Circular Dichroism Study of the Solution Conformation of Luteinizing Hormone Releasing Hormone†

John R. Cann,* K. Channabasavaiah, and John M. Stewart

ABSTRACT: A systematic investigation has been made into the circular dichroic behavior of luteinizing hormone releasing hormone and its peptide fragments and deletion analogues. The results are interpreted to mean that the hormone exists in solution as an ensemble of conformers with different sensitivities to temperature and solvent composition. The far-ultraviolet circular dichroic spectra exhibited by the hormone under different experimental conditions can be simulated satisfactorily by the weighted addition of the spectra of its aliphatic- and aromatic-containing halves. However, the structure of the hormone is not simply the sum of its halves, since some conformational feature of the intact molecule perturbs the near-ultraviolet circular dichroism of its aromatic residues.

Luteinizing hormone releasing hormone (LHRH)[1] is a decapeptide with the amino acid sequence <Glu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2. Despite several different spectroscopic studies, there is as yet no consensus as to the conformation of LHRH in aqueous solution. The ^1H and ^{13}C NMR studies of Wessels et al. (1973) and Deslauriers and co-workers (Deslauriers et al., 1975, 1977; Deslauriers & Somorjai, 1976) indicate an essentially random configuration with increased flexibility of the peptide backbone in the immediate vicinity of the two Gly residues and with no evidence for *strong* intramolecular hydrogen bonding involving CO- - - -NH or for specific side-chain interaction. These conclusions are in accord with those of Mabrey & Klotz (1976) based on CD and fluorescence energy transfer measurements. In contrast, Marche et al. (1973) have interpreted their CD observations on the hormone at neutral and alkaline pH in terms of a trend toward ordered structures, particularly upon increasing the temperature at pH 7.4.

A somewhat different tack has been taken by Donzel et al. (1977), who employed both ^1H NMR and ultraviolet–visible absorption spectroscopy to probe the conformational properties of C^6-nicotinamidium derivatives of [Nva[8]]-, [Nva[8],D-Ala[6]]-, and [Nva[8],L-Ala[6]]LHRH. Even though the NMR indicated the same kind of conformation flexibility of the peptide backbones and side chains as in LHRH, absorption spectroscopy revealed an intramolecular charge transfer complex between the nicotinamidium ring of Nva[8](Nic$^+$) and the indole ring of Trp[3]. This result indicates some kind of order in which the peptide backbone is folded so as to bring the two rings into proper juxtaposition for complex formation, and evidently there is a significant population of the folded conformers.

This is not to say that there is a significant population of the folded states in the case of LHRH itself. It is interesting, however, that the semiempirical calculations of Momany (1976a,b) may be viewed as pointing in that direction. Of the several conformers of LHRH selected for iterative energy minimization, the three of lowest energy seem to be "hinged" at Gly[6] by a modified type II β bend stabilized by a *weak* hydrogen bond.

The present investigation of the CD of LHRH places particular emphasis on its peptide fragments and deletion analogues to gain further insight into the structure of the hormone in aqueous solution.

Materials and Methods

Peptides were synthesized by the Merrifield solid-phase method (Stewart & Young, 1969) using one of two automatic instruments, the Stewart Mark V, which is an improved version of the instrument described earlier (Merrifield et al., 1966), or the Beckman 990 synthesizer. For synthesis of peptide amides, 1% cross-linked benzhydrylamine resin (Beckman) was used as the support to which the C-terminal amino acid was coupled with the aid of DCC. Chloromethylpolystyrene (Lab Systems) (1% cross-linked) was used for synthesis of peptides which are not amides; attachment of amino acids to this resin was done in the standard way (Stewart & Young, 1969). The main steps in one synthetic cycle using the Stewart Mark V instrument were deprotection of the Boc group with trifluoroacetic acid (25% in chloroform and containing 0.1% indole), neutralization with triethylamine (10% in chloroform), and coupling of the next amino acid derivative (2.5-fold) with DCC (2.5-fold) in chloroform (or mixtures of chloroform and dimethylformamide for poorly soluble derivatives). Dichloromethane was used in place of chloroform when the Beckman 990 instrument was employed.

Peptides prepared on the BHA resin were cleaved from the resin by treatment with anhydrous HF containing 10% anisole at 0 °C for 30 min. Of the peptides prepared by using the chloromethyl resin, peptide VI was obtained by transesterification of the corresponding peptide–resin with methanol containing 10% triethylamine, followed by HF treatment; peptide VII was prepared by reaction of VI with dimethylamine; peptide IX was prepared by transesterification of the peptide–resin with methanol, conversion of the resulting protected peptide methyl ester to the dimethylamide by reaction with dimethylamine, and deprotection with HF–anisole.

