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Molecular conformation of gonadoliberin using two-dimensional NMR spectroscopy

Kandala V. R. CHARY[1], Sudha SRIVASTAVA[1], Ramakrishna V. HOSUR[1], Kunal B. ROY[2] and Girjesh GOVIL[1]

[1] Tata Institute of Fundamental Research, Bombay
[2] Department of Biochemistry, All India Institute of Medical Sciences, New Delhi

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Complete resonance assignments of the proton NMR spectrum of gonadoliberin (in its native amide and free acid forms) have been obtained using two-dimensional nuclear magnetic resonance spectroscopy under three different environmental conditions, namely, dimethyl sulphoxide solution, aqueous solution and lipid-bound form in model membranes. The proton chemical shifts in the three cases have been compared to derive information about inherent conformational characteristics of the molecule. It has been inferred that the molecule possesses no short-range or long-range order under any of the three solvent conditions. However, there is a nonspecific increase in the linewidths when gonadoliberin is bound to model membranes, indicating a reduced internal motion in the molecule due to lipid-peptide interactions.

Gonadoliberin is a hypothalamic peptide consisting of a linear chain of ten amino acid residues (M_r = 1170). Its primary sequence has been established to be Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. The hormone, which plays a pivotal role in mammalian reproduction, regulates the secretion of folitropin and lutropin from pitutary. Folitropin and lutropin, in turn, regulate the production of sex steroids, thereby controlling spermatogenesis in the male and ovulation in the female. A major interest in contraception research has therefore focussed on this small peptide [1].

The native peptide lacks free terminal carboxyl groups. The hormone as well as its numerous analogues, agonists or antagonists, have been synthesised and studied. Substitutions with D-amino acids at specific positions, particularly at 2, 6 or 10, drastically alter the biological activity of the peptide [2]. Recently, a bioactive anti-gonadoliberin monoclonal antibody, which blocks ovulation in rats and prevents estrus in dogs, has been shown to recognise, though poorly, gonadoliberin peptide with a free carboxyl group at C-terminal or its different fragments (residues 4–6, 7–10 and 4–10). This may suggest that the relevant epitope comprises a conformation involving the entire molecule [3].

To understand the structure-function relationships of the hormone, extensive studies on the conformation in different solvents have been carried out [4–10]. However, no unequivocal structure could be established for the decapeptide from such studies. Using model building [11, 12] and potential energy calculations [13], a folded structure with a β bend has been suggested for the active form. In this structure, the N- and the C-terminals are in close proximity.

In view of these interesting observations, we have undertaken a comparative study of the native peptide (gonadoliberin-NH_2) and its free acid (gonadoliberin-OH) using two-dimensional NMR spectroscopy. Resonance assignments have been obtained following the usual strategies [15]. We first describe the resonance assignments of gonadoliberin in its native-amide and free-acid forms in fully deuterated dimethyl sulphoxide [$(CD_3)_2SO$] and deuterium oxide (D_2O) and in the lipid-bound forms in model membranes. The chemical shifts under the three different environmental conditions are compared to derive inherent conformational characteristics of the molecule. The spectra of only the native amide are described in detail since the spectral characteristics of the free acid are very similar to those of the amide.

EXPERIMENTAL PROCEDURES

Sample preparation

For all NMR experiments on gonadoliberin-NH_2 in $(CD_3)_2SO$ and D_2O, a concentration of 12 mM was prepared by dissolving 7.5 mg of the sample in 0.5 ml of the solvent. For experiments on gonadoliberin-OH, a solution concentration of 24 mM was used (14.2 mg of the sample in 0.5 ml solvent). Lipid vesicles were prepared using (D_{62})dipalmitoylglycerophosphocholine from Merck Sharp Dohme, Canada.

Gonadoliberin was incorporated in deuterated lipid bilayers by dissolving the required amount into a precooled solvent mixture of doubly distilled ethanol and chloroform. The solvent was evaporated by gentle bubbling of dry nitrogen gas through the solution, which resulted in a thin flim of the sample on the inside wall of the test tube. The sample was dried further under vacuum for 3–4 h and hydrated by 99.6% D_2O and allowed to equilibrate for 30 min. It was then heated in a water bath and vortexed. Unilamellar vesicles were prepared by sonicating the aqueous dispersions at a duty cycle of 50% for 15 min at 333 K. The dispersions showed optical clarity. Lipid/gonadoliberin ratios of 20:1 and 10:1 (mol/mol) were used in the case of free acid and native amide respectively. The NMR experiments were carried out at 333 K which is well above the gel-liquid crystalline phase transition temperature of the dipalmitoylglycerophosphocholine bilayers.

Correspondence to R. V. Hosur, Chemical Physics Group, Tata Institute of Fundamental Research Colaba, Bombay India 400005

Abbreviations. COSY, correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; $(CD_3)_2SO$, ($^2H_6$)dimethyl sulphoxide; Glp, 5-oxoproline or pyroglutamic acid.

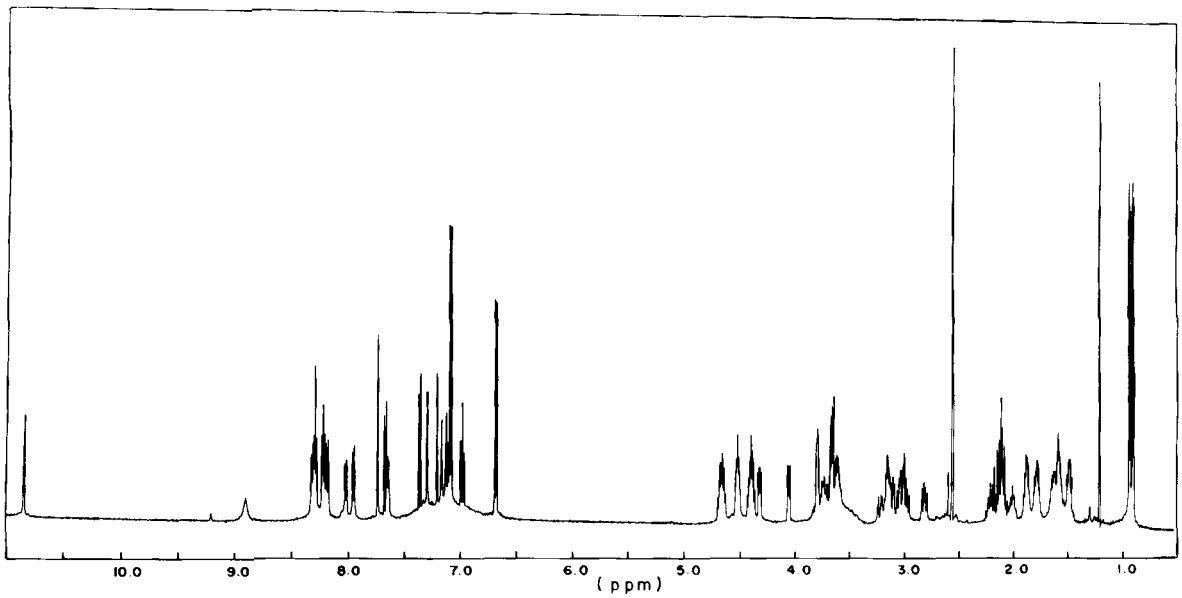

Fig. 1. *500-MHz ^{1}H-NMR spectrum of gonadoliberin-NH_2 dissolved in $(CD_3)_2SO$ at 297 K*

