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NMR Studies of Metal-binding Luteinizing Hormone Releasing Hormone

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Functions of the luteinizing hormone releasing hormone (LHRH) and its induced release by divalent metal ions have received great attention because this neurotransmitter subsequently regulates the secretion of luteinizing hormone (LH). Metal-LHRH complexes were synthesized by addition of various Cu(II),Ni(II),Zn(II) ions into LHRH in order to understand how the induced release of LHRH is possible. The degree of complexation was monitored by ^{1}H, ^{13}C-NMR chemical shifts, and final products were identified by Mass spectrometry. Solution-state structure determination of Zn(II)-LHRH out of metal-complexes was accomplished by using NMR and NMR-based distance geometry (DG). Interproton distance information from nuclear Overhauser effect spectroscopy was utilized for structure determination. Structure obtained in this study has a cyclic conformation exhibiting a specific α-helical turn with residue numbers His[2]-Leu[7] out of 10 amino acids. Comparison of chemical shifts and EPR studies of Ni(II),Cu(II)-LHRH complexes exhibit that these metal complexes have 4-coordination geometry.

Key Words : NMR, LHRH, Metal-LHRH complex

Introduction

Luteinizing hormone-releasing hormone (LHRH, or GnRH = gonadotropin-releasing hormone, MW=1,182) is a decapeptide endocrine biomolecule which regulates the secretion of gonadotrophins, luteinizing hormone (LH) and the follicle stimulating hormone (FSH). The LHRH, best known as a main neurotransmitter, consists of the following 10 amino acid sequences pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2. The synthesis and release of this peptide is regulated by the central nervous system and subject to environmental influences such as age, light, olfactory stimuli, and sexual stimulation.[1-3]

Although numerous LHRH analogs have been synthesized and structurally characterized, relatively little is known about the processes where the LHRH receptor is activated during the hormone action.[4-13] For agonist and antagonist synthesis, the substitution with L-amino acids on the position of Gly[6] decreases activities, whereas the substitution with D-amino acids (D-Ala, D-Val) increases the activity much more than that of native LHRH.[14-16] The properties of dimeric analogs (D-Gly10-[D-Lys6]GnRH-NHEt) indicate that the LHRH receptor is more readily activated by a bivalent ligands.[17] Several antagonists were synthesized by using solid phase peptide synthetic method to test receptor binding on the basis of some cyclic peptide analogs of LHRH. The most potent cyclic peptide antagonist analog is known as Ac-D-Phe(p-Cl)-D-Trp-Ser-Glu-D-Arg-Leu-Lys-Pro-D-ala-NH_2. Energy minimization methods and molecular dynamic studies show that this cyclic antagonist analog has a modified β-bend between residue position [5] and [8].[18-20]

Since Burrows reported that the chelated copper is a highly potent and possibly endogenous agent which includes the release of LHRH from its neurosecretary cells in the hypothalamus, the metal-binding effect of LHRH has been reevaluated in physiological pathway because of their structural unique function.[21] The copper ion, complexed to putative circulating small peptide chelators, is known to markedly stimulate LHRH release. Focusing on the stimuli of metal chelators and on the release of LHRH from the hypothalamus into the portal blood, there have been many papers published to discuss various aspects of conformational dynamics and metal binding effects of LHRH. Although lot of LHRH analogs have been synthesized and theoretically studied, detailed mechanistic roles and functions are not known for the free LHRH and metal-binding complexes of LHRH.

Although dose-response physiological experiments provide information on the efficiency of LHRH analogs, it does not give any detailed structural features, including a mechanism of LHRH releasing and the coordination chemistry of metal-binding LHRH which may provide important features of therapeutic agents. The purpose of this paper is to understand the transition metal-dependent regulation of LHRH releasing from hyperthalamus as well as the coordination chemistry of metal-binding LHRH (or affinities of metal chelators to LHRH) in stimulated LHRH releasing process. In order to understand the induced release of LHRH by divalent metal ions, metal binding LHRH complexes were made by introducing micro aliquot of Ni,Cu,Zn metal ions into LHRH ligand. Complexations were traced by chemical shift changes and NMR signal assignments of complexes were accomplished by homonuclear 2D NMR techniques. Further structural characterization by using NMR and NMR-based Distance Geometry (DG) were also carried out to obtain further insights of the stimulated LHRH releasing process.

