CARBON-13 NUCLEAR MAGNETIC RESONANCE STUDIES ON THYROTROPIN-RELEASING FACTOR AND RELATED PEPTIDES★

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1. Introduction

Thyrotropin-releasing factor (TRF) is a peptide which causes the release of thyroid-stimulating hormone (TSH) from the pituitary in mammals [1]. The sequence of this hormone has recently been determined [1,2] to be L-pyroglutamyl–L-histidyl–L-prolinamide.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) has proven useful in detecting *cis* and *trans* isomers of proline in peptides [3–6]. The purpose of this study was to investigate *cis–trans* isomerism about the histidyl–prolinamide bond in TRF and to measure the spin-lattice relaxation times, T_1, of all the carbons in the hormone. TRF in water is found to have 14% of the proline present as the *cis* isomer – the amount varies with the solvent. The proline resonances are sensitive to the nature of the group attached to the proline nitrogen. ^{13}C NMR is shown to be sensitive to the presence of *D* and *L* forms of histidine. The presence of *D* or *L* histidine in TRF has a profound influence on the ^{13}C chemical shifts of proline, whereas those of pyroglutamate are unaffected.

2. Material 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 at 37° with complete proton noise decoupling. Spin-lattice relaxation times were measured using the inversion-recovery method of Freeman and Hill [7]. Thyrotropin-releasing factor (L-pyroglutamyl–L-histidyl–L-prolinamide, TRF) and the following model peptides were a gift from Dr. R.O. Studer (Hoffman, LaRoche, Bâle): L-pyroglutamyl–*N*-methylamide, < Glu–NHMe; L-pyroglutamyl–L-histidyl-methyl ester, < Glu–His-OMe; L-pyroglutamyl–L-histidyl-*N*,*N*-dimethyl amide, < Glu–His-NMe_2; acetyl prolinamide, Ac-Pro-NH_2. Other samples of TRF were a gift from Wyeth Laboratories or purchased from Bachem Fine Chemicals. TRF-R was synthetized at the Centre de Recherche Paul Pascal by E. Dupart and M. Joussot-Dubien.

3. Results and discussion

3.1. Cis–trans isomerism

The chemical shifts of the compounds studied are given in table 1. Table 2 shows the percentage of *cis* isomer of proline about the His–Pro peptide bond in TRF and Ac-Pro-NH_2 in different solvents. Measurement of *cis* content was made from peak intensities,

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Table 1
Chemical shifts of carbons in TRF and model peptides[+].

Solvent		D_2O									
Compound		< Glu-NHMe	< Glu–His-OMe	< Glu–His-NMe_2			TRF				$AcProNH_2$
pH		7.0	7.2	8.8	5.5	4.7	8.9	6.3	5.5	4.7	7.0
Residue											
< Glu	αCH	58.22	57.90	57.85	57.83	57.84	57.73	57.82	57.80	57.73	
	βCH_2	26.33	26.33	26.31	26.38	26.38	26.24	26.35	26.38	26.43	
	γCH_2	30.40	30.24	30.26	30.30	30.30	30.19	30.30	30.30	30.25	
	C=O	176.30	175.85	175.36	175.48	175.47	172.50	171.89	171.20	170.85	
	C=O	183.21	183.25	183.27	183.36	183.33	183.09	183.38	183.31	183.25	
	CH_3	27.03									
His	αCH		53.94	51.19	50.18	50.08	52.87	52.37	51.91	51.83	
	βCH_2		29.27	29.79	27.85	27.67	29.32	28.38	27.57	27.12	
	C-2		137.06	137.20	135.28	135.03	137.19	136.29	135.38	134.85	
	C-4		118.08	118.08	118.45	118.47	118.35	118.55	118.69	118.89	
	C-5		133.90	134.00	130.48	129.83	133.88	132.00	130.31	129.47	
	C=O		174.22	173.26	172.05	171.90	175.43	175.67	175.69	175.82	
	CH_3		54.18	36.95	37.05	37.05					
	CH_3			38.20	38.24	38.25					
Pro	αCH						61.51	61.68	61.46	61.56	62.66
							61.42				61.12
	βCH_2						32.56	32.80	32.94	33.08	32.90
							30.56	30.72	30.77	30.75	31.36
	γCH_2						25.65	25.75	25.77	25.73	25.45
							22.92	22.96	22.94	22.86	23.81
	δCH_2						48.90	49.17	49.18	49.13	49.69
							48.20	48.44	48.56	48.58	48.30
	C=O						177.77	177.96	177.89	177.81	178.39
	C=O										174.28
	CH_3										22.72, 22.83

