Conformational Characteristics of Luliberin. Circular Dichroism and Fluorescence Studies†

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ABSTRACT: By circular dichroism and fluorescence spectroscopy, the conformation of luliberin (luteinizing hormone-releasing hormone) has been investigated under various conditions of pH and solvents. Several structural parameters have been defined which seem predominant for the maintenance of the hormone in some privileged conformation(s). Formation of an intramolecular hydrogen bond between CO (His) and NH (Ser) seems likely when dissolving the hormone in organic solvent such as dioxane. Energy transfer has been demonstrated between Tyr and Trp residues. Calculation of the energy-transfer efficiency at different pH's allowed us to estimate in the range of 10 Å the distance which separates these residues. Evidence is also provided for a charge-transfer interaction between protonated histidine and tryptophan. These data suggest that, when luliberin has organized structure (under appropriate surrounding conditions), its conformational pattern would resemble that of β-turn structure in which a β bend would exist at the level of the aromatic residues.

The hypothalamic hormone luliberin[1] is a decapeptide (5-oxo-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) which stimulates the secretion of both gonadotrophins lutropin and follitropin (Burgus et al., 1971; Matsuo et al., 1971). Investigations of interactions between luliberin and its pituitary cell receptor(s) first require the characterization of the receptor site(s). For that purpose, tritium-labeled luliberin was prepared (Marche et al., 1972) and permitted to distinguish between two pituitary binding sites (Grant et al., 1973). In a further step, to understand the structure–activity relationship between primary structure and biological activities of the hormone, one has to gain insight into the parameters required both for the specific binding to the receptor(s) and for the triggering of the biological response. One way of approaching these problems is to measure and compare the biological activities of synthetic analogues (Monahan et al., 1973; Yanaihara et al., 1973; Coy et al., 1974; Ling and Vale, 1975). Another way consists of determining the intrinsic conformational characteristics of the hormone or its possible conformational modifications which are necessary for its binding to the receptor target.

Conformational studies of luliberin have been undertaken by circular dichroism and NMR spectroscopy. In a previous communication (Marche et al., 1973), we reported that luliberin exists under a random conformation in acidic medium, and that increasing the pH or adding an organic solvent such as trifluoroethanol (TFE) promoted a marked stabilization of ordered conformation(s) of the peptide. It has also been shown (Wessels et al., 1973; Deslauriers et al., 1975) that the Arg-Pro peptide bond is predominantly in the trans conformation. On the other hand, studies on analogues of luliberin have revealed that tryptophan in position 3 of the decapeptide is one of the most important and interesting residues for its biological activities (Coy et al., 1974).

In order to get more information about the luliberin conformation and more specifically about the environment of its aromatic residues, we have examined this peptide and some of its analogues and peptidic fragments by circular dichroism and fluorescence spectroscopy under various pH conditions (4, 7.8, 11.5) and in organic solvents. In the present publication we report the major characteristics needed by luliberin to adopt some organized structure. In addition we have estimated the distance between Trp and Tyr residues in luliberin, as a function of the surrounding conditions. It also appears that passing from water to trifluoroethanol promotes a conformational modification and a change in the tryptophyl environment.

Materials and Methods

Luliberin, [Leu^8]luliberin, [Lys^8]luliberin, [Pro^6]luliberin, Des-His^2-luliberin (5-oxo-Pro-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), luliberin-(OH) (i.e., luliberin deamidated on its C-terminal glycine), Des-Gly-NH_2^{10}-[Pro-NHEt^9]luliberin (5-oxo-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHEt), and the tripeptidic fragments, 5-oxo-Pro-His-Trp-NH_2 and 5-oxo-Pro-His-Trp-OMe, were generously provided by Drs. R. O. Studer and D. Gillessen (Hoffmann-La Roche, Switzerland). Synthetic luliberin from Bachem has also been used which behaved as that from the preceding source. The tripeptide 5-oxo-Pro-Trp-Pro was a gift of Squibb. *N*-Ac-Trp-NH_2 and *N*-Ac-Tyr-NH_2 were supplied by Sigma. Trifluoroethanol (TFE) and dioxane, for spectroscopy, were purchased from Merck (West Germany) and used without further purification. All other chemicals or solvents were of analytical grade from Prolabo (France).

Circular dichroism measurements were performed at room temperature (21 ± 1 °C) using a Jouan Dichrograph II. The range of peptide concentration used was 0.5 to 1.0 mg/ml, i.e.,

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[1] Abbreviations used: CD, circular dichroism; NMR, nuclear magnetic resonance; TFE, trifluoroethanol; 5-oxo-Pro, pyroglutamyl residue; all optically active amino acids are in the L configuration; as recommended by the *IUB-IUPAC Commission of Biochemical Nomenclature* ((1975) *J. Biol. Chem. 250*, 3215–3216), trivial names such as luliberin, lutropin, and follitropin stand for luteinizing hormone-releasing hormone (LH-RH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), respectively.

$\sim 5 \times 10^{-4}$ to 10^{-3} M with cells of 0.01 to 0.2 cm path length. All the circular dichroism experimental data have been expressed as molar ellipticities $[\theta]$ in deg cm^2 × dmol^{-1}.

Fluorescence spectra were recorded at 21 °C with a Jobin Yvon spectrofluorimeter modified as already described (Brun et al., 1975). They were not corrected for monochromator transmission and photomultiplier response. For each experiment, fluorescence emission of peptides was compared with that of a model system (an equimolar mixture of *N*-Ac-Trp-NH_2 and *N*-Ac-Tyr-NH_2) having the same absorbance as that of the peptide (0.6 at 280 nm, i.e., $\sim 10^{-4}$ M). To investigate energy-transfer processes, experiments were performed at 8 °C on dilute (4×10^{-6} M) solutions with a differential *absolute* spectrofluorimeter FICA 55000 with buffer used as reference and 2.5-nm excitation or emission slits. Spectra recorded with this apparatus were automatically corrected for the wavelength dependence of lamp intensity, monochromator transmission, and photomultiplier response. Fluorescence measurement conditions (excitation and emission wavelengths) will be mentioned on each figure. All compounds were in a 0.1 M NaCl solution (buffer if necessary) and the pH was adjusted with small amounts of 0.1 M NaOH or HCl.

