Conformational Flexibility of Luteinizing Hormone-Releasing Hormone in Aqueous Solution. A Carbon-13 Spin–Lattice Relaxation Time Study†

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ABSTRACT: The carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra of luteinizing hormone-releasing hormone (LH-RH) and lower homologous peptides have been assigned in aqueous solutions at various pH values. ^{13}C spin–lattice relaxation times (T_1) have been measured for all proton-bearing carbons at 25.2 and 67.9 MHz. From the T_1 data the rates of overall molecular motion and intramolecular motion of side chains have been estimated. LH-RH is a flexible molecule in solution, having segmental motion along the backbone as well as in the nonaromatic side chains.

The structural characteristics of the hypothalamic factor which causes the release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary have recently been elucidated (Burgus et al., 1972; Matsuo et al., 1971; Schally et al., 1972). Luteinizing hormone-releasing hormone (LH-RH)[1] stimulates the release of both luteinizing hormone and follicle-stimulating hormone (Kastin et al., 1969, 1971). LH-RH is a polypeptide of sequence <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Schally et al., 1971a–c; Matsuo et al., 1971; Burgus et al., 1972).

Wessels et al. (1973) have done a thorough ^{1}H nuclear magnetic resonance (NMR) study of LH-RH and its component peptides dissolved in H_2O and in deuterated dimethyl sulfoxide ($(CD_3)_2SO$). The assignments were based on studies of model compounds and on spin-decoupling experiments. The chemical shifts of α, β, and other side-chain proton resonances in LH-RH were similar to those observed in the component peptides. The side-chain conformations of serine in LH-RH were analyzed and the rotamer populations about the $C_\alpha H$–$C_\beta H_2$ bond were very similar to those observed for serine in other peptides. The tyrosyl and tryptophyl side-chain conformations were studied in <Glu-His-Trp-Ser-Tyr-Gly and the rotamer populations about the C_α–C_β bond were similar to those measured for aromatic residues in small peptides. The proton chemical shifts of the aromatic rings did not show any stacking of the aromatic rings.

$^3J_{^1HCN^1H}$ values were determined in $(CD_3)_2SO$ in order to elucidate the conformation of the backbone. The coupling constants can be related to the

dihedral angles (Bystrov et al., 1969); an average value of 7 ± 1 Hz is predicted for the distribution of conformations which are possible in a random coil. In LH-RH all the observed values ranged between 6.2 and 7.4 Hz. These values were considered compatible with a random coil backbone conformation. The temperature dependence of the amide proton chemical shifts (Kopple et al., 1969) was followed in order to detect the presence of intramolecular hydrogen bonds in LH-RH. The temperature dependence of the chemical shifts was observed for LH-RH both in D_2O and in $(CD_3)_2SO$ and showed that no strong intramolecular hydrogen bonds are present in LH-RH in these solvents.

Monahan and coworkers (1973) have proposed that the -Ser-Tyr-Gly-Leu- sequences of LH-RH may form a β turn. An L-amino acid is known to destabilize a type II β turn whereas a glycyl residue or a D-amino acid will stabilize the type II β turn (Venkatachalam, 1968). In position 6 replacement of glycine by bulkier L-amino acids leads to decreased activity (Coy et al., 1973) whereas substitution by D-amino acids such as D-lysine or D-alanine (Monahan et al., 1973) leads to compounds possessing higher activity than LH-RH. The doubly substituted D-6-alanine-des-10-glycinamide-LH-RH ethylamide is over 12 times as active as LH-RH (Coy et al., 1974). The combination of two modifications which each yields an increase in hormonal activity results in even higher LH-RH activity. Although LH-RH may have a random coil conformation in solution (Wessels et al., 1973) it appears that LH-RH might have a preferred conformation at the hormone receptor.

We have undertaken ^{13}C NMR studies of LH-RH with the hope of elucidating its conformational characteristics in solution. This is a necessary preliminary to investigation of the conformational characteristics of hyperactive derivatives of LH-RH and to the study of conformationally locked derivatives of LH-RH. The latter should lead to a correlation between the conformational characteristics of the analogs and their activity. Ultimately through ^{13}C enrichment of LH-RH, it should be possible to study the interaction be-

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[1] Abbreviation used is: LH-RH, luteinizing hormone-releasing hormone. The nomenclature used in this paper follows the rules suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry 11*, 1726 (1972)). All optically active amino acids are in the L configuration.

