Conformational Characteristics of Luliberin. Luminescence Properties at Liquid-Nitrogen Temperature†

Pierre Marche,*,‡ Thérèse Montenay-Garestier, Pierre Fromageot, and Claude Hélène

ABSTRACT: The luminescence properties (fluorescence and phosphorescence) of luliberin have been investigated at liquid-nitrogen temperature (77 K) in 50% ethylene glycol–aqueous buffer at various pH's. Calculation of the energy-transfer efficiency between Trp and Tyr residues leads to an evaluation of the distance separating these residues. In alkaline medium, when tyrosine is ionized, a 100% transfer efficiency occurs from Trp to Tyr^- at the singlet level and also from Tyr^- to Trp at the triplet level indicating that the Trp–Tyr distance is less than about 5 Å. When luliberin is at pH 7.8 (or 4.5), transfer efficiency from Tyr to Trp at the singlet level is 85–90% which should correspond to a distance between Trp and Tyr of about 10–12 Å. These results are discussed with respect to the role played by aromatic amino acids both in the hormone conformation and in the hormone biological potency. Moreover, comparison with the data obtained from fluorescence or circular dichroism studies at room temperature allows us to deduce some characteristics of luliberin conformation. Structure–activity relationships in the aromatic region of luliberin are also discussed.

As a result of the actual concepts of "hormone–receptor" interactions structural complementarity should exist between the two entities. Studying the conformational features of the hormone allows an approach of the characterization of the receptor. For this purpose, conformational studies of luliberin,[1] an hypothalamic decapeptide able to stimulate the secretion of both lutropin and follitropin from the anterior pituitary gland, have been undertaken by several laboratories using circular dichroism (CD) and proton or ^{13}C magnetic resonance (Marche et al., 1973; Wessels et al., 1973; Deslauriers et al., 1975).

In the preceding publication, by comparing the CD spectra of luliberin with those of analogues and studying the fluorescence properties of the hormone, some structural characteristics needed for maintaining the peptide in privileged conformation(s) have been reported. Experimental evidence has also been provided for interactions between the tryptophyl and tyrosyl residues. Interpretation of the results by means of the radiationless energy-transfer theory of Förster has allowed us to evaluate the average distance between these residues as a function of pH (Förster, 1965; Eisinger et al., 1969). Since it has been established that luliberin may have different conformations in solution and that dissolving the hormone in alcoholic medium such as trifluoroethanol (TFE) favors a stabilization of ordered conformation(s) (Marche et al., 1973), it was of interest to investigate how the Trp–Tyr distance was modified under conditions preventing conformational motions. For this purpose the luminescence properties (flourescence and phosphorescence) of luliberin have been studied at liquid-nitrogen temperature (77 K) under various pH conditions in 50% ethylene glycol–aqueous solutions. This solvent system has been used because (i) it allows the formation of glasses at 77 K which makes possible the measurement of luliberin emission properties at this temperature and (ii) such an alcoholic mixture could mimic at least partially the particular effect of TFE.

Under these experimental conditions, the distance between Trp and Tyr residues has been deduced from the calculated energy-transfer efficiency. In alkaline medium, total transfer at the triplet level indicates that the maximum distance between Trp and ionized Tyr is 4–6 Å. On the other hand, under neutral or acidic conditions, the Trp–Tyr distance is about 10–12 Å. Agreement between these results and the previously reported conformational and biological characteristics of the hormone or analogues allows us to draw some structure–activity relationships. An hypothesis on the receptor site structure is also discussed.

Experimental Section

Synthetic luliberin (5-oxo-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was a gift of Drs. R. O. Studer and D. Gillessen (Hoffmann-La Roche, Switzerland); *N*-Ac-Trp-NH_2 and *N*-Ac-Tyr-NH_2 were supplied by Sigma. Ethylene glycol and all other chemicals were of analytical grade from Prolabo (France). All measurements reported in this paper were made with solutions in 50% ethylene glycol–0.1 M aqueous buffer (acetate buffer for pH 4 and phosphate buffer for pH 7.5); solutions at pH 11.5 were obtained by mixing (v/v) the compounds dissolved in 0.1 M NaCl with ethylene glycol and then adjusting pH with concentrated NaOH. Luminescence measurements were carried out in a quartz tube of 2-mm inner diameter. For each experiment, luliberin luminescence was compared with that of a model system (an equimolar mixture of *N*-Ac-Trp-NH_2 and *N*-Ac-Tyr-NH_2)

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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; TFE, trifluoroethanol.

having the same absorbance which was usually 0.6 at 280 nm in a 1-cm cell (measured with a Beckman DK2A spectrophotometer). When luliberin was compared with *N*-Ac-Trp-NH_2 (or *N*-Ac-Tyr-NH_2) alone, the absorbance of the model compound was equal to that of the respective residue in the decapeptide.