Accessibility of the Trp residue of luliberin to solvent was studied by measuring its fluorescence quenching by acrylamide (Eftink and Ghiron, 1975). To avoid the screening effect due to acrylamide, solutions were excited at 300 nm and the fluorescence emission was recorded at 360 nm. The results were analyzed according to the modified Stern–Volmer equation (eq 1):

$$I_0/(I_0 - I) = 1/([X]f_a K_Q) + 1/f_a \quad (1)$$

In this equation, I and I_0 are the fluorescence intensities in the presence and absence of acrylamide, respectively, K_Q is the Stern–Volmer quenching constant, [X] the concentration of quencher, and f_a the fractional accessibility of Trp residue. Values of K_Q were calculated from the ratio intercept/slope when $I_0/(I_0 - I)$ is plotted against $1/[X]$ (eq 1). In this representation a value of 1 for the intercept will indicate a total accessibility of the fluorophor to quencher.

Anisotropy measurements were performed at 0 °C in a highly viscous medium (95% glycerol in water) to diminish depolarization arising from thermal motion. Although thermal depolarization might not be completely eliminated under these experimental conditions, these measurements allowed us to compare fluorescence anisotropy of free amino acids and luliberin and to provide evidence for energy transfer from Tyr to Trp. The fluorescence anisotropy, R, as a function of the excitation wavelength, was calculated according to eq 2:

$$R = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp) \quad (2)$$

in which $I_\parallel$ and $I_\perp$ refer to the intensities of the components of the emitted light beam whose polarization is parallel or perpendicular, respectively, to the polarization vector of the polarized incident excitation.

Results and Discussion

Circular Dichroism Spectra of Luliberin, Its Analogues, and Some of Its Peptidic Fragments in Organic Solvent. The physical characteristics of the surrounding medium of the hormone when associated with its receptor are not known. As it has been previously reported that luliberin possessed considerable conformational freedom (Marche et al., 1973), it was of interest to study this hormone under conditions which should reveal the capacities of the molecule to adopt preferential conformations. We have therefore investigated the CD behavior of luliberin and related peptides dissolved in TFE and in dioxane, solvents which have been shown to favor the expression of peptide conformational trends (Madison and Schellman, 1970; Goodman et al., 1971a,b; Cann, 1972; Marche et al., 1973).

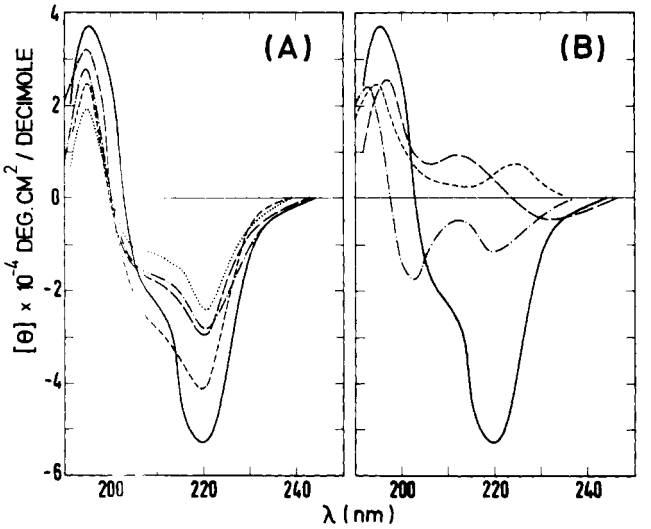

FIGURE 1: Circular dichroism spectra of luliberin and its analogues, recorded in TFE. A: (—) Luliberin; (---) Des-Gly-NH_2[10][Pro-NHEt[9]] luliberin; (— —) luliberin-(OH); (—·—) [Leu[8]]luliberin; (. . .) [Lys[8]] luliberin. B: (—) Luliberin; (—·—) [Pro[6]]luliberin; (---) Des-His[2]-luliberin; (— —) [Gly[3]]luliberin.

Figure 1 represents the circular dichroism spectra of luliberin and analogues, in solution in TFE. All the CD spectra reported in Figure 1A have a similar pattern and present a positive band at 195–197 nm, a negative one at 220 nm, and a negative shoulder centered around 207 nm. So it appears that the substitution of arginine by a lysine or leucine residue (the latter being devoid of the electric charge) and the deamidation of the terminal glycine or substitution of the glycinamide by an ethylamide group are not critical parameters for the CD spectra of the compounds. Intensities of the signals vary somewhat with the peptides but, nevertheless, they are such that, in TFE, [Leu[8]]luliberin, [Lys[8]]luliberin, luliberin-(OH), Des-Gly[10]-NH_2-[Pro[9]NHEt]luliberin, and luliberin could be considered as having a very similar conformation. Except for the presence of the 207-nm shoulder, the above spectra resemble those corresponding to the β-antiparallel-type structure (Goodman et al., 1971a,b). Since luliberin spectral pattern is concentration independent (Marche et al., 1973), it seems likely that this hormone and the above considered analogues possess a secondary structure which would have, at least partially, a feature of cross-β structure (Beychok, 1967; Jirgensons, 1973; Marche et al., 1973; Monahan et al., 1973). On the other hand, Figure 1B shows that the CD spectra of [Gly[3]]-luliberin, Des-His[2]-luliberin, and [Pro[6]]luliberin largely differ from that of luliberin.