Methods

Sample Preparation. A neurohormone LHRH with purity 99% and atomic absorption standard solution of metal ions including $NiCl_2$, $CuCl_2$ and $ZnCl_2$ were purchased from Sigma-Aldrich. LHRH was dissolved in demineralized HPLC grade pure water (pH 5.3), and subsequently standard metal ion were added for complexation by micro titration. During the complexation, the pH of each sample adjusted to pH 6.5-6.8 by titration of 0.001 M NaOH and HCl pertinently. The degree of complexation was monitored by the change of NMR chemical shift, and the final mass of each complex was identified with ESI-Mass and MALDI-TOF. After drying this solution by freeze dryer, water insoluble metal binding LHRH complex samples ca 10 mM were prepared by dissolving synthetic peptide into 500 mL in DMSO-d_6 containing *ca* 5% of D_2O solvent for NMR experiments.

Experiments. All NMR data were collected on Varian 500 MHz and 300 MHz system at 25 ºC by using homo-nuclear and heteronuclear correlation experiments including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), HMQC, and HMBC techniques. COSY: 2x256x1024 raw data matrix size; 16 scans per t1 increment; 1.5 s repetition delay; 6 Hz Gaussian and 90º shifted squared sine bell filtering in the t2 and t1 domains, respectively. TOCSY : 2x256x1024 raw data matrix size; 32 scans per t1 increment; 1.5 s repetition delay; 65 ms MLEV-16 continuous wave spin lock period; 6.25 kHz spin lock field strength, corresponding to 42 μs 90º pulse width; 6 Hz Gaussian and 90o shifted squared sine bell filtering in the t2 and t1 domains, respectively. NOESY: 2x256x1024 raw data matrix size; 64 scans per t1 increment; 2.8 s repetition delay period; 10, 50, 100, 300, 500 ms mixing period for nuclear Overhauser effect (NOE) buildup profile; 6 Hz Gaussian and 90o shifted squared sine bell filtering in the t2 and t1 domains, respectively. X-band EPR spectrum of Ni(II), Cu(II)-LHRH complexes were trcorded at room and 77 K temperature for the comparison of coordination geometry of metal complexes by utilizing Bruker EMX 300 spectrometer with 100 kHz field modulation. Magnet of 13 kW power carrying signal channel of 6-100 kHz with a resolution of 0.01 kHz and 0.05 G step was used.

Since the chelated copper is a highly potent and possibly endogenous agent and putative circulating small copper peptide chelators remarkably stimulate LHRH release, the systametic complexometric titiration was accomplished. Cu(II)-LHRH complex formation was achieved by pH titration curves obtained at 25 ºC with a Titronic universal (SCHOTT) automatic titration system and a CONSORT C831 pH meter. The ligand LHRH concentration was 2×10^{-3} mol·dm^{-3} and the metal ion to ligand ratio was 1:1. The ionic strength was adjusted to 0.1 mol·dm^{-3} with KNO_3 in each case. The titrations were performed over the range pH 2.5-11.0 with NaOH solution. Species distribution curves of metal complexes were calculated with the aid of a BEST program.[22]