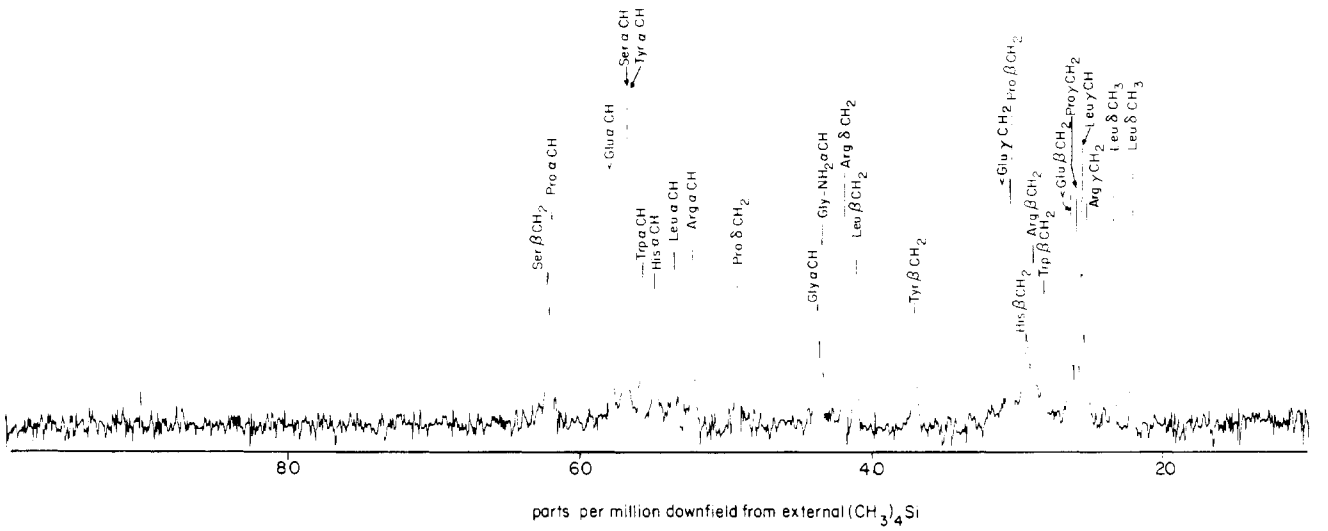

FIGURE 1: ^{13}C NMR spectrum (25 MHz) of aliphatic carbons of LH-RH in D_2O at pH 6.4, 50 mg/ml, 32°, 170,000 scans, cycle time, 0.4 sec, pulse width, 45°.

tween LH-RH and cell membranes or model membranes.

We report the assignment of the ^{13}C resonances of LH-RH, lower analogs, des-2-histidine-LH-RH, des-3-tryptophan-LH-RH, and des-10-glycinamide-LH-RH in D_2O at various pH values, as well as the ^{13}C spin-lattice relaxation times (T_1) of the proton-bearing carbons in the backbone and the side chains. The T_1 values yield information concerning the rate of overall molecular reorientation of LH-RH, the mobility of the peptide backbone, as well as the rates of motion of the various side chains. A detailed review of the technique and its application to peptide hormones will appear shortly (Deslauriers and Smith, 1975).

Experimental Section

Material. <Glu-His-Trp-NH_2 was prepared according to the methods described by Chang et al. (1971) and Schally et al. (1972). Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, $[\alpha]^{20}D$ −34° (*c* 0.5, MeOH), was prepared following Yanaihara et al. (1973a) $[\alpha]^{28}D$ −36.6° (*c* 1, 1 *M* AcOH). Leu-Arg-Pro-Gly-NH_2 was prepared by classical methods, U.S. Patent 3,826,796. The syntheses of des-10-glycineamide-LH-RH, des-2-histidine-LH-RH, and des-3-tryptophan-LH-RH are described in Rivier et al. (1973), Monahan et al. (1972), and Yanaihara et al. (1973b), respectively. LH-RH was supplied by the Contraceptive Development Branch, Center for Population Research, NICHD, NIH.

Methods. Proton-decoupled ^{13}C nuclear magnetic resonance spectra were obtained on a Varian XL-100-15 spectrometer operating in the Fourier-transform mode at 25.16 MHz using a Varian 620L computer with 16K memory and on a Bruker HX-270 spectrometer operating at 67.9 MHz. The Bruker spectrometer was interfaced to a Nicolet 1089 36K computer having a 600K word auxilliary disk memory. Spin-lattice relaxation times (T_1) were measured using the inversion-recovery method as described by Freeman and Hill (1970) using a pulse sequence (T_∞–180°–τ–90°–) where τ is a variable delay time and T_∞ is at least four times longer than the longest T_1 to be measured. The width of the 90° pulse is 14 μsec (XL-100) and 20 μsec (HX-270). T_1 values were determined from a nonlinear two-parameter regression using

$$M(\tau) = M(0)(1 - 2\exp^{-\tau/T_1})$$

where $M(0)$ is the equilibrium value of the magnetization, for $\tau = 0$ M $= -M(0)$, and $M(\tau)$ is the value of the magnetization resulting from a given value of τ. The accuracy of T_1 values is ±15%. Nuclear Overhauser enhancements (NOE) were measured at 25 MHz by comparing the integrated intensities of the ^{13}C resonances in proton noise decoupled spectra and coupled spectra (Levy and Nelson, 1972; Noggle and Schirmer, 1971; Kuhlmann and Grant, 1968). Integrations were performed separately on units consisting of the carbonyl, the aromatic, and the α- and the β-carbon regions. This procedure was required due to the complexity of the coupled spectra.

Samples were studied in tubes of outside diameter 12 mm (25 MHz) and 10 mm (68 MHz). Chemical shifts at 25 MHz are reported downfield from external tetramethylsilane ($(CH_3)_4Si$) contained in a concentric inner tube of outside diameter 5 mm. Spectra at 25 MHz were obtained at 32°. pH values reported in the figures and tables are uncorrected pH meter readings taken in D_2O. (pD = pH meter reading + 0.4, Glascoe and Long, 1960). CD_3COOD, HCl, and NH_4OH diluted in D_2O were used to adjust the pH of samples. Samples used for T_1 measurements were 100 mg/ml (25 MHz) or 200 mg/ml (68 MHz). Temperature control and measurement in ^{13}C NMR at 68 MHz pose substantial practical difficulties; thus the temperatures given in Table III may actually be significantly in error: 35 ± 2°, 40 ± 4°, 55 ± 7°.