The two first analogues present a positive band at 213 and 225 nm, respectively, but of low amplitude. This suggests that we are observing the result of an overlap of two contributions of opposite signs: one, positive, corresponding to the contribution of aromatic chromophores; the second, negative, attributable to a moderate contribution of the n–π^* peptidic transition. These CD spectra indicate that the degree of structuration of [Gly[3]]luliberin and Des-His[2]-luliberin is less than that of luliberin and consequently that the histidyl and tryptophyl residues play a conformational role.

The CD spectrum of [Pro[6]]luliberin presents two negative

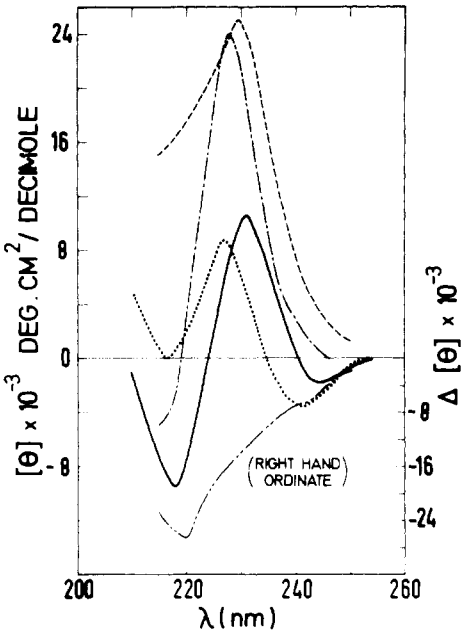

FIGURE 2: Circular dichroism spectra of different tripeptides related to the N-terminal end of luliberin, in dioxane. (---) 5-Oxo-Pro-His-Trp-OMe; (—) 5-oxo-Pro-His-Trp-NH_2; (. . .) *N*-Ac-Trp-NH_2; (—·—) 5-oxo-Pro-Trp-Pro; —· · — represents the difference spectrum between 5-oxo-Pro-His-Trp-NH_2 and 5-oxo-Pro-His-Trp-OMe.

bands located at 203 and 220 nm ([θ] = −18 000 and −12 000, respectively), whereas the parent molecule luliberin shows a spectrum having a shoulder centered around 207 nm and an intense negative signal at 220 nm. Here again the aromatic contribution should be taken into account with the additional remark that, in the [Pro^6]luliberin analogue, the tyrosine precedes a proline residue, which introduces a new constraint into the peptidic backbone. Thus, instead of a shoulder, like in luliberin, it is a negative band which appears in the spectrum of [Pro^6]luliberin, with the concomitant reduction of the amplitude of the 220-nm signal. Moreover, the bend introduced into the peptidic backbone when proline replaces glycine might promote the formation of an arrangement of helical type. In this case, the peptide would be wobbling between two conformations namely, cross-β and helical. The small intensity of the 220-nm signal of the Pro^6 analogue might reflect this situation.

To document the conformational roles played by residues His and Trp and the possible interactions at the level of these residues, the CD spectra of N-terminal tripeptidic fragments of luliberin were recorded in dioxane. Such an aprotic solvent should favor the formation of hydrogen bonds when permitted by the secondary structure of the molecules (Madison and Shellman, 1970; Cann, 1972; Cann et al., 1973). Figure 2 presents the CD spectra of various peptides, namely, 5-oxo-Pro-His-Trp-OMe, 5-oxo-Pro-His-Trp-NH_2, *N*-Ac-Trp-NH_2, and 5-oxo-Pro-Trp-Pro, in dioxane. These spectra can be divided into two types of curves. The first one is that corresponding to 5-oxo-Pro-His-Trp-OMe and 5-oxo-Pro-Trp-Pro: it shows one positive band at 228–230 nm ([θ] = +25 000) which represents the dichroic effect of the indole chromophore (Beychok, 1967); such a CD pattern indicates that no hydrogen-bonding interactions occur within the peptides. Spectra of 5-oxo-Pro-His-Trp-NH_2 and *N*-Ac-Trp-NH_2 constitute the second type of curve which exhibits a negative band at 241–243 nm ([θ] = −2 to 3000), a positive one at 227–231 nm ([θ] = +11 000), and another negative band centered around 218 nm. Moreover these spectra closely resemble those obtained with *N*-Ac-Tyr-NH_2 and Gly-Tyr-Gly (Cann, 1972). As the peptides *N*-Ac-Trp-NH_2 and 5-oxo-Pro-His-Trp-NH_2 both contain a tryptophyl residue, the weak intensity of the 227–231-nm signal as well as the trough at 245 nm suggest the presence of an additional and negative Cotton effect, similar to that described in the case of the *N*-Ac-Pro-NH_2 derivatives (Madison and Schellman, 1970; Cann, 1972), i.e., a well-developed n–π^* Cotton effect exhibited by an intramolecular hydrogen bond. Analogy between spectra of 5-oxo-Pro-His-Trp-NH_2 and *N*-Ac-Trp-NH_2 (Figure 2) suggests that in the amidated tripeptide such an hydrogen bond can be established between the CO of histidine and one hydrogen of the terminal amide, thus forming a seven-membered ring (Madison and Schellman, 1970; Cann, 1972). In Figure 2 is also represented the CD difference spectrum produced by 5-oxo-Pro-His-Trp-NH_2 relative to 5-oxo-Pro-His-Trp-OMe. As the difference spectrum should involve the terminal amide group, its resemblance with the difference spectrum produced by *N*-Ac-Tyr-NH_2 in 100% dioxane referred to *N*-Ac-Tyr-NH_2 in 50% dioxane–water (Cann, 1972) fairly confirms the above hypothesis. If one accepts that the conformational trend of 5-oxo-Pro-His-Trp-NH_2 alone is similar when the tripeptide is incorporated in luliberin, our findings suggest that, in the hormone, under proper conditions, CO (His) and HN (Ser) could be intramolecularly hydrogen bonded, thus favoring a turn in the hormone structure.