Result and Discussion

^{1}H-NMR signal assignments of metal free LHRH and

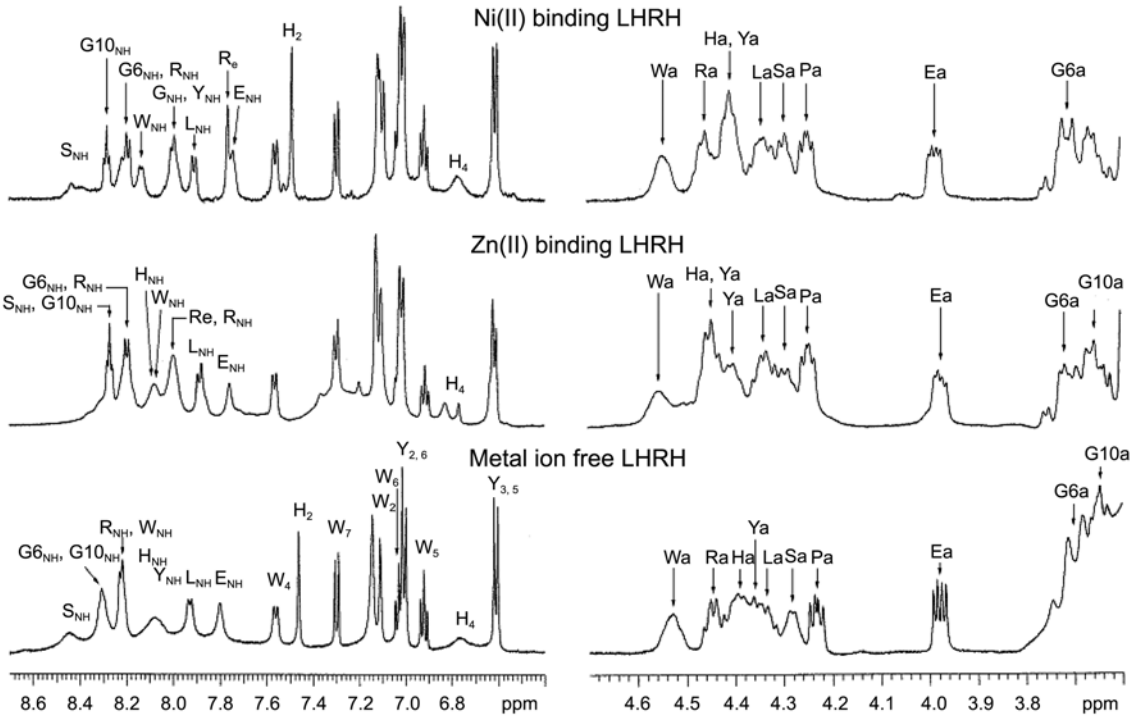

Figure 1. The spectral region of alpha protons of peptide bonds and aromatic protons are shown for LHRH and Ni(II),Zn(II)-binding LHRH.

metal binding LHRH complexes were accomplished by 2D COSY, TOCSY, NOESY experiments.[23,24] The metal binding LHRH complex was made by micro titration and monitored by spectral comparisons for the spectral region of alpha proton and NH proton. Although ^{1}H-NMR signal of Cu(II)-LHRH complex was so broad due to paramagnetic properties, ^{1}H-NMR signal of Ni(II),Zn(II)-LHRH complex was narrow enough to accomplish spectral assignment. The changes of chemical shift provide the possible metal binding site and structural information. As shown in Figure 1, the spectral region of alpha protons of peptide bonds associated with His[2]$_\alpha$ and Tyr[5]$_\alpha$ were clearly changed in Ni(II), Zn(II)-LHRH complex. Spectral comparisons between metal free LHRH and Ni(II),Zn(II)-LHRH complex are also depicted for the spectral region of NH protons of peptide bonds and aromatic protons. Noticeable NH peak changes were appeared in pGlu[1], His[2], Trp[3], Gly[6] and Arg[7] residues by addition of Ni(II) and Zn(II) ions into ligand LHRH. As suggested in previous paper,[25,26] the formation of Cu(II)-LHRH complex may form a coordination bond with three nitrogen donors (a N atom of the imidazole side chain and two amide-N atoms of the pyroglutamyl and histidinyl unit) in the physiological pH range (pH 5-9). The species distribution curves of Cu(II)-LHRH at pH range indicate that two possible protons are ionized during the bond formation with copper ion. However, a deprotonated Cu(II)-H$_{-1}$LHRH complex is major species at physiological pH condition as shown in Figure 2. It is believed that this structural modification induced by stimulus of metal ions is very important in efficient release of LHRH. Except for paramagnetic Cu(II)-LHRH complex, ^{1}H-NMR signal assignments of Ni(II),Zn(II)-LHRH complexes were made by determining scalar connectivities within amino acid residues from COSY and TOCSY spectra, and then correlating the signals of adjacent residues on the basis of dipolar connectivities from 2D NOESY data are summarized in Table 1.

