values for Leu at 0.2 M in D_2O are: α, 2.4; β, 1.7; γ, 1.8; δ, 0.9; δ′, 1.0 ($\times 10^{-11}$ sec).

data in the terms used in the preceding sections.

Consider for example the β, γ, and δ methylene carbons of Pro in MSH-R-IF. If the only motion modulating dipolar couplings were that of a rigid Pro ring, the derived τ values should be equal to one another, and to that of the α-carbon. The shorter τ values (table 2) indicate that rapid ring flexing is taking place, and that the γ carbon does so to a greater extent than the β and δ-carbons. A similar effect has been noted in the Cβ and Cγ Pro resonances of thyrotropin-releasing hormone, < Glu–His–Pro–NH_2 [6]. Examination of molecular models shows that interconversion from a Cγ-*exo* to a Cγ-*endo* conformer changes the angle between a Cγ-H bond and the plane containing Cα, N, and Cδ of approximately 90°; motion of this amplitude causes a very large modulation of the $^{13}C-^{1}H$ dipole coupling.

5. Comparison of chemical shifts with those of the constituent amino acids

Corrected amino acid chemical shifts [34–36] have been used to calculate a spectrum of Pro–Leu–Gly–NH_2. The best agreement is achieved for the Leu and Gly protonated carbons. The protonated carbons of Pro differ in chemical shift by up to 2.0 ppm with the calculated values. To test if these differences are due to peculiarities of Pro, the chemical shifts of Pro in Pro–Leu–Gly–NH_2 were compared with those of Pro–Phe [36]. The agreement is slightly better but differences up to 1.5 ppm are still observed (in the γ carbon). The greatest discrepancies are observed in the carbonyl carbons. The Pro carbonyl chemical shifts of both Pro–Leu–Gly and Pro–Leu–Gly–$N(CH_3)_2$ are found 8.0–9.0 ppm to lower field than expected. The Gly–NH_2 carbonyl resonance is 3.2 ppm to lower field, the Leu carbonyl is within 0.4 ppm of the calculated value. Such large discrepancies might lead one to doubt the validity of the carbonyl carbon assignments. However, a number of substituted derivatives of Pro–Leu–Gly–NH_2 were studied in DMSO-d_6 and D_2O and these confirmed the assignments. For instance, in Pro–Leu–Gly–$N(CH_3)_2$ the Gly carbonyl resonance is shifted upfield 4.0 ppm in D_2O and 3.0 ppm in DMSO-d_6, with little effect on the other two carbonyl groups (Leu and Pro). When Pro–Leu–Gly–NH_2 is converted into Z–Pro–Leu–Gly–NH_2 the Pro carbonyl resonance shifts upfield 2.7 ppm (in DMSO-d_6) and little effect is seen on the Leu and Gly carbonyl resonances. Thus, we conclude that the chemical shifts of Pro in MSH-R-IF and Pro–Leu–Gly–$N(CH_3)_2$ are indicative of special conformational arrangements. Interpretation of these shifts in conformational terms must await a more detailed study of Pro-containing peptides. Therefore, in our opinion, the chemical shift data cannot be taken at this point as evidence for or against the proposed β-turn in MSH-R-IF [26].

6. Conclusion

Measurements of relaxation times (T_1) in D_2O and $(CD_3)_2SO$ reveal that Pro–Leu–Gly–NH_2 is a conformationally flexible molecule. The CH_2 group of Gly is less hindered than the α carbons of Pro and Leu. The side chain of Leu undergoes segmental motion and the CH_3 groups show hindered rotation. The Pro ring exhibits enhanced rotation about the αCH–CO bond of Pro in relation to the remainder of the molecule. In addition, Cγ flips into and out of the plane of the Pro ring, giving rise to a rapid equilibrium between *endo* and *exo* conformations.

Changing solvent from D_2O to $(CD_3)_2SO$ produces an upfield shift of the Cα of Leu and downfield shifts of all other carbons bearing protons. This is probably a result of conformational changes associated with steric compression of the Cα in Leu. The same behavior is observed in Pro–Leu–Gly–$N(CH_3)_2$, which cannot form a β-turn.

T_1 measurements have shown that MSH-R-IF, but not its dimethylamide analog aggregates in $(CD_3)_2SO$. This aggregation is not the cause of the solvent-induced conformational change.

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