Total emission spectra, phosphorescence emission spectra, and excitation spectra were recorded with a Jobin-Yvon spectrofluorimeter equipped with an accessory for liquid-nitrogen-temperature measurements (77 K). A stream of dry nitrogen gas was continuously flushed into the cell compartment to prevent freezing. A rotating can phosphoroscope was used for phosphorescence measurements and was removed for measuring total luminescence. The spectra reported here have not been corrected for the wavelength dependence of monochromator transmission and of photomultiplier response.

For phosphorescence lifetime determination, the phosphorescence decay was observed on a Tektronix oscilloscope screen and photographed. In all cases a strictly exponential decay curve was obtained, where the intensity (I) varies with time (t), according to $I/I_0 = \exp(-t/\tau)$ where I_0 is the initial intensity and τ the triplet state lifetime. A plot of log I vs. t yielded a straight line of slope $1/\tau$.

Results

Total Emission Spectra. The total luminescence spectra (fluorescence and phosphorescence) of luliberin have been recorded at pH 4, 7.5, and 11.5 and compared with those of model compounds under equivalent conditions. Spectra are presented in Figure 1.

At pH 7.5, when excitation wavelength is 280 nm (i.e., where both the Trp and Tyr residues are excited), the spectral distribution of luliberin total emission is essentially identical with that of *N*-Ac-Trp-NH_2 (Figure 1A) with a fluorescence maximum at about 322 nm and shoulders at around 296 and 314 nm and distinct phosphorescence maxima which occur near 405, 432, and 457 nm with a shoulder at 480–485 nm. The pattern of spectra described above did not change by lowering the pH down to 4.

The luminescence emission spectra of luliberin at pH 11.5 is presented in Figure 1B in which spectra of *N*-Ac-Trp-NH_2, *N*-Ac-Tyr-NH_2, and of an equimolar mixture of these compounds are also reported for comparison (excitation wavelength was at 275 nm). In the fluorescence region, spectra of *N*-Ac-Trp-NH_2 alone and of the mixture *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 (Figure 1B, curves 3 and 1) exhibit a similar pattern with a maximum at 322 nm and shoulders around 297 and 310 nm, while ionized tyrosine (curve 4) can be distinguished by a very low intensity maximum at 316 nm as expected from its low fluorescence quantum yield value (Longworth, 1971). The fluorescence of the hormone (curve 2) is characterized by a single band at 320 nm devoid of the above-mentioned shoulders of Trp emission. In the phosphorescence region, the decapeptide spectrum exhibits three maxima at 407, 432, and 457 nm (curve 2) which correspond to the tryptophyl residue emission as can be seen from the spectrum of *N*-Ac-Trp-NH_2 alone (curve 3). In addition to these peaks, the spectrum of the aromatic amino acid mixture (curve 1) possesses a broad shoulder located at around 390 nm which is due to ionized tyrosine phosphorescence as it may be deduced from the spectrum of *N*-Ac-Tyr-NH_2 alone (curve 4).

Phosphorescence Emission Spectra. In Figure 2 are reported the phosphorescence emission spectra of luliberin and of the equimolar mixture *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 at pH 4 and 11.5 and for two exciting wavelengths 277.5 and 297.5 nm). Phosphorescence spectra recorded at pH 4 or 7.5 exhibited similar patterns.