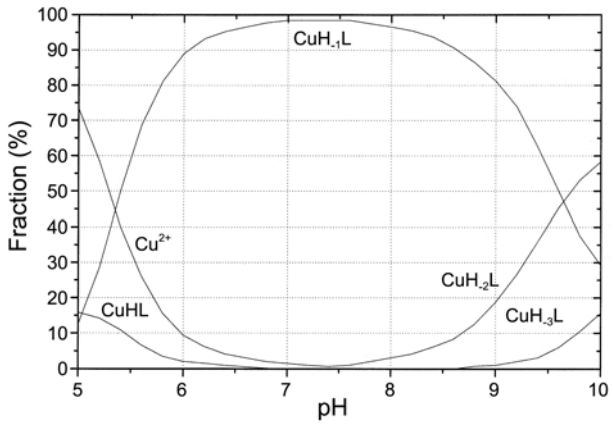

Figure 2. Species distribution curves as a function of pH calculated for the Cu(II)-LHRH complex under condition of $C_{Cu}^{2+} = 1.2 \times 10^{-3}$mol·dm^{-3}, $C_{LHRH} = 1.2 \times 10^{-3}$ mol dm^{-3}. H_n and L refers to the number of hydrogen to be deprotonated and free ligand LHRH, respectively, in CuH_nL complex formation.

Molecular geometry associated with electronic structures of Ni(II),Cu(II)-LHRH complexes exhibiting paramagnetic

Table 1. ^{1}H-NMR signal assignments of LHRH and Ni,Zn-binding LHRH

Residue	NH-proton			α-proton			β-proton			others		
	LHRH	Ni-L	Zn-L	LHRH	Ni-L	Zn-L	LHRH	Ni-L	Zn-L	LHRH	Ni-LHRH	Zn-LHRH
pGlu[1]	7.80	7.77	7.67	3.98	3.98	3.98	1.73,2.14	1.73, 2.14	1.72,2.13	N/A	N/A	N/A
His[2]	8.11	8.00	8.10	4.39	4.41	4.44	2.75,2.80	2.77,2.85	2.80,3.05	H_2 7.46 H_4 6.77	H_2 7.50 H_4 6.78	H_2 N/A H_4 6.77
Trp[3]	8.21	8.14	8.08	4.53	4.53	4.56	2.97,3.14	2.97,3.14	3.06,3.14	W_2 7.11 W_4 7.55 W_5 6.92 W_6 7.01 W_7 7.30 NH_{Ring} 10.84	W_2 7.10 W_4 7.56 W_5 6.92 W_6 7.03 W_7 7.30 NH_{Ring} 10.81	W_2 7.13 W_4 7.56 W_5 6.91 W_6 7.03 W_7 7.30 NH_{Ring} 10.79
Ser[4]	8.42	8.39	8.32	4.28	4.29	4.30	3.49,3.60	3.49,3.60	3.50,3.58	γ_{OH} 5.48	γ_{OH} 5.11	γ_{OH} 5.11
Tyr[5]	8.06	7.99	8.00	4.36	4.40	4.40	2.73,2.95	2.75,2.95	2.74,2.93	$Y_{3,5}$ 6.61 $Y_{2,6}$ 6.99 Y_{OH} 8.99	$Y_{3,5}$ 6.62 $Y_{2,6}$ 7.01 Y_{OH} 9.22	$Y_{3,5}$ 6.62 $Y_{2,6}$ 7.01 Y_{OH} 9.27
Gly[6]	8.23	8.22	8.20	3.70	3.70	3.70	-	-	-	-	-	-
Leu[7]	7.93	7.91	7.89	4.32	4.34	4.33	1.42,1.56	1.42,1.56	1.42,1.55	L_{me}0.82,0.86	L_{me}0.83,0.86	L_{me}0.82,0.86
Arg[8]	8.23	8.19	8.19	4.44	4.46	4.45	1.50,1.67	1.52,1.74	1.51,1.70	γ 1.49,1.67 δ 3.02 $\varepsilon_{(NH)}$ 8.53	γ 1.51,1.73 δ 3.09 $\varepsilon_{(NH)}$ 7.64	γ 1.51,1.70 δ 3.06 $\varepsilon_{(NH)}$ 8.02
Pro[9]	-	-	-	4.23	4.24	4.24	1.80,2.04	1.80,2.03	1.80,2.04	γ 1.80,1.94 δ 3.56,3.65	γ 1.80,1.94 δ 3.55,3.66	γ 1.80,1.94 δ 3.55,3.67
Gly[10]	8.32	8.28	8.27	3.58	3.58	3.58	-	-	-	-	-	-