Fluorescence Study of Luliberin. Several authors have pointed out the importance of His, Trp, and Tyr residues for the biological activities of the hormone (Monahan et al., 1972; Yanaihara et al., 1973; Coy et al., 1974). Moreover the above observations suggest the predominant role of the aromatic residues in the decapeptide conformation. To provide some insight into the origin of such a behavior, a fluorescence study of luliberin has been undertaken.

Influence of the Solvent and Accessibility of the Tryptophyl Residue of Luliberin. In order to check whether structural modification of luliberin may be correlated with the Trp and Tyr aromatic side-chain environment, we have first examined the solvent influence on the emission properties of luliberin. Since we observed (Marche et al., 1973) that addition of TFE to an aqueous solution of luliberin promoted a structuration of the hormone, we have investigated the effect of this alcohol on the hormone fluorescence.

In Figure 3 we have represented the emission spectra of luliberin compared with those of *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2, in water at pH 7.5 and in pure TFE for a 280-nm excitation. Whatever the solvent used, spectra of the aromatic amino acid mixture present two contributions at 300–310 nm and around 350–355 nm corresponding to the fluorescence emission of tyrosine and tryptophan, respectively. The fluorescence maximum of luliberin corresponds to that of Trp but one can also see a weak contribution of Tyr emission at short wavelengths (below 310 nm) in both solvents. Figure 3 also shows that in TFE the emission spectra are shifted toward shorter wavelengths as compared with H_2O. This blue shift, however, is larger in the case of luliberin than for the mixture of model compounds, namely, 13 vs. 4 nm, respectively. In addition to the expected blue shift induced by the change of the solvent dielectric constant (78 for water and 27 for TFE), the difference of shifts suggests that, in luliberin, the Trp residue is located in an hydrophobic environment. In water, the fluorescence intensity of luliberin is much lower than that of the aromatic amino acid mixture, whereas in TFE both spectra only differ in their maxima position.

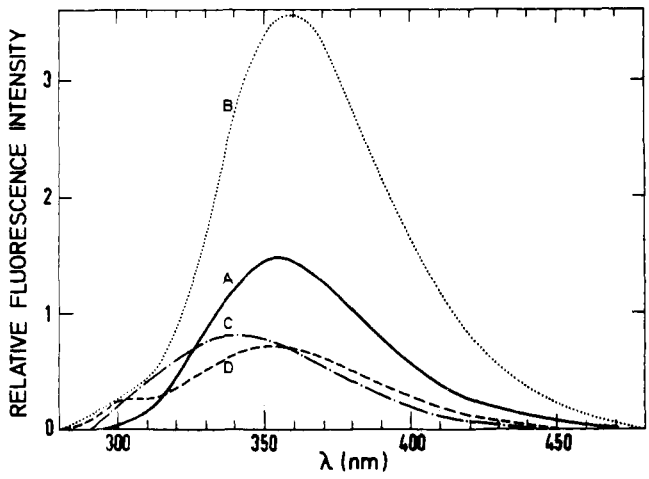

FIGURE 3: Fluorescence emission spectra of luliberin in 0.1 M NaCl, pH 7.5 (curve A), or in TFE (curve C), and of the equimolar mixture *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 in 0.1 M NaCl, pH 7.5 (curve B), or in TFE (curve D). Excitation was at 280 nm. These spectra were recorded with a Jobin-Yvon spectrofluorimeter and were not corrected for monochromator transmission and photomultiplier response.

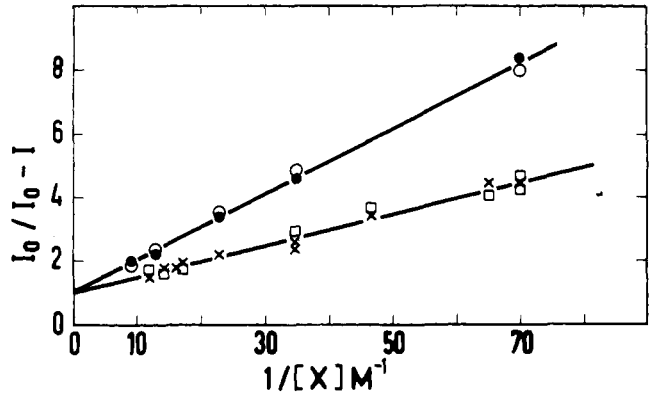

FIGURE 4: Modified Stern-Volmer plot of tryptophan fluorescence quenching by acrylamide, according to eq 1. Excitation was at 300 nm and emission at 360 nm. Luliberin at pH 3.5 (○) or 7.5 (●); *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 at pH 3.5 (X) or 7.5 (□).

TABLE I: Quenching of Tryptophyl Emission by Acrylamide in the Mixture of Aromatic Amino Acids and in Luliberin.[a]

Compound	Solvent Conditions	K_Q (M^{-1})	$10^{-9} k$[b] ($M^{-1} s^{-1}$)
N-Ac-Tyr-NH_2 + *N*-Ac-Trp-NH_2	0.1 M NaCl, pH 3.5	19.6	7.7
	0.1 M NaCl, pH 7.5	19.6	7.7
	TFE	5.4	
Luliberin	0.1 M NaCl, pH 3.5	9.7	11
	0.1 M NaCl, pH 7.5	9.7	8.1
	TFE	5.4	

[a] Excitation and emission wavelengths were 300 and 360 nm, respectively. [b] Determined assuming τ_0 = 2.6 ns for *N*-Ac-Trp-NH_2 (Lehrer, 1971) and proportionality between quantum yield and lifetime for luliberin.

