PROTON NMR STUDIES ON THYROTROPIN RELEASING FACTOR

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1. Introduction

The synthesis of the tripeptide pyroglutamyl-histidyl-prolineamide (TRF), the thyreotropin releasing factor [1, 2], and its labelling [3] have promoted studies of its mode of action at the molecular level [4]. One important step towards this aim is to define the shape of the molecule and the relationships between biological activity and conformations. For this purpose ^{1}H-NMR investigations of TRF and related peptides have been carried out. The conformation of the histidyl residue was deduced from the relationships between the vicinal coupling constant J *(NH–CαH)* and the dihedral angle (CαH–NH). ϕ was derived from the equation $\theta = (\phi - 60)$ [5–8]. The behaviour of the CαH–CβH$_2$ histidyl protons and the non equivalence of the two prolyl Cδ protons, on one hand, the differences between the *cis* and the *trans*-protons of the terminal amide group on the other hand suggested additional conformational restrictions. On this basis a model of TRF conformation is presented.

2. Material and methods

TRF was a gift of Dr. R.O. Studer (Hoffman-La Roche, Basel). The tetrapeptide Val–His–Pro–Phe was kindly provided by Dr. Riniker (Ciba-Geigy, Basel). The dipeptide Pro–Phe, the amino acids L-proline, L-pyroglutamic acid and L-histidine, were from Sigma. Spectra were run on a TSN 250 MHz (Cameca) and PS 100 MHz (Jeol) spectrometers. Assignment of the protons was realized with the double resonance technique (proton–proton decoupling) and by comparison with the free amino acids and related peptides spectra.

The probe temperature was maintained to ±1°. TMS was used as internal or external reference and chemical shifts are defined on δ scale in ppm. Studies were performed on 0.05 to 0.1 M sample solutions.

3. Results

The assignments of the chemical shifts of TRF and of some of the related compounds are reported in table 1. A typical spectrum is shown in fig. 1, where bridges indicate resonance patterns connected by decoupling.

The *trans* and *cis* proton signals belonging to the terminal $CONH_2$ are located, respectively, at 7.94 and 6.89 ppm. The chemical shift difference observed (1.05 ppm) between the *cis* and *trans* protons is large when compared to the values reported for acetamide and propionamide, (0.60 and 0.55 ppm) [9] or observed for the Asn β $CONH_2$ of angiotensinamide II (0.60 ppm) [10]. The imidazole C_2H and C_4H resonances are found at 7.51 ppm and 6.89 ppm. Thus the imidazole C_4 proton overlaps with the *cis* $CONH_2$ proton signal. The chemical shifts of these two imidazole protons are nearly the same as for their homologs in Val–His–Pro–Phe. Their peaks are sharp when the TRF is dissolved in dry DMSO. However,

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Table 1
TRF proton assignments and comparison with several related compounds chemical shifts, TMS as external or internal references.

	$C_\alpha H$	N–H	$C_\beta H$	$C_\gamma H$	$C_\delta H$	Other compounds	
PCA	4.04	7.77	1.82 2.20	2.10			
	4.01	7.82	1.86 2.24	2.10			(6)
His	4.64	8.07	2.89			C_2H 7.51 C_4H 6.89	
	4.68	8.46	3.10			C_2H 7.52 C_4H 6.88	(1)
	3.85		3.13			C_2H 7.77 C_4H 7.01	(4)
	4.07		3.19			C_2H 8.06 C_4H 6.14	(5)
Pro	4.22		2.10 1.82	1.82	3.57 3.23	$-N<^{Hcis}_{Htrans}$ 6.89 7.94	
	4.33		1.60 1.80	1.80	3.59 ≃ 3.30		(1)
	4.59 *cis*		≃ 2.10	≃ 2.10	3.47		(2)
	4.39 *trans*		≃ 2.10	≃ 2.10	3.47		
	4.25		1.90 2.35	1.90	3.35		(3)