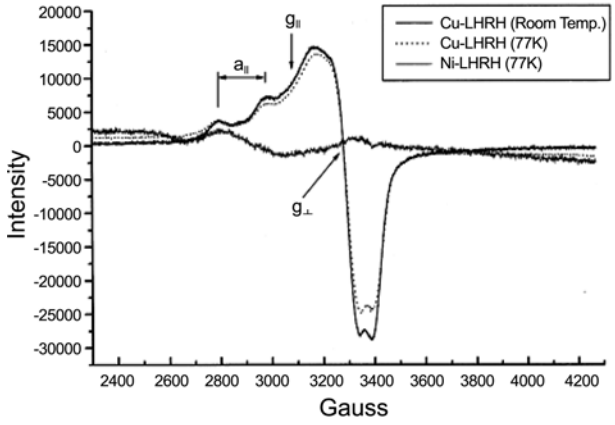

Figure 3. X-band EPR spectrum of Ni(II),Cu(II)-LHRH complexes with $g_{\parallel} = 2.207$, $g_{\perp} = 2.067$, $a_{\parallel} = 192.383$ values were recorded at 77 °K whereas no noticeable peaks were not observed for Cu(II)-LHRH at room temperature.

properties gave rise to signal broadening and change of chemical shifts near binding sites. Although no clear EPR signals was observed at room temperature, the X-band EPR spectra of Ni(II),Cu(II)-LHRH complexes at 77 °K exhibit that metal complexes may have similar coordination geometry as shown in Figure 3. As described in previous reports for various Cu(II),Ni(II)-peptide complexes,[27,28] the EPR spectra of Ni(II),Cu(II)-LHRH with similar values of $g_{\parallel} = 2.207$, $g_{\perp} = 2.067$ and $a_{\parallel} = 192.383$ indicates that these complexes may have 4-coordination geometry.

Structure determinations of Zn(II)-LHRH complex were carried out by using HYGEOM™, HYNMR™. In structural interpretations with distance geometry the experimental NOE constraints are best interpreted as a range of equally

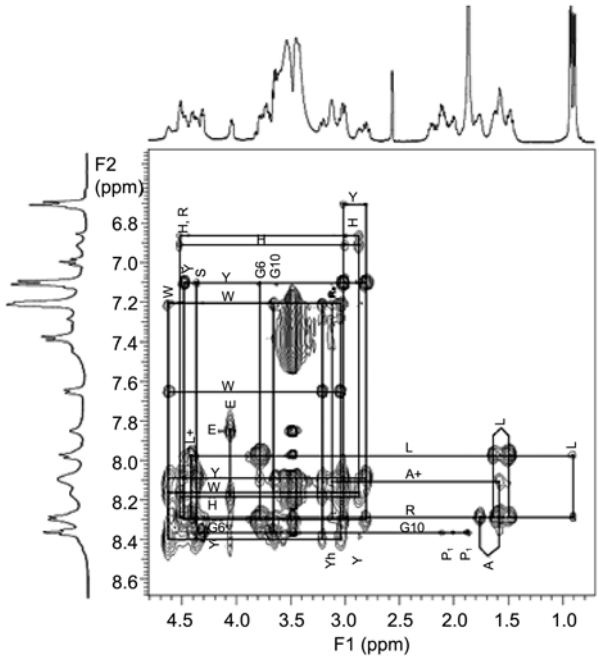

Figure 4. A portion of NOESY spectrum of Zn(II)-LHRH complex showing NOEs between amide NH protons and protons in up-field region. The sample was dissolved in DMSO-d_6 containing 5% of D_2O and measured with mixing time of 300 ms at 25 °C.

probable values for the distances in question. A portion of NOESY spectrum of Zn(II)-LHRH complex showing NOEs between amide NH protons and protons in up-field region is shown in Figure 4. Important NOEs used in NMR based structure determinations are summarized in Table 2. NOE restraints were divided into cross peaks classified as strong,