Additional information on the particular effect of TFE on luliberin fluorescence has been obtained by measuring the emission intensity of its Trp residue (monitored at 360 nm with excitation at 280 nm) in different TFE-H_2O solutions. Adding TFE to aqueous solutions of the mixture of aromatic amino acids strongly decreases the emission intensity which, in pure TFE, is only 20% of that observed in water. On the other hand, the effect of TFE on the Trp residue of luliberin is very different: 25–30% TFE is necessary to modify the hormone emission intensity which, in pure TFE, is about 60% of that in water. The fact that luliberin emits much less than the *N*-Ac-TrpNH_2 + *N*-Ac-Tyr-NH_2 mixture is not surprising and has already been described for a variety of tryptophan-containing peptides in which peptidic bonds are assumed to be responsible for such a quenching (Cowgill, 1970). Nevertheless the different behavior of the hormone and of the model mixture toward the quenching effect of TFE may indicate that in luliberin we are dealing with a superposition of two phenomena: an intrinsic quenching effect of TFE which might be attributed to its electron scavenger property, and a conformational change of the hormone (Cowgill, 1967, 1968). Comparison of the CD behavior of luliberin in the same TFE-H_2O mixtures (Marche et al., 1973) with the fluorescence data clearly indicates that luliberin structuration occurs when passing from water to TFE and that the Trp residue takes part in such an organization.

The involvement of Trp in luliberin structural change and the concomitant formation of an hydrophobic environment raises the question of its accessibility. For this purpose the magnitude of fluorescence quenching by acrylamide has been measured under several experimental conditions: aqueous solutions at pH 3.5 and 7.5, and in TFE (the choice of two pH values resulted from the titration of luliberin; see below) (Steiner and Kirby, 1969; Lehrer, 1971). In a first set of experiments the quenching of Trp fluorescence of luliberin and of the aromatic amino acid mixture at pH 3.5 and 7.5 (without any modification in absorption) is analyzed in Figure 4 according to the modified Stern-Volmer equation (eq 1). A linear dependence of $I_0/(I_0 - I)$ vs. 1/[X] and a value of 1 for the intercept can be observed for both curves. In a second set of experiments similar results were obtained when dissolving substances in TFE. These results indicate: (i) the involvement of a collisional mechanism of quenching; and (ii) the total accessibility of tryptophan both in luliberin and in the mixture of aromatic amino acids. With the collisional quenching mechanism invoked, calling k the second-order rate constant for deactivation of the Trp excited state by quencher and τ_0 the fluorescence lifetime in the absence of quencher, the Stern-Volmer quenching constant K_Q can be written as

$$K_Q = k\tau_0 \qquad (3)$$

The rate constant k represents a measure of Trp accessibility to the quencher. Experimental values of the Stern-Volmer quenching constant (K_Q) calculated from eq 1 are summarized in Table I where the values of k are also reported, assuming τ_0 = 2.6 ns for *N*-Ac-Trp-NH_2 (Lehrer, 1971).

In TFE, the fluorescence quantum yield of *N*-Ac-Trp-NH_2 and of luliberin are nearly identical so that fluorescence lifetimes should also be identical. Since K_Q has the same value for luliberin and the model compound mixture, we can conclude that k is the same for these two compounds. In aqueous solutions, the K_Q value for the mixture is twice that obtained with luliberin. But the fluorescence quantum yield (and therefore the fluorescence lifetime) of *N*-Ac-Trp-NH_2 is about twice that of luliberin. This means that k values are still identical for the two compounds. Whatever the solvent or pH, it appears, therefore, that the tryptophyl residue in luliberin is fully accessible to quencher, even when a conformational change in-

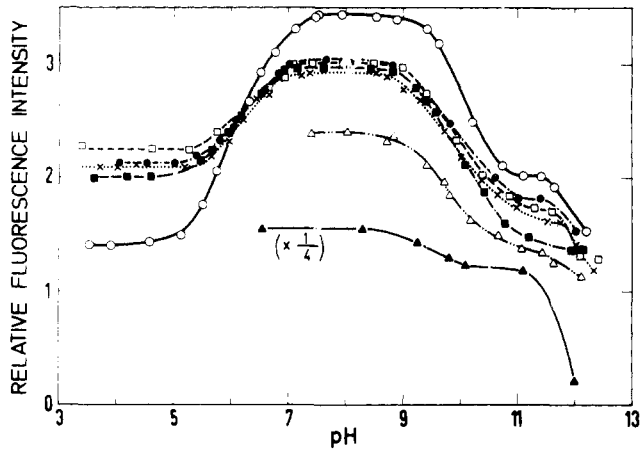

FIGURE 5: The pH dependence of relative fluorescence intensity for luliberin and analogues. The excitation and emission wavelengths were 277.5 and 360 nm, respectively. (●) Luliberin; (○) [Pro^6]luliberin; (X) [Leu^8]-luliberin; (□) [Lys^8]luliberin; (■) luliberin-(OH); (△) Des-His^2-luliberin; (▲) *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2.

duced by specific solvation conditions promotes an hydrophobic environment.

Fluorometric Titration of Luliberin and Related Compounds. Correlations between the emission properties of the Trp residue of luliberin (or of its analogues or fragments) and the hormone conformation are possible due to the existence of interactions between this residue and other parts of the molecule. For these reasons emission measurements of luliberin and related peptides appear to be another way of approaching the structural parameters in the neighborhood of its Trp, His, and Tyr residues. Also it was of interest to study the fluorescence behavior of the hormone under various pH conditions which are known to induce conformational change of the peptide (Marche et al., 1973). Moreover, such an investigation may allow the evaluation of the distance between interacting chromophores (Edelhoch et al., 1967; Shinitzky and Fridkin, 1969).