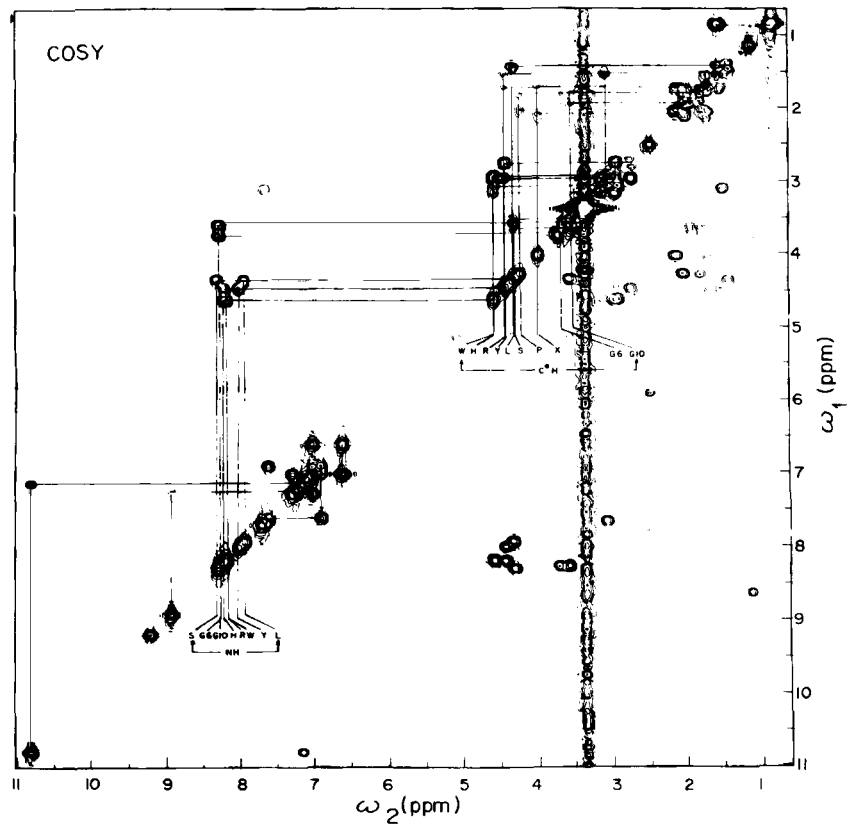

Fig. 2. *Contour plot of 500-MHz ^{1}H-COSY spectrum of gonadoliberin-NH_2 dissolved in $(CD_3)_2SO$ at 297 K.* The backbone NH and CαH protons are shown by the one-letter symbols of the respective amino acids in their respective regions. The glycines are distinguished as G6 and G10, where the numbers correspond to their positions in the sequence. The time domain data was collected in approximately 2 h and the digital resolution is 11.1 Hz/point. The spectrum contains all the expected *J*-coupling correlations and provides a complete fingerprint of the decapeptide. The solid straight lines connecting two distinct diagonal peaks through a cross peak show the *J*-coupling correlation. These lines depict complete spin system connectivities involving NH, CαH, CβH, CγH, etc. protons of all the residues in the peptide

NMR

^{1}H-NMR spectra were recorded on a Bruker AM 500 FT-NMR spectrometer whose operational frequency is 500 MHz for protons. The techniques used in these studies include two-dimensional proton-correlated spectroscopy (COSY) [14], super COSY [16, 17] and nuclear Overhauser enhancement spectroscopy (NOESY) [18, 19].

In the case of COSY experiments, the conventional COSY pulse sequence was modified to [---90-t_1-Δ-90-Δ-t_2] (Δ is a fixed delay time), so as to enhance the cross-peak intensities [16, 17]. A value of 10 ms was used for Δ. The t_1 value was

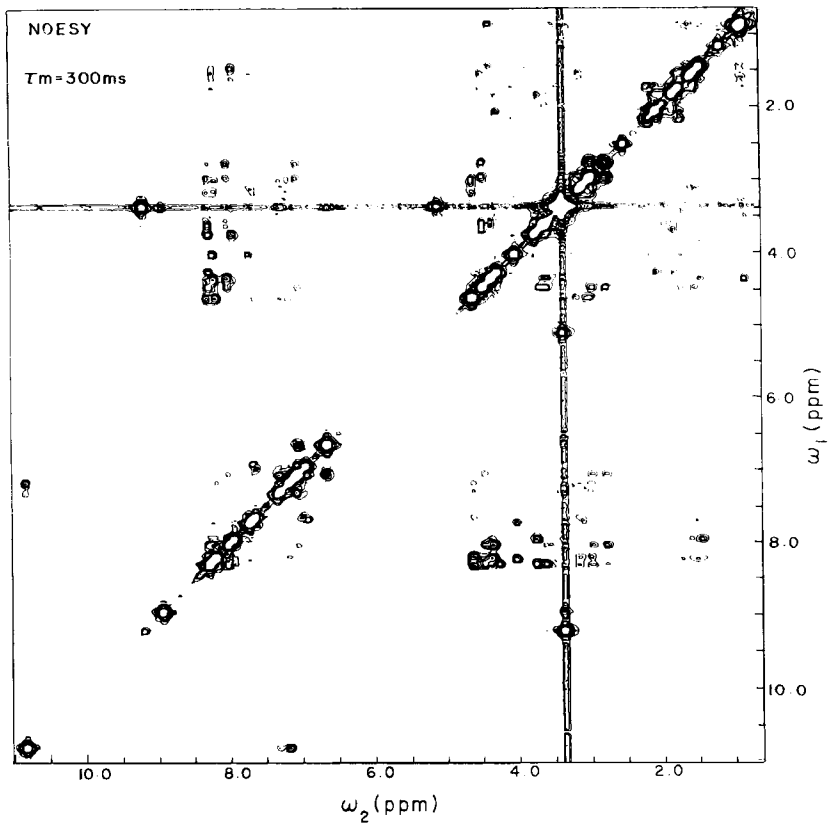

Fig. 3. *Contour plot of 500-MHz symmetrized ^{1}H-NOESY spectrum of gonadoliberin-NH_2 dissolved in $(CD_3)_2SO$ at 297 K.* The time domain data was collected in approximately 8 h and the digital resolution is 11.1 Hz/point. The mixing time is 300 ms