Results

Spectral Assignments. The calculated and observed ^{13}C chemical shifts of LH-RH, des-His^2-LH-RH, des-Trp^3-LH-RH, des-Gly^{10}-LH-RH, and lower homologs of LH-RH dissolved in D_2O at various pH values are given in Tables I and II. The aliphatic carbon resonances of LH-RH are shown in Figure 1. The ^{13}C spectrum calculated for LH-RH at pH 6.4 was based on the ^{13}C chemical shifts of the free amino acids in D_2O (Horsley et al., 1970, Voelter et al., 1971). The effects of peptide bond formation (Christl and Roberts, 1972; Keim et al., 1973a,b, 1974) and amino acid titration (Freedman et al., 1971; Gurd et al., 1971; Christl and Roberts, 1972; Reynolds et al., 1973; Keim et al., 1973a; Quirt et al., 1974) were also considered in calculating the ^{13}C chemical shifts of LH-RH. The assignments of the observed ^{13}C resonances in LH-RH were based mainly on studies of lower homologs with corrections made

Table I: ^{13}C Chemical Shifts[a] of Peptide Components of Luteinizing Hormone-Releasing Hormone.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

Peptide	Calcd 1–10	1–3[c]			7–10				3–10	1–9			
pH[b]	6.4	1.0	5.1	7.6	1.0	4.5	7.5	9.6	7.0	3.2	6.3	8.7	10.5
Residue													
<Glu													
αCH	57.7	57.69	57.69	57.70						57.63	57.55	57.61	57.64
βCH_2	26.4	26.23	26.23	26.15						26.14	26.08	26.08	26.15
γCH_2	30.3	30.32	30.32	30.23						30.19	30.18	30.16	30.19
δC=O	183.1	183.18	183.18	183.25									
C=O	175.6	175.57	175.63	175.75									
His													
αCH	53.8	53.48	53.49	54.86						53.39	54.16	55.19	55.15
βCH_2	28.8	27.30	27.33	29.37						27.31	27.62	29.66	29.73
γC	132.2	129.36	129.64	132.68						129.43	130.89	133.94	134.03
δCH	118.6	118.31	N.O.	N.O.						118.25	118.05	117.87	118.07
εCH	136.5	134.64	134.64	N.O.						134.64	135.45	137.09	137.07
C=O	172.6	172.14	172.23	173.40									
Trp													
αCH	56.0	55.14	55.14	55.03					55.45	55.45	55.48	55.64	55.61
βCH_2	28.2	28.30	28.30	28.08					29.45	28.35	28.21	27.82	27.85
C-2	127.6	125.65	125.66	125.67					126.03	125.45	125.50	125.60	125.59
C-3		110.03	110.03	109.93					N.O.	109.81	109.61	109.44	109.59
C-4	120.5	119.50	119.50	119.48					119.37	119.32	119.22	119.26	119.23
C-5	124.5	123.11	123.11	123.11					123.23	123.03	122.97	123.06	123.00
C-6	121.8	120.48	120.50	120.48					120.57	120.41	120.34	120.42	120.37
C-7	114.2	113.04	113.05	113.05					113.10	112.94	112.92	112.98	112.97
C-8	128.9	128.00	128.08	128.09					N.O.	128.06	127.98	128.03	128.04
C-9	138.1	137.29	137.35	137.36					N.O.	137.29	137.19	137.22	137.07
C=O	173.3	176.91	N.O.	177.01									
Ser													
αCH	57.4								56.19	56.57	56.57	56.75	56.91
βCH_2	61.3								62.36	62.31	62.05	61.94	61.89
C=O	171.6												
Tyr													
αCH	57.3								56.73	56.43	56.57	56.75	56.91
βCH_2	37.5								37.05	37.04	36.82	36.79	36.84
γC									129.14	129.02	128.93	128.67	128.08
ortho CH	130.5								131.73	131.59	131.59	131.49	131.41
meta CH	117.5								116.69	116.57	116.54	116.74	118.07
para C	156.3								N.O.	155.73	155.67	156.18	159.58
C=O	173.5												
Gly													
αCH	43.5								43.53	43.53	43.52	43.58	43.49
C=O	172.6												
Leu													
αCH	54.7				52.90	52.88	53.11	53.88	53.45	53.39	53.63	53.40	53.35
βCH_2	41.0				40.99	41.06	41.78	44.31	40.93	40.97	40.85	40.85	40.93
γCH	25.4				25.25	25.24	25.23	25.25	25.45	25.40	25.33	25.34	25.34
δCH_3	23.2				22.91	22.83	22.93	23.21	23.27	23.22	23.20	23.25	23.29
δCH_3	22.1				21.95	22.31	22.41	22.70	22.00	21.96	21.90	21.91	21.94
C=O	175.1				175.75	175.74	175.81						
Arg													
αCH	53.8				52.52	52.51	52.43	52.10	52.19	51.95	51.91	51.94	51.91
βCH_2	28.1				28.66	28.67	28.67	28.68	28.76	28.77	28.58	28.61	28.66
γCH_2	24.6				24.97	24.97	25.08	25.27	25.18	25.03	25.02	25.05	25.08
δCH_2	41.2				41.84	41.77	41.78	41.76	41.73	41.72	41.63	41.66	41.73
εC	157.2				N.O.	N.O.	N.O.	157.96	N.O.	157.79	157.77	N.O.	157.92
C=O	172.9				171.35		172.52						
Pro													
αCH	61.6				61.96	61.88	61.90	61.89	61.88	61.90	63.13	63.15	63.11
βCH_2	29.7				30.44	30.44	30.45	30.43	30.45	30.19	30.18	30.16	30.40
γCH_2	24.4				25.86	25.85	25.88	25.85	25.82	25.60	25.69	25.57	25.54
δCH_2	46.5				49.27	49.19	49.23	49.18	49.10	48.67	48.63	48.67	48.58
C=O	173.1				172.42		172.80						
Gly													
αCH	43.3				43.33	43.32	43.23	43.88	43.30				
C=O	175.2				175.34		175.34						

[a] Chemical shifts are reported in parts per million downfield from external $(CH_3)_4Si$. Accuracy of chemical shifts: ±0.05 ppm. Carbonyl carbons which were not assigned are not reported. [b] pH represents the uncorrected pH meter reading in D_2O. [c] Represents <Glu-His-Trp-NH_2.

