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On the Optical Activity of Ionized Tyrosyl Residues in Ovine Lutropin

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The effect of alkali on the circular dichroic (CD) spectra of ovine lutropin and its subunits has been studied. Mild alkaline pH induces the appearance of a new optically active band in the 250-nm region of the spectra of lutropin without any detectable alteration in the secondary structure of the protein. This change is reversible and can be correlated with ionization of 2–3 exposed tyrosyl residues in the intact hormone. In a previous report from this laboratory it was concluded that the three exposed tyrosyl residues are located in the α subunit, in positions 21, 92 and 93 [Burleigh, B. D., Liu, W.-K. and Ward, D. N. (1976) *J. Biol. Chem. 251*, 308–315]. Nitration of these residues lowers the pH at which the intensity of the 250-nm band is maximal.

The importance of the tyrosyl residues of lutropin α (as opposed to those of lutropin β) is also supported by the similarity of the effect of alkali on the CD spectra of lutropin and lutropin α. Further evidence for this involvement was also obtained by a comparison of the alkali-induced changes of refolded lutropin (α + β recombinant) and the product obtained by recombination of des-(92–96)-lutropin α (obtained from carboxypeptidase treatment of the α-subunit) and lutropin β. The results indicate that removal of tyrosines α92 and α93 results in a decrease of the intensity of the 235-nm band of ovine lutropin (at pH 7.5) as well as that of the 250-nm band observed under alkaline conditions. It is therefore concluded that the 250-nm band observed in alkaline solutions of lutropin arises (at least partially) from the red shift produced in the short-wavelength optically active band of tyrosines α21, α92, and α93 upon ionization.

Ovine lutropin is one of the glycoprotein hormones produced by the anterior pituitary gland. The hormone is composed of two non-identical subunits of similar size designated α and β. The primary structure of both subunits has been established [1–4]. Lutropin α contains five tyrosyl residues (in positions 21, 30, 41, 92 and 93) and five disulfides. Lutropin β contains two tyrosyl residues (in positions 37 and 59) as well as six disulfides.

The CD spectra of ovine lutropin and its subunits at neutral or acid pH have been studied in detail by several authors [5–10]. Analysis of the far-ultraviolet CD spectra revealed the absence of significant amounts of α-helicity in the native hormone or isolated subunits. A recent report on the contributions of different chromophores to the near-ultraviolet CD spectra of the intact homone shows significant tyrosyl contributions (mainly those from the α subunit) to the 230–240-nm region of the spectra and disulfide contributions (mainly those from the β subunit) to the region above 250 nm [10].

We have investigated the effects of tyrosine ionization on the near-ultraviolet CD spectra of lutropin and subunits in an effort to learn more about the contribution of these residues to the CD spectra of the homone and subunits.

Abbreviation. CD, circular dichroism.

Enzymes. Carboxypeptidase A (EC. 3.4.12.2); carboxypeptidase B (EC 3.4.12.3).

Trivial names. Tri-atyrosyl lutropin, a derivative of lutropin in which tyrosines α21, α92 and α93 have been nitrated by tetranitromethane so that they react to produce either an unidentified derivative or 3-nitrotyrosine; tris(aminotyrosyl) lutropin, lutropin in which tyrosines α21, α92 and α93 have been converted to 3-aminotyrosine; des-(92–96)-lutropin α, lutropin α in which the C-terminal pentapeptide 92–96 has been removed.

MATERIALS AND METHODS

Chemicals

Carboxypeptidase A (42.6 U/mg) and B (101 U/mg) were from the Worthington Biochemical Corporation (Freehold, New Jersey). *N*-α-Tosyl-L-lysyl-chloromethane and L-1-tosylamide-2-phenylethyl chloromethyl ketone were from the Sigma Chemical Company (St Louis, Missouri).

Hormones and Derivatives

Ovine lutropin, its subunits and oxytocin were prepared by procedures previously described [11–15]. The preparation and characterization of tri-atyrosyl-lutropin and nitro-oxytocin have been reported recently [16,17]. Tris(aminotyrosyl)lutropin was prepared by reduction of tri-atyrosyl lutropin according to the method of Sokolovsky *et al.* [18].

