^{1}H NUCLEAR MAGNETIC RESONANCE STUDIES OF THYROTROPIN RELEASING FACTOR (TRF)

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Received 25 February 1974

1. Introduction

Recently there has been much speculation about whether or not thyrotropin releasing factor (Pyroglu-His-ProNH_2) has a preferred conformation in solution stabilised by hydrogen bonding and some inferences that this might have relevance to its biological activity. Grant and co-workers [1] noted anomalous pK values for the imidazole protonation in TRF and interpreted this in terms of hydrogen bonding structures involving the nitrogen of the imidazole ring and the α-NH of the histidine to form a six-membered ring. Fermandjian et al. [2], from proton NMR measurements in DMSO-d_6 solution, have proposed a structure with this hydrogen bond and a further hydrogen bond between the His peptide carbonyl oxygen and the trans proton of the Pro amide group. Other workers [3], on the basis of semi-empirical energy calculations and the pH dependence of the proline δ-protons have proposed that this second hydrogen bond acceptor is the carbonyl oxygen of the pyroglutamyl* residue (rather than that of the histidine) making a β-turn in TRF. Deslauriers et al. [4] have also considered this possibility on the evidence of a high trans population of the Pro-NH_2 residue as deduced from ^{13}C spectral studies, although in a related paper some of the same authors [5] report that ^{13}C T_1 measurements give no suggestion of intramolecular hydrogen bonds.

We have compared the ^{1}H chemical shifts of the NH protons of TRF and N-acetyl proline amide in water and find no evidence for hydrogen bonding. Furthermore the temperature dependence of the NH proton chemical shifts for TRF in DMSO shows normal behaviour and does not support the presence of hydrogen bonded structures.

2. Experimental

The ^{1}H spectra were obtained at 100 and 220 MHz using Varian HA100 D, XL-100 and HR 220 spectrometers. The samples were examined as solutions containing 15–30 mg of the peptides in 0.5 ml of solvent (H_2O, $(CD_3)_2SO$ and $CDCl_3$). Tetramethylsilane and sodium 4,4 dimethyl-4-silapentane were used as internal references for the DMSO and H_2O solutions respectively. Variable temperature measurements were made using the Varian temperature control unit and the pH measurement were made at 22°C using a glass electrode Radiometer model 26 pH meter. The reported pH values are uncorrected for deuterium isotope effects.

Proline amide and N-acetyl proline amide were obtained from Sigma Chemical Company. The TRF and □-GluHisOCH$_3$ samples were supplied by Drs M. J. Smithers and H. Gregory (ICI Pharmaceuticals Ltd, England) and a sample of TRF was provided by Dr R. O. Studer (Hoffman-La-Roche, Switzerland).

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*□-Glu is the symbol used for the pyroglutamyl residue.

3. Results and discussion

Proton NMR measurements on TRF, □-GluHisOCH$_3$ and *N*-acetyl proline amide were carried out in DMSO-d_6 and aqueous solutions.

3.1. *No NMR evidence for intramolecular hydrogen bonding*

Fig. 1. shows the ^{1}H spectrum of TRF in DMSO-d_6; the intense bands have been assigned previously by Fermandjian and co-workers[2] and correspond to the molecules in the trans proline configuration. ^{13}C NMR studies by Deslauriers and co-workers [4,5] have established the presence of ~ 6% of the cis isomer in DMSO-d_6 solution and the small bands in the ^{1}H spectrum are assigned to the TRF isomer

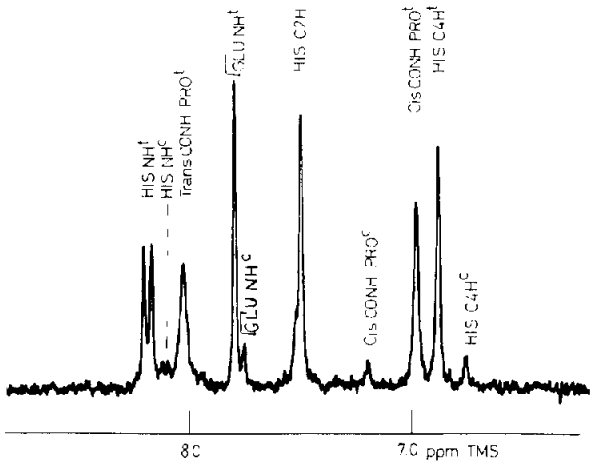

Fig. 1. The low-field region of the ^{1}H spectrum of TRF in DMSO-d_6 at 220 MHz. The superscripts t and c refer to the trans- and cis-proline configurations.

with the cis–Pro configuration. The possibility that they are due to racemisation at the α-carbon histidine as noted in the ^{13}C spectrum of one of the samples studied in reference 5 is discounted, since these peaks were visible in two samples examined by us from different sources. It is interesting that His-C2H, C4H, NH and α-CH all have different shifts in the two isomers: the His-C2H and NH assignments in the cis-Pro-containing isomer were confirmed by double resonance experiments in which the C4H and α-CH protons were irradiated respectively. By measuring the spectra at different temperatures it was found that all the observed peptide NH and amide proton absorptions have essentially the same temperature coefficients for their chemical shifts as indicated in table 1. Furthermore, the observed temperature coefficients are similar to values normally expected from non-intramolecularly bonded NH protons in DMSO solution which is direct evidence against the possibility of any of the NH protons being involved in structure forming intramolecular hydrogen bonds.