CCD was used for the purification of all the peptides except VII, VIII, and IX, which were purified by chromatography on Sephadex G-25. The homogeneity of the freeze-dried peptides was established by high-voltage electrophoresis. The

† From the Department of Biophysics and Genetics (J.R.C.) and the Department of Biochemistry (K.C. and J.M.S.), University of Colorado Medical Center, Denver, Colorado 80262. *Received January 10, 1979.* Supported in part by Research Grant 5R01 HL 13909-27 from the National Heart, Lung, and Blood Institute (J.R.C.) and Contract N01-HD-6-2843 from the National Institute of Child Health and Human Development, National Institutes of Health, U.S. Public Health Service. This publication is No. 728 from the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, CO 80262.

[1] Abbreviations used: LHRH, luteinizing hormone releasing hormone; NMR, nuclear magnetic resonance; CD, circular dichroism; Nva(Nic$^+$), C^6-nicotinamidiumnorvaline; BHA, benzhydrylamine; DCC, dicyclohexylcarbodiimide; CCD, countercurrent distribution; F_3AcOH, trifluoroacetic acid.

0006-2960/79/0418-5776$01.00/0

Table I: Methods of Purification and Electrophoresis Data for Synthetic Peptides

no.	peptide	purification method[a]	electrophoretic mobility[b]
I	<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	CCD [BuOH-HOAc-H_2O (4:1:5); $k = 0.25$]	0.56
II	<Glu-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	CCD [BuOH-HOAc-H_2O (4:1:5); $k = 1.22$]	0.38
III	<Glu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	CCD [BuOH-HOAc-H_2O (4:1:5); $k = 0.60$]	0.43
IV	<Glu-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	CCD [BuOH-HOAc-H_2O (4:1:5); $k = 0.5$]	0.42
V	Gly-Leu-Arg-Pro-Gly-NH_2	CCD [BuOH-1% F_3AcOH (1:1); $k = 0.1$]	0.92
VI	Gly-Leu-Arg-Pro-Gly-OCH_3	CCD [BuOH-1% F_3AcOH (1:1); $k = 0.25$]	0.94
VII	Gly-Leu-Arg-Pro-Gly-$N(CH_3)_2$	gel chromatography [Sephadex G-25/1 M HOAc]	0.92
VIII	Arg-Pro-Gly-NH_2	partition chromatography [Sephadex G-25/BuOH-HOAc-H_2O (4:1:5)]	1.15
IX	Arg-Pro-Gly-$N(CH_3)_2$	partition chromatography [Sephadex G-25/BuOH-HOAc-H_2O (4:1:5)]	1.10
X	<Glu-His-Trp-Ser-Tyr-Gly-NH_2	CCD [BuOH-HOAc-H_2O (4:1:5); $k = 0.43$]	0.39

[a] Solvent ratios are expressed by volume. Only 1-butanol was used. [b] Ratio of the mobility of peptide to that of lysine at pH 5.0 in pyridine–acetic acid buffer.

purification methods employed and the electrophoretic mobilities are listed in Table I.

Acid hydrolysis of peptides was done by heating in sealed tubes at 110 °C for 22 h with 6 N HCl containing 1 mg/mL each of β-mercaptoethanol and phenol. The amino acid ratios in the hydrolysates were determined on a Beckman 120C amino acid analyzer and were those expected for the structures synthesized.

The synthetic LHRH was assayed by Dr. C. Y. Bowers, Tulane University, for activity in isolated rat hemipituitaries and was found to possess full biological activity.

Its ultraviolet absorption in water at pH 7.0 was found to obey Beers law over the 450-fold concentration range 7.06×10^{-3} to 3.20 mg/mL. Measurements on a standard solution, which had been analyzed for its amino acid composition to establish the concentration of the hormone, gave a molar extinction coefficient at 280 nm of $\epsilon = 7.19 \times 10^3$ M^{-1} cm^{-1}. The ultraviolet absorption of related peptides containing aromatic residue(s) was calibrated with solutions whose concentrations were given by the percentage of peptide by weight in the freeze-dried preparation, as determined from amino acid analysis. The concentration of LHRH and the aromatic-containing peptides was routinely determined spectroscopically. The concentration of nonaromatic peptides was reckoned from the percentage of peptide in the dry material.

The *N*-acetyl-L-tyrosine amide was obtained from Schwarz/Mann; *N*-acetyl-L-tryptophan amide and *N*-acetyl-L-histidine *N'*-methylamide were from Cyclo Chemical Co.

CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment, equipped with a thermostatable cell holder. The cell temperature at equilibrium is specified by Cary to be stable within 0.03 °C at 90 °C. The temperature controller of the cell holder is connected to a constant-temperature (±0.02 °C) circulating water bath via insulated tubing. The temperature of the solution in the cell was determined from a calibration curve of sample vs. bath temperature. The calibration curve had been previously established by inserting a thermistor probe into a water-filled CD cell in situ and recording the temperature in the cell as a function of the bath temperature (10–92 °C) measured with a calibrated thermometer, against which the thermistor probe had been checked. Occasionally the temperature of the solution in the cell was checked by inserting the probe into the cell in situ after recording the CD spectrum. The instrument had been modified by Cary Instruments in the field to eliminate possible artifactual signals on passing through intense absorption bands (Cary Model 6003100 modification kit for changing the carrier frequency from 60 to 325 Hz). Slits were programmed for 1.5-nm bandwidth at each wavelength. Concentration and path length were dictated by the absorbance of the solution; the sample absorbance was generally less than 1 OD unit and only in a few experiments reached an OD of 2–2.5. Mean residue ellipticities, $[\Theta]_{mrw}$ [(deg cm^2)/dmol], were calculated in the usual fashion by using the mean residue weight reckoned from the molecular weight of the peptide. Each spectrum is the average of at least two determinations. Except where noted, all experiments in water and salt solutions were done at pH 7.0.

Results

Our interest in the CD of LHRH stems from the observation that, whereas in aqueous solution its spectrum over the wavelength range 260–225 nm is dominated by positive aromatic bands (Marche et al., 1973; Mabrey & Klotz, 1976; curve a in our Figure 1A), the spectrum of the C-terminal pentapeptide fragment of LHRH, Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2, exhibits a relatively weak negative band with an extremum at 231 nm (curve b in Figure 1A). The questions posed are "Is this negative band a spectral component of LHRH itself but masked by overlying aromatic contributions?" and "What is its conformational significance?" This study of the CD behavior of LHRH and related short-chain peptides and derivatives, whose structures and properties are given in Table I, was designed to provide answers to these questions.

C-Terminal Aliphatic Peptide Fragments of LHRH. The aforementioned pivotal observation on the C-terminal pentapeptide fragment of LHRH invited elaboration with respect to the effect of solvent composition and temperature. These experiments reveal that in the wavelength region of interest, the CD spectrum shows two bands with different sensitivities to environmental parameters, a negative band in the region of 230 nm and a positive band at about 218 nm (parts A and

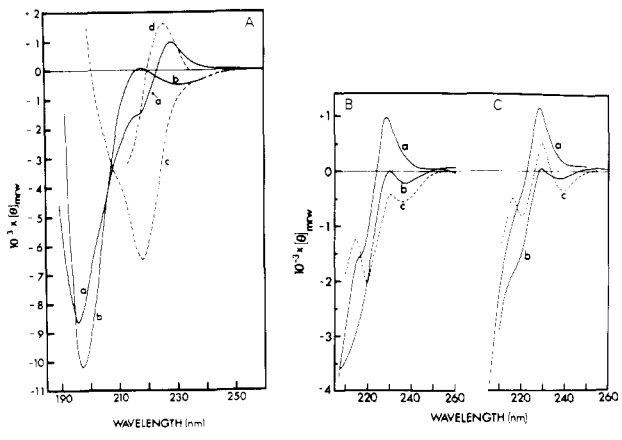

FIGURE 1: Far-ultraviolet CD spectra. (A) LHRH and its C-terminal pentapeptide fragment: curve a, LHRH in water; curve b, Gly-Leu-Arg-Pro-Gly-NH_2 in water; curve c, LHRH in trifluoroethanol; curve d, LHRH in a 20% trifluoroacetic acid–80% trifluoroethanol mixture. Temperature, 27 °C. (B) LHRH: curve a, in water at 27 °C; curve b, in water at 65.5 °C; curve c, in a 90% dioxane–water mixture. (C) Simulated spectra of LHRH; designation of curves is the same as that described in (B); positions of bands and shoulders are within 1 nm of experimental values except for the negative 238-nm band of curve c (experimental, 236 nm).

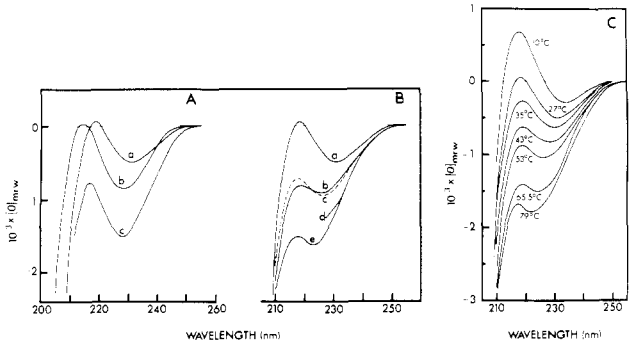

FIGURE 2: Effect of solvent and temperature on the CD spectrum of Gly-Leu-Arg-Pro-Gly-NH_2. (A) Comparison of water and organic media: curve a, water; curve b, trifluoroethanol; curve c, a 90% dioxane-water mixture. (B) Effect of salts: curve a, water; curve b, 1 M $(NH_4)_2SO_4$; curve c, 1 M NaCl; curve d, 1 M NaBr; curve e, 1 M $NaClO_4$. Temperature, 27 °C. (C) Effect of temperature in water at pH 7.0. Virtually the same results were obtained in exploratory experiments at pH 4.9, thus eliminating ionization of the α-amino group as the origin of the effect.