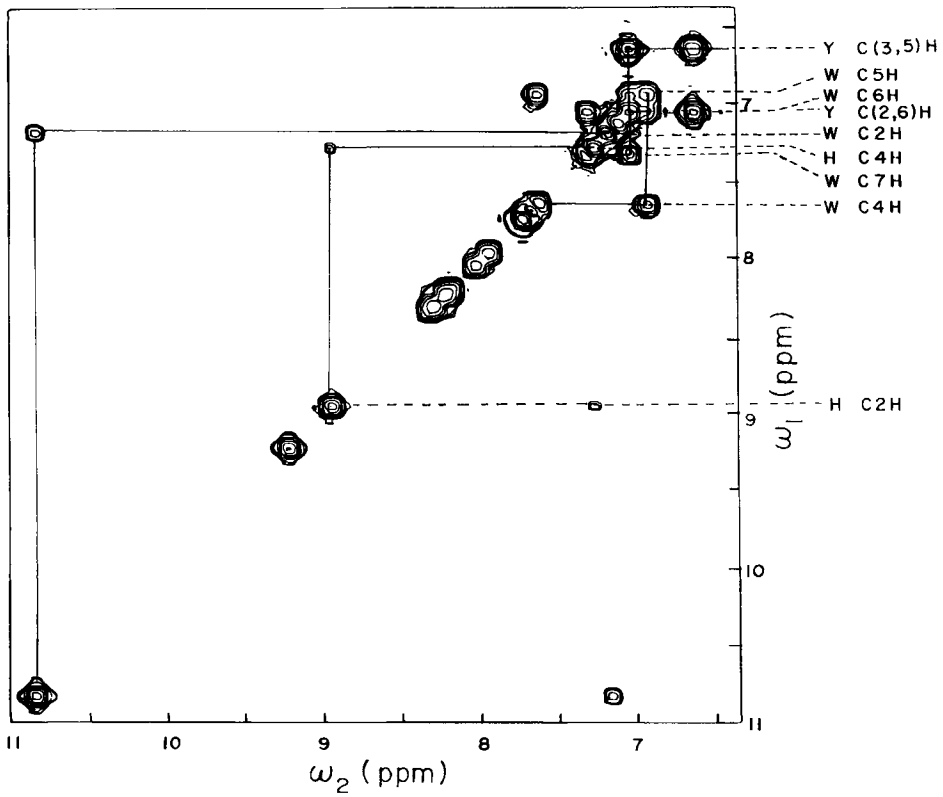

Fig. 4. *Expanded aromatic spectral region of the COSY spectrum (Fig. 2).* Unambiguous *J*-coupling correlations are shown for the aromatic spin systems of His, Trp and Tyr

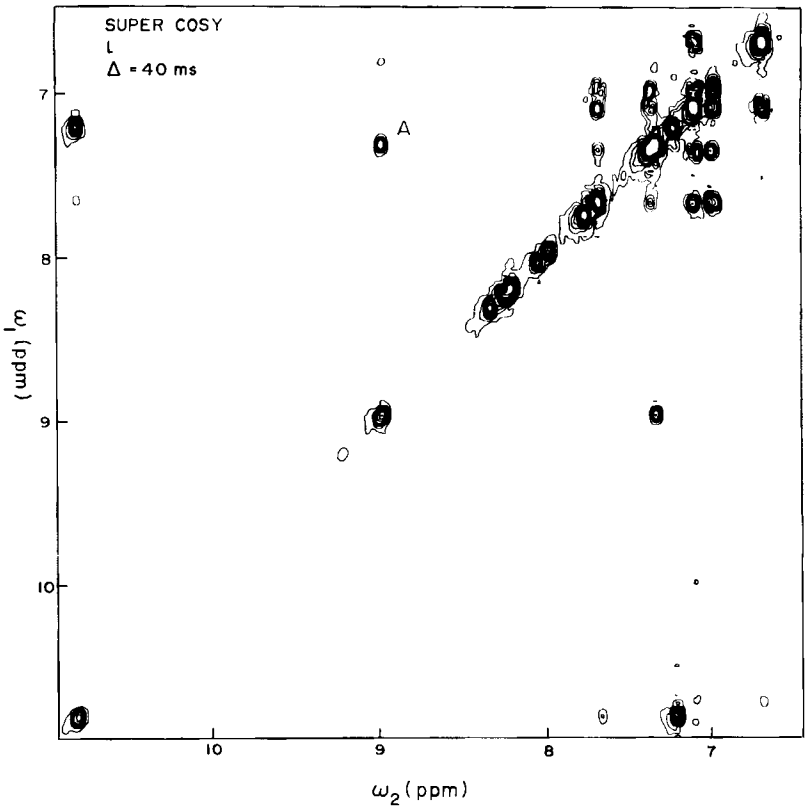

Fig. 5. *Aromatic spectral region of 500-MHz ^1H-super-COSY spectrum of gonadoliberin-OH dissolved in $(CD_3)_2SO$ at 297 K.* The pulse sequence used was [$\cdots$-90-t_1-Δ-180-Δ-90-Δ-180-Δ-t_2], where Δ is fixed delay. The fixed delay was 40 ms. The cross peak A depicts four-bond *J*-coupling between C2H and C4H protons of the His residue