Des-(92–96)-lutropin α was prepared as follows: equal amounts of carboxypeptidase A and B (in 10% LiCl, 0.04 M sodium borate) were mixed with equal amounts of *N*-α-tosyl-L-lysyl-chloromethane and L-1-tosylamide-2-phenylethyl chloromethyl ketone (in methanol) to give a 0.6 ratio (by weight) of inhibitors to enzymes and incubated at 37 °C for 30 min. An aliquot of the enzyme/inhibitor mixture was then added to 15 mg of lutropin α (in 1.0 ml of 10% LiCl, 0.04 M sodium borate) to give an enzyme/substrate ratio of 1/15 (by weight). The mixture was then incubated at 37 °C for 2 h. At the end of the incubation an aliquot was saved for amino acid analysis, and the mixture was chromatographed on a 1 × 100-cm column of Sephadex G-100 equilibrated and eluted with 0.126 M NH_4HCO_3. The fractions containing the α subunit were pooled and lyophilized. The recovery was 85%. The extent of the carboxypeptidase reaction was determined by amino acid analysis of an aliquot of the unhydrolyzed reaction mixture. Recombination of subunits was performed and quantitated as previously described [13,19].

Bioassays

The assay of lutropin activity was based on the stimulation *in vitro* of testosterone production by collagenase-dispersed rat Leydig cells [20]. The assays were carried out as previously described [21,22] except that the cells were suspended in medium 199 instead of Krebs-Ringer bicarbonate buffer. Relative potencies and 95% confidence limits were calculated by parallel-line assays [23] and are expressed in terms of the reference preparation from the National Institutes of Health, NIH-LH-S19 (1 U/mg).

Spectroscopy

CD spectra were obtained using a Durrum-Jasco Model CD-SP dichrograph. Near-ultraviolet (330–230 nm) CD spectra were determined at 25 ± 0.5 °C using quartz cells of 1 cm pathlength, protein concentrations of 0.4–1.0 mg/ml, and scale settings of 2×10^{-5} or 5×10^{-5} differential dichroic absorbance (ΔA)/cm on the recorder chart. The near-ultraviolet CD spectra of lutropin β were recorded using a protein concentration of 0.2 mg/ml and a scale setting of 1×10^{-5} ΔA/cm. Far-ultraviolet (230–195 nm) spectra were recorded at room temperature (25–27 °C) using cells with pathlengths of 0.05 cm and 0.1 cm and protein concentrations of 0.1–0.2 mg/ml. The scale setting was 5×10^{-5} ΔA/cm. CD spectra are reported as mean residue ellipticity (in $deg \cdot cm^2 \cdot dmol^{-1}$) based on the polypeptide moiety only. The concentrations of lutropin or subunits were calculated from the absorption spectra (corrected for light scattering) and the reported absorption coefficients [7]. The absorption coefficients of des-(92–96)-lutropin α and des-(92–96)-lutropin α + lutropin β were calculated (based on tyrosine contributions) to be 4818 M^{-1} cm^{-1} and 8871 M^{-1} cm^{-1} respectively.

Absorption and difference spectra were obtained using a Cary 14 spectrophotometer. The ionization of tyrosyl residues was measured from the difference spectra (230–320 nm) of a solution of lutropin at a given pH and that of lutropin at pH 7.0. The spectra were recorded at room temperature, using quartz cells of 1.0 cm pathlength and lutropin concentrations of 1.0 mg/ml. The difference spectra showed extrema at 245 nm and 295 nm. The number of ionized tyrosines and their apparent p*K* values were calculated using the molar absorption coefficient changes and methods described by Tachinaba and Murachi [24].

All difference and CD spectra were recorded immediately after adding 1.0 ml of 100 mM buffer at the desired pH to 1.0 ml of the hormone or subunit solution in H_2O. The following buffers were used: pH 7.0–10.0, Tris-Cl; pH 10.5–11.5, triethylamine-Cl; pH 12.0–13.6, NaOH.

Other Methods

The fractional amounts of α-helix and β-structure in the different preparations of lutropin and subunits were calculated by a least-square fit of the mean-residue ellipticity at 3-nm intervals between 210 nm and 243 nm, using the reference spectra of Grosse *et al.* [25] with a computer program kindly provided by Dr L. A. Holladay (Vanderbilt University, Nashville, Tennessee).

RESULTS

Characterization of Hormone Derivatives

Table 1 shows the amino acids released upon carboxypeptidase digestion of lutropin α. The amino acids released are those expected from the known carboxyl-terminal sequence of lutropin α [1,3] with the exception of the release of arginine. The latter indicates some tryptic-like internal cleavage in spite of the use of trypsin inhibitors to treat the carboxypeptidase enzymes. The amino acids in Table 1 are presented in the order in which their release is expected according to the amino acid sequence.