C of Figure 2). As shown in Figures 2A and 3, changing the solvent from water to either trifluoroethanol or a dioxane–water mixture, 90%:10% by volume, causes a strong enhancement of the negative band with marginal, if any, effect on the positive one. The difference spectra (curves a and b in Figure 3) reveal the negative band to be located at 224–226 nm, while the spectra displayed in Figure 2C show that, because of the overlap of the two bands, the negative one superficially appears to be centered at 231 nm at 27 °C. (For the sake of clarity, we shall simply refer to the "negative 231-nm band" from now on.)

Elevating the temperature of aqueous solutions also has a pronounced effect on the CD (Figure 2C), but, in sharp contrast to the organic solvents, it acts to decrease the amplitude of the positive 218-nm band without discernible effect on the negative 231-nm band (curve c in Figure 3). The temperature-induced transition appears to be essentially complete at 79 °C and is reversible. Thus, for example, a solution, which had been incubated at 65.5 °C and then returned to 27 °C, gave the same CD spectrum as peptide never exposed to the higher temperature. Since an isoellipticity point has

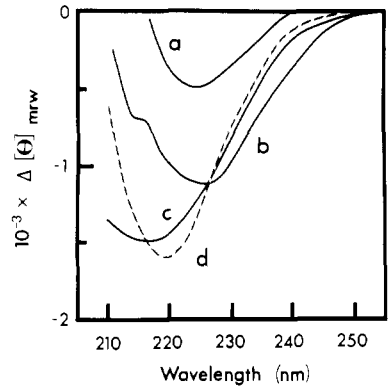

FIGURE 3: Difference CD spectra of Gly-Leu-Arg-Pro-Gly-NH_2. Curve a, CD at 27 °C in trifluoroethanol referred to water (at wavelengths below 216 nm $\Delta[\theta]_{mrw}$ was positive and increased monotonically down to 205 nm, below which measurements were not made); curve b, CD at 27 °C in a 90% dioxane–water mixture referred to water; curve c, CD in water at 65.5 °C referred to 27 °C; curve d, CD at 27 °C in 1 M $NaClO_4$ referred to water. Wavelength of extremum: (a) 244.5; (b) 226; (c) 217; (d) 219 nm.

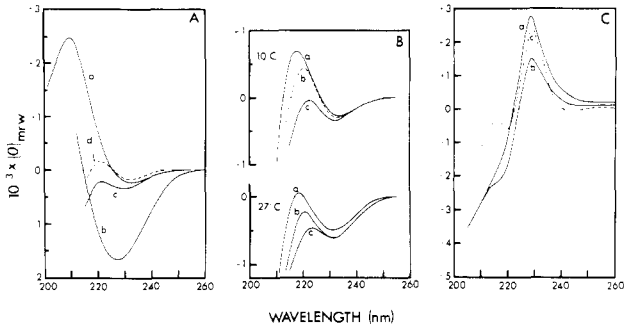

FIGURE 4: CD spectra of peptide fragments. (A) C-Terminal tripeptide and its dimethyl derivative: curve a, Arg-Pro-Gly-NH_2 in water at 27 °C; curve b, Arg-Pro-Gly-NH_2 in a 90% dioxane–water mixture at 27 °C; curve c, Arg-Pro-Gly-$N(CH_3)_2$ in water at 27 °C; curve d, Arg-Pro-Gly-$N(CH_3)_2$ in water at 10 °C. (B) C-Terminal pentapeptide fragment and its derivatives in water at the indicated temperature: curve a, Gly-Leu-Arg-Pro-Gly-NH_2; curve b, Gly-Leu-Arg-Pro-Gly-OCH_3; curve c, Gly-Leu-Arg-Pro-Gly-$N(CH_3)_2$. (C) <Glu-His-Trp-Ser-Tyr-Gly-NH_2: curve a, in water at 27 °C; curve b, in water at 65.5 °C; curve c, in a 90% dioxane-water mixture at 27 °C.

not been observed,[2] further characterization of the transition in terms of thermodynamic parameters is not warranted.

Finally, the CD is sensitive to the addition of salts. As shown in Figure 2B, salts at high concentration and 27 °C simulate elevated temperatures, their order of increasing effect on the spectrum following a Hofmeister series: $(NH_4)_2SO_4 \simeq NaCl < NaBr < NaClO_4$. Moreover, as illustrated in Figure 3, for $NaClO_4$ (curve d), salts behave like temperature in exerting their effect on the positive 218-nm band with indiscernible effect on the negative 231-nm band.

Although we have focused on the C-terminal pentapeptide fragment, the C-terminal tripeptide fragment, Arg-Pro-Gly-NH_2, exhibits a similar CD spectrum (Figure 4A) with a negative band at 232 nm and a positive one at 209 nm, albeit of much greater amplitude. Likewise, the spectrum is strongly solvent dependent. This is the smallest fragment examined which shows the two bands of interest here; neither Arg-Pro nor Arg-Pro-NH_2 do so (Cann et al., 1973). The chemical structural requirements for expression of the two bands were examined further with the derivatized peptides Gly-Leu-

[2] It was technically impossible with our thermostatable cell holder to extend the measurements, particularly those at elevated temperatures, to wavelengths below 210 nm.