Table 1 (continued)

Solvent		DMSO-d_6			Pyridine-d_5		
Compound		$AcProNH_2$	$<Glu-His-NMe_2$	TRF	$AcProNH_2$	$<Glu-His-NMe_2$	TRF
Residue							
< Glu	αCH		56.87	56.83		60.69	60.54
	βCH_2		30.61	30.65		33.51	33.56
	γCH_2		26.65	26.57		29.68	29.58
	C=O (peptide)		173.49	173.56		176.55	176.55
	C=O		179.03	181.03		182.29	182.37
His	αCH		50.38	53.67		53.72	55.95
	βCH_2		31.28	32.63		34.42	34.53
	C-2		136.26	136.50		139.35	139.56
	C-4		N.O.	N.O.		N.O.	N.O.
	C-5		N.O.	N.O.		N.O.	N.O.
	C=O		172.27	171.46		175.41	175.04
	CH_3		38.97			40.35	
	CH_3		36.76			39.09	
Pro	αCH	61.88		61.59	65.55		64.90
		60.69			63.80		
	βCH_2	33.26		N.O.	36.09		33.56
		31.10		30.83	33.12		
	γCH_2	25.71		25.85	28.86		28.48
		24.05		23.45	26.78		
	δCH_2	48.96		48.29	51.74		51.15
		47.67		47.69	50.53		
	C=O	175.52		175.54	179.80		179.12
	C=O (acetyl)	170.15			174.40		
	CH_3	23.85			26.01		
		23.52					

+ Chemical shifts are reported in ppm downfield from external tetramethylsilane (TMS).
N.O.: Not observable in the solvent used.

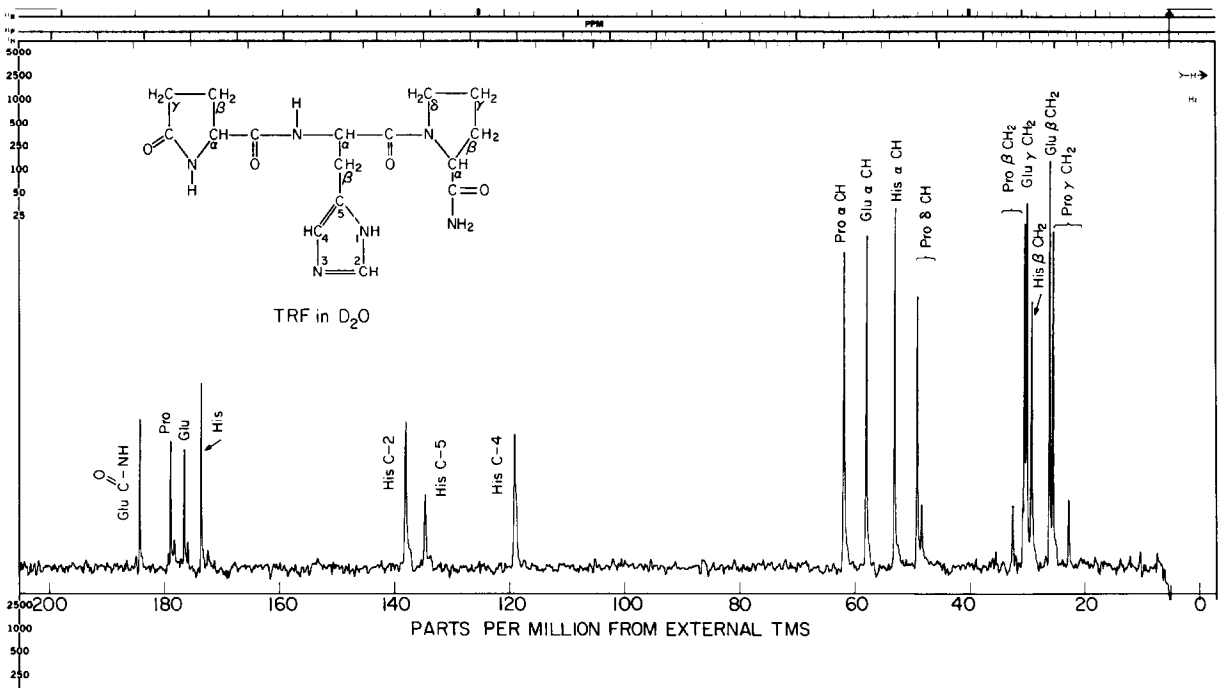

Fig.1. Carbon-13 NMR spectrum of TRF in D_2O, 80 mg/ml, 1 ml in 12 mm outside diameter sample tube, pD 8.9, 7,379 transients, pulse width 30°, cycle time 0.8 sec, 8192 data points.