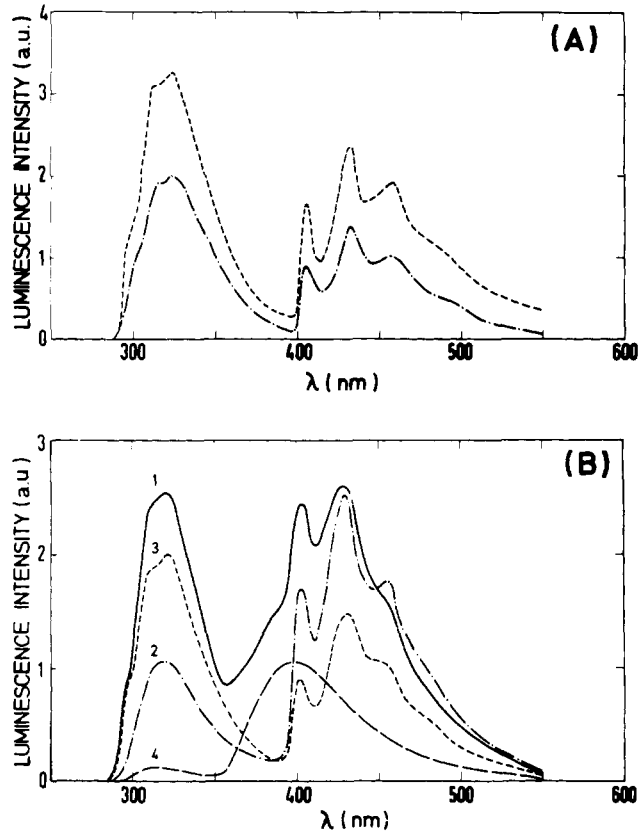

FIGURE 1: Total emission spectra of luliberin and model compounds. (A) At pH 7.5 and excitation at 280 nm: luliberin (—·—) and *N*-Ac-Trp-NH_2 (- - -). (B) At pH 11.5 and excitation at 275 nm: curve 1, *N*-Ac-Tyr-NH_2 + *N*-Ac-Trp-NH_2; curve 2, luliberin; curve 3, *N*-Ac-Trp-NH_2; curve 4, *N*-Ac-Tyr-NH_2.

At pH 4 (or 7.5), both residues Trp and Tyr absorb when solutions are excited at 277.5 nm. Only Trp absorbs for an excitation wavelength of 297.5 nm. Phosphorescence spectra of luliberin and of the aromatic amino acid mixture are similar when excited at 297.5 nm (Figure 2A, curves 2 and 4). In this case, spectra are characteristic of the Trp emission, mainly showing three maxima at 405–407, 429–432, and 453–455 nm, respectively (Weinryb and Steiner, 1970; Longworth, 1971). For an excitation wavelength of 277.5 nm, the phosphorescence spectrum of *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 presents a broad band at 380–390 nm, in addition to the Trp maxima (Figure 2A, curve 1). This band reflects the phosphorescence contribution of un-ionized tyrosine (Hélène et al., 1968; Longworth, 1971). In contrast, the phosphorescence spectrum of the hormone is unchanged whether the peptide is excited at 277.5 or at 297.5 nm (Figure 2A, curves 3 and 4). The low intensity observed in the 380–390-nm range, which is the region where tyrosine emits phosphorescence, indicates that luliberin tyrosyl residue does not contribute very much to the peptide phosphorescence.

At pH 11.5, when the phenol ring of tyrosine is ionized, both residues Trp and Tyr^- nearly absorb the same fraction of the exciting light at 297.5 nm, while Trp absorbs four to five times more than Tyr^- if excitation is at 277.5 nm. Thus the phosphorescence emission spectrum of *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 excited at 277.5 nm (Figure 2B, curve 1) resembles a Trp emission spectrum on which has been added the smaller

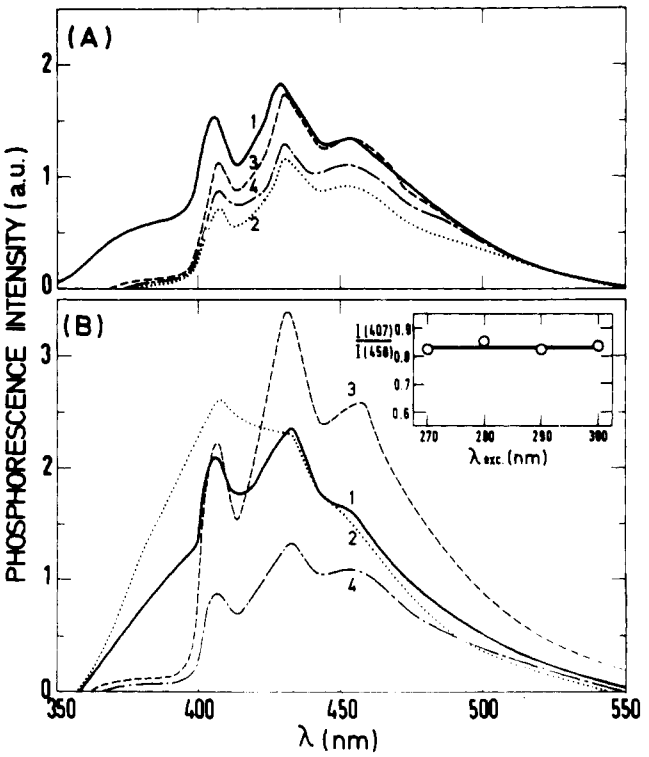

FIGURE 2: Phosphorescence emission spectra of luliberin (curves 3 and 4) and of the equimolar mixture *N*-Ac-Tyr-NH_2 + *N*-Ac-Trp-NH_2 (curves 1 and 2). At pH 4 (or 7.5) for A and at pH 11.5 for B. Excitation was at 277.5 nm (curves 1 and 3) or 297.5 nm (curves 2 and 4). (Inset) The ratio between emission intensities at 407 and 458 nm, as a function of the excitation wavelength.