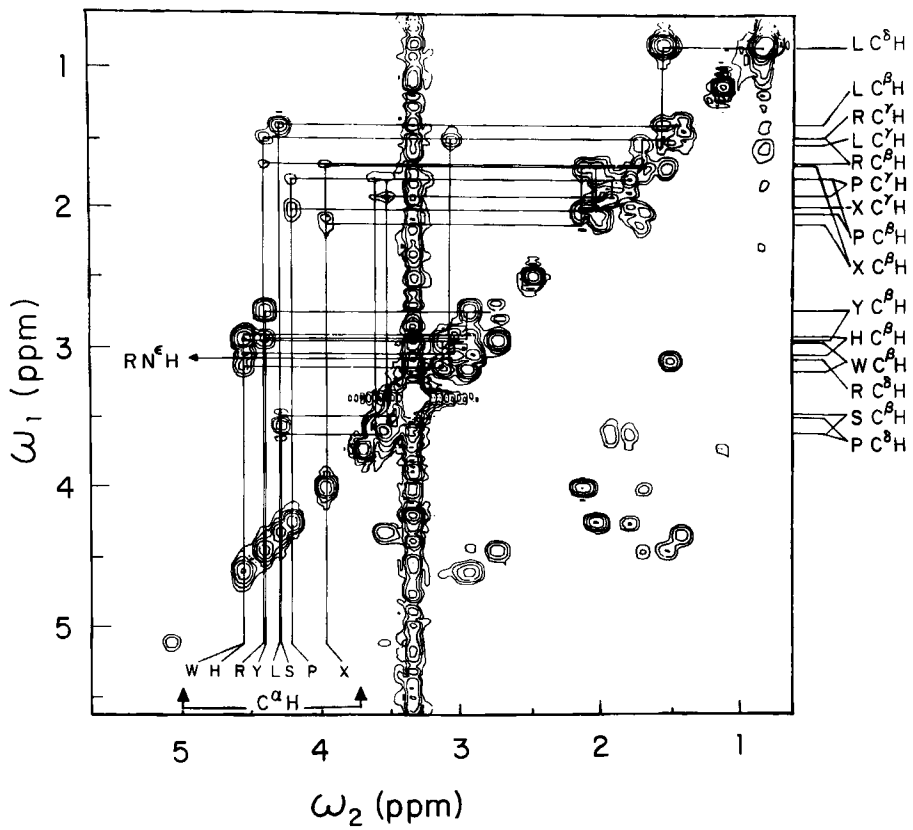

Fig. 6. *Expanded high-field region of the COSY spectrum (Fig. 2).* *J*-coupling correlations are shown for the aliphatic spin systems involving CαH, CβH, CγH, etc. protons. The side-chain methyl protons of Leu residue are not resolved in the COSY spectrum. However their different chemical shifts could be measured in the one-dimensional spectrum. The CγH protons and one of the CβH protons of the Arg residue are equivalent

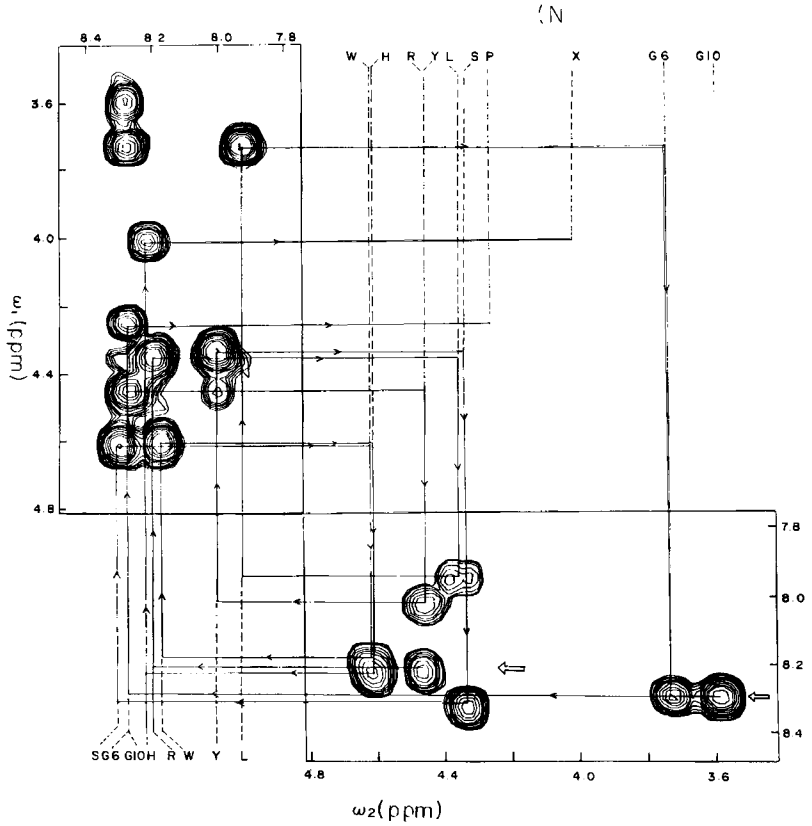

Fig. 7. *Combined COSY-NOESY connectivity diagram of gonadoliberin-NH_2 dissolved in $(CD_3)_2SO$ at 297 K for sequential resonance assignments via NOEs between the backbone NH protons and the CαH protons of the preceding residue (from NH_{i+1} to $C\alpha H_i$) d1 connectivity).* In the upper left of this diagram the region (ω_1 = 3.4–4.8 ppm) * (ω_2 = 7.8–8.5 ppm) from the NOESY spectrum (Fig. 3) is presented. In the lower right the region (ω_2 = 3.4–4.8 ppm; ω_1 = 7.8–8.5 ppm) from the COSY spectrum (Fig. 2) is shown. The solid lines and arrows indicate the connectivities between neighbouring residues in the decapeptide. The large arrows (bottom right) identify the starting point of the d1 connectivity pattern

varied from 10 ms to 55 ms in equidistant steps. In the case of NOESY experiments, the pulse sequence used was the conventional [---90-t_1-90-τ_m-90-t_2]. Here t_1 was varied from 10 μs to 45 ms in equal steps and a value of 300 ms was chosen for the mixing time τ_m. For COSY experiments, 16 transients were accumulated for each t_1 value, while the corresponding number was 32 for NOESY experiments. A relaxation delay of 1 s was used. For experiments in D_2O and lipid vesicles, continuous low-power irradiation of the HDO signal was carried out to avoid dynamic range problems. The two-dimensional spectra were obtained from 512 measurements along the t_1 axis. For each value of t_1, 1024 data points were collected along the t_2 axis. To enhance the digital resolution, the time domain data matrix was expanded by zero filling to 2048 and 1024 data points along the t_2 and t_1 axes, respectively, and it was multiplied by sine-square bell and sine bell window functions along the t_2 and t_1 directions prior to their respective Fourier transformations. Thus the frequency domain spectra consisted of a 512*512 point data matrix.