The pH dependence of the fluorescence intensity of luliberin and some of its analogues in aqueous solution is reported in Figure 5 where the titration curves of peptides are also compared with that of the mixture of *N*-acetylcarboxamides. Since fluorescence intensity has been measured at 360 nm, this figure represents only the fluorescence of Trp residue. The excitation wavelength was chosen at 278 nm which is the isosbestic point for ionized and un-ionized *N*-Ac-Tyr-NH_2. On this figure we first remark that [Leu^8]luliberin, [Lys^8]luliberin, luliberin-(OH), and luliberin all exhibit superimposable titration curves, whereas the Pro^6 and Des-His^2 analogues behave differently. Keeping in mind the forementioned CD behavior of these peptides, it can be assumed that the conformation of these compounds could be correlated with the fluorescence properties of their tryptophyl residue. It should also be noted that an aqueous solution of luliberin at pH 7.5 shows an emission spectrum (Figure 3, curve A) which presents an uncorrected maximum at 355–357 nm and is pH independent. The luliberin spectrum appears characterized by a low tyrosine emission in the 305-nm region. This observation indicates either that a radiationless energy transfer may occur from the excited singlet level of Tyr to that of Trp or that Tyr fluorescence is quenched in luliberin by some interaction with neighboring groups. This point will be discussed later in this report. In Figure 5 is also shown the decrease of the Trp emission of luliberin and analogues during ionization of the phenol and imidazole rings of tyrosine and histidine, respectively. The curves thus obtained allow the determination of the pK_a values of these residues (see Table II) and suggest (i) in acidic medium the formation of a charge-transfer complex between the protonated imidazole and the indole ring (Shinitzky and Fridkin, 1969) and (ii) in alkaline medium an energy-transfer process from indole to tyrosinate anion (Edelhoch et al., 1967). In the latter case the interaction between Trp and Tyr promotes a 47% quenching of the tryptophyl emission. Since the tyrosinate emission contribution has not been taken into account, such a value is an underestimated measure of the efficiency of energy transfer occurring between the singlet levels of these residues. Several luliberin conformations may be envisaged in alkaline medium; thus the above value constitutes an average one. According to Eisinger calculations of critical energy-transfer distances (Eisinger et al., 1969), these experimental data allow us to estimate that the separating distance between Trp and Tyr residues is of the order of 8–10 Å when luliberin is at pH 11.5.

TABLE II: Characteristic Data on the Fluorometric Titration in 0.1 M NaCl of Luliberin, Some of Its Analogues, and Related Peptides.[a]

	pK_a (His)	% Q (His$^+$)[b]	pK_a (Tyr)	% Q (Tyr$^-$)[b]
Luliberin[c]	6.05	29	10.05	47
[Leu^8]Luliberin	6.25	29	9.95	45
[Lys^8]Luliberin	6.25	25	9.95	44
Luliberin (OH)	6.25	29	9.95	41
[Pro^6]Luliberin	6.10	59	10.05	41
Des-His^2-Luliberin			9.90	46
5-Oxo-Pro-His-Trp	6.70	36		
5-Oxo-Pro-His-Trp-OMe	6.45	26		
5-Oxo-Pro-Trp-NH_2	6.25	32		

[a] Emission intensity was read at 360 nm with an excitation wavelength of 278 nm. [b] % Q (His$^+$) and % Q (Tyr$^-$) represent the ratio $(I_{max} - I_{min})/I_{max}$ in which I_{max} is the intensity of the upper plateau of the titration curve (pH 7.5–9) and I_{min} that of the lower plateau corresponding to the ionization of His and Tyr, respectively (see Figure 5). [c] By potentiometric titration the pK_a values of the His and Tyr residues of luliberin have been found to be 6.20 and 9.95, respectively. Absorption spectroscopy measurements at 245 and 295 nm have given pK_a (Tyr) = 10.00.

Table II reports the fluorometric titration data obtained with several analogues of luliberin and related peptides. As already observed (Edelhoch et al., 1967; Shinitzky and Fridkin, 1969), pK_a values obtained from fluorometric titration perfectly agree with those found by potentiometric or absorbance measurements. In Table II one may first observe that pK_a values of His (6–6.2) and Tyr (9.9–10) residues are similar both in luliberin and in its analogues. For all peptides, ionization of tyrosine promotes a fluorescence decrease of about 45% whereas protonation of the histidine side-chain produces a fluorescence quenching of about 25–30% except in the case of the [Pro^6]-luliberin where it reaches 60%. Among the N-terminal tripeptides presently examined, only the amidated one (5-oxo-Pro-His-Trp-NH_2) exhibits characteristics (pK_a and percentage of quenching promoted by protonation of the imidazole) similar to those of luliberin. Keeping in mind the CD behavior of this tripeptide, one may conclude that the 5-oxo-Pro-His-Trp-NH_2 structure could mimic that of the N-terminal part of luliberin.

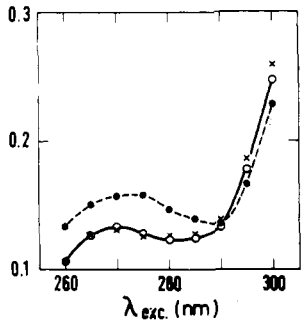

FIGURE 6: Variation of fluorescence anisotropy with excitation wavelength (see eq 2), at 0 °C in 95% glycerol-aqueous buffer solutions. Emission was at 350 nm. Luliberin at pH 4.5 (○) or 7.8 (X); *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 (●) at pH 8. All solutions were 10^{-4} M.