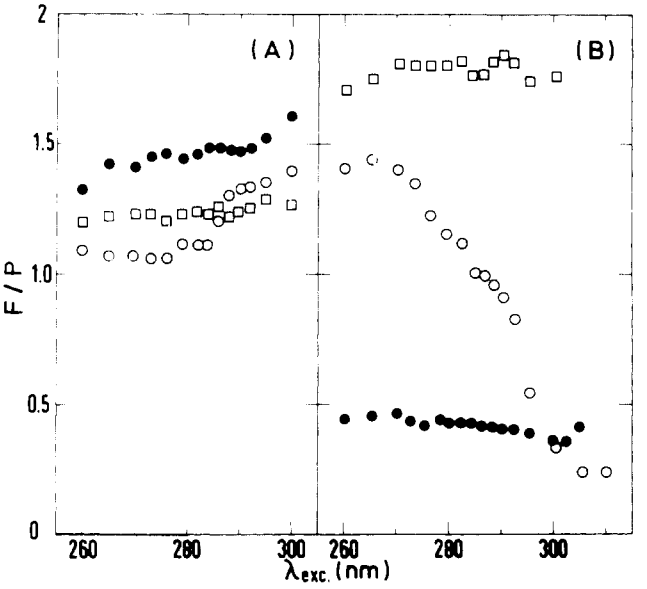

FIGURE 3: Value of the ratio *F/P* of the fluorescence and phosphorescence excitation spectra. (A) At pH 7.5 and (B) at pH 11.5. Fluorescence and phosphorescence emission intensities were monitored at 330 and 430 nm, respectively. (□) *N*-Ac-Trp-NH_2; (○) *N*-Ac-Tyr-NH_2 + *N*-Ac-Trp-NH_2; (●) luliberin.

contribution of Tyr^- (Longworth, 1971). In contrast, when the aromatic amino acid mixture is excited at 297.5 nm (Figure 2B, curve 2), the emission maxima corresponding to Trp are masked by the intense broad emission band of Tyr^-. In spite of the difference between the phosphorescence quantum yields of Trp and Tyr^-, emission spectra of luliberin (Figure 2B, curves 3 and 4) were unchanged for excitation wavelengths of 277.5 or 297.5 nm and typically corresponded to the phosphorescence emission spectrum of Trp alone, with the same maxima as observed in Figure 2A. Varying the excitation wavelength from 270 to 300 nm did not alter the spectrum shape. Moreover, it can be seen in Figure 2B that the ratio between the emission intensities at 407 and 458 nm does not depend on the excitation wavelength, although the contribution of Trp and Tyr^- at these two wavelengths are quite different (see Figure 2B). This indicates that phosphorescence of luliberin is only due to the emission of its tryptophyl residue.

Phosphorescence Lifetime. Phosphorescence lifetimes are summarized in Table I. Results for the mixture of aromatic amino acids are included for comparison. These data are in good agreement with those of the literature (Hélène et al., 1968; Steiner and Kolinski, 1968; Weinryb and Steiner, 1968, 1970; Longworth, 1971). The phosphorescence lifetime of tryptophan was measured by exciting at 280 nm and monitoring emission at 426 nm where the contribution of Tyr is weak; values found either for luliberin or *N*-Ac-Trp-NH_2 are 6.2–6.4 s and are independent of the pH conditions. Un-ionized tyrosine phosphorescence lifetime was obtained by exciting at 280 nm and recording its emission at 370 nm. In the case of *N*-Ac-Tyr-NH_2 (in the mixture), values found are 3.0–3.1 s. In luliberin, measurements were hindered by very low intensities of tyrosine contribution in the phosphorescence emission of the peptide. At pH 11.5 tyrosinate phosphorescence lifetime in the model mixture could be measured by exciting at 300 nm and monitoring its emission at 370 nm; the value found is 1.65 s. Such a measurement was not possible with the decapeptide since the contribution of Tyr^- could not be detected. Even when the peptide was excited at 280 nm, the decay curve observed at 426 nm could be fitted with a single exponential whose lifetime was characteristic of the tryptophyl residue (Table I).