Table 2. Important NOE connectivities used for the structure determination of Zn-binding LHRH

^{1}H-NMR Signals	Chemical Shift (PPM)	NOE Connectivities
Trp[3](NH)	8.32	W_α (s), W_β (vw)
Gly[10](NH)	8.28	P_α (s), $G10_\alpha$ (s)
Gly[6](NH)	8.22	$G6_\alpha$ (s), Y_β (w)
Arg[8](NH)	8.18	R_α (s), R_β (m), L_β (m), L_α (s)
His[2](NH)	8.09	W_α (w), H_α (s), E_α (w), W_β (w), H_β (w)
Tyr[5](NH)	8.02	W_α (m)
Ser[4](NH)	8.02	W_α (w), Y_α (m), S_α (s), $G6_\alpha$ (vw), W_β (w), T_β (m)
Leu[7](NH)	7.90	L_α (m), $G6_\alpha$ (s), Lβr(s), L_β(m), E(NH)(w), G6(NH)(m)
Glu[1](NH)	7.77	E_α (m)
$Trp[3]_4$	7.57	W_α (w), W_β (w)
$Trp[3]_7$	7.57	W_α (w), W_β (w)
$Trp[3]_6$	7.14	W_α (w), W_β (w)
$Tyr[5]_2$	7.03	Y_α (w), Y_β (m), Y3(s)
$Tyr[5]_6$	7.03	Y_α (w), Y_β (m), Y5(s)
$His[2]_4$	6.83	H_α (w), H_β (vw)
$Leu[7]_\alpha$	4.35	Lm(m)
$Tyr[5]_{2,6}$	7.02	W2,5(m), Y3.5(s), W(NH,ring)(m)
$Trp[3]_{2,5}$	6.91	W(NH,ring)(w), W4,7(s)
His[2](NH,ring)	10.8	W(NH,ring)(m), G10(NH_2)(m), W6(s), W(NH)(vw), G6(NH)(vw), E(NH)(vw)

[a]Important NOEs are classified as strong(s), medium(m), weak(w), respectively, for NOE restraints in structure determination.

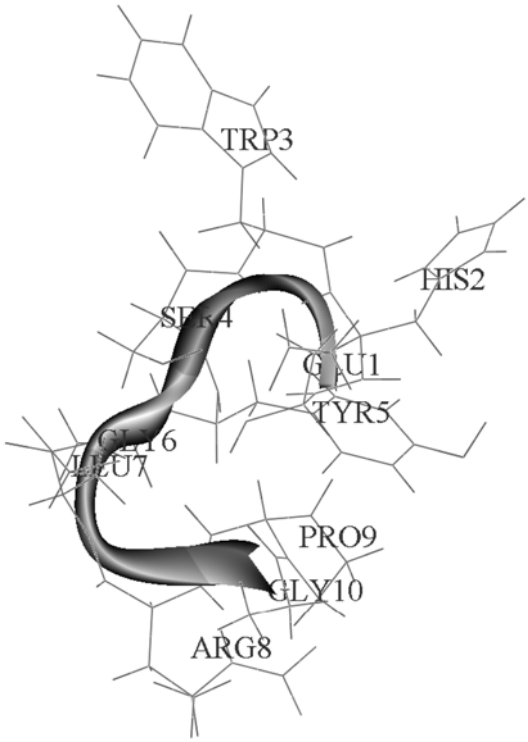

Figure 5. A NMR based solution state structure of Zn(II)-LHRH complex exhibiting a helical form with residue numbers His[2]-Leu[7].

