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Study of the Secondary Structure of the Luteinizing Hormone Releasing Factor Using Intramolecular Charge Transfer Complexes†

Bernard Donzel,* Jean Rivier, and Murray Goodman

ABSTRACT: The syntheses and conformational properties of three analogues of the luteinizing hormone releasing factor (LRF)—the C^{δ}-nicotinamidium derivative of [8-norvalyl]-LRF, the C^{δ}-nicotinamidium derivatives of [8-norvalyl,6-D-alanyl]- and [8-norvalyl,6-L-alanyl]-LRF—are reported. This series of analogues was designed for the purpose of analyzing the probability of folding of the LRF peptide skeleton as a function of the steric nature of the residue in position 6 by measuring the charge-transfer interaction between the nicotinamidium ring on residue 8 and the side chain of tryptophan in position 3. Nuclear magnetic resonance studies in water show evidence for a pronounced conformational flexibility of the peptide backbones and side chains, similar to the case of LRF itself. The ultraviolet–visible electronic spectra of all three compounds contain a shoulder in the long wavelength region which is identified as the *intramolecular* charge transfer spectrum of the nicotinamidium–indolyl complex. The relative population of the molecules in the complexed state was evaluated in each case from the intensity of the charge transfer band at 380 nm, using the molar extinction coefficient of the *intermolecular* complex between acetyltryptophanamide and *N*-methylnicotinamide chloride. It is highest in the [6-D-alanyl] analogue (ca. 15%) and lowest in the [6-L-alanyl] analogue (ca. 9%). This trend is in qualitative agreement with the theoretical predictions of the stability of β turn of type II, provided that the amino acid substitution was made at the third residue of the turn.

Studies on structure–activity relationships of the luteinizing hormone releasing factor (LRF)[1] (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) have shown that most residues present in the peptide molecule are necessary for full biological potency (Vale and Rivier, 1975). Substantial efforts have been made to delineate both the amino acid residues responsible for binding to the receptor and also those for triggering the biological response, but no simple relationships have been found. From these studies, however, some positions along the peptide chain were found to be critical for biological activity. Among them are:

(a) Histidine in Position 2. Deletion or substitution of this residue by D-amino acids with aromatic side chains produces analogues with antagonist properties. Their affinity constant to the receptor sites, however, was found to be lower than in the case of the hormone (LRF) itself (Vale et al., 1976).

(b) Glycine in Position 6. Replacement of this residue by any L-amino acid drastically decreases the biological potency, whereas replacement by a D-amino acid residue dramatically increases the activity. The nature of the side chain in this case was found to have a profound influence, as reflected in the biological potencies of LRF (100%), [D-Ala^6]-LRF (450%), [D-Phe^6]-LRF (750%), [D-Tyr^6]-LRF (1500%), and [D-Trp^6]-LRF (3600%) (Monahan et al., 1973; Rivier et al., 1975; Coy et al., 1975).

(c) Glycinamide in Position 10. Substitution of this residue by a variety of alkylamide groups (Fujino et al., 1972b) provokes a substantial increase of the LH-releasing activity, with a maximum obtained for the ethylamide derivative (300%).

Simultaneous modifications at positions 6 and 10, as well as at positions 2 and 6, produce effects which vary in a geometric fashion while modifications at positions 2 and 10 apparently are not interrelated.

These modifications have been used in our laboratories in an attempt to determine whether such drastic variations in the biological activities could be correlated with conformational changes. This paper deals with the conformational effects caused by the replacement of glycine in position 6 with L-alanine or D-alanine. A preliminary report has been previously presented (Donzel et al., 1975).

Recently, several conformational studies of LRF have been reported. From ^{1}H and ^{13}C NMR studies on LRF and component fragments, Wessels et al. (1973) concluded that the hormone, when dissolved in polar solvents such as water or dimethyl sulfoxide, adopts a random structure. No intramolecular hydrogen bonding and no stacking of the aromatic side chains could be detected. Such an interaction between tyrosine-5 and tryptophan-3 was previously proposed by Chang and co-workers (1972) based upon model considerations. Furthermore, ^{13}C spin–lattice relaxation studies (Deslauriers et

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[1] Abbreviations used: LRF, luteinizing hormone releasing factor; LH, luteinizing hormone; NMR, nuclear magnetic resonance; UV, ultraviolet; vis, visible; CD, circular dichroism; CT, charge transfer; DEAE, diethylaminoethyl; CM, carboxymethyl; Boc, *tert*-butoxycarbonyl; HMDS, hexamethyldisiloxane; CPK, Corey–Pauling–Koltun.

TABLE I: Amino Acid Analyses and Yields.

Compound	Yield (%)[a]	Glu	His	Trp	Ser	Tyr	Gly	Ala	Leu	Orn	Pro	NH_3	% peptide
[Orn^8]LRF	11	1.02	0.96	0.79	0.79	0.96	2.00	—	1.00	1.01	1.01	1.32	97
[D-Ala^6,Orn^8]-LRF	24	1.06	1.12	1.01	0.77	1.03	1.04	1.04	1.00	1.11	1.02	1.08	95
[L-Ala^6,Orn^8]-LRF	21	1.00	0.91	0.92	0.87	1.00	1.00	1.00	1.00	0.99	1.03	1.03	101

[a] Calculated from the degree of substitution of the resin.

al., 1975; Deslauriers and Somorjai, 1976) were also consistent with a high mobility for the backbone and side chains of LRF. Using a variety of spectroscopic techniques, especially circular dichroism and fluorescence energy transfer measurements, Mabrey and Klotz (1976) investigated the conformation of LRF and various analogues in solution. Their studies in water did not reveal any characteristic conformational features for these compounds. Somewhat different interpretations of the circular dichroism results in water were given by Marche et al. (1973). At neutral and basic pHs, these authors found some evidence for a trend toward ordered structures. Both CD investigations, however, have revealed that a conformational transition does occur when trifluoroethanol is added to the water solutions. In pure trifluoroethanol solution, Marche et al. (1973) found evidence for a two-state mixture of β and helical structures, whereas Mabrey and Klotz described a distribution of conformers characteristic of a mixture of β and random coil structures.