Arg-Pro-Gly-OCH_3, Gly-Leu-Arg-Pro-Gly-$N(CH_3)_2$, and Arg-Pro-Gly-$N(CH_3)_2$. The conclusion drawn from the CD behavior of these compounds (parts A and B of Figure 4) is that the C-terminal amide group per se is not required for the peptide conformation(s) which give rise to the two bands. It is particularly striking that the wavelength and amplitude of the negative band shown by the derivatives are about the same as those for the corresponding parent peptide.

N-Terminal Aromatic Fragment. The CD properties of <Glu-His-Trp-Ser-Tyr-Gly-NH_2 are illustrated by the spectra presented in Figure 4C. Two features are noteworthy: (1) the intensity of the positive 228-nm band, which is attributable to the long-wavelength half of the 225- and 223-nm bands of Tyr and Trp and the tail of the 217-nm band of His, decreases by about 50% upon raising the temperature from 27 to 65.5 °C, while (2) a well-defined negative shoulder appears at about 217 nm, presumably due to a decrease in amplitude of the His band as judged from subsidiary experiments on the model compound acetylhistidine methylamide.

LHRH and Its Deletion Analogues. A twofold approach was adopted to ascertain whether or not the two CD bands exhibited by the C-terminal pentapeptide fragment of LHRH are spectral components of LHRH itself: (1) examination of the effect of elevated temperature and solvent change on the CD of LHRH and a homologous series of deletion analogues and (2) simulation of the CD of LHRH under various experimental conditions by summation of the CD of its aliphatic- and aromatic-containing halves.

The first peptide in the aforementioned series of deletion analogues is <Gly-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (peptide IV in Table I) from which the other members of the series can be generated by successive insertion between <Glu and Tyr of Ser (peptide III), Trp (II), and His (I; i.e., LHRH) in that order. A priori one would expect that, if the two CD bands in question are in fact spectral components of the analogues, they would be at least partially masked by overlying aromatic band(s). At best, only the long wavelength half of the negative 231-nm band might be evident. On the other hand, raising the temperature or changing the solvent from water to a 90% dioxane-water mixture would be expected to accentuate the vestige of the possible negative CD component or partially unmask its concealed presence. This presumption is based on (a) the effect of these environmental parameters on the CD of the C-terminal pentapeptide fragment of LHRH (parts A and C of Figure 2) and (b) the likelihood that increasing the temperature from 27 to 65 °C may decrease by at least 25% the amplitude of the overlying, positive 225-nm tyrosine band (but possibly not the 223-nm tryptophan band) as judged from subsidiary experiments on the model compounds acetyltyrosine amide and acetyltryptophan amide.

As shown in Figures 5 and 1B, these expectations are borne out by experiment. It seems justified to conclude that (1) the negative 231-nm and positive 218-nm CD bands of the C-terminal pentapeptide fragment are spectral components of the deletion analogues and LHRH itself and (2) in LHRH these spectral components are masked by overlying aromatic bands, particularly the Trp band (compare curve a in Figure 5C with curves a of parts A and B of Figure 5).[3]

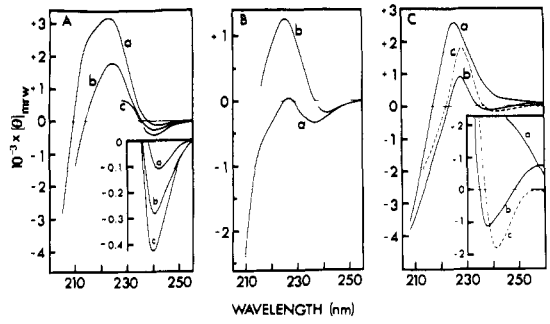

FIGURE 5: CD behavior of a homologous series of deletion analogues of LHRH. (A) Peptide IV in Table I: curve a, in water at 27 °C; curve b, in water at 65.5 °C; curve c, in a 90% dioxane–water mixture at 27 °C. (B) Peptide III: curve a, in water at 27 °C; curve b, in 4 M guanidine hydrochloride (see footnote 3 to the text). (C) Peptide II: curve a, in water at 27 °C; curve b, in water at 79 °C; curve c, in a 90% dioxane–water mixture at 27 °C; the ordinate of the insert is $10^{-2}[\Theta]_{mrw}$.

The effect of temperature and solvent change is particularly striking in the case of LHRH (Figure 1B). In water at 27 °C the spectrum shows a positive band at 229 nm and a well-defined, negative shoulder at 217 nm. Raising the temperature to 65.5 °C causes the appearance of a negative band at 237 nm and, in agreement with Marche et al. (1973), loss of the positive band and intensification of the 217-nm shoulder. Changing the solvent from water to a 90% dioxane–water mixture causes the appearance of a negative band at 236 nm with loss of the positive band and the appearance of a negative band at 219 nm.