RESULTS AND DISCUSSIONS

Resonance assignment in $(CD_3)_2SO$

The one-dimensional 500-MHz ^{1}H-NMR spectrum of gonadoliberin-NH_2 in $(CD_3)_2SO$ at 298 K is shown in Fig. 1. This spectrum shows two well-separated spectral regions, one at 0.5–5.0 ppm and another at 6.5–11.0 ppm. The low-field region contains well-resolved resonances of the backbone NH, side-chain NH and the aromatic ring protons, while the high-field region contains all other protons.

Fig. 2 shows the contour plot of the COSY spectrum of gonadoliberin-NH_2 in $(CD_3)_2SO$. As usual, the diagonal of the COSY spectrum represents peaks corresponding to the resonances in the one-dimensional spectrum. Off-diagonal peaks indicate *J*-connectivities between two distinct diagonal peaks. One clearly sees seven out of the eight expected cross peaks connecting the CαH and the NH regions of the spectrum. The cross peaks belonging to His and Trp overlap. However, the sequential connectivities (see Fig. 6) show a small difference in the NH and CαH proton chemical shifts of these residues. The CαH of Pro residue does not show any *J*-coupling correlation to the low-field region because of the absence of the NH proton. Also, the N-terminal 5-oxoproline (pyroglutamic acid) of the peptide chain which is exposed to the solvent does not show a cross peak because of the rapid exchange of the labile backbone NH proton with water in $(CD_3)_2SO$ solution.

Fig. 3 shows the contour plot of the NOESY spectrum of gonadoliberin-NH_2. In this spectrum, a number of cross peaks are seen which show through-space connectivities.

The resonance assignment in the two forms of decapeptide has been carried out in three steps.

Aromatic ring proton identification. Fig. 4 shows the low-field region of the COSY spectrum with clear connectivities between the aromatic ring protons of Trp, His and Tyr residues. This spectrum provides information needed for unambiguous identification of all the side-chain spin systems in the

Table 1. *Chemical shifts of the assigned proton spin systems of gonadoliberin in its two forms in three different environments*
Chemical shifts in $(CD_3)_2SO$ have been measured with respect to external tetramethyl silane, those in D_2O and (D_{62})dipalmitoylglycerophosphocholine have been measured with respect to external sodium 3-trimethylsilyl(2,2,3,-^{2}H)propionate

Residue	Proton	$(CD_3)_2SO$		D_2O		Lipid-bound	
		$-OH$	$-NH_2$	$-OH$	$-NH_2$	$-OH$	$-NH_2$
		ppm					
Glp	NH	–	–	–	–	–	–
	α	4.03	4.03	4.18	4.20	4.20	4.20
	β	1.66, 2.07	1.65, 2.07	1.59, 2.28	1.64, 2.38	1.65, 2.35	1.70, 2.34
	γ	2.01	2.00	2.20	2.28	2.20	2.25
His	NH	8.21	8.20	–	–	–	–
	α	4.63	4.63	4.64	4.64	4.68	4.68
	β	3.01, 3.15	2.93, 3.08	3.02, 3.10	3.04, 3.12	3.05, 3.16	3.07, 3.18
	C2H	8.96	8.94	8.54	8.42	8.55	8.54
	C4H	7.29	7.26	7.10	7.15	7.18	7.20
Trp	NH	8.18	8.18	–	–	–	–
	α	4.64	4.64	4.69	4.70	4.75	4.75
	β	3.03, 3.18	2.98, 3.18	3.04, 3.12	3.12, 3.20	3.16, 3.27	9.16, 3.28
	C2H	7.10	7.15	–	–	–	–
	C4H	7.65	7.61	7.46	7.49	7.50	7.50
	C5H	6.94	6.91	7.21	7.24	7.22	7.21
	C6H	7.05	7.03	7.14	7.14	7.10	7.10
	C7H	7.17	7.26	7.56	7.58	7.60	7.60
	NH	10.83	10.83	–	–	–	–
Ser	NH	8.30	8.30	–	–	–	–
	α	4.35	4.35	4.34	4.34	4.33	4.31
	β	3.56, 3.64	3.50, 3.62	3.68, 3.60	3.74, 3.66	3.70	3.70
Tyr	NH	8.02	8.01	–	–	–	–
	α	4.48	4.47	4.44	4.45	4.46	4.50
	β	2.77, 2.98	2.75, 2.94	2.88, 3.02	3.94, 3.06	2.95, 3.07	2.95, 3.05
	C(2,6)H	7.08	7.03	7.08	7.11	7.10	7.10
	C(3,5)H	6.68	6.61	6.81	6.83	6.83	6.83
Gly	NH	8.28	8.27	–	–	–	–
	α	3.74	3.65	–	–	–	–
Leu	NH	7.94	7.91	–	–	–	–
	α	4.38	4.36	4.36	4.36	4.35	4.31
	β	1.44	1.42	1.59	1.63	1.59	1.60
	γ	1.58	1.58	1.54	1.60	1.50	1.60
	δ	0.87, 0.89	0.87, 0.90	0.86, 0.91	0.88, 0.93	0.90, 0.95	0.87, 0.90
Arg	NH	8.15	8.18	–	–	–	–
	α	4.47	4.47	4.51	4.51	4.35	4.60
	β	1.50, 1.68	1.50, 1.68	1.63, 1.72	1.66, 1.80	1.60	1.74
	γ	1.50	1.50	1.54	1.60	1.63	1.60
	δ	3.10	3.10	3.08	3.16	3.19	3.18
	ε	7.70	7.70	–	–	–	–
Pro	NH	–	–	–	–	–	–
	α	4.35	4.27	4.37	4.36	4.39	4.40
	β	1.82, 2.01	1.78, 2.03	1.92, 2.20	2.28, 1.96	1.95, 2.22	2.00, 2.30
	γ	1.82, 2.01	1.77, 1.91	2.05, 2.12	2.04, 1.94	1.80, 2.00	1.77, 1.91
	δ	3.52, 3.62	3.53, 3.62	3.50, 3.62	3.56, 3.70	3.59, 3.67	3.60, 3.73
Gly	NH	8.17	8.27	–	–	–	–
	α	3.82, 3.67	3.60	–	–	–	–

three aromatic residues. To illustrate, we may consider the Trp ring spin system in which the ring NH proton can be easily assigned to the low-field peak at 10.83 ppm. The cross peak at ω_1 = 7.15 ppm and ω_2 = 10.83 ppm depicts connectivity between the ring NH and the C2H protons. The proton at 7.61 ppm is coupled to proton at 6.91 ppm which also shows a *J*-coupling to another proton at 7.03 ppm, which in turn is *J*-coupled to the proton at 7.26 ppm. From the very nature of such a COSY pattern, it can be concluded that the pattern belongs to the Trp ring protons, i.e. C4H, C5H, C6H and C7H. The positon of C7H proton has been identified from NOESY experiments where one observes a strong NOESY peak (at ω_1 = 7.26 ppm, ω_2 = 10.83 ppm) between the ring NH and C7H protons. Starting from C7H, the other ring protons can be easily identified. At the end of assignment of the aromatic spin systems there remains one cross peak in the aromatic spectral region which can be attributed to the C2H and C4H protons of the His residue. The low intensity

