^{13}C NUCLEAR MAGNETIC RESONANCE STUDIES OF THE CONFORMATION OF THE X-PRO BOND IN THE OLIGOPEPTIDE HORMONES, THYROTROPIN-RELEASING HORMONE, LUTEINIZING HORMONE-RELEASING FACTOR, ANGIOTENSIN AND MELANOCYTE-STIMULATING HORMONE RELEASE-INHIBITING FACTOR

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Received May 18, 1973

Summary: Studies by carbon-13 NMR of small proline-containing peptide are useful in determining *cis-trans* isomerism about the X-Pro bond, in which X is acylating the secondary amino group of the proline moiety. In tripeptides the ratio of the isomers depends upon the conformationa constraints induced by the acylating moiety, but is subject to solvent and temperature perturbations. Thyrotropin-releasing hormone (TRH) wa studied in particular detail using chemical shift and T_1 measurements for *cis-trans* isomerism about the amide bond of the proline residue an for mobility of the individual carbon atoms forming the proline ring. The results were related to those obtained with MSH-release-inhibiting factor, Val^5-angiotensin II β-amide, and luteinizing hormone-releasing factor. The His-Pro residues in angiotensin II and TRH give rise to similar spectra, implying that this sequence experiences the same environment in both hormones. The His spectrum of LRF is different fr that of angiotensin II and TRH, indicating a conformational difference LRF.

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One of the most significant contributions of ^{13}C nuclear magnetic resonance spectroscopy to conformational analysis of peptides has been in the detection of *cis* and *trans* isomerism about the X-Pro bond (1-7). With the exception of proline the chemical shifts of residues in peptides were thought to be insensitive to the nature of the neighboring residues in the primary sequence (8). Further exceptions have since been encountered with histidine-containing peptides (7) and diastereomeric peptides (7,9). In addition, studies on peptides have demonstrated that when an acyclic peptide chain is cyclized via a disulfide linkage, perturbations of chemical shifts are observed in the α-carbons located in the ring (2,3,9-13). Due to the greater conformational freedom of linear peptides, ^{13}C chemical shift data have not been as easy to interpret in conformational terms as those of cyclic peptides. Lyerla *et al.* (14) studied poly-L-glutamic acid in aqueous solution and found that the pH-induced transition from helix to coil resulted in deshielding of the carbonyl (-2.2 ppm) and

α-carbons (-2.3 ppm) and shielding of the β-carbons (+0.8 ppm). Similar results have been reported by Boccalon *et al.* (15) for poly (N^{δ}-carbobenzoxy-L-ornithine) which undergoes a helix-coil transition as the percentage of acid is varied in a trifluoroacetic acid-deuteriochloroform solvent system. However, because of possible complications due to solvent effects on the resonances, this type of experiment is difficult to interpret. Recent studies on poly-L-lysine in aqueous media (9,16) have also shown downfield shifts of the carbonyl and α-carbons in lysine accompanying the transition to the α-helix. However, in the oligomer $(Lys)_{12}$, which does not undergo a helix-coil transition, similar effects were noted (9,16). Thus, it appears that both pH-induced and conformational effects are manifest in α- and carbonyl carbon resonances, but that they are difficult to separate.

Because ^{13}C NMR is sensitive to *cis* and *trans* isomers of proline in an unequivocal way, we have studied the following linear proline-containing hormones: [Val^5] angiotensin II β-amide, Asn-Arg-Val-Tyr-Val-His-Pro-Phe (17); luteinizing hormone-releasing factor (LRF), <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (9,18); thyrotropin-releasing hormone (TRH), <Glu-His-Pro-NH_2 (7,19,20); and melanocyte-stimulating hormone release-inhibiting factor (MSH-R-IF), pro-Leu-Gly-NH_2 (9,21,22).

Materials and Methods: LRF and [des-His^2]LRF were gifts of Wyeth Laboratories, and angiotensin II of Ciba-Geigy (Hypertensin$^{(R)}$, Ciba); TRH was a product of Bachem. MSH-R-IF was from the same preparation used earlier (26). Samples were prepared and NMR spectra obtained by procedures described in detail previously (2,7,9,10,12,13).