Fluorescence Anisotropy Measurements and Excitation Spectra. To provide evidence for energy transfer from Tyr to Trp when luliberin is in acidic or neutral medium (pH 4 and 8), a more detailed investigation using anisotropy measurements and excitation spectra has been undertaken (Longworth, 1971). As the hormone excitation spectra (recorded with emission at 350 nm) were similar at pH 4 and 8, we will only consider the results obtained at pH 8 in the following paragraphs.

In a first experiment fluorescence anisotropy of the decapeptide has been studied as a function of the excitation wavelength. Since the fluorescence emitted by luliberin in solution at room temperature is depolarized due to fast rotational motion, the polarization measurements were carried out in a much more viscous solvent, namely, 95% glycerol at 0 °C. As shown in Figure 6 the fluorescence emission of luliberin at 350 nm is depolarized as compared with *N*-Ac-Trp-NH_2 (or to the equimolar mixture *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2) when excitation is performed at 275 nm. Moreover this figure clearly shows that luliberin anisotropy is of the same magnitude both at pH 7.8 and at pH 4.5. Experimental conditions do not allow us to assume that the limit value corresponding to a complete absence of thermal depolarization has been reached. Nevertheless the observed depolarization of luliberin compared with *N*-Ac-Trp-NH_2 under the same experimental conditions indicates that most of the energy absorbed by Tyr is transferred to Trp and shows that the absorption transition moment of Tyr and the emission transition moment of Trp are not parallel to each other. As a matter of fact, the rotational correlation time of Trp residue in luliberin is certainly longer than that of *N*-Ac-Trp-NH_2; therefore, the depolarization of Trp emission from luliberin as compared with *N*-Ac-Trp-NH_2 is certainly underestimated.

In a second set of experiments, excitation spectra of the hormone have been recorded for two emission wavelengths, namely, 305 and 350 nm and compared with spectra of *N*-Ac-Trp-NH_2 (Figure 7). Excitation spectra of the model compound are identical at these two emission wavelengths. In contrast it can be seen that the excitation spectrum monitored at 305 nm markedly differs from that of *N*-Ac-Trp-NH_2 (Figure 7A) and from that monitored at 350 nm (Figure 7B). Since Tyr emission maximum is 305 nm, one should expect that the excitation spectrum of luliberin shows more of Tyr absorption than of Trp absorption when the emission wavelength is 305 nm. This is seen on Figure 7A where the characteristic shoulder of Trp at 287.5 nm—clearly observed in the spectra recorded at 350 nm (Figure 7B)—is no longer visible in the 305-nm spectrum of the hormone.

Additional information has been obtained by recording

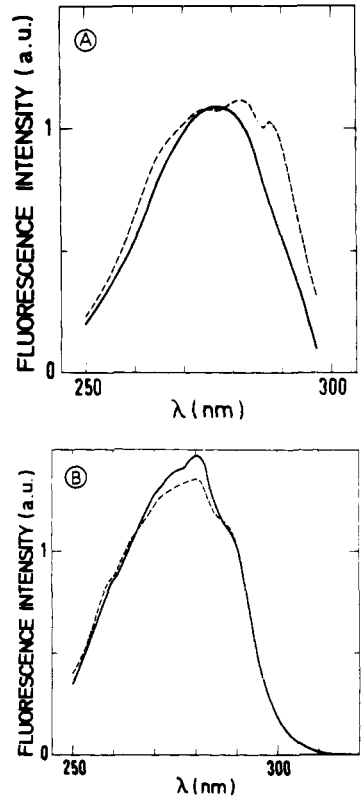

FIGURE 7: Excitation spectra of luliberin (—) and *N*-Ac-Trp-NH_2 (---). (A) Emission wavelength, 305 nm. Solutions were 2×10^{-5} M. Spectra were normalized at 275 nm. (B) Emission wavelength, 350 nm. Solutions were 4×10^{-6} M. Spectra were normalized at 290 nm. All solutions were in 10^{-3} M borax buffer at pH 8, 0.1 M NaCl; excitation slit width, 2.5 nm; temperature was 8 °C.

emission spectra of diluted solutions of luliberin. After normalization of the emission at 352.5 nm, the contribution of Tyr at 305 nm is observed upon excitation at 275 nm, whereas it is absent when excitation is at 290 nm. These results indicate that part of the light absorbed by Tyr is reemitted as Tyr fluorescence.

Confirmation of the existence of an energy transfer from Tyr to Trp in luliberin has been provided by comparing its absorption and excitation spectra monitored at 350 nm. In the case of *N*-Ac-Trp-NH_2 alone, absorption and excitation spectra are identical within experimental error between 260 and 295 nm. In the case of the hormone, the ratio of the excitation spectrum relative to its absorption spectrum is not constant in the same wavelength range but shows a minimum at about 275 nm. Such a result clearly proves that energy transfer from Tyr to Trp is not total. Assuming that the absorption spectra of both Tyr and Trp residues are not perturbed when incorporated in the decapeptide, the efficiency α of such a transfer can be calculated according to the procedure described by Eisinger (1969).

The fluorescence quantum yield of luliberin (ϕ) when excited at wavelength λ is given by eq 4:

$$\phi = \phi_{Trp}[f_{Trp}(\lambda) + \alpha f_{Tyr}(\lambda)] \quad (4)$$

where $f_{Trp}(\lambda)$ and $f_{Tyr}(\lambda)$ are the fraction of light absorbed by Trp and Tyr, respectively, in the hormone. ϕ_{Trp} is the fluorescence quantum yield of Trp in luliberin (measured under conditions where only this residue is excited).

