CONFORMATIONAL ANALYSIS OF AN ANALOGUE OF THYROLIBERIN (T.R.F.)

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Received November 23,1976

S U M M A R Y

A preferential conformation of <Glu-(2-thienyl)-Ala-Pro-NH_2 (Thi^2-TRF) in DMSO-d_6 and D_2O is proposed from 270 MHz proton magnetic resonance spectra and is compared to that of TRF. Thi^2-TRF which retains 30 % of the activity of TRF in vivo has in solution a three-dimensional structure different from that of TRF. The differences between the NMR spectra of Thi^2-TRF and TRF itself can be explained by a change in the preferred conformation of the side chain of the second residue. The results are discussed in relation to the biological activity of TRF-analogs.

I - INTRODUCTION

Numerous analogs of thyroliberin, TRF (<Glu-His-Pro-NH_2), have been synthetized and tested for thyrotropin (TSH) and prolactin release (1-7). In general, even small chemical modifications result in a substantial loss of hormonal activity and so far only two peptides, N_ε-Me-His^2-TRF (8) and Pyr(1)Ala^2-TRF(9) have been found to be more potent than TRF. Replacement of the imidazole ring of the histidine residue with a neutral aromatic ring (phenyl, thienyl) leads to potent analogs.

Another approach to elucidate the structure-activity relationships of TRF is to investigate the spatial structure of the hormone and the relation between conformation and biological activity (10,22). Several of these studies have shown that the central residue is in an extended conformation (10,12,16,20). Moreover, it has been found that an interaction maintains in TRF, the histidine side chain preferentially folded back over the prolinamide residue (16,20). We have shown that the hyperactive analogue N_ε-Me-His^2-TRF exhibits all these structural features (21).

In order to analyze how important the conformation of the aromatic imidazole ring is for the activity of TRF, we have investigated the structure of <Glu-β(2-thienyl)-Ala-Pro-NH_2 (Thi^2-TRF) and <Glu-β(2-thienyl)-Ala-Pro-OMe.

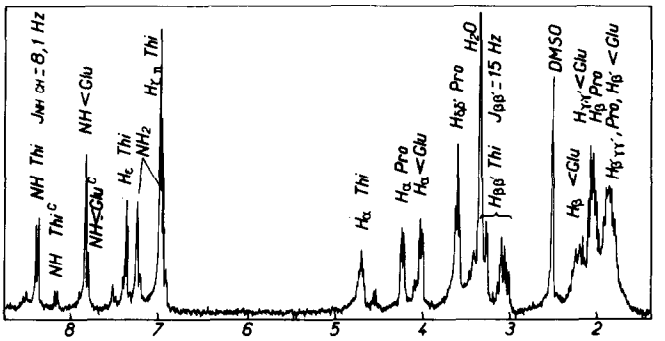

Fig. 1. 270 MHz spectrum of Thi^2-TRF in DMSO-d_6 at 18°C.

SIEVERTSSON et al. have shown that Thi^2-TRF retains 30 % of the activity of TRF in vivo and 75 % in vitro while the corresponding ester is inactive. In this paper, we report on the conformational behavior of these analogs determined by proton nuclear magnetic spectroscopy.

II - MATERIALS and METHODS

- Synthesis :

The peptides have been synthetized by classical methods in solution as previously described by SIEVERTSSON et al. (6).

- Spectroscopic determinations :

The spectra were obtained at 270 MHz using a BRUKER W.H. 270 spectrometer. Samples were examined in deuterated dimethylsulfoxide (DMSO-d_6) and D_2O at concentrations of 10 to 20 mg/ml. Chemical shifts are reported downfield from an internal reference of tetramethylsilane (TMS) or sodium trimethyl silyl-3propane sulfonate (TMPS).

III - RESULTS

^{1}H NMR spectrum of Thi^2-TRF in solution in DMSO-d_6 is presented in figure 1. Unambiguous assignments of the resonances were obtained by the double resonance technique. Interpretation of the spectra was also facilitated by comparison with those of TRF (16,20) and model compounds containing the Thi residue, such as N-formyl-Thi,N-formyl-Thi-Pro-NH_2.Details on chemical shifts and coupling constants are given in tables I and II.