Table II: ^{13}C Chemical Shifts[a] of Luteinizing Hormone-Releasing Hormone (LH-RH) and Modified Synthetic Peptides.

Peptide	Calcd	LH-RH					des-His[2]-LH-RH	des-Trp[3]-LH-RH
pH[b]	6.4	3.6	4.7	5.2	6.4	8.8	6.4	4.8
Residue								
<Glu								
αCH	57.7	57.70	57.72	57.66	57.73	57.73	57.88	57.82
βCH_2	26.4	26.23	26.22	26.17	26.20	26.18	26.04	26.32
γCH_2	30.3	30.34	30.32	30.30	30.25	30.33	30.07	30.40
δC=O	183.1							
C=O	175.6							
His								
αCH	53.8	53.45	53.57	53.53	54.79	54.80		53.99
βCH_2	28.8	27.27	27.40	27.40	29.21	29.76		27.71
γC	132.2	129.50	129.86	130.89	N.O.	N.O.		130.89
δCH	118.6	118.27	118.27	118.22	N.O.	N.O.		118.25
εCH	136.5	134.74	134.74	134.70	N.O.	N.O.		137.74
C=O	172.6		172.64		173.49			
Trp								
αCH	56.0	55.48	55.51	55.43	55.62	55.64	55.48	
βCH_2	28.2	28.36	28.36	28.20	28.01	28.10	28.20	
C-2	127.6	125.58	125.60	125.54	125.63	125.52	125.54	
C-3		109.85	109.85	109.82	109.65	109.55	110.07	
C-4	120.5	119.39	119.42	119.36	119.34	119.32	119.39	
C-5	124.5	123.14	123.16	123.09	123.16	123.14	123.15	
C-6	121.8	120.51	120.54	120.44	120.52	120.47	120.56	
C-7	114.2	113.03	113.06	112.98	113.06	113.04	113.08	
C-8	128.9	128.04	128.02	128.05	128.13	128.11	128.18	
C-9	138.1	137.31	137.33	137.27	137.32	137.31	137.31	
C=O	173.3							
Ser								
αCH	57.4	56.63	56.64	56.62	56.74	56.81	56.60	56.40
βCH_2	61.3	62.22	62.21	62.18	62.13	62.04	62.16	62.09
C=O	171.6							
Tyr								
αCH	53.7	56.63	56.64	56.62	56.74	56.81	56.60	56.61
βCH_2	37.5	37.05	37.00	36.90	36.88	36.84	36.92	37.27
γC		129.09	129.11	129.03	129.11	128.72	129.11	129.28
ortho CH	130.5	131.66	131.68	131.61	131.61	131.56	131.66	131.64
meta CH	117.5	116.64	116.65	116.61	116.64	116.83	116.62	116.60
para C	156.3	155.74	155.74	155.73	155.73	156.31	155.72	155.66
C=O	173.5							
Gly								
αCH	43.5	43.59	43.61	43.59	43.60	43.60	43.54	43.56
C=O	172.6							
Leu								
αCH	54.7	53.45	53.46	53.43	53.44	53.41	53.42	53.48
βCH_2	41.0	40.95	40.95	40.83	40.93	40.95	40.93	40.91
γCH	25.4	25.42	25.45	25.39	25.44	25.39	25.42	25.44
δCH_3	23.2	23.26	23.27	23.23	23.30	23.29	23.25	23.20
δCH_3	22.1	21.96	21.98	21.95	21.96	21.95	21.95	21.96
C=O	175.1							
Arg								
αCH	53.8	52.16	52.17	52.13	52.17	52.16	52.14	52.16
βCH_2	28.1	28.72	28.74	28.68	28.76	28.70	28.71	28.70
γCH_2	24.6	25.12	25.13	25.10	25.12	25.12	25.09	25.18
δCH_2	41.2	41.72	41.73	41.68	41.73	41.72	41.70	41.73
εC	157.2	157.80	157.79	157.85	157.83	157.88		157.88
C=O	172.9							
Pro								
αCH	61.6	61.87	61.88	61.84	61.87	61.87	61.85	61.88
βCH_2	29.7	30.34	30.32	30.30	30.38	30.33	30.36	30.32
γCH_2	24.4	25.80	25.82	25.76	25.82	25.78	25.86	25.83
δCH_2	46.5	49.08	49.11	49.03	49.09	49.08	49.10	49.13
C=O	173.1							
Gly								
αCH	43.3	43.28	43.29	43.25	43.29	43.29	43.27	43.27
C=O	175.2							

[a] Chemical shifts are reported in parts per million downfield from external $(CH_3)_4Si$. Accuracy of chemical shifts: ±0.05 ppm. [b] pH represents the uncorrected pH meter reading in D_2O.

for the incorporation of each new residue. The assignments presented here differ in some instances from those reported previously (Smith et al., 1973; Wessels et al., 1973). The present assignments are based on a larger number of model peptides as well as on the effect of titration on the chemical shifts of specific residues. Confirmation of some of the as-

signments must await the synthesis of peptides containing specifically deuterated or ^{13}C-enriched amino acids. The assignments given in Tables I and II are based on the following considerations.