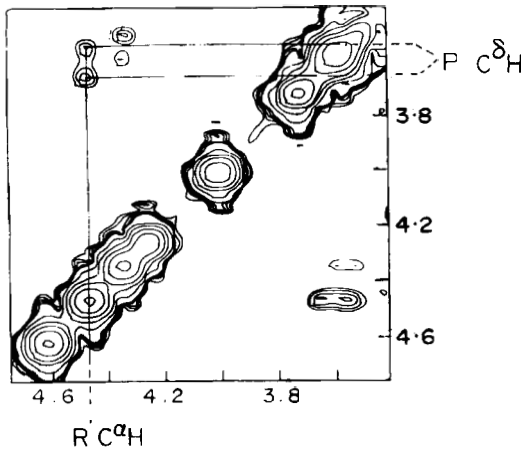

Fig. 8. *Contour plot of a portion of the NOESY spectrum depicting through-space NOESY connectivity between CαH proton of Arg residue and the CδH protons of the proline*

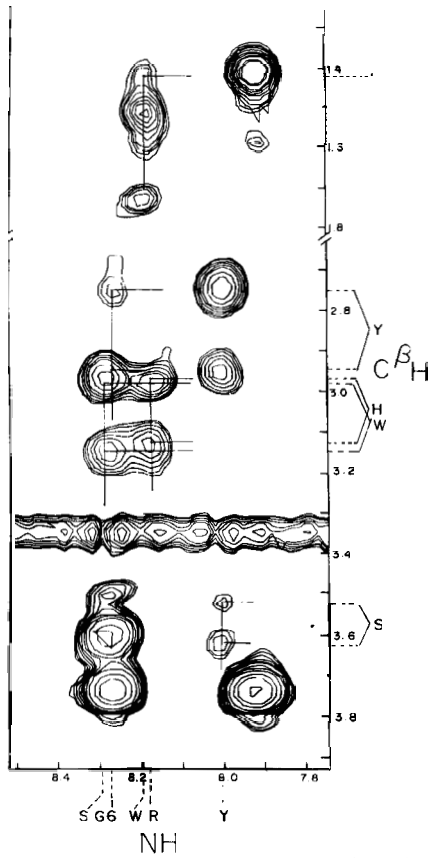

Fig. 9. *Contour plot of the NOESY spectrum (Fig. 3) depicting d3 connectivities (from NH_{i+1} to $C\beta H_i$)*

of this cross peak is the result of the relatively small value of the four-bond coupling constant. This has been confirmed using a super COSY experiment designed to enhance cross peaks with small values of coupling constants (cross peak A in Fig. 5). The individual assignments of the two protons could be easily obtained on the basis of chemical shifts, C2H being down field with respect to C4H.

Identification of aliphatic spin system. Fig. 6 also shows connectivities involving aliphatic protons of various amino acid residues of the decapeptide. The spectrum contains all the expected *J*-coupling correlations and thus provides a complete

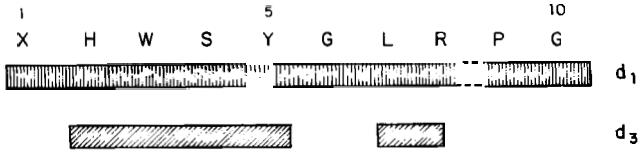

Fig. 10. *Amino acid sequence of gonadoliberin-NH_2 and survey of the sequential connectivities from which individual spin-system assignments were obtained.* Identical connectivities were obtained for gonadoliberin in its two forms. The vertically hatched parts indicate d1 connectivities (from NH_{i+1} to $C\alpha H_i$) and the diagonally hatched parts indicate d3 connectivities (from NH_{i+1} to $C\beta H_i$). The dotted box shows the NOE between the Arg CαH proton and the Pro CδH protons

fingerprint of the decapeptide. It is seen that the CβH protons of all the amino acid residues are well resolved and the CαH-CβH connectivities can be established in all cases. The CβH protons of the aromatic residues are identified on the basis of NOESY peaks from the aromatic protons to the CβH protons. Among the long side-chain residues, the Leu spin system is identified starting from the CH_3 resoances which could be assigned on the basis of their chemical shifts. In the case of Arg, the NεH proton could be identified from the observation of a COSY cross peak (at $\omega_1 = 3.10$ ppm and $\omega_2 = 7.70$ ppm) and forms the starting point for the identification of the remaining side-chain protons. However, the terminal NH and NH_2 protons of this residue could not be assigned because they do not show any through-bond *J*-connectivities in the COSY spectrum. Finally, observation of NOESY cross peaks between the CαH proton of Arg and CδH protons of Pro (see Fig. 8) provides a straightforward identification of CδH protons of Pro and consequently of the remaining side-chain protons, from the COSY spectrum. The observed chemical shifts of all the proton spin systems in the case of gonadoliberin in its two forms are listed in the Table 1.

Sequential connectivities. Sequential connectivities between successive residues provide individual assignments of the spin system. This is achieved through a combined use of COSY (Fig. 2) and NOESY (Fig. 3) spectra of the molecule recorded under identical conditions [20, 21]. Fig. 7 shows the COSY-NOESY connectivity diagram for the decapeptide in its amide form. The vertical lines into the NOESY spectrum indicate through-space NOE connectivities between NH_{i+1} and $C\alpha H_i$, while the lines (horizontal or vertical) into the COSY indicate the through-bond *J*-connectivities between $C\alpha H_i$ and NH_i. However, such an assignment is truncated at Cα-H proton of Pro residue because of the absence of a backbone amide proton in the Pro residue. This discontinuity could be bridged with the observation of strong NOEs from CδH protons of Pro residue to the CαH proton of Arg residue (Fig. 8). Additional support for the assignments has been obtained from the so-called d3 connectivities which are shown in Fig. 9. A summary of all the sequential connectivities is given in Fig. 10. It is worth mentioning here that the experiments on gonadoliberin-OH also revealed a similar kind of sequential connectivities.