The Thi^2-TRF spectrum does not exhibit all the typical spectral

TABLE I

CHEMICAL SHIFTS OF Thi^2-TRF AND MODEL COMPOUNDS IN DMSO-d_6 AND D_2O AT 20°C

	HCO-Thi	HCO-Thi-Pro-NH_2		<Glu-Thi-Pro-NH_2		<Glu-Thi-Pro-OMe		TRF (16)	
	DMSO	DMSO	D_2O	DMSO	D_2O	DMSO	D_2O	DMSO	D_2O
<Glu									
H_α				4,03	4,29	4,0	4,29	4,04	4,30
H_β				2,20	2,48	2,20	2,47	2,20	2,48
H_γ				2,08	2,00	2,08	2,01	2,10	1,97
NH				7,80	--	7,82	--	7,85	--
Thi									
H_α	4,52	4,78	4,98	4,71	4,95	4,72	4,92	4,64	4,90
H_β	3,30	3,30	3,41	3,34	3,41	3,23	3,41	3,0	3,06
	3,14	3,00	3,23	3,05	3,23	3,05	3,23	2,83	3,06
H_ε	7,35	7,33	7,35	7,33	7,34	7,34	7,41	7,58	7,66
H_δ	6,95	6,95	7,00	6,95	7,00	6,95	7,07	6,95	7,00
H_η	6,91	6,94	7,00	6,94	7,00	6,94	7,07	--	
NH	8,43	8,53	--	8,35	--	8,39	--	8,25	
Pro									
H_α		4,23	4,38	4,25	4,40	4,31	4,45	4,22	4,40
H_β		2,08	2,29	2,08	2,34	2,08	2,34	2,08	2,35
H_γ		1,86	2,00	1,87	2,00	1,81	2,01	1,80	1,97
H_δ		3,62	3,88	3,62	3,83	3,63	3,83	3,57	3,80
		3,62	3,66	3,62	3,67	3,48	3,59	3,23	3,50
NH_2		7,19		7,21				8,06	
NH_2		6,94		6,94				7,00	

features of TRF. Differences are observed for some resonances of the prolinamide residue, in particular :

a) an upfield shift of the "anti" trans carboxamide resonance of ~ 0,75 ppm

b) the disappearence of the non equivalence of the H_δ-Pro protons in DMSO-d_6 and a downfield shift of 0,4 ppm of one of these protons.

TABLE II

COUPLING CONSTANTS AND POPULATIONS OF Thi SIDE CHAIN ROTAMERS

	DMSO-d_6				D_2O			
	*$J_{\alpha\beta}$-$J_{\alpha\beta'}$	a	b	c	$J_{\alpha\beta}$-$J_{\alpha\beta'}$	a	b	c
HCO-Thi	4,75 8,50	28	19	53				
HCO-Thi-Pro-NH_2	4,0 9,25	26	13	61	6,0 8,0	20	31	49
<Glu-Thi-Pro-NH_2	4,5 9,0	24	17	59	5,5 8,75	18	26	56
<Glu-Thi-Pro-OMe	5,2 8,0	27	24	49	6,0 8,5	16	31	53
<Glu-His-Pro-NH_2	7,6 5,3	36	42	22	6,4 8,4	19	32	49

**In all the Thi peptides, the high field coupling constant $J_{\alpha\beta}$, is larger than the low field coupling constant $J_{\alpha\beta}$.*

These spectral results are quite similar to those of Phe^2-TRF reported by DONZEL et al. (20). The H_β-Thi protons are widely separated into two distinct patterns at 3,34 and 3,05 ppm. This split is much larger than that observed for the corresponding protons of histidine in TRF. Also the vicinal coupling constants in the C_α-C_β fragment of the second residue side chain are much more non equivalent in Thi^2-TRF (Table II). The aromatic proton resonances observed at 7,35 and 6,95 ppm in N-formyl-Thi are not influenced by the addition of the two other residues, <Glu and Pro-NH_2.

Comparison between the spectra of Thi^2-TRF and TRF reveals also some similarities. Thus, the weak peaks due to the "cis"-Pro isomer are observed. The value of the vicinal coupling constant J_{NH-CH} for the second residue is close to that measured for TRF. All the backbone amide NH resonances manifest a significant upfield shift with increasing temperature. Measurement of their chemical shifts at different temperatures (20-60°C) indicates that the temperature coefficients $\Delta\delta$ NH/ΔT are quite similar for corresponding protons of TRF and Thi^2-TRF (NH<Glu = $4,66.10^{-3}$ppm/°C ; NH Thi = $6,3.10^{-3}$ppm/°C ; NH_2"trans" in Pro-NH_2 = $5,3.10^{-3}$ppm/°C ; NH_2"cis" in Pro-NH_2 = $5,0.10^{-3}$ppm/°C). Replacement of the pyroglutamyl residue in Thi^2-TRF by a formyl group (N-formyl-Thi-Pro-NH_2) does not affect the resonances of the two other residues. Changing solvent from DMSO-d_6 to D_2O produces a downfield shift of one of the H_δ-Pro protons and a slight modification of the vicinal coupling constants J_{CH-CH} of the second residue.

The spectrum of <Glu-Thi-Pro-OMe is different from that of Thi^2-TRF but it is essentially similar to those of <Glu-Leu-Pro-OMe (24) and <Glu-His-Pro OMe (20).