medium, weak and very weak. The ranges of NOE restraints were assigned with 2.0-2.9 (strong peaks), 2.0-3.5 (medium peaks), 3.5-4.5 (weak peaks), 3.5-5.0 (very weak peaks). Standard pseudo atom corrections were applied to the NOE restraints. Distance geometry (DG) structures were generated and refined by using primary restraints. Trial distances generated by selecting random distances between the upper and lower bounds of each element were embedded. These DG structures were then subjected to conjugate gradient minimization (CGM), affording new structures with penalties in the range of *ca.* 0.8-2.0Å^2. When additional CGM was unable to further reduce the penalty for a particular structure, 2D NOESY back calculations were also performed, and new distance restraints dictated by discrepancies between the experimental and back-calculated spectra were added to the experimental restraint list. Freshly embedded DG structures minimized with the modified restraints list generally exhibited penalty values lower than those of the previously refined structures and the new DG structures generally gave back-calculated NOESY spectra that were more consistent with experimental data. This cycle of (1) random embedding, (2) minimal simulated annealing and CGM, (3) back calculation and (4) restraints modification was repeated iteratively until structures consistent with the experimental data could be obtained. The structure was calculated using the DG algorithm HYGEOM^{TM}, and 30 separate structures were generated using all constrains and random input. No further refinement by energy minimization was carried out on the output of the DG calculations. RMSD (root-mean-square distances) deviations between the NMR structures were 0.65 Å for the backbone. Back-calculation was made by using GNOE calculation in order to generate the theoretical NOEs. A consecutive serial files, obtained from GNOE calculation were incorporated into HYNMR^{TM} to generate NOE back-calculation spectra which can be directly compared with experimental NOESY spectra.[29] One of the resultant solution state 30 structures of zinc-binding LHRH exhibiting 1.32 Å of root mean square deviation was shown in Figure 5.

Conclusion

The ^{1}H-NMR studies of Zn-LHRH complex represents that the coordination of Zn-imidazole ring is in changes of the chemical shift of the C2-H proton. The stability constant studies with potentiometric technique as well as NMR for Zn(II)-LHRH complex show that zinc binds to the imidazole nitrogen and the peptide oxygen of the His-Trp bond, and no deprotonation of amide nitrogens occurs. Imidazole ring of histidine residue seems to be primary target for metals ions, but the identities of rest of N donor are still unknown.

Recent spectroscopic and potentiometric experiments demonstrated that Cu(II) (or Ni, Zn) forms a stable complex with LHRH.[25,26] These experiments indicated that an released LHRH may interact with Cu(II) ions in the vicinity of LHRH releasing sites. This peptide exhibits a metal-binding pattern that is stabilized both by coordination of a histidine and couple of deprotonated nitrogens to metal. Spectroscopic (UV/vis, CD) results provided that Cu-H$_{-1}$-LHRH has 3 N donors from LHRH (two amide nitrogens and histidine residue) in the pH range (pH 6-9), whereas Ni-H$_{-1}$-LHRH has 3 N/4 N from residues (pGlu, His, and deprotonated Nitrogens) and Zn-LHRH has a nitrogen and an oxygen donor from imidazole nitrogen and peptide oxygen of His-Trp bond, respectively. The results of our experiments including microtitration and EPR and NMR measurements are consistent with previous reports in metal binding LHRH studies. Although signal assignments of Cu(II)-LHRH complex could not be made because of paramagnetic signal broadening, the 4-coordination geometry in complex formation was observed by comparing the low temperature EPR experiments of Ni(II)-LHRH.[27,28]

Complete ^{1}H-NMR signal assignments for Ni(II),Zn(II)-LHRH complexes were accomplished by utilizing 2D NMR techniques. NMR-based DG computation enabled us to determine the solution state structure of zinc binding LHRH. The final 30 structures of LHRH having 0.19-0.28 Å^2 of penalty value, and 0.2-0.3 Å of root mean square deviations were obtained. Zinc binding sites of LHRH were observed to be imidazole nitrogen of His[2], oxygen in peptide bond between His[2] and Trp[3], and terminal NH_2 of Arg[8]. Refined structure of Zn(II)-LHRH having 0.18-0.30 Å^2 of penalty value were obtained. The backbone shape of metal complex resemble free LHRH's, like cyclic form. So far metal-LHRH studies were focused on understanding the nature of metal-LHRH (Ni,Cu,Zn-LHRH) which may pro-

vide insight into the specific biological meanings of these transition metals. The neurotransmitter LHRH targeting the release of luteinizing hormone can form metal complexes with transition metal elements so that the structural modification by specific 4-coordination geometry may facilitate the release of LHRH in the vicinity of hyperthalamus. Metal chelator, for example specifically designed Cu(II)-ligand, can be used in regulating the secretion of gonadotrophins, luteinizing hormone (LH), and the follicle stimulating hormone (FSH), and further biological studies are necessary.

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