Excitation Spectra and Energy-Transfer Calculation. In the preceding paper (Marche et al., 1976) it was mentioned that luliberin excitation spectra at room temperature were similar either at pH 4 or 7.8 and different from that at pH 11.5. Therefore excitation spectra at low temperature have been measured at pH 7.8 and 11.5. Whatever the pH, fluorescence excitation spectra were monitored at 330 nm, whereas phosphorescence excitation spectra were monitored at 430 nm. Results are reported in Figure 3, where only the ratio *F/P* of the fluorescence and phosphorescence spectra is shown, as a function of the excitation wavelength. Such a ratio is a quite good approximation of the ratio between the quantum yields of fluorescence and phosphorescence, respectively. As expected, in the case of *N*-Ac-Trp-NH_2 alone, this ratio does not depend upon the excitation wavelength. On the other hand, the *F/P* ratio of the mixture *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2 markedly varies with λ_{exc} since there is no or little interactions between the two fluorophors within the mixture.

It can be seen that the *F/P* ratio of luliberin at pH 11.5 is wavelength independent (Figure 3B). This result indicates that fluorescence and phosphorescence of the hormone each originates from a single emitting state. From the phosphorescence spectra and lifetime given in Figure 2B and Table I, it is clear that luliberin phosphorescence is entirely due to tryptophan. The fluorescence could be due either to Trp or Tyr^-. The fluorescence spectrum shown in Figure 1B and the much lower F/P ratio of luliberin as compared with *N*-Ac-Trp-NH_2 strongly suggest that Tyr^- is the fluorescence emitter in the hormone at pH 11.5. If Trp was the fluorescence emitter one would have to assume that the vibronic structure of Trp fluo-

TABLE I: Phosphorescence Lifetime (τ_p) for Luliberin and for the Mixture *N*-Ac-Trp-NH_2 + *N*-Ac-Tyr-NH_2.

Compound	pH[a]	Excitation Wavelength (nm)	Emission Wavelength (nm)	τ_p[b] (s)
N-Ac-Trp-NH_2 +	4	280	426	6.3
N-Ac-Tyr-NH_2	4	280	370	3.1
	7.5	280	426	6.3
	7.5	280	370	3.0
	11.5	280	426	6.2
	11.5	300	370	1.65
Luliberin	4	280	426	6.4
	7.5	280	426	6.4
	11.5	280	426	6.4

[a] The solvent was ethylene glycol-aqueous buffer (1:1, v/v) as described in the Experimental Section; pH was measured at room temperature for mixed solvent system. [b] Estimated precision: ±0.3 s.

rescence spectrum is lost in luliberin and that the fluorescence quantum yield of Trp is greatly decreased (~50%) without any marked change either in intersystem crossing or in triplet state population and decay. These latter possibilities seem to be very unlikely. Fluorescence could have been emitted from an exciplex state. However, the shape and λ_{max} of the luliberin fluorescence spectrum are not markedly different from those of Tyr^-. This is not in agreement with an exciplex emission. Therefore it can be concluded that, in luliberin at pH 11.5, fluorescence is emitted by Tyr^- and phosphorescence by Trp. This implies that at this pH Trp → Tyr^- energy-transfer efficiency is 100% at the singlet level and that the reverse transfer efficiency (Tyr^- → Trp) is also 100% at the triplet level.

When the hormone is at pH 7.8, total energy transfer at the singlet level would imply that both fluorescence and phosphorescence of luliberin arise from its tryptophyl residue only. In this case the *F/P* ratio of the peptide should be wavelength independent. As observed in Figure 3A, our experimental results show a slight but significant dependence of *F/P* vs λ_{exc}. This indicates that neither the fluorescence nor the phosphorescence of luliberin could be exclusively attributed to the Trp residue and suggests that singlet energy transfer from Tyr to Trp is not 100% at pH 7.8. Let $A_{Trp}(\lambda)$, $A_{Tyr}(\lambda)$, and $A_t(\lambda)$ be the wavelength-dependent absorbances of the Trp residue, Tyr residue, and of luliberin, respectively. If we call $I_0(\lambda)$ the intensity of the incident beam and $I_1(\lambda)$ and $I_2(\lambda)$ the intensities emitted by the indole ring in *N*-Ac-Trp-NH_2 and when incorporated in luliberin, respectively, we can write the following equations:

$$I_1(\lambda) = KI_0(\lambda)[1 - 10^{-A_{Trp}(\lambda)}]\phi_{Trp}^{N\text{-Ac-Trp-NH}_2} \quad (1)$$

$$I_2(\lambda) = KI_0(\lambda)[1 - 10^{-A_t(\lambda)}] \times \phi_{Trp}^{lulib}\left[\frac{A_{Trp}(\lambda)}{A_t(\lambda)} + \alpha\frac{A_{Tyr}(\lambda)}{A_t(\lambda)}\right] \quad (2)$$

in which K is a constant from the apparatus and α is the wavelength-independent efficiency with which excitation energy is transferred from Tyr to Trp. In these equations, $\phi_{Trp}^{N\text{-Ac-Trp-NH}_2}$ is the fluorescence quantum yield of Trp in the model compound *N*-Ac-Trp-NH_2 and ϕ_{Trp}^{lulib} that of the tryptophyl residue when incorporated in the decapeptide and in the absence of energy transfer (i.e., when the excitation wavelength