Table 2

Percentage of *cis* isomer about the histidyl–prolinamide bond in TRF and Ac-Pro-NH_2 in different solvents.

Solvent	TRF *% cis*	Ac-Pro-NH_2 *% cis*
D_2O	14	25
DMSO-d_6	6	34
Pyridine-d_5	0	31

cis Content in TRF was measured at pH 4.7, 5.5, 6.3, 8.9 and found not to vary more than 3% within this range.

assuming that *cis* and *trans* isomers had the same relaxation times and nuclear Overhauser enhancements [8]. The percentage of each isomer [3,4,6] could be measured on the δ, β or γ carbons of proline in each spectrum. Results agreed within 1% in each spectrum. TRF in pyridine-d_5 shows no *cis* isomer, but in the same solvent Ac-Pro-NH_2 exists as 31% *cis* isomer. The largest amount of *cis* isomer (15%) in TRF is found in deuterium oxide, D_2O (fig.1), whereas the least amount of *cis* isomer of Ac-Pro-NH_2 (25%) occurs in this solvent. The assumptions above can lead to errors in estimating the absolute amounts of the two isomers but the relative ratios of *cis* content in TRF and Ac-Pro-NH_2 are significant. All measurements were done on the same sample of TRF and Ac-Pro-NH_2 in the order given in table 2. As a further check, the samples were rerun in D_2O and identical results were obtained. Thus, we conclude the relative amounts of each isomer are solvent-dependent and that the solvent effects are different in the hormone and in the model peptide Ac-Pro-NH_2. It was not possible to measure accurately the spin-lattice relaxation times of the carbons in the *cis* isomers due to their weak intensities.

3.2. Chemical shift assignment

In order to identify all the resonances in TRF, and to look for possible conformational peculiarities in TRF, model peptides were studied. The major differences in the pyroglutamyl resonances of < Glu–His-OMe when compared with those of < Glu-NHMe are in the α carbon and carbonyl carbon of the peptide link; the other carbon resonances of the pyroglutamic acid residue are identical. The spectra of < Glu–His-OMe and < Glu–His-NMe_2 are quite similar. The α carbon of histidine and the carbonyl carbon of histidine and pyroglutamate are the only resonances which differ significantly. These differences are attributed to substituent effects [8,9]. In the case of the

α carbon of histidine, replacing an O-methyl group by an *N*,*N*-dimethylamide group in the γ position produces an upfield shift of 3 parts per million (ppm); the upfield shift of the carbonyl carbon can also be explained on the same basis.

Changing the pH from 8.8 to 4.7 in <Glu–His-NMe_2 produced no effect on the ^{13}C resonances of the pyroglutamyl residue. The changes observed in the histidine residue are caused by protonation of the imidazole ring [10].

The presence of a proline residue in TRF causes the α carbon of histidine to shift downfield 1.7 ppm with respect to its position in < Glu–His-NMe_2. This demonstrates the importance of the γ steric effect of proline [11,12]. When Ac-Pro-NH_2 is compared to TRF, small differences are seen in the proline ring carbon resonances. This is not unexpected. However, carbons in the *cis* and *trans* isomers are not affected to the same extent. This could mean that the *cis* and *trans* proline rings have slightly different conformations [12] or conformational equilibria [13] due to different interactions of the two isomers with the histidine residue.

3.3. Racemized histidine in TRF

Fig.2 shows that one sample of TRF (TRF-R) gave a spectrum indicative of racemization at the α-carbon of histidine. Major effects of racemization are also seen on the proline resonances, but there is no observable effect on those of the pyroglutamate residue. A doubling of the resonances of the α-carbons of histidine and proline is evident. Doubling can clearly be seen in the δ carbon of proline, and in the β and γ carbon region of the spectrum four extra lines are visible. The two extra lines appearing in the δ CH_2 region of proline are attributed to the *cis* and *trans* isomers of proline in the D-His–L-Pro-NH_2 residue; the separation between the lines is different from that of the *cis* and *trans* isomers of proline in the L-His–L-Pro-NH_2 sequence. In the imidazole ring, the D form of histidine is clearly manifest in the resonance of C-4; C-5 shows a slight broadening but no effect is seen on C-2. An important observation here is that the nature of the doubling of the α-CH resonances of proline by *cis–trans* isomerism depends heavily on the nature of the next residue. In Ac-Pro-NH_2 (in D_2O)