With respect to conformation, modifications at position 6, which cause large variations in biological potencies, are very attractive. As Monahan et al. (1973) already proposed, most of these variations could be consistent with a secondary structure of LRF containing a β bend at the sequence Ser-Tyr-Gly-Leu. Based upon semiempirical energy calculations, Némethy and Printz (1972) have shown that a β turn of type II (Venkatachalam, 1968) is destabilized by the introduction of an L-amino acid residue at the third position of the turn, whereas this conformation is considerably stabilized by the introduction of a D residue. In the case of LRF, the presence of a bend between positions 4 and 7 would be best explained if the remaining residues would also contribute to the overall stability of the turn as in the case of an antiparallel β-pleated sheet structure stabilized by intramolecular hydrogen bonding. Examination of molecular models shows that tryptophan in position 3 and arginine in position 8 are in close proximity in this structure. Thus, our first attempt was to determine if a short-range interaction between these two positions could be detected at all and, if this were the case, to analyze the conformer distribution as a function of the substituent in position 6. For this purpose, arginine in position 8 was replaced by ornithine, and the δ-amino group of ornithine was then quaternized to a nicotinamidium residue.

Interaction between the side chain of tryptophan and the nicotinamidium residue, if it occurs, can be identified as a typical *intramolecular* charge-transfer (CT) complex (Shifrin, 1964; Deranleau and Schwyzer, 1970; Bosshard, 1970; Donzel, 1971). This approach is very selective in that a CT transition can only occur during the lifetime of the nicotinamidium-indolyl complex; therefore, all the conformers which do not allow formation will not be detected (Donzel, 1971). This method also has the potential to give precise information about the statistical occurrence of the folded conformer, regardless of the dynamic properties of the peptide molecule. It was thus hoped that the presence of the folded conformer could be detected even if it does not represent the most stable conformational state of the molecule. And, last but not least, it was hoped that the analogues would have some activity in the release of LH and FSH, as previous reports have shown that [Orn^8]-LRF possesses 5–12% potency (Fujino et al., 1972a) and that the positive charge in this position appears to contribute to the hormonal action (Chang et al., 1972).

Semiempirical energy calculations have recently been performed on the LRF molecule and various analogues (Momany, 1976a,b). Three low-energy conformers were selected, which are all characterized by a modified β-bend type II at the sequence Tyr-Gly-Leu-Arg. One of these conformers was proposed as the active three-dimensional structure of the molecule.

Materials and Methods

Synthesis of LRF Analogues. The protected amino acids used for the peptide syntheses were purchased from Bachem, Inc. The compound, 1-chloro-2,4-dinitrobenzene, was obtained from Aldrich and was purified by high vacuum distillation. Nicotinamide (Aldrich) was purified by recrystallization from ethyl acetate (mp 128–129 °C). DEAE-cellulose and CM-cellulose were obtained from Bio-Rad Laboratories and Sephadex G-25 was purchased from the Sigma Chemical Co. Elemental analyses were determined by Galbraith Laboratories, Inc.

The protected decapeptides were synthesized in a stepwise manner on a benzhydrylamine resin (Pietta and Marshall, 1970; Rivier et al., 1973) of 0.15 mequiv/g substitution, using dicyclohexylcarbodiimide as coupling agent. The coupling steps were carried out in dichloromethane or, in some cases, in a mixture of dichloromethane–dimethylformamide (1:1) using a three- to fourfold excess of the Boc amino acid. A standard coupling time of one-and-one-half hour was usually used. Completion of reaction was assured after each coupling by a ninhydrin test. If this test were positive, a second coupling was performed. The amino acid side chains were protected as follows: N$^{\delta}$Z-Orn, OBzl-Tyr, N^{im}Tos-His, N$^{\alpha}$Z-pGlu, and OBzl-Ser. Deblocking of the N$^{\alpha}$Boc protecting groups was performed by the action of a 50% solution of trifluoroacetic acid in dichloromethane for 20 min at room temperature, followed by neutralization with a 12% solution of triethylamine in dimethylformamide. Cleavage from the resin and concomitant deprotection of the side chains was accomplished by the action of doubly distilled HF (10 mL/g resin) in the presence of anisole (1.5 mL/g resin) for 1 h at 0 °C. The peptides were purified by chromatography on carboxymethylcellulose in a gradient of ammonium acetate (0–0.3 M) and then by partition chromatography on Sephadex G-25 in a butanol–pyridine–acetic acid (1%)–water (5:3:11 v/v). The products were found to be homogeneous by thin-layer chromatography in both basic and acidic systems and were characterized by quantitative amino acid analysis (Table I).

Quaternization of the Ornithyl Side Chain. Before use, the

TABLE II: Elementary Analysis.