(1) Val–His–Pro–Phe/DMSO, 20°. (2) *N*-acetyl-proline/D_2O, 40°, neutral pH, from McDonald and Phillips. (3) Pro–Phe/D_2O, 20°, neutral pH. (4) Histidine/D_2O, 20°, neutral pH. (5) L-His–L-Leu/D_2O, 40°, neutral pH, from Mc Donald and Phillips. (6) PCA/DMSO, 20°.

when a drop of water is added to the solution, the C_4H signal broadens whereas the C_2H signal remains unchanged. Moreover, one observes at about 12 ppm, a broad assigned to the imidazole NH resonance, and of course at about 3.4 ppm the very intense water peak. A correlation between these two peaks had been established with double resonance technique.

The amide cyclic proton of the pyroglutamyl residue appears as a singlet at 7.77 ppm and irradiation in the $C_\alpha H$ area (4.04 ppm) does modify only its intensity. Comparison with the free pyroglutamic acid spectrum recorded in the same conditions shows no significant difference in signal positions without or with irradiation experiments.

The doublet at 8.07 ppm for which a coupling constant of 7.5–8 Hz is measured, belongs to the histidyl amide proton signal. Upon irradiation at 4.64 ppm this doublet coalesces as a singlet. It should be noted that histidyl amide resonance in Val–His–Pro–Phe is located more downfield, at 8.46 ppm. In the $C_\alpha H$ area the multiplet at 4.64 ppm assigned to the histidyl $C_\alpha H$ is transformed upon irradiation of the NH doublet in a triplet (X part of an ABX system) with a coupling constant of about 6 H corresponding to JC_α H–$C_\beta H_A$, nearly equal to JC_α H–$C_\beta H_B$. With double resonance technique the AB part (histidyl $C_\beta H_2$ centered at 2.89 ppm) of the ABX system, is changed to a very well defined quadruplet with $JC_\beta{<}^{H_A}_{H_B}$ nearly equal to 16 Hz. By using the Pachler assumption [11] and the values proposed by Abraham and McLaughan [12], we can determine that the mole fraction of the staggered conformer (fig. 2b) could be present at about 39%. This means that this conformation which would appear to be least favored sterically is slightly stabilized.