Results and Discussion: The ^{13}C chemical shifts and spin-lattice relaxation times (T_1) are shown in Table 1. The spectrum of Ac-Pro in deuterated dimethylsulfoxide ($(CD_3)_2SO$) (1) and Ac-Pro-NH_2 in $(CD_3)_2SO$, deuterated pyridine, and deuterium oxide (D_2O) have shown the presence of *cis* and *trans* forms in equilibrium (7). The ratio of the *cis* form to the *trans* form was 30:70 for Ac-Pro (1). In Ac-Pro-NH_2 the ratio varied slightly with the solvent; 25:75 in D_2O, 31:69 in pyridine-d_5 and 34:66 in $(CD_3)_2SO$. Thomas and Williams (1) concluded that the bulk of the side chain in the amino acid preceding proline in dipeptides in aqueous solution did not affect the *cis:trans* ratio, since they found that Gly-Pro, Ala-Pro and Val-Pro all exhibited a 40:60 *cis:trans* isomer ratio.

Our studies on the tripeptide TRH (7,9) in various solvents demonstrated a remarkable solvent sensitivity of the *cis-trans* populations of the Pro-NH_2 residue; in D_2O, $(CD_3)_2SO$, and pyridine-d_5 the

Table 1

^{13}C Chemical Shifts[a] and Spin-Lattice Relaxation Times[b] of Individual Carbon Atoms

	MSH-R-IF		TRH		Angiotensin II	LRF	[Des-His^2]LRF
	Chemical Shift	T_1	Chemical Shift	T_1	Chemical Shift	Chemical Shift	Chemical Shift
Pro							
αCH	61.07	0.86	61.56	0.50	61.93	61.88	61.85
β CH_2 (*cis*)	31.63	1.01	33.08				
(*trans*)			30.75	0.57	30.35	30.32	30.32
γ CH_2 (*trans*)	26.48	1.69	25.73	0.65	25.63	25.82	25.80
(*cis*)			22.86		22.94		
δ CH_2 (*trans*)	47.62	1.03	49.13	0.39	49.15	49.11	49.05
(*cis*)			48.58				
C=O	178.99	10.0	177.81	11.3	N.A.	N.A.	N.A.
His							
αCH			51.83		51.02	53.67	
βCH_2			27.12		27.13	28.28	
C-5 (Cγ)			129.47		N.O.	129.86	
C-4 (Cδ)			118.89		118.83	118.10	
C-2 (Cε)			134.85		134.72	134.80	
C=O			170.85		170.76	172.64	
Arg							
αCH					51.65	52.17	52.14
βCH_2					29.47	28.74	28.71
γCH_2					25.45	25.13	25.09
δCH_2					41.73	40.98	40.93

a) Chemical shifts are expressed in parts per million downfield from the resonance of tetramethylsilane in a 5 mm tube concentric to the 12 mm sample tube. Samples were run at a pH-meter value of 4.0.

b) T_1 values are expressed in seconds.

N.A. Not assigned.

N.O. Not observable.

cis populations were 14, 6, and 0%, respectively. It is likely that in TRH the increase in content of the *trans* X-Pro isomer is a consequence of the Pro being present in an acyclic peptide larger than a dipeptide with concomitant steric constraints introduced by the <Glu-His moiety. The high content of *trans* isomer in TRH is not in disagreement with the preferred conformation proposed by Boilot *et al.* (23) and Fermandjian *et al.* (24), where the amide hydrogen of TRH is intramolecularly hydrogen-bonded to the carbonyl group of histidine; the *cis* isomer is unable to assume such a conformation. The fact that Ac-Pro-NH_2 exhibits a *cis-trans* ratio of about 30:70 depending on the solvent, and that this compound in a polar solvent is not expected to form a hydrogen bond between a proton of the carboxamide and the carbonyl oxygen of the acetyl residue (25) (which is analogous to the hydrogen bond between the carboxamide and the His carbonyl in TRH (23,24)), raises the possibility that the proposed hydrogen bond acceptor is the carbonyl group of the <Glu residue rather than that of the His, making a β-turn in TRH analogous to that proposed for Pro-Leu-Gly-NH_2 (26).

^{13}C NMR of angiotensin II in aqueous solution (17) has provided the assignments of the Cα and side-chain carbon resonances. The pK values of the titratable groups have not indicated any direct interactions between these groups and their local environments (17). The terminal tripeptide sequence of angiotensin II is His-Pro-Phe; therefore, on the basis of studies on TRH one should be able to determine the conformation about the His-Pro bond. We have found all four Pro carbon resonances in angiotensin II (40 mg/ml water) to superimpose with those of the *trans* isomer of Pro in TRH in water, pH 4.0. Furthermore a small resonance on the highfield side of the Cγ resonance of Pro in angiotensin II superimposes with that from the *cis* isomer of Pro in TRH. In $(CD_3)_2SO$ the *cis* isomer increases to 50 percent; upon a return of the sample to water, the resonances associated with the *cis* isomer decreased to their original size. However, not more than one resonance each is seen for each of the β and δ carbons of Pro in angiotensin II. We can conclude that the Pro residue is mainly in the *trans* conformation and, that if the *cis* isomer is present, it represents less than 20% of the total population in D_2O. This observation militates against the γ-turn suggested by Printz *et al.* (27) for angiotensin II in aqueous solution at a concentration of 40 mg/ml, because a *cis* His-Pro bond in angiotensin II with a γ-turn conformation is energetically favored (27). However, angiotensin II with a β-turn, also proposed by Printz *et al.* (27), remains a possibility; in