Compound	Formula	% C calcd/exptl	% H calcd/exptl	% N calcd/exptl	% Cl calcd/exptl
[Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH(4H$_2$O)	$C_{63}H_{89}N_{16}O_{20}Cl$	52.67/52.96	6.34/6.37	15.84/15.67	2.50/1.64
[L-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH(4H$_2$O)	$C_{63}H_{91}N_{16}O_{20}Cl$	52.99/52.88	6.42/6.38	15.69/15.78	2.48/1.65
[D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH(4H$_2$O)	$C_{63}H_{91}N_{16}O_{20}Cl$	52.99/52.98	6.42/6.46	15.69/15.71	2.48/1.78

deprotected decapeptides were neutralized on a DEAE-cellulose column and lyophilized to a white powder. In a typical experiment, [D-Ala^6,Orn^8]-LRF free base (88 mg) was dissolved in redistilled methanol (5.5 mL). A solution of 2,4-dinitrophenylnicotinamidium chloride (25 mg, mp 125–128 °C) (Lettré et al., 1953; Donzel, 1971) in methanol (0.7 mL) was then added dropwise under nitrogen atmosphere. After each drop, the red solution turned progressively yellow, at which point the next drop was added. The peptide was then precipitated by adding peroxide-free ether (50 mL). After decantation, the residue was redissolved in methanol and precipitated again with ether. After three cycles, most of the nitroaniline was eliminated from the reaction mixture. The crude material was then purified by partition chromatography on Sephadex G-25 (column size 2, 5 cm × 40 cm) in a 1-butanol–acetic acid–water system (4:1:5 v/v). The fractions (2 mL) were analyzed by thin-layer chromatography in 1-butanol–acetic acid–water (4:1:5 v/v) and 1-butanol–pyridine–0.1% acetic acid–water (5:3:11 v/v) (upper phases). A homogeneous product was found in fractions 65–96, from which 96 mg (88%) of yellow peptide was isolated.

The NMR assignment of the aromatic region is shown in Figure 1, and elementary analyses are shown in Table II.

Biological Activities. The effect of the analogues on the rate of release of LH by primary cultures of dispersed rat anterior pituitary cells was found to be ca. 4% for [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$ and less than 1% for [L-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$ and [Nva^8(Nic$^+$)]-LRF·Cl$^-$. Quaternization of the ornithyl side chain causes an approximately tenfold decrease in the activity of the corresponding Orn^8 analogue.

^{1}H NMR Measurements. The NMR spectra were recorded on a Varian HR-220 Fourier transform system equipped with a Nicolet TT-100 computer and disk, and with a standard variable temperature unit. The temperature in the sample zone was determined from the chemical shifts of an ethylene glycol sample and maintained within ±1 °C. Chemical shifts were related to the internal standard hexamethyldisiloxane (HMDS). A standard solute concentration of 0.4% v/w was used.

UV–Vis Measurements. The charge-transfer measurements were recorded on a Cary 118 spectrophotometer fitted with a thermostated cuvette cell holder. All measurements were carried out at 25 °C.

In a typical experiment, a stock solution of the peptide (10^{-2} M) in doubly distilled (quartz) water was prepared. Eight aliquots were then taken and diluted to the appropriate volumes with water to incremental concentrations in the range of 10^{-2} to 10^{-4} M. The optical densities of the solutions were recorded from 520 to 220 nm, using doubly distilled water as reference. The full scale response of the pen was varied for OD values between 2 and 0.1 so as to maintain the recorder amplitude in an optimal range. Two separate series of measurements were recorded for each LRF analogue and the OD values were then averaged. Graphic representations of the optical density as a function of concentration at various wavelengths were established (Beers' law test). In an attempt to minimize *intermolecular* CT effects, the apparent extinction coefficients were calculated from the linear portion of these plots only. The values of ϵ_{app} in all three analogues were normalized at the λ_{max} of 271 nm.

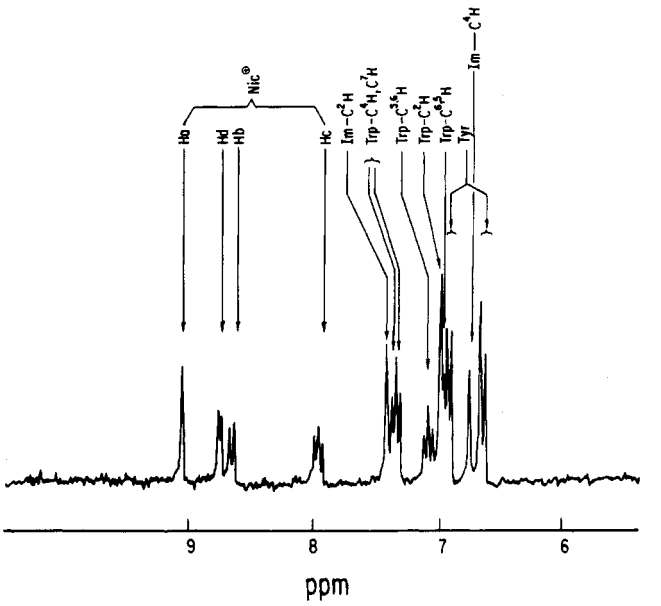

FIGURE 1: The 220-MHz FT spectrum (low-field region) of [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH in D_2O at 39 °C. (Internal reference: hexamethyldisiloxane.)

Results

NMR Studies. The 6.5–7.5-ppm region of the NMR spectrum of the luteinizing hormone releasing factor (LRF) in D_2O contains all the resonances of the nonexchangeable aromatic protons of tryptophan, tyrosine, and histidine residues. Their assignments are straightforward. The resonance positions of the tryptophan and tyrosine residues at three different pH values are indicated schematically in Figure 2. The same resonances in the model compounds Ac-Trp-NH_2 and Ac-Ser-Tyr-Gly-Leu-$NHCH_3$ are also reported. At acidic pH, the tryptophan resonances in LRF are slightly shifted compared with the model compounds, particularly in the case of the C_2–H, C_5–H, and C_6–H resonances. Deprotonation of the histidyl side chain, however, causes strong perturbations of the indole spectrum, especially in the case of the C_7–H resonance, which coalesces, at pH 8.5, with the C_4–H resonance. This shift follows the titration of the imidazole residue and can thus be attributed to a reciprocal influence of the two adjacent side chains of histidine in position 2 and tryptophan in position 3.