Decoupling measurements and comparison with the

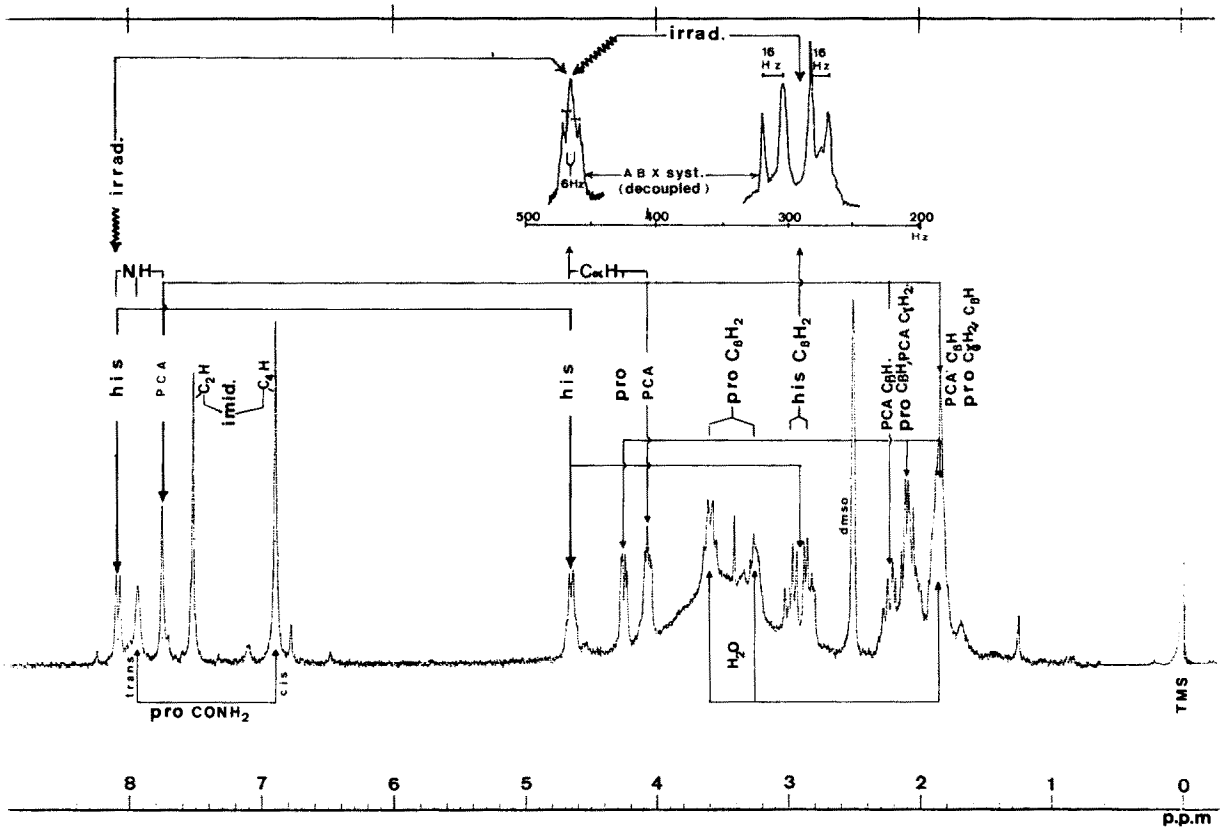

Fig. 1. 250 MHz ^{1}H-NMR spectrum of TRF dissolved in d_6 DMSO at 20°. Bridges indicate the coupled protons as determined by irradiation.

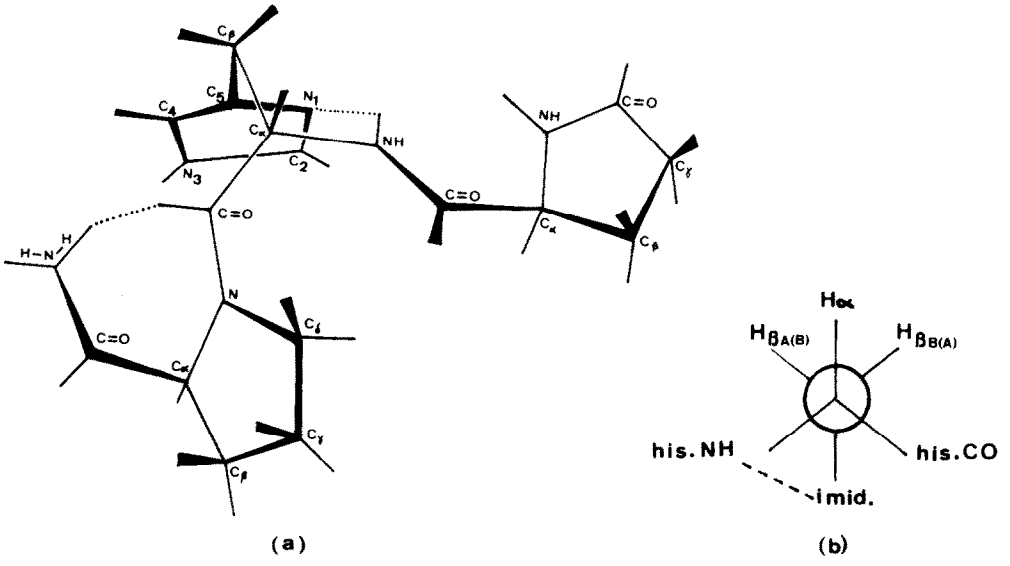

Fig. 2. (a) A proposed model for TRF (PCA-His–Pro-NH_2). (b) A preferential conformation of *His* side chain.

spectra of free amino acids and related compounds, lead to the assignments of prolyl $C_\alpha H$ (4.22 ppm), $C_\beta H_2$ (2.10 and 1.82 ppm), $C_\gamma H_2$ (1.82 ppm) and pyroglutamyl $C_\alpha H$ (4.04 ppm), $C_\beta H$ (1.82 and 2.20 ppm) in spite of the overlapping of the upfield peaks.