Simulated spectra of LHRH for the three different experimental conditions were constructed by the weighted addition of the spectra of Gly-Leu-Arg-Pro-Gly-NH_2 and <Glu-His-Trp-Ser-Tyr-Gly-NH_2 under identical conditions.[4] The simulated and experimental spectra are compared in parts B and C of Figure 1. The agreement is quite reasonable considering that the intensities of the spectra of the polypeptide fragments cannot be expected to sum quantitatively to the absolute band intensities because of the different asymmetrical environments and residue interactions in the intact hormone molecule. Clearly, the simulations provide further support for the conclusion that the two CD bands exhibited by the C-terminal pentapeptide fragment are spectral components of LHRH itself.

Near-Ultraviolet CD of LHRH. Despite the relative success of the foregoing spectral simulations, it cannot be concluded that the conformation of LHRH is simply the sum of its aliphatic- and aromatic-containing halves. This is evident from observations on the near-ultraviolet CD associated with the tyrosine and tryptophan residues in the hormone molecule. Before proceeding to these measurements, however, it is necessary in the way of background to reiterate the findings of other investigators with regard to the effects of the structure-promoting solvent trifluoroethanol (Marche et al., 1973; Mabrey & Klotz, 1976) and the structure-disrupting solvent trifluoroacetic acid (Mabrey & Klotz, 1976) on the far-ultraviolet CD of LHRH. Whereas highly ordered conformers (which have been ascribed to helical and/or β structures) predominate in trifluoroethanol (curve c of Figure 1A), they are virtually disrupted in a 20% trifluoroacetic acid–80% trifluoroethanol mixture (curve d in Figure 1A).

The near-ultraviolet CD spectra are displayed in Figure 6A. In trifluoroethanol (curve a) LHRH shows negative bands,

[3] The analogue, <Glu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is aberrant in that its water solutions show a light Tyndall effect indicating aggregation. Solutions in 4 M guanidine hydrochloride are devoid of this effect, and their ultraviolet absorption at 275 nm obeys Beers law over the concentration range 0.30–0.025 mg/mL. The plot of absorbance vs. concentration is a straight line passing through the origin. Thus, the peptide is evidently not aggregated in 4 M guanidine hydrochloride.

[4] Within experimental error, the same results were obtained either by averaging the mean residue ellipticities or by taking one-tenth of the sum of the molar ellipticities of the two fragments.

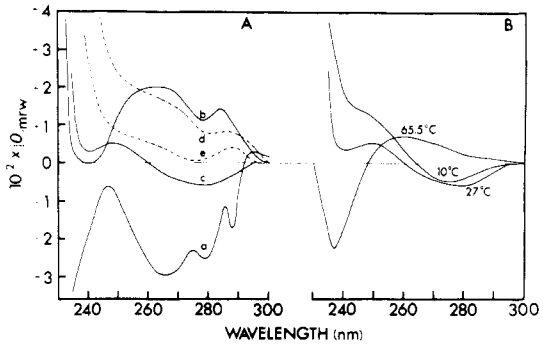

FIGURE 6: Near-ultraviolet CD spectra. (A) Curve a, LHRH in trifluoroethanol; curve b, LHRH in a 20% trifluoroacetic acid–80% trifluoroethanol mixture; curve c, LHRH in water; curve d, <Glu-His-Trp-Ser-Tyr-Gly-NH_2 in water; curve e, one-tenth the sum of the molar ellipticities of acetyltyrosine amide and acetyltryptophan amide; temperature, 27 °C. (B) LHRH in water at the indicated temperatures.

while in a 20% trifluoroacetic acid–80% trifluoroethanol mixture (curve b) it shows positive bands. This spectral difference is evidently conformationally related, since there is no correlation with the behavior of model compounds. Both acetyltyrosine amide and acetyltryptophan amide show negative CD in both of these solvents. When water is used as the solvent, the deletion analogue, <Glu-His-Trp-Ser-Tyr-Gly-NH_2, which contains all of the aromatic residues in LHRH, shows positive CD (curve d). Its spectrum is in qualitative agreement with the simulated spectrum of a disordered hexapeptide containing one Tyr and one Trp residue (curve e multiplied by ~1.7) as constructed from the spectra of acetyltyrosine amide (negative CD band at 275 nm) and acetyltryptophan amide (positive CD from about 297 nm down). In contrast to its aromatic-containing deletion analogue and the expectation for a disordered decapeptide containing one Tyr and one Trp (curve e), the CD of LHRH is negative (curve c).[5] Comparison of these several spectra invites the conclusion that in water LHRH enjoys some privileged conformational feature which perturbs the CD of aromatic residues, in particular, Trp^3. Supporting evidence is provided by the striking effect of elevating the temperature from 27 to 65.5 °C (Figure 6B), which reverses the sign of the CD in the wavelength region of concern here.