IV - DISCUSSION

These NMR data indicate that Thi^2-TRF has in solution a three-dimensional structure different from that of TRF at least concerning the spatial arrangement of the second and third residues. Analysis of the chemical shifts of the prolinamide (H_δ, NH_2 protons) and thienyl (H_δ, H_ε, H_η) resonances show that the interaction which was found in TRF between the side chain of the second residue and the prolinamide residue does not occur in the case of Thi^2-TRF. This is evidenced by the fact that the thienyl ring does not cause any magnetic anisotropic effect on the proline resonances neither in DMSO-d_6 nor in D_2O ; the sulfur aromatic ring is expected to produce a ring current effect of the same order of magnitude as that due to imidazole. The values of the $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ coupling constants of the $-CH_\alpha-CH_{2\beta}-$ fragment of the thienyl side chain can be used to estimate the fractional populations a,b,c of the three rotamers A,B,C, respectively, defined by the χ_1 angle = 60°, 180° and 300°(25). Usually one cannot assign the β and β' protons and thus it is impossible to choose between rotamers B and C. However the absence of an interaction between the thienyl ring and the prolinamide residue suggests that the C rotamer is preferred in both examined solutions. Thus, in the analogue the thienyl side chain is preferentially "trans" to the proline residue while in TRF the histidine side chain is "gauche". Rotation about the $C_\beta-C_\gamma$ bond of the thienyl residue is probably hindered judged by the pronounced non equivalence of the H_β-Thi resonances. However we are not able to precise the value of the χ_2 angle.

Moreover, the NMR data reveal that Thi^2-TRF exhibits some of the structural features of TRF. In DMSO-d_6 the analogue presents a "cis-trans" isomerism of proline, and evaluated from the relative heights of the NH peaks the "cis" content appears to be $\sim$ 20 %. This amount is larger than that found for TRF ($\sim$ 6%) but it is less than that observed in AC-Pro-NH_2 ($\sim$ 34%) (17). The increased "cis" content is probably due to the absence of an interaction between the thienyl and proline residues. Comparison between N-formyl-Thi-Pro-NH_2 and Thi^2-TRF shows that the <Glu residue is exposed to solvent molecules. No interaction of the lactam ring with the backbone or the thienyl side chain occurs. The temperature dependence experiments support this conclusion and suggest that no strong intramolecular hydrogen-bonding stabilizes the conformation of Thi^2-TRF. One important

source of backbone conformational information in the proton spectrum of peptides is the J_{NH-CH} coupling constant from which the torsional ϕ angle can be deduced. In DMSO-d_6, structures defined by ϕ_2 = - 150° or - 90° are consistent with the measured J_{NH-CH}-Thi coupling constant.

The differences between the NMR spectra of Thi^2-TRF and the corresponding ester could be explained by a change in the preferred backbone conformation.

The observed conformational differences between TRF and Thi^2-TRF do not seriously impair the biological activity, since the analogue retains 30 % of the effect of TRF *in vivo* and ∿ 75 % *in vitro* (23). This means that the three dimensional appearance of the side chain in the second position might not be the only crucial factor for the recognition of the molecule by the receptor. On the contrary, this process seems to depend more on the structural characteristics of the <Glu and the Pro-NH_2 moieties, since high biological activity appears to be intimately connected with the rigidity and functionality in these positions. However, at a more advanced position in the receptor binding process specific chemical characteristics of the side chain of the second residue, such as aromaticity and polarisation, may become important. This interpretation might be supported by the finding that <Glu-Leu-Pro-NH_2 has about 2 % of the activity of TRF *in vivo* (23), while its conformational picture is similar to that of TRF (22).

ACKNOWLEDGEMENT

Supported by the Swedish Medical Research Council 4135

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MOLECULAR EVENTS IN LYMPHOCYTE DIFFERENTIATION: STIMULATION OF NONHISTONE NUCLEAR PROTEIN SYNTHESIS IN RABBIT PERIPHERAL BLOOD LYMPHOCYTES BY ANTI-IMMUNOGLOBULIN

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Received November 23,1976

SUMMARY: Using a sensitive double-labelling technique, changes in protein synthesis were investigated in rabbit peripheral blood lymphocytes at early times following stimulation with heterologous anti-immunoglobulin serum (GARG) or concanavalin A (Con A). At four hours following stimulation with either GARG or Con A, a striking increase in the synthesis of a nonhistone nuclear protein of apparent molecular weight 30-40,000 was observed. These results suggest that lymphocyte activation by both anti-immunoglobulin and Con A includes as an early event synthesis of a nonhistone nuclear protein.

INTRODUCTION

The binding to surface immunoglobulin (Ig) molecules on rabbit peripheral blood lymphocytes (PBL) by anti-Ig sera is followed by blastogenic transformation of up to 90% of the cells (1-3). The high percentage of responding cells, in contrast to the low levels obtained with antigen, allows the investigation of events following interaction of ligand with antigen receptor Ig (4).

Mitogen stimulation of lymphocytes from several species initiates rapid changes in several parameters, one of the earliest being the synthesis of nonhistone nuclear proteins (5,6), which have been implicated in the regulation of DNA transcription (7). In this paper, we will report quantitative changes in nonhistone protein synthesis within four hours of anti-Ig stimulation of rabbit PBL.

MATERIALS AND METHODS

Cell preparation and culture. PBL were prepared under sterile conditions at room temperature from the blood of adult outbred rabbits from the colony at the Walter and Eliza Hall Institute of Medical Research. Buffy coat cells were separated from whole blood in heparin (10^5 units/liter) and re-suspended in RPMI 1640 (Grand Island Biological Co., San Francisco, CA)