Table 1
Temperature dependencies of NH proton chemical shifts in TRF

Proton assignment	Temperature coefficient* (ppm/10°C)	
	DMSO	H_2O (pH 1.5)
His NH^t	0.065	0.071
Trans Pro NH^t	0.067	0.075
Cis Pro NH^c	0.06†	–
Cis Pro NH^t		0.065
□-Glu NH^t	0.043	0.065

* Errors ± 0.005 ppm
† Error ± 0.01 ppm

Fermandjian and co-workers [2] had originally suggested intramolecular hydrogen bonding to explain the large shift difference (1.05 ppm) between the cis- and trans-amide protons of TRF (trans Pro) in DMSO solution. Examination of table 2 reveals that the chemical shift differences between the cis and trans amide protons in proline amide, *N*-acetyl proline amide and TRF in different solvents behave somewhat erratically. It is likely that solvent interactions make important contributions to the chemical shift differences between the amide protons. Without a detailed understanding of these effects it is dangerous to interpret observed chemical shifts in terms of the absence or presence of intramolecular hydrogen bonds.

In aqueous solutions the amide protons of proline amide, *N*-acetylproline amide and TRF have similar chemical shift differences to each other and to other amides: this suggests that in aqueous solutions (pH 1.5 to 7.55) of TRF there is no intramolecular hydro-

Table 2
The ^{1}H chemical shifts* of the amide protons in TRF and related molecules

Compound	Solvent	δ cis ppm	δ trans ppm	(δ cis − δ trans) ppm
Proline amide	DMSO	7.866	8.415	0.549
Proline amide	H_2O	7.603	8.163	0.560
N-acetyl proline amide (trans Ac)	DMSO	7.038	7.417	0.379
	$CDCl_3$	6.174	7.460	1.286
N-acetyl proline amide (cis Ac)	DMSO	7.306	7.695	0.389
	$CDCl_3$	6.677	7.020	0.343
N-acetyl proline amide	H_2O	7.256	7.928	0.672
TRF (trans Pro)	DMSO	6.894	7.944	1.050
TRF (cis Pro)	DMSO	7.127	–	–
TRF	H_2O (pH 1.5)	7.37	8.052	0.682
	H_2O (pH 4.55)	7.38	8.06	0.657
	H_2O (pH 7.55)	7.38	8.01	0.625

* Chemical shifts measured from TMS (DMSO and $CDCl_3$ solutions and DSS (H_2O solutions) reference compounds.

gen bonding involving the proline amide protons. This is supported by the observed normal temperature dependencies of the chemical shifts of the amide protons in aqueous solutions of TRF.

3.2. *Backbone Conformation*

The only source of backbone conformational information in the proton spectrum of TRF is the J_{NC} coupling constant (7.5 Hz) between the His αCH and NH protons measured from the His-NH resonance band. This value is consistent with the random-coil configuration calculated from the ψ/ϕ potential energy map of Blagdon and co-workers [3] which in conjunction with the Bystrov-Karplus relationship for J_{NC} and ϕ dihedral angles [6] gives a calculated value of 7.5 to 8.0 Hz. However, the observed value could also result from some other distribution of conformations or from a fixed conformation with any of the values −151, −89, 45 or 74*. This is consistent with a minimum energy conformation of TRF calculated by Burgess and co-workers [7] in which an extended form of the molecule has $\phi = -150°$. It could not however have the 'hairpin-turn' conformation (ϕ = −54°) suggested as a possibility by the energy calculations of Blagdon and co-workers [3]. In the absence of additional information it is not possible to assume the molecule to have other than a random-coil backbone configuration.

3.3. *Side-chain conformation*

The HisαCH-βCH$_2$ absorption bands are clearly resolved in the ^{1}H spectrum of TRF recorded under various conditions and the two vicinal proton coupling constants ($J_{H_\alpha H_{\beta_1}}$ and $J_{H_\alpha H_{\beta_2}}$) can be extracted from an ABX analysis of the spectra. Table 3 summarises the His side chain proton–proton coupling constants for TRF and related molecules in different solvents and under different ionisation conditions.