The spectrum of the N-terminal tripeptide, <Glu-His-Trp-NH_2, was obtained and compared with that of <Glu-His-Pro-NH_2 (Deslauriers et al., 1973a, 1974a) in order to assign the resonances of the pyroglutamate. The resonances of the histidyl residue were assigned by varying the pH of the solution from 7.6 to 1.0. This results in protonation of the histidyl residue and causes upfield shifts in the resonances of histidine (Reynolds et al., 1973). The remaining resonances in the spectrum of <Glu-His-Trp-NH_2 were assigned to the tryptophyl residue. The assignments obtained in the above manner were in good agreement with those calculated from the corrected amino acid shifts. The C-terminal tetrapeptide Leu-Arg-Pro-Gly-NH_2 was examined in D_2O between pH 1.0 and 9.6. Raising the pH above 9.0 should lead to deprotonation of the amino group of leucine (pK_a of amino group in free leucine = 9.74, Dawson et al., 1969). This leads to downfield shifts of the leucyl resonances. The α-carbon resonance of the glycyl residue was assigned by comparison with the spectrum of Pro-Leu-Gly-NH_2 (Deslauriers et al., 1973b). The resonances of the arginyl and prolyl residues were then assigned by comparing the remaining unassigned resonances with the spectra of free arginine and proline as well as with these resonances in the octapeptide hormone arginine-vasotocin (Walter et al., 1973). The chemical shift of the α carbon of arginine was shifted upfield ca. 2.0 ppm from the position expected in a peptide. This has been attributed to the "γ steric effect" of the adjacent proline residue (Christl and Roberts, 1972). The assignment of leucyl resonances was further confirmed by observing the effect of incorporating the tetrapeptide Leu-Arg-Pro-Gly-NH_2 into the octapeptide Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. The leucyl resonances all undergo slight chemical shift changes: C_α, +0.3 ppm; C_β, −0.9 ppm; C_γ, +0.2 ppm; C_δ, −0.4 ppm; C_δ, +0.3 ppm, a negative sign indicating an upfield shift. This assignment of the leucyl C_β resonance agrees with that of Wessels et al. (1973). In a previous assignment (Smith et al. 1973) the leucyl C_β and arginyl C_δ resonances were interchanged. The assignment of the leucyl C_α resonance differs from that of Wessels et al. (1973); the resonance assigned by them to leucine C_α has now been shown by titration to be that of the α carbon of histidine (vide infra). The tryptophyl resonances in the octapeptide were assigned by examining the peptide at different pH values. Upon varying the pH over the range 4.5–10.6 the tryptophyl resonances were shifted downfield (pK_a of the α-amino group in tryptophan is 9.44; Dawson et al., 1969). Incorporating the octapeptide Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 into the decapeptide <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 should permit assignment of the tryptophyl resonances in LH-RH. However, two C_α resonances are perturbed and this renders difficult the assignment of the tryptophyl and seryl α carbons. Therefore we examined spectra of LH-RH analogs which lacked one residue. By comparing the spectrum of des-3-tryptophan-LH-RH at pH 4.8 with that of LH-RH at pH 4.7 we were able to assign the resonances of tryptophan in LH-RH. Deletion of the tryptophyl residue leads to perturbations only in the resonances of serine: C_α and C_β shift upfield 0.2 and 0.1 ppm, respectively. Comparing the spectrum of des-2-histidine-LH-RH with that of LH-RH at pH 6.4 confirms the assignment of the histidine assignments in

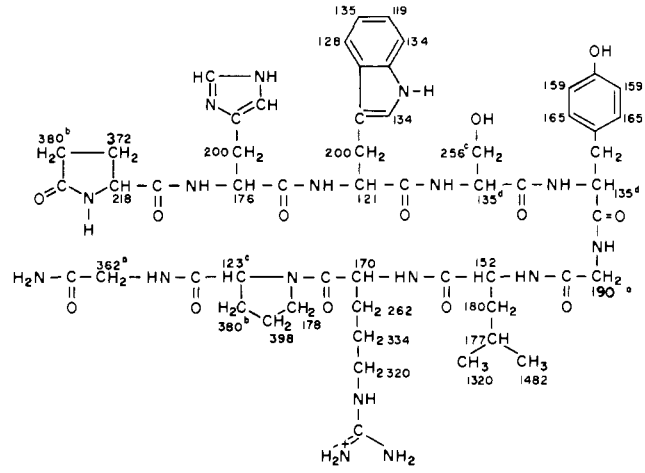

FIGURE 2: Primary sequence of luteinizing hormone-releasing hormone showing NT_1 values obtained at 25.16 MHz for carbons bearing protons in D_2O at pH 6.4. NT_1 values are in milliseconds; N is the number of directly attached protons. Sample concentration, 100 mg/ml. Temperature, 32°. T_1 values determined using 20 spectra, 10,000 scans/spectrum, T = 2.0 sec, τ = 10–250 msec. Superscripts indicate overlapping resonances.

LH-RH. The pyroglutamyl or tryptophyl residues adjacent to histidine show only slight perturbations in chemical shift. The C_α, C_β, and C_γ resonances of pyroglutamate shift downfield 0.1 ppm and upfield 0.2 and 0.2 ppm, respectively. The C_α resonance of tryptophan shifts upfield 0.1 ppm. Deletion of the C-terminal glycinamide moiety in des-10-glycinamide-LH-RH confirms the assignment of the terminal glycine as well as that of proline. All the proton-bearing carbons of proline are affected: C_α, +0.2 ppm; C_β, −0.2 ppm; C_γ, 0.1 ppm; and C_δ, −0.5 ppm. We believe that the study of des-2-histidine-LH-RH, and des-10-glycinamide-LH-RH provides a firm basis for the assignment of the C_α resonances. We have found that deletion of one residue in a nonterminal position of a peptide results in only small changes in the chemical shifts of other residues. The residues adjacent to the amino acid which has been deleted undergo shifts of less than 0.3 ppm whereas the other residues show no change. Comparing the present assignments of the C_α resonances with those calculated from the corrected amino acid shifts we find the order of the chemical shifts to be identical in both cases (i.e., no cross-over of resonances is seen between the observed and the calculated chemical shifts).