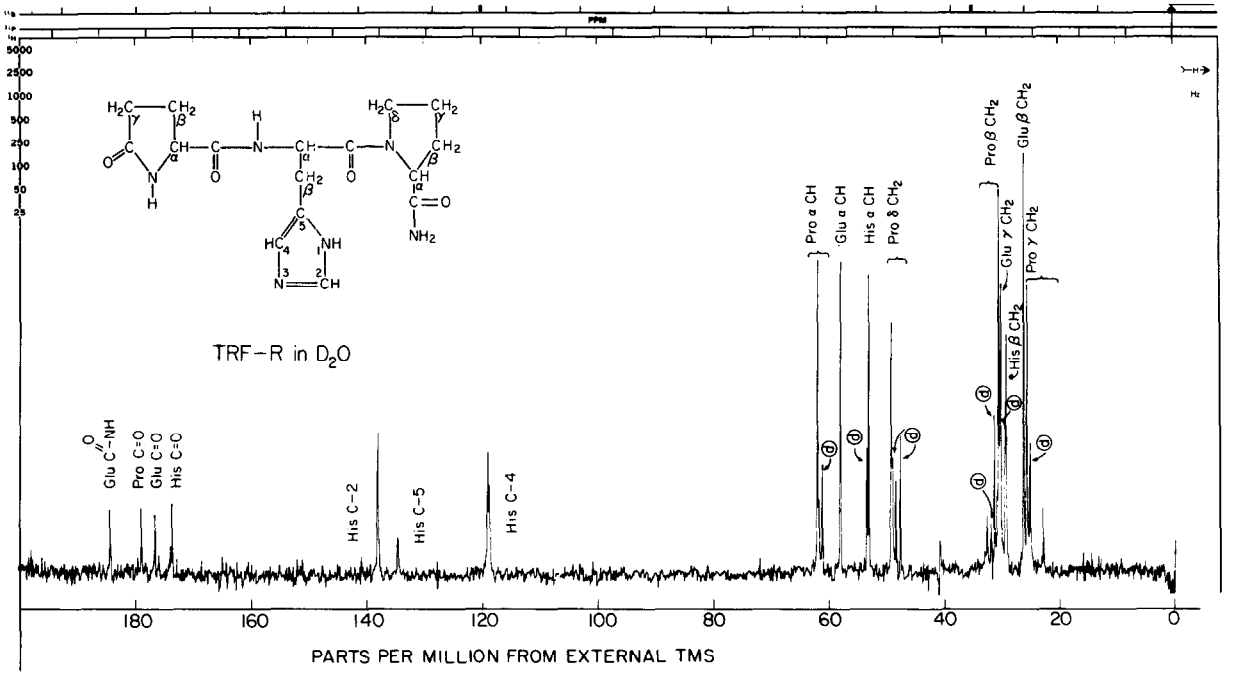

Fig.2. Carbon-13 NMR spectrum of sample of TRF containing both D and L isomers of histidine. Resonances assignable to species containing D-histidine are marked d. Experimental conditions same as fig.1 except 60 mg/ml, 10,000 transients, pulse width 40°, cycle time 0.4 sec, 4096 data points.

and Z-Pro–Leu–Gly-NH_2 [4,5] the resonance of the *cis* C-α is to low field of that of the *trans* C-α. In TRF (in D_2O) the resonance of the *cis* C-α is seen as a shoulder on the high field side of the *trans* C-α resonance. When a D-histidine residue is present next to the proline (in TRF-R), the C-α resonance shifts upfield. It is not possible at present to assert whether the predominant form of proline in this case is *cis* or *trans*. However if one looks at the C-δ resonance of proline in TRF-R in D_2O four lines are present. The two extra lines (which have been attributed to the presence of a D-histidine next to the proline) are almost of equal intensity. This implies that the *cis* and *trans* isomers are almost equally favored in the sequence –D-His–L-Pro-NH_2 in contrast to 14% *cis* isomer in the sequence –L-His–L-Pro-NH_2.