The coupling constants can be used to estimate the fractional populations p_I, p_{II} and p_{III} of rotamers I to III for the His side chains. The measured vicinal coupling constants J_{AX} and J_{BX} are averaged values of the gauche (J_g) and trans (J_t) vicinal coupling constants in rotamers I to III weighted according to the fractional populations. It is assumed that the J_g and J_t values are the same in the different rotamers and that the values are J_g = 2.56 and J_t = 13.6 Hz as obtained in model peptide studies [8]. Usually one cannot assign the A and B protons and thus it is impossible to distinguish between rotamers I and II.

* For definition of ϕ and ψ see IUPAC-IUB. Commission of Biochemical Nomenclature Report (Biochemistry, 1970, 9, 3471).

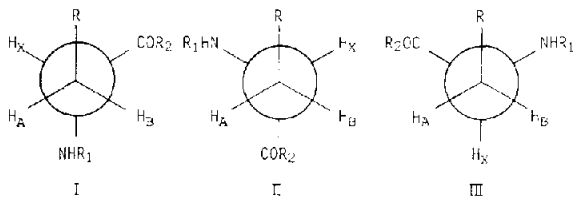

Table 3. contains the rotamer fractional populations calculated by assuming that a mixture of rotamers is present. Thus it is seen that TRF and $\square$GluHisOCH$_3$ do have some conformational differences mainly for the populations of rotamers I and II: this could simply be a reflection of the larger size of the group R_2 in TRF (in $\square$GluHisOCH$_3$, R_2 = OCH$_3$: in TRF, R_2 = ProNH$_2$) which is known to increase the population of the rotamer with the bulky substituent gauche to H_A and H_B (rotamer II) [9]. Some workers [3] have considered the possibility that the His side chain in TRF is in a fixed conformation resulting from a hydrogen bonding interaction between an imidazole nitrogen and the His peptide NH proton. Such an interaction would give rise to an exclusive population of rotamer III and would be expected to be influenced by changing the ionisation of the imidazole ring: however it is seen from table 3. that the His $H_\alpha H_\beta$ coupling constants are essentially the same above and below the pK value for the imidazole ring. The same authors [3] have also cited the change in shift of the δ proline protons on protonation of the imidazole ring as evidence of the hydrogen bond being broken. Even in a random-coil configuration, the proline ring will have a significant lifetime adjacent to the imidazole ring and may thus be expected to reflect the ionisation behaviour of the histidine residue. Considering the constancy of the His$H_\alpha H_\beta$ coupling constants with changing pH together with the normal temperature dependence of the His peptide NH proton, it seems unlikely that such hydrogen bonding exists in this system.

4. Conclusions

The normal temperature dependence of the ^{1}H NMR spectrum of TRF does not provide evidence for intramolecular hydrogen bonding. No evidence for other than a random-coil configuration for TRF could be found in the spectrum. Similar findings have been reported for other small linear peptides such as pentagastrin [9] and luteinizing hormone releasing hormone [10]. It would appear that for these peptides where the active groups are in close proximity to each other, the hormone structure can possibly organise itself into its bound conformation during the actual binding process to the receptor. However, one cannot exclude the possibility that the receptor molecule binds to a small fractions of molecules with the correct conformation for binding present in the random-coil equilibrium mixture of conformers.

Table 3
^{1}H–^{1}H spin-coupling constants (Hz) between the His side chain protons in TRF and $\square$GluHisOCH$_3$

Compound	Solvent	H–H Coupling Constants (Hz)			Fractional Populations*		
		J_{AB}	J_{BX}	J_{AX}	p_I	p_{II}	p_{III}
TRF	D_2O (pH 7.9)	14.6	6.2	8.4	0.33	0.53	0.14
TRF	D_2O (pH 1.1)	15.4	6.2	7.8	0.33	0.48	0.19
TRF	DMSO-d_6	13.5	5.6	7.9	0.28	0.48	0.24
$\square$GluHisOCH$_3$	D_2O (pH 7.1)	15.4	4.4	9.2	0.17	0.60	0.23
$\square$GluHisOCH$_3$	D_2O (pH 1.1)	15.3	4.7	9.2	0.19	0.60	0.21
$\square$GluHisOCH$_3$	DMSO-d_6	14.4	4.3	8.7	0.16	0.56	0.28

* It is not possible to assign A and B unequivocably and rotamer populations for I and II may be interchanged.

Errors on coupling constants ± 0.2 Hz.

Acknowledgements

We thank Dr M. J. Smithers (ICI Pharmaceuticals Ltd, England) and Dr. R. O. Studer (Hoffman-la-Roche, Switzerland) for generously supplying the TRF samples and the Science Research Council for providing facilities to make the measurements at 220 MHz.

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