No significant changes in the tryptophan spectrum are observed upon ionization of the tyrosine side chain (pH 11) revealing the absence of a preponderant interaction between the

TABLE III: Chemical Shifts in ppm[b] of Various Aromatic Protons in the LRF Analogues.[a]

Compound	Tryptophan					Tyrosine		Nicotinamide			
	H_7	H_4	H_2	$H_{5,6}$	$H_{6,5}$	$H_{\bar{A}}$	$H_{\bar{B}}$	H_a	$H_{b,d}$	$H_{d,b}$	H_c
[Nva^8 (Nic^+)]-LRF·Cl^-	7.34	7.31	6.96	7.07	6.95	6.63	6.91	9.01	8.72	8.60	7.91
[D-Ala^6, Nva^8 (Nic^+)]-LRF·Cl^-	7.33	7.30	6.97	7.06	6.94	6.65	6.88	9.01	8.70	8.60	7.90
[L-Ala^6, Nva^8 (Nic^+)]-LRF·Cl^-	7.32	7.32	6.95	7.08	6.95	6.62	6.94	9.04	8.75	8.63	7.93
LRF	7.36	7.36	7.00	7.13	7.00	6.68	6.96				

[a] D_2O, T = 22 °C, concentration = 4 mg/mL, internal reference is HMDS. [b] Given as center of the multiplets.

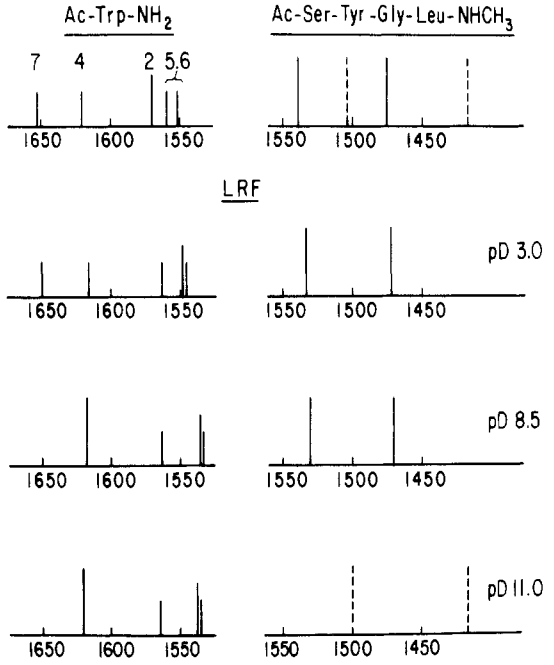

FIGURE 2: Schematic representation of the tryptophan and tyrosine aromatic resonances in LRF as a function of pH (D_2O, T = 22 °C, internal reference was hexamethyldisiloxane). Dashed lines indicate the resonance positions of the tyrosinate ring. For practical reasons, only the centers of the resonance multiplets are given.

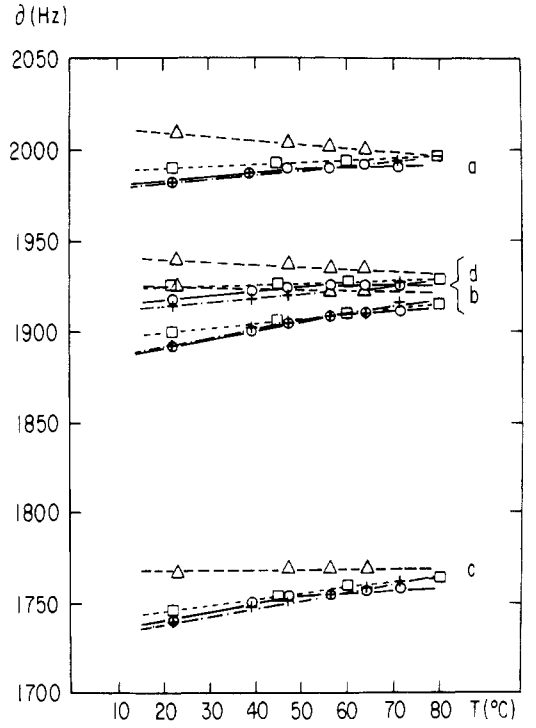

FIGURE 3: Temperature dependence of the nicotinamidium resonances in the charge-transfer-labeled LRF analogues (in D_2O, internal reference was hexamethyldisiloxane): (+) [D-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (○) [Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (□) [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (△) reference compound, *N*-methylnicotinamide chloride. (Resonance assignment is given in Table III.)

two side chains. This observation is further substantiated by the fact that the aromatic resonances of the tyrosine side chain in LRF coincide with the same resonances in the reference Ac-Ser-Tyr-Gly-Leu-$NHCH_3$ at all three pH values, independent of the ionization state of the phenolic group. This clearly shows that the tyrosine side chain in LRF dissolved in water is not involved in any strong intramolecular interaction with the other aromatic side chains present in the LRF molecule.

NMR studies on the marked LRF analogues, [Nva^8-(Nic^+)]-LRF·Cl^-, [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-, and [D-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-, are summarized in Table III and Figure 3. The resonance positions of the aromatic protons of tyrosine and tryptophan in the three analogues do not vary significantly, generally appearing at slightly higher fields than in LRF itself. The differences may be attributed mainly to the average anisotropic effect of the nicotinamidium residue. Within the limits of sensitivity of the NMR method, however, there is no indication of any marked perturbation of the environment of these aromatic side chains; these data would rather suggest that the conformational flexibility which characterizes the LRF molecule is still present in the labeled analogues.