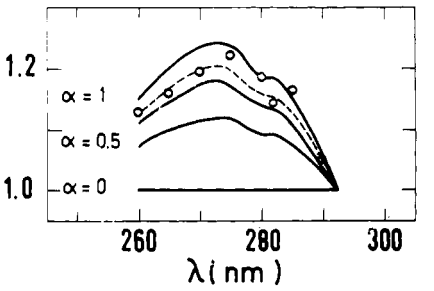

FIGURE 4: Evaluation of the energy-transfer efficiency (α) in luliberin at pH 7.8 according to eq 3 (see text). Theoretical curves correspond to different values of α (0, 0.5, 0.7, 1, full lines and 0.85 broken line); (○) experimental data.

is such that only Trp absorbs). These quantum yields are wavelength independent. The functions given by eq 1 and 2 are the fluorescence excitation spectra of *N*-Ac-Trp-NH_2 and luliberin, respectively, monitored at a wavelength at which only Trp emits. In the present case of dilute solutions, $1 - 10^{-A_i(\lambda)}$ can be replaced by $2.3A_i(\lambda)$. Then the ratio $I_2(\lambda)/I_1(\lambda)$ can be expressed as:

$$I_2(\lambda)/I_1(\lambda) = [1 + \alpha f_{Tyr}(\lambda)/f_{Trp}(\lambda)] \times [\phi_{Trp}^{lulib}/\phi_{Trp}^{N\text{-Ac-Trp-NH}_2}] \quad (3)$$

where $f_{Tyr}(\lambda)$ and $f_{Trp}(\lambda)$ are the fractions of light absorbed by Tyr and Trp, respectively ($f_i(\lambda) = A_i(\lambda)/A_t(\lambda)$). From the knowledge of the functions $f_{Tyr}(\lambda)$ and $f_{Trp}(\lambda)$, one may draw the theoretical curves $I_2(\lambda)/\gamma I_1(\lambda)$ corresponding to different values of the transfer efficiency α (Figure 4) and where $\gamma = \phi_{Trp}^{lulib}/\phi_{Trp}^{N\text{-Ac-Trp-NH}_2}$. On the other hand, the ratio between the fluorescence excitation spectra of luliberin and *N*-Ac-Trp-NH_2 (after normalizing spectra for λ_{exc} = 293 nm) allows us to obtain the experimental values of $I_2(\lambda)/\gamma I_1(\lambda)$. Comparison between theoretical and experimental data provides an estimation of the efficiency of singlet energy transfer. From Figure 4 it can be seen that α reaches a value of 85–90% for experiments at pH 7.8. Such a large efficiency was expected from data of Figure 3A which presented only a very slight wavelength dependence of *F/P* and also from the nearly total absence of Tyr phosphorescence in luliberin (Figure 2A). One cannot exclude that the difference between this experimental value (85–90%) and 100% is due to some impurity present in our luliberin preparation. However, the increase in transfer efficiency when going from room temperature (70%) to 77 K (85–90%) is expected since the critical Förster distance is larger at 77 K than at room temperature (Eisinger et al., 1969).

Discussion and Conclusion

Containing one tryptophan and one tyrosine as emitting amino acids, luliberin luminescence will reflect the microenvironment of each of these residues and consequently the folding of the peptide. The specific interactions of individual residues, including intramolecular hydrogen bonding as well as the degree of shielding from the solvent between fluorophors whose emission and absorption spectra overlap, may also strongly affect the luminescence of the peptide.

When the hormone is studied at pH 11.5, i.e., when its tyrosyl residue is ionized, the fluorescence spectrum of the decapeptide seems to occur solely from its Tyr^- residue. From the arguments developed above, it could be concluded that total energy transfer from Trp to Tyr^- occurs at the singlet level in luliberin. On the other hand, Figures 1B and 2B clearly show

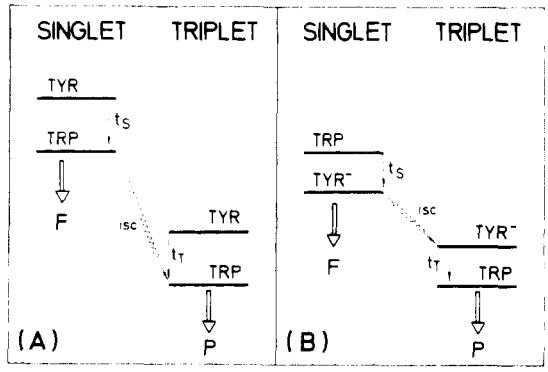

FIGURE 5: Schematic representation of the excited-state energy levels of tryptophan (Trp) and tyrosine (Tyr). In neutral or acidic medium (A) or in basic medium (B) i.e., when tyrosine is ionized.