Discussion

The results presented above indicate that the seemingly simple CD spectrum of LHRH is actually a composite of bands which can be unraveled, at least in part, by examining the effects of temperature and solvent composition on the CD of the hormone, related short-chain peptides, and model compounds. The question as to the conformational relatedness of the bands was already posed by the pivotal observation on the C-terminal pentapeptide fragment of LHRH, which shows a negative band at 231 nm and a positive one at 218 nm. The same qualitative profile is shared by the C-terminal tripeptide fragment. Comparison of the tripeptide's spectrum (curve a in Figure 4A) with theoretical spectra for β bends (Woody, 1974) arouses the suspicion that in aqueous solution the molecule may assume a β-bend-like configuration stabilized by a 4 → 1 type hydrogen bond involving the Arg^9 carbonyl oxygen and the Gly^{10}-NH_2 amide proton. However, this possibility has evidently been eliminated by experiments on derivatives of both the pentapeptide and tripeptide fragments in which the C-terminal amide is modified in such a way that it cannot participate in hydrogen bonding.

The simplest interpretation of the CD takes cognizance of the fact that the profiles of the C-terminal fragments of LHRH resemble the spectrum exhibited under certain conditions by the random-coil conformation of polypeptides chains (Mattice, 1974; Woody, 1977). Interpretation of the CD of random-coil peptides is still not settled. The literature has recently been reviewed by Woody (1977), who concludes that the exact assignment of the bands is not as yet clear, although the positive 218-nm band is very likely due in part or in whole to an n–π^* transition. Some investigators, for example, would attribute the weakly negative 238-nm band to at least a modicum of some kind of ordered structure, while others would not or are noncommittal. Thus, our present state of understanding can accommodate the following interpretation of the differential effects of temperature, salts, and solvent on the 218- and 231-nm bands of the C-terminal fragments. Each of the peptides is considered as existing in solution as an ensemble of conformers with different spectral properties and different sensitivities to environmental parameters. In the case of the C-terminal pentapeptide, for example, we propose that the CD spectrum of one subset of conformers shows a strong negative band located at 197 nm and a weak positive band at 218 nm but does not show the negative 231-nm band. The 218-nm band is sensitive to temperature and salts but insensitive to changing the solvent from water to either trifluoroethanol or a 90% dioxane–water mixture. The effect of temperature and salts is consistent with their effect on the positive 218-nm band of random-coil polypeptides (Mattice, 1974; Woody, 1977). Increasing temperature or addition of salts evidently decreases the population of conformers showing the 218-nm band.

Another subset of conformers is presumed to have a strong negative band at 197 nm and a weak negative band at 231 nm but not the positive 218-nm band. The amplitude of the 231-nm band, while insensitive to temperature and salts, is enhanced by changing the solvent from water to trifluoroethanol or to a 90% dioxane–water mixture. This may be a classical solvent effect and/or, possibly, an increase in the population of these conformers. Given the Pro-containing sequence of these peptides, it seems relevant to note that the intramolecularly hydrogen-bonded conformation adopted by acetylproline methylamide in organic solvents [Madison & Schellman (1970a,b); Cann (1972); Figure 1 in Cann et al. (1973)] and the γ-turn conformer of cyclo(Pro-Gly)$_3$ (Madison et al., 1974) show a negative CD band at about 230 nm as predicted by theory.

The effects of elevated temperature and solvent change on the CD of LHRH and its deletion analogues indicate that the conformational features proposed for the C-terminal fragments of LHRH are conserved in the hormone itself, which is also considered to be an ensemble of conformers. In addition, the near-ultraviolet measurements point to a privileged conformational feature of the hormone (or one of its conformers) which perturbs the CD of aromatic residues, in particular Trp^3 as judged from inspection of the spectra of relevant peptides and model compounds. There are at least two possible configurations which could conceivably give rise to such an effect. First, in one of the theoretical low-energy conformations calculated for LHRH (conformer CC) by Momany (1976a,b), the His^2 ring shows nearly planar ring–ring stacking with the Trp^3 ring, at a distance of ~4.5Å. While this distance is too large for significant ring-current effects in NMR, it is short

[5] In the wavelength region 300 nm to about 260 nm, the spectrum of LHRH determined in a 90% dioxane–water mixture is quite similar to that obtained in water.

enough to affect the CD of the Trp. (Magnetic fields fall off approximately as $1/r^3$ and electric fields due to charged groups fall off approximately as $1/r$. His is partially protonated at pH 7.) The second possibility is that there is a significant population of folded backbone conformers in which the Arg^8 guanido group and Trp^3 are brought into proximity. A proximate guanido group would be expected to perturb the CD of Trp not only because of its charge per se but also via coupling of electronic transitions. Such a conformation is analogous to the folding of the C^5-nicotinamidium derivatives of [Nva^8]-, [Nva^8,D-Ala^6]-, and [Nva^8,L-Ala^6]LHRH (Donzel et al., 1977) mentioned early on. Our results give no indication of a β bend at the sequence of Tyr-Gly-Leu-Arg, but as stressed by Donzel et al. (1977) this type of bend is not the only possible mode of folding. The analysis of chain reversals in protein by Zimmerman & Scheraga (1977) indicates that, while a $4 \rightarrow 1$ hydrogen bond is a common feature of bends, such a hydrogen bond is not an important factor in stabilizing most bends.