Spin–Lattice Relaxation Times. The NT_1 values at 25 MHz for the protonated carbons of LH-RH dissolved in D_2O at pH 5.3 are given in Figure 2 (where N is the number of directly attached hydrogens). The NT_1 values obtained at 68 MHz are shown in Figure 3 and Table III. The NT_1 values for the backbone carbons are longest at the N and C terminal residues. The exception at each temperature is the NT_1 value for the α carbon of the 6-glycine, which is always longer than adjacent backbone carbon NT_1's. At 25 MHz, the glycine-6 α-carbon resonance was incompletely resolved from the C-terminal glycine resonance and the resulting NT_1 values can be greatly in error. At 68 MHz, however, the two resonance lines were sufficiently resolved to give individually accurate T_1 values. The 68-MHz T_1 data clearly indicate that the internal glycine residue has a longer NT_1 than for adjacent α carbons. This is probably a result of increased internal motional freedom for the unsubstituted glycine residue relative to all other amino acid resi-

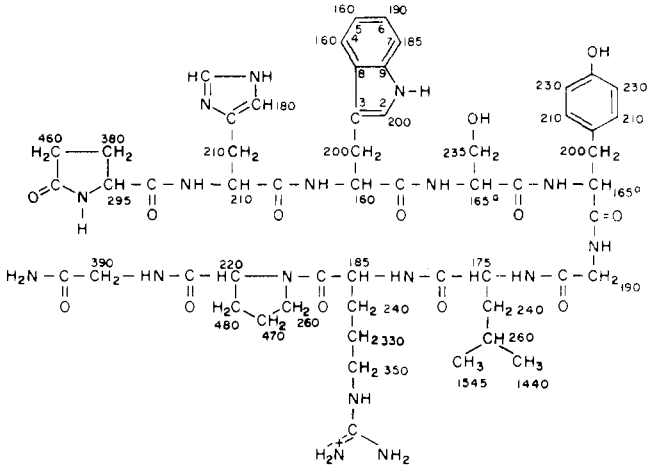

FIGURE 3: Primary sequence of luteinizing hormone-releasing hormone showing NT_1 values obtained at 68 MHz for carbons bearing protons in D_2O at pH 6.4. NT_1 values are in milliseconds; N is the number of directly attached protons. Sample concentration, 200 mg/ml; 2000 scans/spectrum; $T = 2.0$ sec, $\tau = 10$–500 msec, temperature 35°. Superscripts in figure indicate overlap of resonances.

dues which are constrained by side-chain mass and conformational considerations.

It is also possible that a slight lengthening of the α-carbon C–H bond for the CH_2 group in glycine relative to the CH groups of the other residues might be partly responsible for the observed effect. Since the NT_1 differential for the α carbon of glycine-6 increases at higher temperatures (see Table III) the explanation based on increased internal motional freedom is preferred.

Although direct and exact comparison of the 25- and 68-MHz data is not possible because of the different sample concentrations used, it should be pointed out that the expected magnetic field dependence (Komoroski et al., 1975a,b) is observed for carbons with $NT_1 \lesssim 400$ msec. For correlation times longer than 1×10^{-10} sec (corresponding to $NT_1 \simeq 475$ msec) the extreme narrowing condition begins to break down for ^{13}C NMR at 68 MHz. For $NT_1 >$ 200 msec the differences between the 68- and 25-MHz data are small, increasing as motion becomes slower. For LH-RH the central residue α-carbon NT_1 values at 68 MHz and 35° (~170 msec) correspond to $NT_1 \simeq 130$ msec at 25 MHz. The side chains of the aromatic carbons in LH-RH do not show a great increase in NT_1 values from those in the backbone; however the ortho and meta carbons of the tyrosine residue show evidence of some internal motion (Figures 2 and 3). In the cyclic amino acids, pyroglutamate and proline, we observe longer NT_1 values for certain carbons in the ring than for the backbone α carbons; this has been observed in other peptide hormones (Deslauriers et al., 1974c). There is a gradation in NT_1 values only between the β and γ carbons of arginine; those of the γ and δ carbons are not significantly different. This is characteristic of arginine in peptides (Keim et al., 1974; Deslauriers et al., 1975); similar behavior has been found for the ornithine residue in gramicidin S (Komoroski et al., 1975b). The C-terminal glycine residue shows an NT_1 value which is greater than that of the α carbon on the N-terminal pyroglutamate. At 68 MHz the observed NT_1 for the C-terminal glycine α carbon indicates that the effective motional correlation time at the C-terminus is ca. one-half of that for the central residues, including the glycine-6 residue. This observation may reflect greater intrinsic conformational flexibility of the glycine residue, which bears no side chain.

Table III: Spin–Lattice Relaxation Times,[a] and Effective Correlation Times of Carbons Bearing Protons in LH-RH Observed at 67.9 MHz.