3.4. Solvent effects on histidine

Changing solvent from D_2O to DMSO-d_6 or pyridine-d_5 caused the disappearance of the resonances from the C-5 and C-4 carbons of histidine. It is known that histidine forms strong complexes with paramagnetic metal ions [14, 15]. Proton magnetic resonance studies have shown that copper ions (Cu^{2+}) cause the two imidazole proton resonances to disappear [16]. The effect in this case is not likely to be due to complexing with a paramagnetic metal ion impurity in the solvent because the C-2 resonance was unaffected, and the C-4 and C-5 resonances reappeared when the sample was redissolved in D_2O. A possible explanation resides in the tautomeric equilibrium of the proton on N-1 or N-3 of the imidazole ring of histidine. The resonance of C-2 would be unaffected if the chemical shift of this carbon is unchanged by the presence of the proton on either nitrogen atom. However C-4 and C-5 would have substantially different chemical shifts depending on whether the proton is on N-1 or N-3. If the lifetime of the proton on each nitrogen is similar to the inverse of the separation of the two chemical shifts only a single broadened line will appear. Reynolds et al. [17] have found that the N-3 tautomer is predominant in histidine in basic solution; it would appear this is not true in DMSO-d_6 or pyridine-d_5.

With increasing time in solution the C-2, C-4 and C-5 resonances of TRF, < Glu–His-OMe, and < Glu–His-NMe_2 in D_2O broadened and finally disappeared. Because all three resonances were affected it was thought that paramagnetic metal ions from solvents or syringe needles were responsible. To remove interfering ions H_2S was bubbled through the solutions for 2 min. The solutions were left standing overnight and then centrifuged. After this procedure the imidazole ring carbon resonances reappeared in the correct positions, and no others in the spectra were affected. This confirms that the effect is due to metal ions, as has been demonstrated in ^1H NMR studies [16].

3.5. Spin-lattice relaxation time measurements

Results of T_1 measurements are given in table 3. In < Glu–His-OMe the relaxation time of the O-methyl carbon varies by a factor of three before and after treatment with H_2S. Before treatment it was shorter than that of an α CH group, behaviour which is expected for partial immobilization [8,18] or interaction with a paramagnetic ion. After H_2S treatment the relaxation time of the methyl group is twice as long as that of an α CH carbon. This provides further confirmation that before H_2S treatment the His residue was interacting with paramagnetic metal ions.

If dipole–dipole relaxation is the main relaxation mechanism, CH_2 groups should relax twice as fast as CH groups [8,18]. This is generally observed for the CH and CH_2 groups of histidine in all the compounds studied before and after H_2S treatment, but not in the pyrrolidine and pyrrolidone rings of the prolyl and pyroglutamyl residues, respectively. There is evidence for conformational flexibility in the < Glu ring since the C-β and C-γ T_1's are unequal and larger than expected relative to that of C-α. The gradual decrease in the C-α T_1's from < Glu to Pro suggests that the Pro end of the molecule has less mobility than the < Glu end.

Carbonyl carbon relaxation times are difficult to measure at these concentrations due to their length. Preliminary results indicate that the shortest carbonyl relaxation time in TRF before H_2S treatment is that of the histidyl residue. However, this may be mainly due to complexation with a paramagnetic impurity. No anomalies are noted in the carbonyl T_1 values measured after H_2S treatment. Thus far the T_1 measurements give no suggestion of intramolecular hydrogen bonds which have been suggested for TRF [16,20,21].

Table 3
T_1 Measurements on TRF and model peptides in D_2O, before and after treatment with hydrogen sulfide.[+]

Compound		< Glu–His-OMe		< Glu–His-NMe_2	TRF	
		Before	After	After	Before	After
< Glu	αCH	.67	1.04	1.51	1.01	.93
	βCH_2	.95	.95	1.04	.64	.64
	γCH_2	.88	1.02	.88	.69	.71
	C=O (peptide)				7.9	8.9
	C=O				9.7	10.5
	CH_3					
His	αCH	.85	.73	.98	.46	.75
	βCH_2	.44		.40	.30	.44
	C-2			1.18		
	C-4			.92		
	C-5			.86		
	C=O				4.7	9.4
	CH_3	.56	1.69	> 2.		
	CH_3			> 2.		
Pro	αCH				.69	.50
	βCH_2				.63	.57
	γCH_2				.65	.65
	δCH_2				.40	.39
	C=O				9.2	11.3
	C=O (acetyl)					
	CH_3					

[+] Relaxation times are given in sec.

The present data, and those for oxytocin [4–6,19, 22], angiotensin [10], lysine and arginine vasopressin [5,19] arginine vasotocin [19], melanocyte-stimulating hormone release-inhibiting factor, and luteinizing hormone-releasing factor [19] indicate that Fourier transform ^{13}C NMR is a very sensitive and powerful means of studying the conformations of peptides without resort to isotopic enrichment.

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