that luliberin phosphorescence emission exclusively arises from its Trp residue. Interpretation of these observations can be made with the help of the energy diagram reported in Figure 5B, which shows that the tyrosinate singlet state energy is lower than that of tryptophan, while the reverse situation applies to the triplet states. Thus, as expected in the case of total energy transfers (at both singlet and triplet levels) between the Trp and Tyr residues of luliberin, the hormone fluorescence arises from its ionized tyrosine residue which is sensitized by tryptophan and phosphorescence emission results from the tryptophan triplet state populated by a sensitization from tyrosinate triplet. Confirmation of such a situation is provided by the fact that the ratio (F/P) between the fluorescence and phosphorescence intensities of the hormone does not depend upon the excitation wavelength (Figure 3B). Moreover, the ratio of $(F/P)_{N\text{-Ac-Trp-NH}_2}$ relative to $(F/P)_{\text{lulib}}$ obtained from Figure 3B reaches a value of 4.4 which can be related to the value of 4.6 obtained by Hélène et al. (1968) for the ratio of $(F/P)_{\text{Trp}}$ relative to $(F/P)_{\text{Trp-Tyr}}$ a dipeptide in which total energy transfer at the triplet level has been well established. As triplet transfer proceeds by an electron-exchange mechanism requiring orbital overlap between the donor and acceptor, the ionized phenol ring and the indole ring of luliberin must be very close to each other (4–5 Å) at pH 11.5. The slight red shift (4 nm) of the fluorescence spectrum of luliberin as compared with free *N*-Ac-Tyr-NH_2 (Figure 1B) is likely due to the stacking of Tyr^- and Trp aromatic rings.

A quite different situation is observed in neutral medium. Tyrosyl fluorescence and phosphorescence are hardly detected in luliberin. From the excitation spectra analysis, we have concluded that about 85–90% of the energy absorbed by tyrosine is transferred at the singlet level to tryptophan. Moreover the phosphorescence lifetime of the peptide has been found to be identical with that of its tryptophyl component (Table I). Since the energies for both the singlet and triplet levels of tryptophan are lower than those of tyrosine (Figure 5A), a 85–90% energy-transfer efficiency occurring at the singlet state from Tyr to Trp clearly explains the above experimental observations. The Förster critical distance for energy transfer from Tyr to Trp at 77 K is larger than that determined at room temperature due to an increased overlap between Tyr fluorescence and Trp absorption spectra. The average distance separating Trp and Tyr at 77 K is therefore similar under both experimental conditions, i.e., around 10 Å, although this might represent only a rough estimation due to the possible superposition of different conformations with different Tyr-Trp distances (Marche et al., 1976). Since similar luminescence and phosphorescence spectra were found when luliberin was at pH 4, and since similar excitation spectra for both pH 4 and 7.5 have been reported at room temperature (Marche et al., 1976), it can be reasonably assumed that the distance separating the aromatic residues is similar at pH 4 and 7.5.

Our CD investigations have revealed a random conformation of the peptide at pH 4 and at room temperature (Marche et al., 1973). Therefore, under these pH conditions (4–8), an average distance of about 10 Å between Trp and Tyr well reflects the fact that all orientations of both chromophores are possible. Moreover our data indicate that ionization of the histidyl side chain does not affect the distance between aromatic residues. On the other hand, this distance depends upon the ionization state of the tyrosyl residue since raising the pH up to 11.5 at 77 K favors a stacking between Tyr and Trp.

Comparison of low-temperature results (rigid medium) with other observations at room temperature is often difficult. However, as far as the pH dependence is concerned, data presented in this and in the preceding paper fairly agree with each other.

It so appears that a main structural characteristic of the hormone is the presence at short distance of two electronically rich regions, namely, the indole and phenol rings of the tryptophyl and tyrosyl residues, respectively. Thus, an electrophilic region must be specifically located on the receptor for permitting its association with the hormone and then the triggering of the biological response. Consistent with this hypothesis is the fact that the [Trp^2, His^3]luliberin analogue (in which the Trp-Tyr distance likely should be increased as compared with that in luliberin) is devoid of biological activities (Yanaihara et al., 1973). According to this conclusion, one may remark that luliberin analogues whose indole and/or phenol ring(s) have been either deleted ([Gly^3]luliberin) or electronically modified (e.g., [5-F-Trp^3]luliberin, [Me_5-Phe^3]-luliberin, [*p*-NO_2-Phe^3]luliberin, [*p*-NH_2-Phe^3]luliberin, [*p*-NO_2-Phe^5]luliberin, [*p*-NH_2-Phe^5]luliberin) possess biological activities which increase as a function of the electronic richness of the Trp-Tyr region (Monahan et al., 1972; Coy et al., 1973, 1974).