Table 1. *Amino acids released from ovine lutropin α by hydrolysis with carboxypeptidases A and B*
Results are corrected for 12% moisture content

Amino acid	Amount released in preparation	
	1	2
	mol/mol lutropin α	
Serine	0.91	0.46[b]
Lysine	0.63[a]	0.88[a]
Histidine	0.85[a]	0.71[a]
Tryosine	1.98	1.87
Arginine	0.51	0.14

[a] These values are probably low estimates due to a baseline anomaly in the lysine-histidine area of the chromatogram.

[b] The low serine value in this preparation is probably due to the C-terminal heterogeneity of some lutropin α preparations [16].

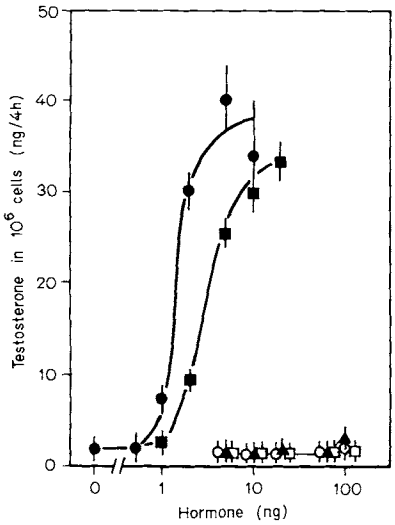

Fig. 1. *Dose-response curves for lutropin (●), tri-atyrosyl lutropin (○), tris(aminotyrosyl)lutropin (□), lutropin α + lutropin β recombinant (■) and des-(92–96)-lutropin α + β recombinant (▲).* Each point represents the average of two different samples. The bars extend to the individual values of these samples

As previously shown by Cheng *et al.* [8] it was found that removal of the C-terminal pentapeptide of lutropin α does not affect its ability to recombine with lutropin β. The yield of recombination of lutropin α + lutropin β and des-(92–96)-lutropin α + lutropin β was 76% in both cases.

In a previous report from this laboratory it was shown that in tri-atyrosyl lutropin tyrosines α21, α92 and α93 are nitrated in such a way that they react to produce either an unidentified derivative or 3-nitrotyrosine [16]. This derivative was reduced with sodium hydrosulfite to yield tris(aminotyrosyl)lutropin. Amino acid analysis showed that the reduction was complete, as judged by the absence of nitrotyrosine.

The ability of these derivatives to stimulate testosterone production in collagenase-dispersed rat Leydig cells is shown in Fig. 1. Only lutropin and the α + β recombinant produced a response over the range of hormone concentrations tested. The calculated potencies of native lutropin and the α + β recombinant are 2.31 (1.77–3.03) U/mg and 0.83 (0.64–1.02) U/mg respectively. The potencies of the other three derivatives cannot be calculated from the data shown. It is safe to conclude, however, that they retain less than 2% of the biological activity of the native hormone.

These results confirm previous reports from this [17] and other laboratories [8] on the importance of tyrosines α21, α92 and α93 (especially the last two) on the expression of the biological activity of lutropin.

CD of Lutropin and Derivatives in Alkaline Solutions

Fig. 2 shows the near and far-ultraviolet CD spectra of lutropin at different alkaline pH values. It can be seen that when the pH is raised above 7.5, a new band, centered at 248–250 nm, appears, and the band centered at 235 nm vanishes. The negative band centered at 280 nm is red-shifted by 5–10 nm, and its intensity is slightly reduced. The left panel in Fig. 2 shows that these changes are not accompanied by any significant changes in the secondary structure of the hormone. The effects of alkali on the near-ultraviolet CD spectra of lutropin are similar to those reported for ribonuclease A [26] and seem to arise from the absorption bands of ionized tyrosyl residues.