	Temp					
	35°		40°		55°	
Residue	$NT_1 \times 10^3$	$\tau_{eff} \times 10^{10}$	$NT_1 \times 10^3$	$\tau_{eff} \times 10^{10}$	$NT_1 \times 10^3$	$\tau_{eff} \times 10^{10}$
<Glu						
αCH	295	1.7	340	1.4	400	1.2
βCH_2	380	1.3	420	1.1	800	0.58
γCH_2	460	1.0	420	1.1	680	0.69
His						
αCH	210	2.6	205	2.7	270	1.9
βCH_2	210	2.6	216	2.5	340	1.4
δCH	180	3.3	N.O.		N.O.	
ϵCH	N.O.		N.O.		N.O.	
Trp						
αCH	160	4.1	195	2.9	240	2.2
βCH_2	200	2.8	200	2.8	280	1.8
C-2	200	2.8	N.O.		N.O.	
C-4	160	4.1	N.O.		N.O.	
C-5	160	4.1	N.O.		N.O.	
C-6	190	3.1	N.O.		N.O.	
C-7	185	3.2	N.O.		N.O.	
Ser						
αCH	165	3.8	190	3.0	260	1.9
βCH_2	234	2.2	224	2.3	420	1.1
Tyr						
αCH	165	3.8	190	3.0	260	1.9
βCH_2	200	2.8	234	2.2	266	1.9
ortho CH	230	2.3	N.O.		N.O.	
meta CH	210	2.6	N.O.		N.O.	
Gly						
αCH_2	190	3.0	240	2.2	340	1.4
Leu						
αCH	175	3.4	205	2.7	270	1.9
βCH_2	240	2.2	286	1.8	400	1.2
γCH	260	1.9	313	1.6	440	1.1
δCH_3	1545	0.31	1590	0.29	1680	0.27
δCH_3	1440	0.32	1650	0.28	1860	0.25
Arg						
αCH	185	3.2	218	2.5	330	1.5
βCH_2	240	2.2	270	1.9	390	1.2
γCH_2	330	1.5	416	1.1	680	0.69
δCH_2	350	1.4	400	1.2	710	0.66
Pro						
αCH	220	2.4	240	2.1	380	1.3
βCH_2	470	1.0	500	0.95	660	0.70
γCH_2	480	1.0	496	0.96	680	0.69
δCH_2	260	2.0	280	1.8	460	1.0
Gly						
αCH_2	390	1.2	424	1.1	780	0.60

[a] NT_1 values given in seconds. Effective correlation times (τ_{eff}) are given in seconds. Concentration 200 mg/ml, pH 6.4. N.O., not observed.

The NT_1 values of LH-RH obtained at 25 MHz and at a concentration of 100 mg/ml can be compared with those obtained for [5-isoleucine]-angiotensin II (Deslauriers et al., 1975), oxytocin, and lysine-vasopressin (Deslauriers et al., 1974b). These hormones are octapeptides which have molecular weights of ca. 1000. LH-RH has a molecular weight of ca. 1200 and consequently the T_1 values obtained under conditions similar to those used for the octapeptides should be shorter. This, however, is not observed. The most restricted α carbons on the octapeptides have NT_1 values of the order of 95 msec. In LH-RH the average value of T_1 for the α carbons of the optically active residues is ca. 150 msec. These significantly longer values may result from the

presence of increased segmental motion in the backbone of LH-RH which is less significant in the cyclic octapeptides, oxytocin or lysine-vasopressin.

T_1 values can be related directly to molecular motion under certain conditions. In the limit of rapid motion on the ^{13}C NMR time scale, the greater the NT_1 value, the greater is the mobility of the C–H moiety. In the usual case, when dipole–dipole interactions provide the dominant relaxation mechanism, the observed T_1 value for a protonated carbon can be related to the effective correlation time (τ_{eff}) for overall molecular reorientation (Allerhand et al., 1971; Levy, 1973):

$$\frac{1}{T_1} = \frac{1}{T_1^{DD}} = \left\langle \frac{1}{r^6} \right\rangle \frac{N\hbar^2\gamma_C^2\gamma_H^2}{10} \times [f(\omega_H - \omega_C) + 3f(\omega_C) + 6f(\omega_H + \omega_C)] \quad (1)$$

where $f(\omega) = \tau_{eff}/(1 + \omega^2\tau_{eff}^2)$; ω_H and ω_C are the angular resonance frequencies of ^{1}H and ^{13}C, respectively; $\langle r^{-6} \rangle$ is the vibrationally averaged inverse sixth power of the ^{1}H–^{13}C internuclear distance; N is the number of directly attached hydrogens; $\hbar$ is Planck's constant/2π.

In LH-RH, dipole–dipole interactions provide the dominant relaxation mechanism, as is true for most carbon nuclei in large molecules (Lyerla and Levy, 1974; Doddrell et al., 1972). Nuclear Overhauser enhancements of 3.0 for the proton-bearing carbons of LH-RH observed at 25 MHz support this statement. Therefore eq 1 can be used to obtain an estimate of τ_{eff}.

The value of τ_{eff} thus contains contributions from overall rotational diffusion of the molecule (τ_{mol}) as well as from intramolecular motions (τ_{int}).

Recently it has been possible to estimate values for τ_{int} and τ_{mol} in the case of gramicidin S (Allerhand and Komoroski, 1973) and angiotensin (Deslauriers et al., 1975) in which the peptide backbone was considered to undergo no rapid internal motion, and to analyze T_1 data in terms of the degree of anisotropy of rotational diffusion (Becker et al., 1974; Deslauriers et al., 1975). In the case of LH-RH, however, it is clear that segmental motion occurs in both the backbone and the side chains of the hormone, and a detailed analysis is extremely complex. This is currently being attempted, and the results will be described elsewhere. For the present we shall discuss the relative mobilities (rates and amplitudes of motion of C–H moieties) in terms of net effective motion—the greater is NT_1, the greater the rate and/or angular amplitude of motion. Table III shows the NT_1 values observed at 68 MHz for LH-RH at a concentration of 200 mg/ml and the derived τ_{eff} values.