Finally, it is instructive to summarize the proposed spectral contributions to the CD of LHRH under various experimental conditions. (1) The resultant of the far-ultraviolet, positive aromatic bands overlying the positive 218-nm and negative 231-nm bands associated with the C-terminal pentapeptide moiety, when superimposed on the tail of the strongly negative band centered at 196 nm, is the positive 229-nm band exhibited by LHRH in water at 27 °C. Raising the temperature to 65.5 °C decreases the intensity of both the positive 218-nm band and certain of the aromatic contributions, thereby partially unmasking the negative 231-nm band. Changing the solvent to a 90% dioxane–water mixture reveals the presence of the negative band largely because of its increased amplitude in the mixed solvent, although perturbation of aromatic bands, and perhaps other factors, also plays a role. (2) The negative 217-nm shoulder shown in water at 27 °C may be largely a reflection of the local maximum in the spectrum of the C-terminal pentapeptide moiety (see Figure 2C). Raising the temperature increases the intensity of the shoulder for two reasons: (a) intensification of the CD contribution of the C-terminal pentapeptide moiety (which overall is negative at elevated temperatures) without loss of a local maximum nor change in its position and (b) diminution of the amplitude of the His band. In other words, our results indicate that the growth of the shoulder with temperature is not to be interpreted in terms of a trend toward ordered structures as proposed by Marche et al. (1973) but rather to depopulation of conformers which show a positive band at 218 nm and to a change in the CD of His. Changing the solvent to a 90% dioxane–water mixture causes the appearance of a distinct negative band at 219 nm. This is attributed to (a) the intensified and slightly blue-shifted negative CD profile of the C-terminal pentapeptide moiety in this solvent (Figure 2A) superimposed on (b) the strongly diminished, negative CD of the aromatic-containing N-terminal moiety in the spectral region 220–110 nm (Figure 4C), both of which show a local maximum at about 216 nm. This assessment of the CD of LHRH finds support in the relatively successful simulation of its spectra under different experimental conditions (parts B and C of Figure 1).

Acknowledgments

The authors acknowledge the technical assistance of Robert O. Coombs, Esperanza Alvarez, and Virginia Sweeney.

References

Cann, J. R. (1972) *Biochemistry 11*, 2654.
Cann, J. R., Stewart, J. M., & Matsueda, G. R. (1973) *Biochemistry 12*, 3780.
Deslauriers, R., & Somorjai, R. L. (1976) *J. Am. Chem. Soc. 98*, 1931.
Deslauriers, R., Levy, G. C., McGregor, W. H., Sarantakis, D., & Smith, I. C. P. (1975) *Biochemistry 14*, 4335.
Deslauriers, R., Levy, G. C., McGregor, W. H., Sarantakis, D., & Smith, I. C. P. (1977) *Eur. J. Biochem. 75*, 343.
Donzel, B., Rivier, J., & Goodman, M. (1977) *Biochemistry 16*, 2611.
Mabrey, S., & Klotz, I. M. (1976) *Biochemistry 15*, 234.
Madison, V., & Schellman, J. (1970a) *Biopolymers 9*, 511.
Madison, V., & Schellman, J. (1970b) *Biopolymers 9*, 569.
Madison, V., Atreyi, M., Deber, C. M., & Blout, E. R. (1974) *J. Am. Chem. Soc. 96*, 6725.
Marche, P., Margat, J.-L., & Fromageot, P. (1973) *Eur. J. Biochem. 40*, 513.
Mattice, W. L. (1974) *Biopolymers 13*, 169.
Merrifield, R. B., Stewart, J. M., & Jernberg, N. (1966) *Anal. Chem. 38*, 1905.
Momany, F. A. (1976a) *J. Am. Chem. Soc. 98*, 2990.
Momany, F. A. (1976b) *J. Am. Chem. Soc. 98*, 2996.
Stewart, J. M., & Young, J. D. (1969) *Solid-Phase Peptide Synthesis*, W. H. Freeman, San Francisco.
Wessels, P. L., Feeney, J., Gregory, H., & Gormley, J. J. (1973) *J. Chem. Soc., Perkin Trans. 2*, 1691.
Woody, R. W. (1974) in *Peptides, Polypeptides, and Proteins* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) p 338, Wiley, New York.
Woody, R. W. (1977) *J. Polym. Sci., Macromol. Rev. 12*, 181.
Zimmerman, S. S., & Scheraga, H. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4126.