The wavelength dependence of luliberin quantum yield calculated according to eq 4 and assuming different values of α is shown on Figure 8. It can be seen that the best agreement

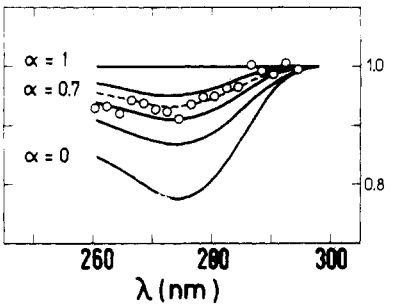

FIGURE 8: Variation of the fluorescence quantum yield of luliberin (ϕ/ϕ_{Trp}) vs. excitation wavelength (O) and theoretical curves calculated according to eq 4 (see text) for different values of the transfer efficiency α (0, 0.4, 0.6, 0.7, 0.8, 1). The broken line corresponds to $\alpha = 0.7$. All calculated curves and experimental data were normalized at 295 nm.

with experimental values is obtained for $\alpha = 0.7$. This means that 70% of light absorbed by Tyr is transferred to Trp. The equimolar mixture *N*-Ac-TrpNH_2 + *N*-Ac-TyrNH_2 does not give any evidence of energy transfer at the low concentration used in this investigation ($\sim 10^{-6}$ M). On the other hand, as indicated by the above results, it appears that most of the energy which is not transferred is reemitted as Tyr fluorescence.

In the first UV absorption band of Trp there are two electronic transitions whose transition moments are probably nearly perpendicular to each other. In the determination of Tyr → Trp energy-transfer efficiency, it is difficult to estimate the individual overlap integrals for these two transitions. The critical Förster distances calculated by Eisinger (1969) do not separate the contributions of the two transitions. This situation means that energy transfer from Tyr to Trp may still occur when the emission transition moment of Tyr is perpendicular to the absorption moment of the lowest energy transition of Trp because transfer will then involve the second absorption transition. This also means that energy transfer will take place whatever the relative orientation of Tyr and Trp. In other terms, κ^2, the orientation factor in the expression of the transfer rate constant cannot be very small. Since the 6th root of κ^2 intervenes in the calculation of R_0, we have used the average value of $\kappa^2 = \frac{2}{3}$ which is usually introduced for a random distribution of donor and acceptor molecules in an isotropic medium.

Assuming such a value for the orientation factor, the Förster critical distance between Tyr and Trp for a 50% energy transfer efficiency has been calculated to be 12–15 Å depending on the donor fluorescence quantum yield (Eisinger et al., 1969). In the present case, the experimental results obtained with luliberin may thus correspond to an average distance around 10 Å between the Trp and Tyr residues. However, it should be kept in mind that luliberin may have different conformations in solution. Thus the above distance must be considered as only an estimation of an average distance.

Conclusion

^1H and ^{13}C NMR investigations (Wessels et al., 1973; Deslauriers et al., 1975) as well as a preliminary CD study (Marche et al., 1973) have reported the flexibility and polymorphism of luliberin in aqueous solution. Moreover it has also been reported that luliberin could adopt privileged ordered conformation when dissolving the hormone in alcoholic medium (Marche et al., 1973). With the aim of determining which were the predominant parameters of the luliberin structure(s) and which were the specific interactions responsible for such structuration(s), two kinds of studies have been presently reported.

First, comparison between the CD spectra of luliberin and of various analogues and fragments, in structure-promoting solvents such as trifluoroethanol or dioxane, leads to the following interpretations: (i) the side chain of the eighth residue (Arg) does not seem important for the hormone structure which, moreover, appears to be slightly affected by deamidation of the terminal glycine or by substituting this residue with an ethylamide group; (ii) residues His, Trp, and Gly, at the 2nd, 3rd, and 6th positions, respectively, seem essential for the structure of the decapeptide; (iii) the formation of an hydrogen bond between the CO of tryptophan and the NH of serine likely stabilizes a folded conformation.

Secondly by measuring the fluorescence emission and excitation spectra of luliberin as well as its fluorescence polarization spectrum, several conclusions can be reasonably drawn: (i) conformation was obtained of the predominant roles played by His^2, Trp^3, and Gly^6 residues in the hormone structure, thus confirming the preceding CD data; (ii) also it appears that the tryptophyl side chain is fully accessible to a quenching agent, whatever the pH and solvent conditions, and that the luliberin Trp residue is located in an hydrophobic environment when dissolving the hormone in TFE; (iii) a strong interaction exists between Trp and His residues (at least when the latter is protonated) as reflected by the quenching of Trp fluorescence by protonated histidine; (iv) energy transfer occurs between aromatic residues from Tyr to Trp at neutral pH and from Trp to Tyr^- in alkaline medium.

As already mentioned, it should be pointed out that the evaluated distance between Tyr and Trp rings (8–12 Å) represents an average value. Construction of a molecular model of luliberin with a CPK kit indicates that our experimental data are consistent with a β-turn-like structure allowing an interaction between Trp and Tyr residues by contrast to the model proposed by Monahan et al. (1973) and Ling and Vale (1975) which excluded the possibility of such an interaction. From the data presently reported, existence of a β bend located at the level of the luliberin aromatic residues seems likely. On the other hand, the above evaluated distances are large enough to explain the absence of chemical-shift differences of the aromatic protons or carbons due to anisotropic ring current effects (Wessels et al., 1973; Deslauriers et al., 1975).

While this manuscript was being typewritten, a publication reported fluorescence studies of luliberin. Although we cannot explain some discrepancy in the quantitative data, our results are in rather good qualitative agreement with those presented by Mabrey and Klotz. It should be pointed out that we have obtained identical results with two luliberin preparations obtained from Hoffmann La Roche and Bachem, respectively.

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