Discussion

Chemical Shifts. The observed chemical shifts of LH-RH and those calculated show a good agreement. No cross-over of resonances is seen between the two sets of results. This demonstrates the usefulness of calculated spectra in obtaining a preliminary set of assignments, which then must be confirmed by other methods such as substitution of specific amino acid residues with ^{13}C (Sogn et al., 1974) or ^{2}H (Brewster et al., 1973). The limited availability of amino acids specifically enriched in ^{13}C or ^{2}H renders this approach impractical at present. Another possibility is specific deletion of one residue from the peptide, and comparison of the ^{13}C NMR spectrum with that of the parent peptide. We have found this approach quite reliable; only the resonances of residues immediately neighboring that deleted are perturbed, and the perturbations are less than 0.3 ppm. Also useful is the successive addition of residues to short peptide fragments of known assignment. On comparing fragments in the LH-RH sequence with the spectrum of LH-RH itself, no significant differences in chemical shifts are observed (Smith et al., 1973; Wessels et al., 1973). In LH-RH in D_2O the Arg-Pro peptide bond is in the trans conformation (Wessels et al., 1973; Smith et al., 1973; Deslauriers et al., 1973c). It has been suggested that the His-Trp peptide bond of LH-RH is in the cis conformation (Humphries et al., 1974b). This was based on the chemical shift difference between the ^{13}C NMR resonances of histidine in thyrotropin-releasing hormone, <Glu-His-Pro-NH_2, and in LH-RH. However, we believe this difference is mainly attributable to the presence of a proline residue next to histidine in TRH. A proline residue is known to produce a "γ-steric effect" which results in an upfield shift of ca. 1 ppm in the residue attached to the nitrogen of proline (Christl and Roberts, 1972). Deletion of histidine in des-2-histidine-LH-RH should result in the loss of a cis His-Trp peptide bond and the more common trans peptide bond would be expected in the αGlu-Trp sequence. ^{13}C NMR spectra have been shown to be sensitive to cis and trans peptide bonds in proline (Thomas and Williams, 1972; Voelter and Oster, 1972, 1973; Smith et al., 1972; Deslauriers et al., 1972, 1973a; Dorman and Bovey, 1973; Grathwohl et al., 1973). This sensitivity to peptide bond conformation is reflected in all the resonances of the proline residue. If a cis $\rightarrow$ trans transition were to occur about the peptide bond preceding tryptophan, one would expect changes in the chemical shifts of the tryptophan. Such changes were not observed in des-2-histidine-LH-RH and therefore we do not find any evidence for the presence of a cis peptide bond in the His-Trp moiety of LH-RH. To test further for the existence of cis peptide bonds in the His-Trp moiety, one could synthesize the *N*-methyl derivative of tryptophan and incorporate this into a peptide. The presence of a methyl group is expected to lead to an equilibrium between cis and trans peptide bonds and the assignments might be used to characterize a cis or trans peptide bond in LH-RH.

The ^{13}C chemical shifts of LH-RH in aqueous solution do not provide any evidence for stacking of the aromatic rings of tryptophan and tyrosine. Deletion of tryptophan in des-3-tryptophan-LH-RH does not perturb the spectrum of the tyrosyl residue. Similar conclusions have been reported from ^{1}H NMR studies of LH-RH (Wessels et al., 1973).

Spin–Lattice Relaxation Times. The NT_1 values observed at 25 MHz for LH-RH at a concentration of 100 mg/ml are longer than those found for smaller, more rigid molecules such as oxytocin and lysine-vasopressin (Deslauriers et al., 1974b) under similar conditions. Based on these findings, we assume that the NT_1 values observed for the peptide backbone of LH-RH comprise contributions from both overall molecular motion as well as internal or segmental motion. In this work segmental motion is defined as motion of two moieties of fixed conformation about a bond connecting the moieties. This definition assumes that the motion is independent of the conformations of the two moieties with respect to all other bonds, and that these other conformations do not change during motion about the bond in question (Deslauriers et al., 1975). The backbone of LH-RH is quite mobile. The side chains of the aliphatic residues in LH-RH show additional segmental motion. Motion is more restricted at the α carbon due to "anchoring" of this carbon in the peptide backbone. Internal motion in-

creases from the point of attachment of the side chain. The cyclic residues pyroglutamate and proline show intracyclic motion. The intracyclic motion in proline is most pronounced for the β and γ carbons as has been observed in other proline residues occupying nonterminal positions in peptides (Deslauriers et al., 1974c) and polypeptides (Torchia and Lyerla, 1974). The aromatic residues do not show a large degree of segmental motion; this may result from the bulkiness of the side chain rather than from steric hindrance of neighboring residues. The N- and C-terminal residues show a greater degree of mobility than the backbone. The C-terminal glycine residue is much more mobile than the α carbon of the N-terminal pyroglutamyl residue. This may be attributed to the lack of a side chain on glycine which would allow more flexibility of the α carbon relative to those of optically active amino acids. The greater mobility of glycine is also apparent within the peptide backbone. The glycine residue at position 6 shows an NT_1 value for the α carbon which is significantly greater than those of the residues in positions 5 and 7.

Conclusion

The spectra of LH-RH in aqueous solution do not indicate any strong conformational preferences relative to those of its component peptides. The spectrum calculated from the constituent amino acids closely resembles that observed from LH-RH. The Arg–Pro peptide bond is trans. The arginyl residue demonstrates an upfield shift due to the "γ-steric effect" of the adjacent proline residue. A previously proposed cis His–Trp peptide bond in the LH-RH does not receive support from the ^{13}C spectra of LH-RH and des-2-histidine-LH-RH. There is an absence of ring-current effects on the chemical shifts of the aromatic residues in LH-RH.

The spin–lattice relaxation time measurements reveal that in aqueous solution LH-RH is a flexible molecule with segmental motion in the peptide backbone. segmental motion is also observed in the side chains of the aliphatic residues whereas the aromatic residues are more restricted. The glycine residue in position 6 shows more mobility than other α carbons in the peptide backbone.

These studies should now lead to the study of the chemical shifts and spin–lattice relaxation times of hyperactive [D-6-alanine]-LH-RH and [D-6-lysine]-LH-RH. By comparing these spectra with those of the reference compounds [L-6-alanine]-LH-RH and [L-6-lysine]-LH-RH it is hoped insight will be gained into the conformation of LH-RH which is active at the hormone receptor.

Acknowledgment

We are grateful for material supplied by the Contraceptive Development Branch (N.I.H.C.C.D.), National Institutes of Health.

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