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Kinetics of Ribosome Dissociation and Subunit Association Studied in a Light-Scattering Stopped-Flow Apparatus†

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ABSTRACT: The association-dissociation kinetics of ribosomes from *Escherichia coli* have been studied under various conditions in a light-scattering stopped-flow apparatus. The dissociation reaction at 2 mg/ml at 25 °C, induced by lowering the $MgCl_2$ concentration from 18 to 3 mM, can best be described by three independent first-order processes with rate constants of 15 s^{-1}, 0.9 s^{-1}, and 3×10^{-2} s^{-1}, the slowest process comprising about 60% of the overall reaction. The fraction of ribosomes dissociating with the fastest rate (15 s^{-1}) is concentration dependent and becomes negligible at 0.1 mg/ml. Ribosomes treated with puromycin also show three dissociation rates with essentially the same rate constants as the nontreated samples. The dissociation induced by a high KCl concentration (0.85 M KCl, 18 mM MgCl) also shows three first-order phases with the same rate constants as for the dissociation induced by lowering the $MgCl_2$ concentration. The formation of 70S ribosomes from 30S and 50S subunits, induced by increasing the $MgCl_2$ concentration from 2 to 21 mM, follows second-order biphasic kinetics. A detailed analysis of the kinetic results shows that the two principal ribosomal forms must have one type of subunit in common. When the association data are analyzed assuming that the kinetic heterogeneity arises from two forms of only one subunit, the rate constants are found to be 6.4×10^6 and 1.05×10^6 M^{-1} s^{-1}. Sequential flow experiments show that the rapid and slow association species are to be identified, respectively, with phases II (0.9 s^{-1}) and III (0.03 s^{-1}) of dissociation. Relaxation measurements show that these correspond to type B ("loose") and A ("tight") ribosomes, respectively. Tight and loose ribosomes were isolated by sucrose density centrifugation, and dissociation and association kinetic studies confirmed the above assignments. Furthermore, the rate constants for these ribosomes agreed within experimental error with the rate constants derived from analysis of the multiphasic kinetic data. The association rate constants are for ribosomes dissociated by dilution with the appropriate buffer immediately before recording the kinetics of association. Ribosomes dissociated by dialysis overnight against 2 mM $MgCl_2$ show an association rate constant for the slower association reaction (type-A ribosome) that is about four times smaller, whereas the rate constant for the faster process is roughly the same. The activation energies of the dissociation reactions, whether induced by lowering the $MgCl_2$ concentration or increasing the KCl concentration, and the association reaction induced by increasing the $MgCl_2$ concentration are less than 3.5 kcal/mol. The rate constants of the dissociation at 3 mM $MgCl_2$ and of the association reaction at 21 mM $MgCl_2$ do not vary between pH 7.2 and 8.4. When 30S and 50S subunits are flowed against buffer containing 20 mM spermidine, the association process is *monophasic,* with an association constant $k = 6 \times 10^6$ M^{-1} s^{-1}. Approach-to-equilibrium studies show that k_{assoc} varies with $(Mg^{2+})^n$ and k_{dissoc} varies with $(Mg^{2+})^{-m}$ (n = 2.3-3.7, m = 2-3) for 4 mM < (Mg^{2+}) < 8 mM.

The magnesium ion-dependent association of *Escherichia coli* 30S and 50S subunits to form 70S ribosomes is known to be reversible (Tissieres et al., 1959) and Nomura and Lowry (1967) discussed the physiological importance of this association-dissociation process. Various aspects of the magnesium ion-dependent reversible association have been studied by ultracentrifugation methods (Belitsina and Spirin, 1970; Ball et al., 1973; Spirin, 1971; van Diggelen and Bosch, 1973; Noll et al., 1973a) and even polyacrylamide gel electrophoresis (Talens et al., 1970). Ball et al. (1973) demonstrated that 70S ribosomes are in equilibrium with their subunits. In an elegant study, Zitomer and Flaks (1972) determined the equilibrium constant of the magnesium ion-dependent reversible association and examined, also, the influence of temperature, monovalent cations, and polyamines upon the equilibrium by light-scattering experiments. Since light-scattering measurements involve a minimum perturbation of the system under

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