Resonance assignments in D_2O

The one-dimensional 500-MHz ^{1}H-NMR spectrum of gonadoliberin-NH_2 in D_2O at 298 K is show in Fig. 11. This spectrum shows two very well separated spectral regions, one at 0.5–5.0 ppm and the other at 6.5–8.5 ppm. The low-

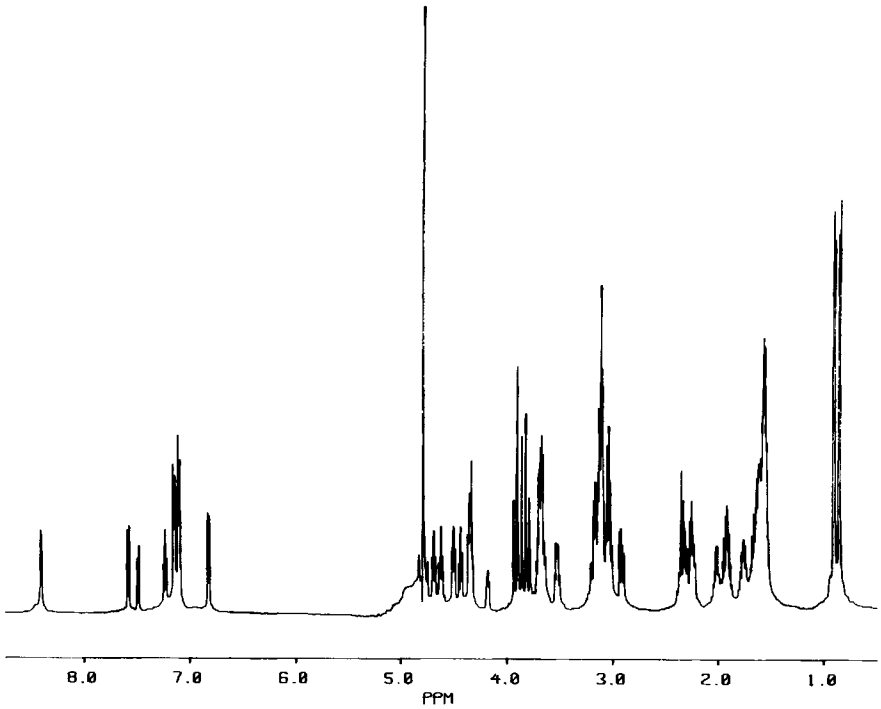

Fig. 11. *500-MHz ^{1}H-NMR spectrum of gonadoliberin-NH_2 dissolved in D_2O at 298 K*

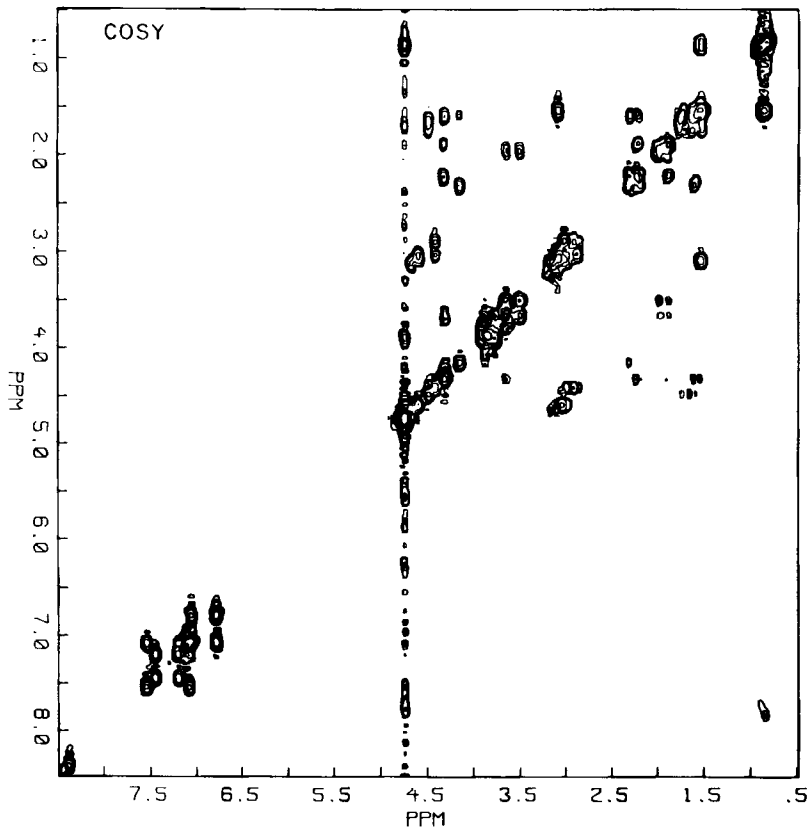

Fig. 12. *Contour plot of 500-MHz ^{1}H-COSY spectrum of gonadoliberin-NH_2 dissolved in D_2O at 298 K.* The digital resolution in this spectrum is 8.5 Hz/point

field region contains well-resolved resonances of aromatic ring protons, while the high-field region includes those of all other protons.

Fig. 12 shows the contour plot of the COSY spectrum of gonadoliberin-NH_2 at 298 K. Comparison of this spectrum with that in $(CD_3)_2SO$ indicates that COSY connectivity patterns involving CαH, CβH, CγH, etc. protons (including aromatic ring protons) of all the individual amino acid residues are similar in the two cases. Thus, all the spin systems, except CαH protons of Gly residues have been identified. The