this model the His-Pro bond would be present in the *trans* conformation. Fermandjian *et al.* (28) have pointed out that in "concentrated aqueous solution" or in $(CD_3)_2SO$ the hormone is aggregated. In the aggregated state a large proportion of the molecules adopt an antiparallel β-type structure in which a first turn involves Val^3 and Tyr^4 and a second turn the His-Pro linkage. The His-Pro linkage is in the *cis* conformation, with the His and Pro carbonyls pointing in the same direction. In aqueous solution at the concentrations used in our experiments, aggregates of the nature proposed by Fermandjian *et al.* (28) are not the predominant species, but in $(CD_3)_2SO$ these aggregates may involve 50% of the molecules.

It is noteworthy that the resonances of the His carbons in angiotensin II are similar to those from the same residue in TRH at the same pH. Moreover, the His-Pro-X sequence in both hormones appears to possess an identical conformation.

LRF contains the N-terminal sequence <Glu-His-, which also occurs in TRH, and the C-terminal sequence Arg-Pro-Gly-NH_2. Grant and Vale (29) proposed that the LRF decapeptide exists in a configuration such that the distal ends of the molecule form a "face" (29) with an appearance similar to that of TRH. The amino acid residues located in the center portion of the molecule were proposed to fold back in a pleated sheet which hinged on Gly^6, thereby allowing the carboxyl terminal amide group to be close to the N-terminal dipeptide. Our studies on LRF (9) allow us to conclude that the Arg-Pro peptide bond must be in the *trans* conformation. Comparing the chemical shifts of the <Glu-His moiety in LRF with those arising from the same residues in TRH at the same pH, we find a striking difference. The <Glu resonances in both hormones are the same, but in LRF the α, β and carbonyl carbons of His are shifted downfield 1.8, 1.2 and 1.8 ppm, respectively. Our assignments of the His resonances in LRF were confirmed by taking a spectrum of a peptide of the same sequence as LRF, but lacking the His residue, [des-His^2]LRF. The shift in the α and β carbons of the His residue was not due to a variation in the pK_a of the imidazole ring because the spectra of LRF and TRH were compared at pH 4.0 where the imidazole ring is fully protonated. One possible explanation is that in TRH the Cα resonance of the His residue has an abnormally high chemical shift because of its proximity to the Pro residue. A Pro residue can cause a steric compression shift in the preceding residue (8). Comparis of the α carbons of Arg in angiotensin II and LRF shows that the steric compression shift in LRF amounted to 0.5 ppm, which agrees with the

findings of Christl and Roberts for Phe-containing peptides (8). We conclude that differences in chemical shifts of His reflect a conformational difference between LRF, TRH and angiotensin II.

Spin-lattice relaxation time measurements (T_1) can provide information regarding the various types of motion within a molecule, and give an estimate of the overall correlation time for rotational reorientation (7,9,16,30,31). We have examined two proline-containing peptides with this technique, TRH and MSH-R-IF (Table 1). MSH-R-IF is a peptide in which Pro is the N-terminal residue and therefore cannot exhibit *cis-trans* isomerism. The T_1 measurements show that the Pro ring in MSH-R-IF is quite mobile; rotation occurs about the αC-CO bond of Pro and the γ carbon of Pro moves rapidly in and out of the plane formed by the N, Cα, Cβ, and Cδ of the ring (9,32). In TRH the T_1 data demonstrate that the His-Pro link is quite rigid, as judged from the presence of observable *cis* and *trans* isomers as well as from the similar relaxation times of the α carbons (7,9). In TRH the β and γ carbons are more mobile than the δ carbon, which may be a result of alternate rapid movement of the β and γ carbons above and below the plane of the ring.

Acknowledgment: We are very grateful to Drs. H. McGregor and D. Sarantakis of Wyeth Laboratories for the generous gift of LRF and [des-His^2]LRF, and to Drs. A.J. Plummer and W.E. Wagner of Ciba-Geigy for angiotensin II. This work was supported in part by the National Research Council of Canada and by USPHS grant AM-13567.

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