Finally, an important feature concerns the two quadruplets observed at 3.57 ppm for the propyl $C_\delta H_2$. Such a pattern is also found in the Val–His–Pro–Phe spectrum but never in that given by proline alone.

3. Discussion

Let us first consider the peptide backbone: the dihedral angle corresponding to the $C_\alpha H$–NH coupling constant $JC_\alpha H$–NH (7.5–8 Hz) for histidine has been derived on the basis of Bystov et al. [13] calculations and empirical curves, and from Gibbons et al. [7] and Ramachandran et al. [6] work on peptide conformations. To choose between the three possibilities offered for Φ, $-150°$, $-90°$ and $+60°$, Dreiding models have been made. Maximum stability is found for $\Phi = -150°$. In this case, interaction between $N^{1\,im.}$ and the histidyl amide proton might be possible if the side chain of this residue is in the staggered conformation seen above (fig. 2b). Evidences from the literature are in agreement with this preferential conformation a) the observation of an anomalous pK (6.25) for the imidazole ring [14], b) a loss or a potentialization of the biological activity, respectively, for $N^{1\,im.}$ Me-TRF and $N^{3\,im.}$ Me-TRF [15]. This indicates a probable involvement of the $N^{1\,im.}$ in a hydrogen bond and confers to this nitrogen an important structural role.

The C-terminal amide protons yielded two separate signals ($\Delta\delta$ *cis–trans* = 1.05 ppm). The anomalous downfield shift of the *trans* proton seems to indicate some interaction. It has long been recognized that protons involved in a hydrogen bond exhibit a very marked deshielding [16–18]. In the case of TRF, examination of the molecule suggests immediately a hydrogen bonding involving both the *trans* C-terminus amide proton and the histidyl carbonyl group. In this situation with the prolyl residue in *trans* configuration, a stable equatorial seven-membered ring is created [19–21]. Such a conformation would produce a lock of the prolyl residue. Moreover the two signals given by the prolyl $C\delta H_2$ indicate a high asymmetry at the ring level and could be the result of the nitrogen free doublet upfield effect on the axial anticoplanar Cδ proton [22].

The pyroglutamyl NH is not involved in a hydrogen bond and this result is in agreement with those reported [14] showing that N-Me PCA-His–Pro-NH_2 presents no modification of the imidazole pK.

The last observation is the broadening of the imidazole C_4H signal only, without change of the C_2H signal, in presence of water. This phenomenon is not yet clearly understood, and is upon investigation. The proton exchange occurring between the nitrogen and a water molecule could be responsible for the modification observed.

4. Conclusion

The TRF model proposed here (fig. 2a) presents as essential features two hydrogen bonds: the first holds the imidazole ring of histidyl in a well defined orientation (maybe in relation with the biological activity). The second hydrogen involving one C-terminal $CONH_2$ proton and the histidyl carbonyl group, stabilizes the peptide backbone defining a seven membered-ring. Interesting is the comparison with the results obtained for the octapeptide angiotensine II and Val–His–Pro–Phe [10] which both exhibit a hydrogen bond between the C-terminal phenylalanine NH and the histidyl carbonyl group. This would mean that in even short peptides a stabilizing event could occur around the propyl residue. If this generalization would be true it would account for the essential role of proline in a variety of biologically active peptides. Thus, it could well be that the resulting conformations correspond to structural requirements associated with the characteristics of more than a few types of receptor sites.

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