The changes in the 250-nm band as a function of pH, as well as the ionization of tyrosyl residues of ovine lutropin, are shown in Fig. 3. The ellipticity at 250 nm shows a marked increase between pH 8 and pH 10, followed by a plateau between pH 10.5 and pH 12, and a sharp decrease above pH 12. The midpoint of the first transition occurs at pH 9. The pH-dependent changes observed up to pH 11 occurred immediately after addition of buffer, did not change with time and were totally reversible. Above pH 12, however, the pH-dependent changes were time dependent and only partially reversible. At these pH values both the 250-nm and 280-nm bands decreased in intensity as the time of exposure increased, and the far-ultraviolet spectra became less negative. These results are probably due to the dissociation of the hormone observed at pH 11–12 [27]. The spectrophotometric titration of the tyrosyl residues of ovine lutropin is also shown in Fig. 3. It can be seen that the increase in ellipticity at 250 nm correlates well with the ionization of 2–3 exposed tyrosyl residues. Analysis of the titration data indicates the presence of 3.4 exposed tyrosyl residues with an apparent pK of 9.9 and 3 buried residues with an apparent pK of 11.8.

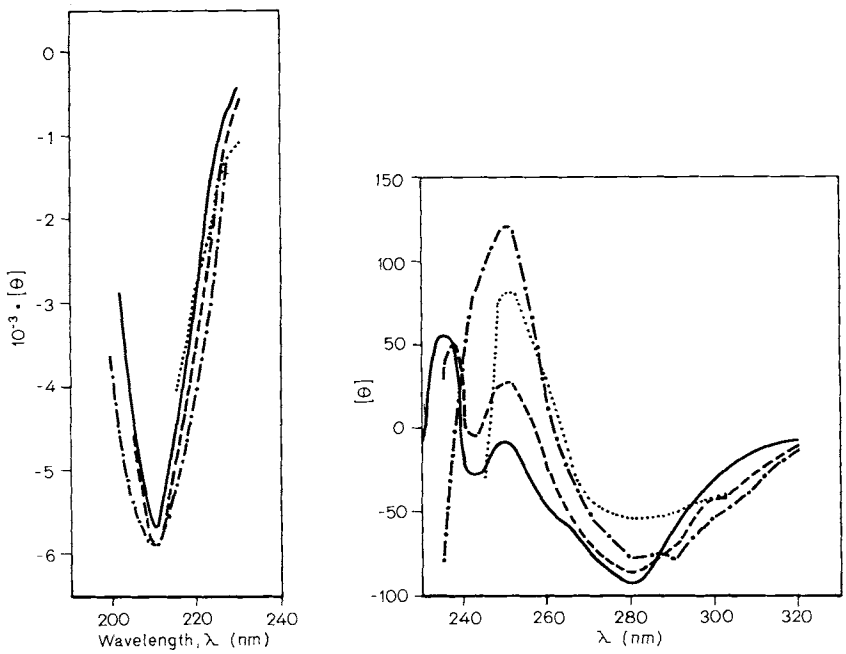

Fig. 2. *Effect of alkaline pH on the CD spectra of ovine lutropin.* (— — —) 50 mM Tris-Cl, pH 7.5; (- - - -) 50 mM Tris-Cl, pH 8.5; (-·-·) 50 mM Tris-Cl, pH 9.5; (·····) 50 mM NaOH, pH 12.8

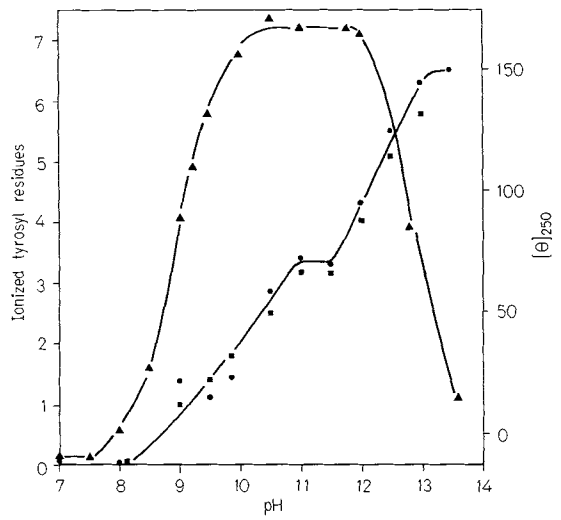

Fig. 3. *Spectrophotometric titration of the tyrosyl residues of ovine lutropin.* The number of ionized tyrosyl residues was determined from ΔA_{245} (■) or ΔA_{295} (●) as described in Materials and Methods. The value of $[\theta]_{250}$ (▲) as a function of pH is also shown

These results are in good agreement with the titration data and thermal perturbation studies of Garnier *et al.* [28].