The resonance positions of the nicotinamidium protons (Table III) and their temperature dependence (Figure 3) are similar for [Nva^8(Nic^+)]-LRF·Cl^- and [D-Ala^6,Nva^8-(Nic^+)]-LRF·Cl^-. In [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-, the four resonances appear at slightly lower field (0.04 ppm). In all three cases, however, they are shifted upfield compared with the same resonances in the model compound, *N*-methylnicotinamide chloride. Furthermore, their temperature dependences are positive, in contrast to the model compound for which slightly negative slopes are observed. These differences suggest that the nicotinamidium residue in the LRF analogues is involved in some kind of interaction and that the effect is least pronounced in the [L-Ala^6] analogue as compared with the other two analogues.

Charge-Transfer Studies. Each of the labeled LRF an-

alogues contains two amino acid residues, Trp[3] and Tyr[5], which have the potential to act as donors in a charge transfer interaction with the nicotinamidium ring. In general, the *intermolecular* CT spectrum of a solution containing these chromophores consists of overlapping, nonidentical absorption bands, in proportion to the amount of each kind of complex. At a given wavelength, the average charge-transfer extinction coefficient of the solution is defined as:

$$\bar{\epsilon} = \epsilon_{CT_{Trp}} s_{Trp} + \epsilon_{CT_{Tyr}} s_{Tyr} \quad (1)$$

The saturation fractions of the donors s_{Trp} and s_{Tyr} are related to the complex binding constants K_{Trp} and K_{Tyr} by the relationship

$$s = \frac{[AD]}{[D_0]} = \frac{K([A_0] - [AD])}{1 + K([A_0] - [AD])} \quad (2)$$

where $[A_0]$ and $[D_0]$ denote the initial concentrations of the acceptor and donor and [AD] is the concentration of the CT complex. Thus, at given initial concentrations, the relative contributions of the tryptophan and tyrosine complexes are essentially a function of their respective K and ϵ values at the chosen wavelength. From extensive studies on model compounds, Deranleau and co-workers (Deranleau and Schwyzer, 1970; Deranleau et al., 1969, 1975; Hinman et al., 1974; Coan et al., 1975) determined the following parameters for the complexes in water: $K_{Trp} = 4$ L/mol, $K_{Tyr} = 0.95$ L/mol; at 360 nm, $\epsilon_{CT_{Trp}} \simeq 650$ L mol^{-1} cm^{-1} and $\epsilon_{CT_{Tyr}} \simeq 100$ L mol^{-1} cm^{-1}.

In a 10^{-3} equimolar solution of the two donors and the acceptor, for instance, the contribution of the tyrosine residue to the overall charge transfer band intensity at 360 nm is deduced to be about 3%. At wavelengths above 380 nm, its contribution becomes negligible. The reference wavelength for the comparison of the *intramolecular* CT intensities in the three LRF analogues was chosen as 380 nm.

Definition of an Apparent Folding Constant of the Peptide Backbone. If the molar extinction coefficient at a given wavelength of the *intramolecular* CT band between the tryptophan side chain and the nicotinamidium ring is known, a minimum value for the folding of the peptide backbone in the LRF analogues can be approximated from the experimental CT spectrum, using the definition:

$$K_{intra} = \frac{[AD]}{[A]} = \frac{\overline{OD}}{\overline{OD}_{max} - \overline{OD}} \quad (3)$$

where [A] is the molar concentration of the uncomplexed acceptor molecules, which is equal to the total population of all the peptide conformers which do not allow complex formation, and $\overline{OD}_{max}$ is the absorbance of the CT band which would be observed if all peptide molecules were in the complexed state ($\overline{OD}_{max} = \epsilon_{intra}[A_0]l$).

Clearly such a definition does not provide great insight into the folding mechanism of the peptide since it is expected that only a fraction of the "folded" conformers are participating in the CT interaction. Based on the similarity of their primary structure, it can be assumed, however, that the ensemble of folded conformers will be similar in the three analogues. If this is the case, any differences in the CT intensities can be analyzed, at least qualitatively, in terms of changes in the population of the folded backbone conformers.

Estimation of the Overall Energy Contribution of the Peptidic Framework in the Folding Process of the LRF Analogues. Assuming that the charge-transfer method described above is sensitive enough to detect folding in the LRF analogues, it would be of interest to have an estimate of the effects of the geometrical factors inherent in the chemical nature of the peptide backbone. In other words, our goal is to evaluate the CT effect that would be observed in these analogues, if the acceptor and the donor would be linked together by a hypothetical framework of infinite flexibility. The volume of unity, which contains only one peptide molecule, was chosen to be a sphere of radius equal to the distance between the acceptor and the donor moieties characteristic of a fully extended conformation of the peptide backbone.

Expressed in molar units, the effective concentration of the peptide becomes

$$[A_0] = \frac{V}{\frac{4}{3}\pi r^3 N} \quad (4)$$

where N represents the Avogadro constant, $V = 10^{27}$ Å^3 and $r = 19.075$ Å.

It is assumed furthermore that the acceptor and donor molecules interact pairwise inside the sphere, and that no interaction takes place between the peptide chains. Consequently the equilibrium state can be defined in an analogous manner to the case of an intermolecular complex formation between an equimolar amount of acceptor and donor, i.e.:

$$K = \frac{[AD]}{([A_0] - [AD])^2} \quad (5)$$

where [AD] represents the concentration of the CT complex. Using for K the experimental value of the *intermolecular* association constant between N-methylnicotinamide chloride and acetyltryptophanamide ($K = 4$ L/mol) (Deranleau and Schwyzer, 1970), the saturation fraction was found to be $s = [AD]/[A_0] = 0.16$. This represents the probability of *intramolecular* complex formation in the model described. The value s is an intrinsic parameter of the molecule which is independent of concentrations and therefore represents an appropriate proportionality constant in the Beer's law expression:

$$\overline{OD} = \epsilon s [A_0] l \quad (6)$$

The graphic representation of eq 6 using for ϵ the experimental value of the *intermolecular* complex (Deranleau and Schwyzer, 1970) is shown in Figure 4 (uppermost curve).