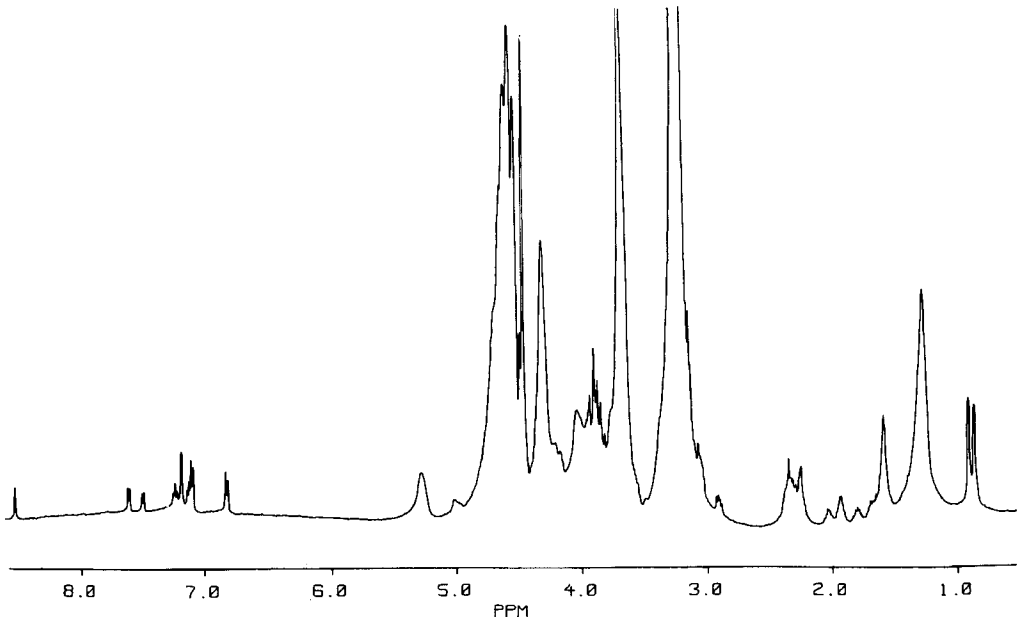

Fig. 13. *500-MHz ^{1}H-NMR spectrum of gonadoliberin-NH_2 entrapped in (D_{62})dipalmitoylglycerophosphocholine vesicles at 333 K*

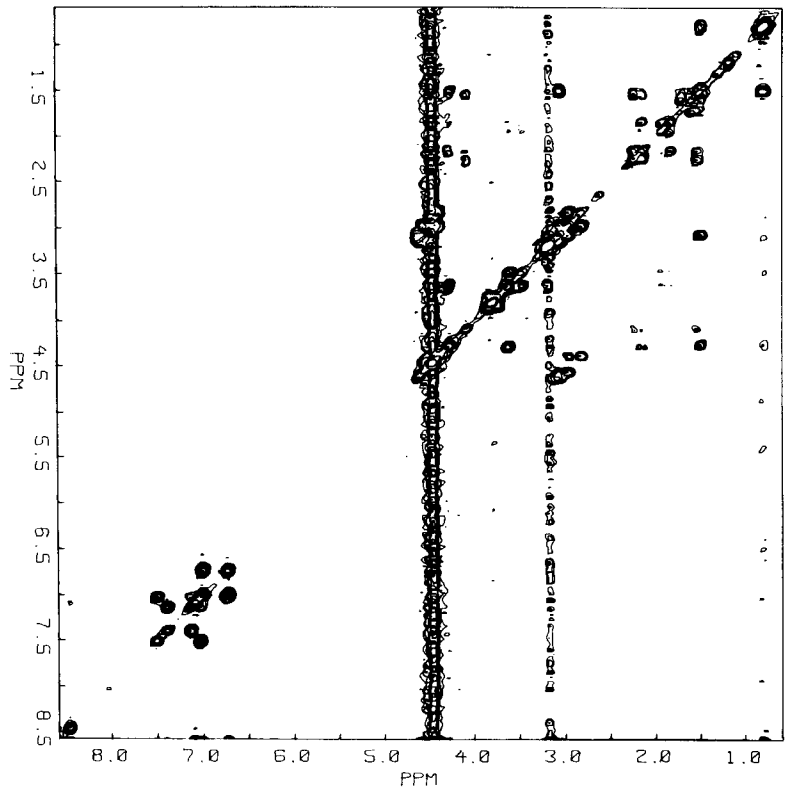

Fig. 14. *Contour plot of 500-MHz ^{1}H-COSY spectrum of gonadoliberin-NH_2 in the lipid-bound form at 333 K.* The digital resolution in this spectrum is 8.5 Hz/point

CαH protons of glycines could not be assigned unambiguously because of the extensive overlap in this region. Also in D_2O solutions, sequential d1 and d3 connectivities are not observed. Table 1 shows all the observed chemical shifts in the case of gonadoliberin-NH_2 and gonadoliberin-OH.

The NOESY experiments on these molecules at various mixing times ranging over 50 – 500 ms in D_2O did not provide additional information on spin system correlations.

Resonance assignments in lipid-bound gonadoliberin

The one-dimensional 500-MHz ^{1}H-NMR spectrum of gonadoliberin-NH_2 in lipid vesicles at 333 K is shown in Fig. 13. This spectrum shows substantial line broadening and many of the resonances are shifted with respect to their positions in free gonadoliberin, indicating that these protons are in a different chemical environment when the hormone is entrapped in dipalmitoylglycerophosphocholine vesicles.

Fig. 14 shows the contour plot of the COSY spectrum of lipid-bound gonadoliberin-NH_2 at 333 K. Comparison of this spectrum of the decapeptide with that in $(CD_3)_2SO$ shows very similar COSY connectivities involving the non-exchangeable proton spin systems of the individual amino acid residues. From such connectivities, all the spin systems except CαH protons of Gly residues, have been identified unambigously. The CαH protons of glycines could not be assigned unambiguously because of the extensive overlap in this region. Also, in the lipid-bound environment no sequential d1 and d3 connectivities are observed. Table 1 lists all the observed chemical shifts in the case of lipid-bound gonadoliberin in its two forms.

As in the case of results in D_2O, here also the NOESY experiments at various mixing times ranging over 50–700 ms provided no additional information for the correlations of spin systems.

CONCLUSIONS

The chemical shifts of the various protons in gonadoliberin in their three different environments, viz. $(CD_3)_2SO$, D_2O and (D_{62})dipalmitoylglycerophosphocholine vesicles are found to be similar indicating that the molecular conformation is not significantly influenced by solvent conditions. Further, most of the chemical shifts are very close to the corresponding shifts in free amino acid residues. The NOESY experiments at various mixing times in D_2O as well as in lipid vesicles show only self NOEs. An absence of long-range NOEs suggests the absence of any short-range or long-range order in the structure in the two environments. This conclusion is in sharp contrast with the earlier speculations [8, 9] which have suggested that the decapeptide structure involves two half γ turns resulting from the hydrogen bonding interactions between Ser and His. This also contradicts the hypothesis of Grant and Vale [22] that the decapeptide adopts a conformation such that the two ends of the molecule come together and form a hydrogen bond across the chain as in a β-pleated sheet. The speculation of Chang et al. [7] that there could be stacking of the indole and phenolic rings parallel to each other as a result of π-bond interactions is also inconsistent with the present observations. Our results indicate that the molecule is essentially a random coil under all the three solvent conditions used here and adopts predominantly extended conformations. It has, however, different mobilities under the three different environmental conditions. The mobility is significantly reduced when the hormone is bound to the lipid vesicles, as is evident from the consistent line broadening of the signal.

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