Based on the reactivity of the tyrosyl residues to tetranitromethane, it was concluded in a previous report from this laboratory [16] that the three exposed tyrosyl residues of ovine lutropin are α21, α92 and α93. The data shown in Fig. 2 and 3, suggest that the 250-nm band observed in the CD spectra of alkaline solutions of ovine lutropin is due mainly to the 245-nm absorption band resulting from the ionization of these residues.

It is well established that the phenolic group of nitrotyrosine has a lower pK than that of tyrosine, and that reduction of the nitro group returns this pK toward normal values [18]. If the 250-nm band arises from the ionization of the exposed tyrosyl residues, the magnitude of this band, at neutral pH should be higher in tri-atyrosyl lutropin (in which tyrosines α21, α92 and α93 are nitrated) than in native lutropin. Moreover, the magnitude of the 250-nm band should return to normal upon reduction of the nitrotyrosyl residues to aminotyrosine. The data shown in Fig. 4 indicate that this is the case.

CD spectra (not shown) obtained at pH 9.5 showed that the intensity of the 250-nm band of tri-atyrosyl lutropin is 1.4 times greater than that of lutropin. This result is consistent with the relative degree of ionization of nitrotyrosyl and tyrosyl residues at this pH.

In order to make this conclusion, however, it is necessary to know if the ionized nitrotyrosyl residues are optically active in this region of the spectra. This was shown by comparing the CD spectra of oxytocin (a disulfide-containing cyclic nonapeptide with a single tyrosyl residue in position 2) and its fully nitrated derivative at neutral and alkaline pH.

The results shown in Fig. 5 A show the expected [29] red shift of the 228-nm tyrosyl band produced by the ionization of the tyrosyl residue. The data shown in Fig. 5 B show that the ionized nitrotyrosyl residue is also optically active in the 240—250-nm region of the

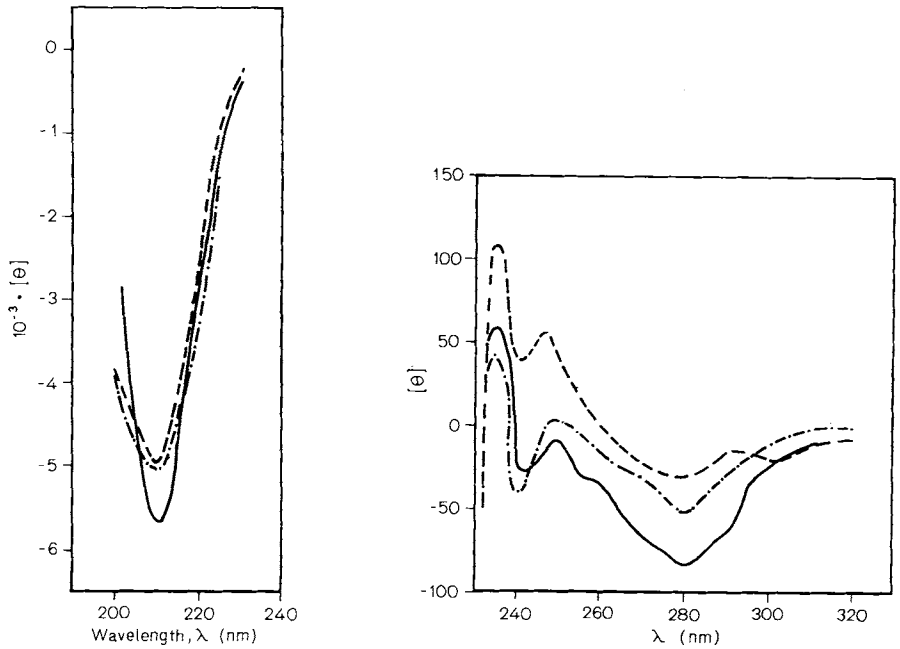

Fig. 4. *CD spectra of native lutropin (———), tri-atyrosyl lutropin (– – – –), and tris(aminotyrosyl)lutropin (–·–·) in 50 mM Tris-Cl, pH 7.5*