Using eq 3, the apparent association constant of this hypothetical *intramolecular* charge transfer model was found to be $K_{intra} = 0.19$.

Charge-Transfer Measurements. The electronic absorption spectra of [L-Ala[6],Nva[8](Nic$^+$)]-LRF·Cl$^-$, [Nva[8](Nic$^+$)]-LRF·Cl$^-$, and [D-Ala[6],Nva[8](Nic$^+$)]-LRF·Cl$^-$ between 240 and 500 nm are shown in Figure 5. The addition spectrum of the unmarked [D-Ala[6],Orn[8]]-LRF analogue and N-methylnicotinamide chloride is also reported as a reference. For the evaluation of the apparent extinction coefficients, the experimental values for the absorbance were essentially taken from the linear portion of the $\overline{OD}$ vs. concentration plot (Figure 4). For comparison, the absorbances were normalized at the $\lambda_{max} = 271$ nm. Interestingly enough, all three analogues show a definite charge-transfer band which appears as a shoulder in the long wavelength region of the spectra. Even more interesting is the fact that the intensity of the CT band is different in all cases, being the highest in the case of the [D-Ala[6]] analogue, and the lowest in the case of the [L-Ala[6]] analogue. The small difference between the [Gly[6]] and the [D-Ala[6]] analogues (best observed in Figures 4 and 6) was found to be reproducible and significant. The net charge-transfer effects obtained by subtracting the residual absorption of the un-

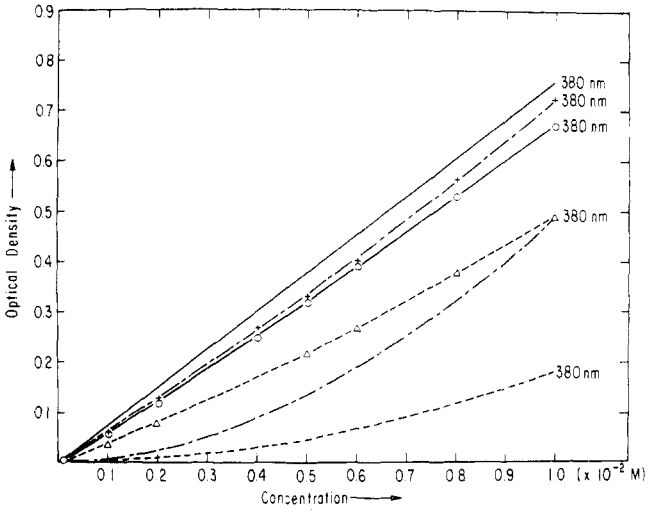

FIGURE 4: Charge-transfer absorbance at 380 nm, as a function of concentration (H_2O, T = 25 °C). (○)[Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (+) [D-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (△) [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (—) theoretical curve calculated from the hypothetical intramolecular CT model; (— - —) hypothetical intermolecular 1:1 CT complex calculated for ϵ_{380} = 472 L mol^{-1} cm^{-1} and normalized at 10^{-2} M with the absorbance of [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (- - - -) intermolecular 1:1 CT complex of $AcTrpNH_2$ + N-MeNic$^+$Cl calculated for ϵ_{380} = 472 L mol^{-1} cm^{-1} and K = 4 L/mol.

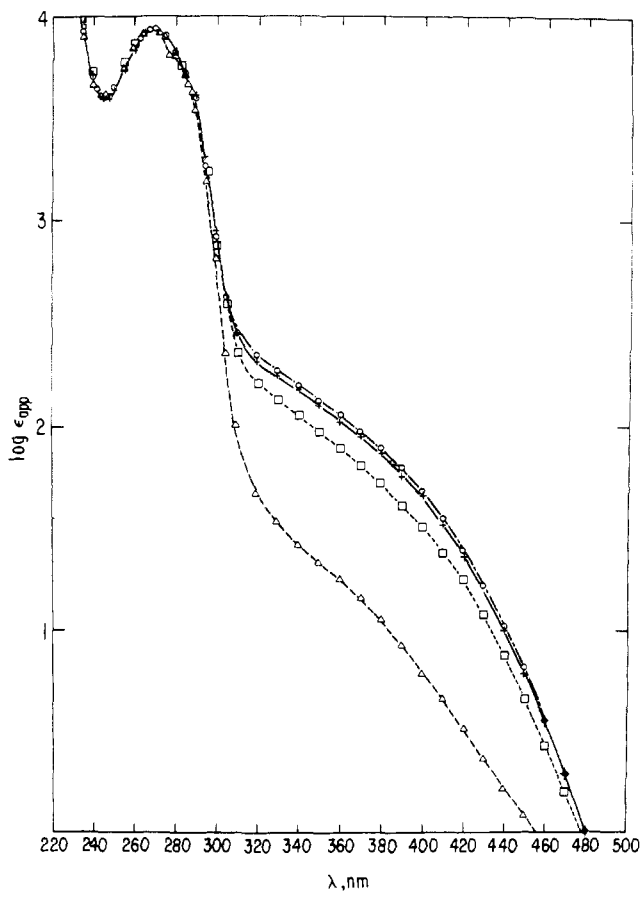

FIGURE 5: Apparent molar extinction coefficients as a function of wavelength (H_2O, T = 25°C): (+) [Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (○) [D-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (□) [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^-,AcOH; (△) addition spectrum of [D-Ala^6,Orn^8]-LRF·2AcOH and N-methylnicotinamide chloride.

marked peptides and of the acceptor are represented in a linear scale in Figure 6.

Compared with the ϵ values of the *intermolecular* complex between acetyltryptophanamide and nicotinamide chloride, these effects are relatively small. At 360 nm, the apparent molar extinction coefficient in [D-Ala^6,Nva^8(Nic^+)]-LRF·Cl^- is about six times smaller than the intrinsic extinction coefficient of the *intermolecular* complex. In the high concentration region, *intermolecular* charge-transfer contributions arising from self-association of the peptide analogues are observed in all three cases (Figure 4). The increase in optical density with concentration is linear only up to 4-5 × 10^{-3} M. At these concentrations, the lines start to deviate from linearity which is evidence of the cumulative effect of *intra-* and *intermolecular* charge-transfer interactions. Quantitative estimation of the contribution of the *intermolecular* CT effect is quite difficult. Association between decapeptide molecules in a given solvent is governed by several forces such as van der Waals interactions between side chains (the same type of interaction which is responsible for the CT complex formation), hydrogen bonding between the peptide skeletons, external entropy effects due to changes in solvation, and others. As a result, an equilibrium between several aggregated forms will take place. Among them, only the ones which have the correct geometries which allow the tryptophan side chain and the nicotinamidium residue to be in close vicinity will contribute to the overall *intermolecular* charge-transfer spectrum.

Simplifying the system to the extreme, we have considered two cases of *intermolecular* associations which are represented graphically in Figure 4. The lowermost curve represents the charge-transfer absorbance of the *intermolecular* model complex between acetyltryptophanamide and N-methylnicotinamide chloride at 380 nm as a function of concentration, for a ratio of acceptor to donor concentrations equal to 1 (eq 5) and using the known intrinsic characteristics of the model complex (Deranleau and Schwyzer, 1970), K = 4 L/mol and ϵ_{380} = 472 L mol^{-1} cm^{-1}. Clearly, the calculated intensities are much smaller than the experimental values in [L-Ala^6, Nva^8(Nic^+)]-LRF·Cl^-. Furthermore, the deviation from linearity is detectable at the lowest concentrations. The second lowest curve represents the absorbances of an *intermolecular* complex involving the same chromophore in a 1:1 ratio, but characterized by an association constant K, such that the charge-transfer absorbance at the highest concentration (10^{-2} M) coincides with the experimental value in [L-Ala^6, Nva^8(Nic^+)]-LRF·Cl^- (K = 13.4 L/mol). In this case also, the shape of the curve is quite different from the experimental curve. As a matter of fact, the two calculated curves have similar slopes in the low concentration region. Thus, assuming that the molar extinction coefficients for the model complex and for the *intermolecular* peptide complex are identical (both interactions are *intermolecular*), it can be concluded that the observed charge-transfer spectrum in [L-Ala^6,Nva^8(Nic^+)]-LRF·Cl^- is predominantly *intramolecular* in nature.

Let us now consider the shape of the charge-transfer spectra observed in the three LRF analogues. Intramolecular CT interactions of the type described in this paper have been extensively studied (Shifrin, 1964; Bosshard, 1970; Donzel, 1971; Deranleau and Bosshard, in preparation). In most cases, model compounds of restricted conformational flexibility were used, such as the nicotinamidium derivative of tryptophanamide. The CT spectra of such compounds were found to be somewhat different from the spectrum of the *intermolecular* model complex, viz., shift of the wavelength at maximum intensity (λ_{max}), lower molar extinction coefficient at this particular wavelength, and some differences in the overall shape of the spectra. One common geometric characteristic of these com-

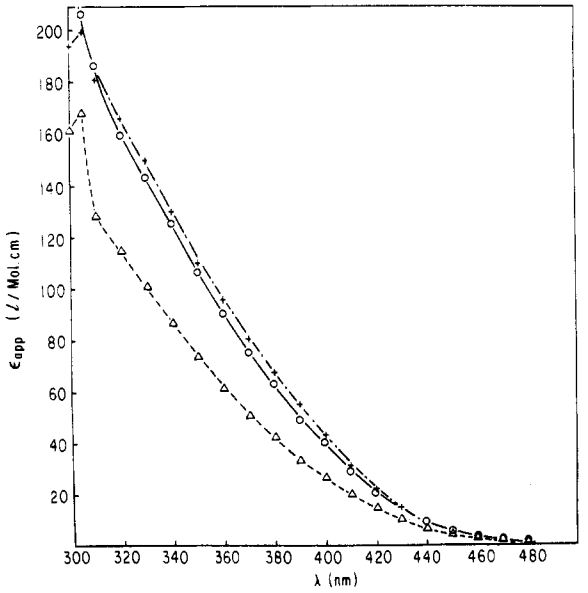

FIGURE 6: Intramolecular charge transfer spectra (H_2O, T = 25 °C): (+) [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH; (○) [Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH; (△) [L-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH.

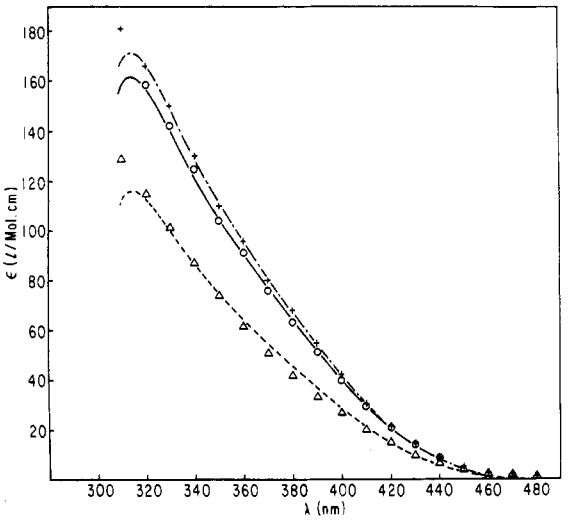

FIGURE 7: Comparison between the experimental and simulated intramolecular CT spectra of the marked LRF analogues. [The parameters used in the simulations were taken from the intermolecular CT spectrum between $AcTrpNH_2$ and N-MeNic$^+$Cl$^-$ (Deranleau and Schwyzer, 1969).] (—) Simulation for [Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH; (— · —) simulation for [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH; (- - - -) simulation for [L-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$,AcOH.

plexes is that the rings of the acceptor and donor chromophores are at a defined angle in the "best-complex" conformation, a situation which causes a decrease in the probability of charge-transfer transition. A quantitative relationship between angle and transition probability was recently proposed (Deranleau and Bosshard, in preparation). In our case, it can be expected that a complex geometry more similar to the one found in the *intermolecular* complex can take place, resulting primarily from the high conformational flexibility of the ornithyl side chain. In fact, the three-dimensional CPK molecular models of the peptides suggest that a coplanar arrangement of the acceptor and donor is possible. It is thus tempting to compare the spectra of the LRF analogues with the *intermolecular* spectrum of the *N*-methylnicotinamide chloride-acetyl tryptophanamide complex. For this purpose, the *intermolecular* and *intramolecular* intensities were normalized at a chosen wavelength (chosen to give the best fit) and the spectral distribution compared with the experimental values. The results are shown in Figure 7. Interestingly enough, this curve-fitting procedure gives fairly satisfactory results, especially in the case of [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$ (for the [L-Ala^6] analogue, one observes a small discrepancy in the middle wavelength region). This confirms our previous assumption that the observed charge-transfer bands, at least in the cases of the [Gly^6] and [D-Ala^6] analogues, are substantially caused by the nicotinamidium–tryptophan interaction. At least as a first approximation, these results support the use of the *intermolecular* CT spectrum as a reference for the estimation of the complex population in the LRF analogues.

Discussion

Study of the conformational behavior of peptide hormones in solution is often considered as a prerequisite for the understanding of the mode of receptor binding. Besides this biological significance, such studies, quite generally, present an attractive challenge to delineate the various forces which determine the allowed conformations of a peptide in a given environment. This task is certainly not easy, considering the multiplicity of these allowed conformational states, especially in the case of linear peptides. The LRF molecule is not an exception and most spectroscopic studies have evidenced the high degree of flexibility in the backbone as well as in the side chains. The use of specific charge-transfer labels, first proposed by Schwyzer and co-workers (Carrion et al., 1968), offers a means of visualizing binary side-chain interactions with an unequivocal "yes" or "no" answer and allows one, consequently, to investigate in successive steps, the main conformational features of the peptide backbone. If qualitative conclusions can be easily drawn, a quantitative interpretation of the CT data, at this stage, is not straightforward and several assumptions have to be made.

We have assumed in our calculations that the extinction coefficient of the *intramolecular* CT complexes at 380 nm is similar to the value determined for the typical *intermolecular* complex at the same wavelength (ϵ_{380} = 472 L mol^{-1} cm^{-1}, Deranleau and Schwyzer, 1970). This assumption is difficult to verify since it implies studies on rigid molecules with a coplanar arrangement of donor and acceptor. The observation that the CT spectra of the LRF analogues can be fitted with the *intermolecular* spectrum is a good indication that this could be the case, although not a proof, mainly because the observed CT intensities are small.

Furthermore, it would be convenient to define a particular reference conformational state of the molecule and evaluate the CT effect of the molecule in this chosen state. For our purpose, an adequate choice would be the random structure of the peptide backbone. In the present study, we have first considered an *intramolecular* model consisting of an acceptor and donor joined by a hypothetical chain of infinite flexibility, but of length equal to the dimensions of the peptide backbone in its extended conformation. Inside the sphere of unity, the probability of complex formation was evaluated from the known characteristics of the *intermolecular* complex. Such a model can only give a lower limit for the expected *intramolecular* CT effect. The value for the *intramolecular* complex binding constant characteristic for this model (K_{intra} = 0.19) using ϵ = 472 L mol^{-1} cm^{-1} is of similar magnitude to the value found for [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$ (K_{intra} = 0.17) (Figure 4). Since it is expected that the geometrical constraints

of the peptide strongly diminish the probability of complex formation, it appears justified to assume, according to the proposed model, that the primary sequence of the LRF molecule does contain some energy factors favoring the folding of the peptide chain.

The apparent binding constants for the three analogues derived from eq 3 are: [D-Ala^6,Nva^8(Nic$^+$)]-LRF·Cl$^-$, K = 0.17 or ca. 15% apparent folding; [Nva^8(Nic$^+$)]-LRF·Cl$^-$, K = 0.15 or ca. 13% apparent folding; and [L-Ala^6,Nva^8-(Nic$^+$)]-LRF·Cl$^-$, K = 0.10 or ca. 9% apparent folding. It should be emphasized again that these values represent only an approximation of the CT complex concentration and do not indicate the actual amount of folding of the backbone. The mode of folding is necessarily a multiple-step mechanism involving conformational changes in the backbone as well as in the side chains of ornithine and tryptophan; in order to account for the observed CT effects, the folded backbone conformers apparently have to be significantly populated. We are currently trying to obtain more quantitative results using analogues which are chemically "frozen" in folded structures. At this stage, however, the most interesting results remain the observation that the intensity of the charge-transfer band between the donor in position three and the acceptor in position eight varies with the configuration and the steric nature of the residue in position six. This trend is in qualitative agreement with the theoretical prediction of the stability of a β^{II} turn (Venkatachalam, 1968), provided that the turn in LRF is located at the sequence Ser-Tyr-Gly-Leu. However, we must stress that this type of turn is not the only possible mode of folding which is consistent with our results.

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