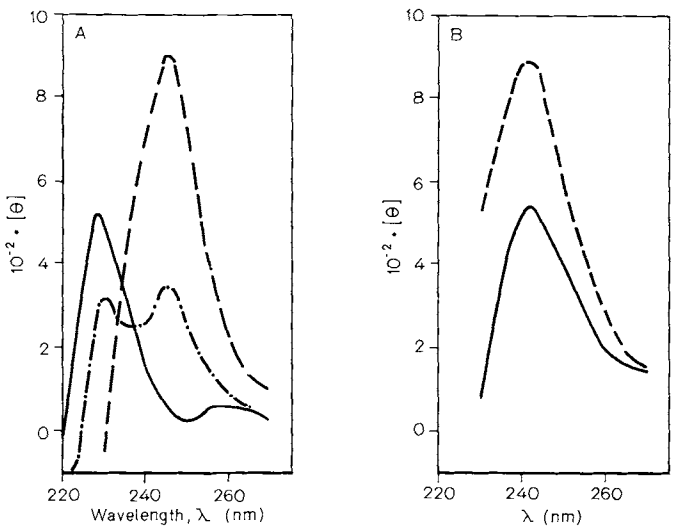

Fig. 5. *Effect of alkaline pH on the near-ultraviolet CD spectra of oxytocin and nitro-oxytocin.* (A) Oxytocin in 50 mM Tris-Cl, pH 7.5 (———); 50 mM Tris-Cl, pH 9.5 (–·–·); 50 mM NaOH, pH 12 (– – – –). (B) Nitro-oxytocin in 50 mM Tris-Cl, pH 7.5 (— — —); 50 mM Tris-Cl, pH 10.0 (– – – –)

spectra. Due to the lower pK of nitrotyrosine [18] the intensity of the 245-nm band of nitro-oxytocin is maximal at pH 10. That of oxytocin reaches a maximum at pH 12.

CD of Lutropin Subunits and Derivatives in Alkaline Solutions

Fig. 6 shows the effect of alkali on the CD spectra of lutropin α and des-(92–96)-lutropin α. The CD spectra of des-(92–96)-lutropin α confirms the data of Cheng *et al.* [8], who showed that removal of the C-terminal pentapeptide of bovine thyrotropin α (whose sequence is identical to that of ovine lutropin α) produces a 50–60% decrease in the intensity of the 232-nm band and has no effect on the ellipticity above 240 nm. The data also show that the effect of pH on the CD spectra of this subunit is qualitatively similar to that on the spectra of native lutropin. The pH values at which the intensity of the 250-nm band is maximal, however, are shifted compared to those of native lutropin. This finding correlates well with the expected

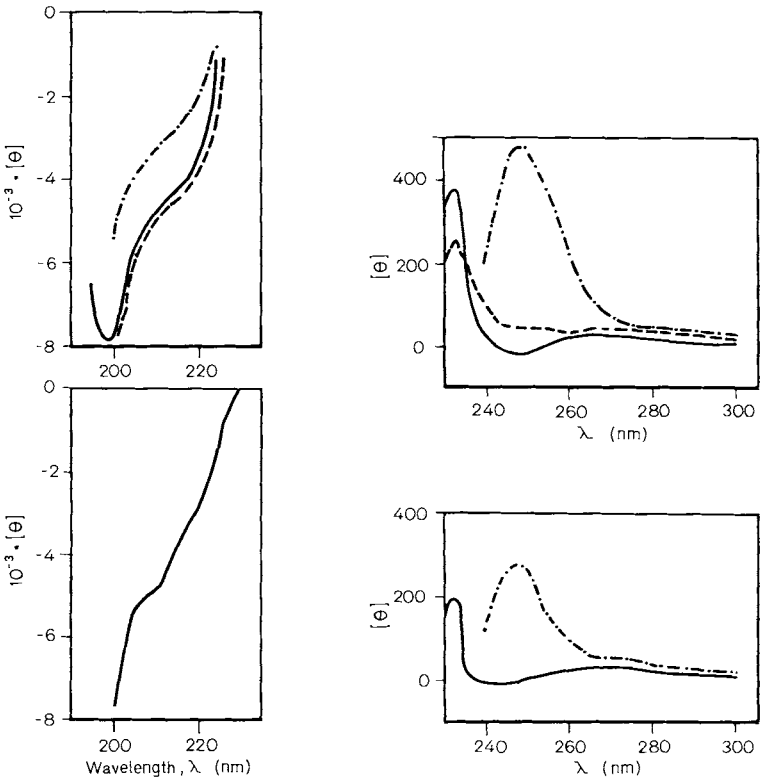

Fig. 6. *CD spectra of lutropin α (upper panels) and des-(92–96)-lutropin α (lower panels).* (— – –) 50 mM Tris-Cl, pH 7.5; (– – – –) 50 mM Tris-Cl, pH 9.5; (–·–·) 50 mM NaOH, pH 12.8

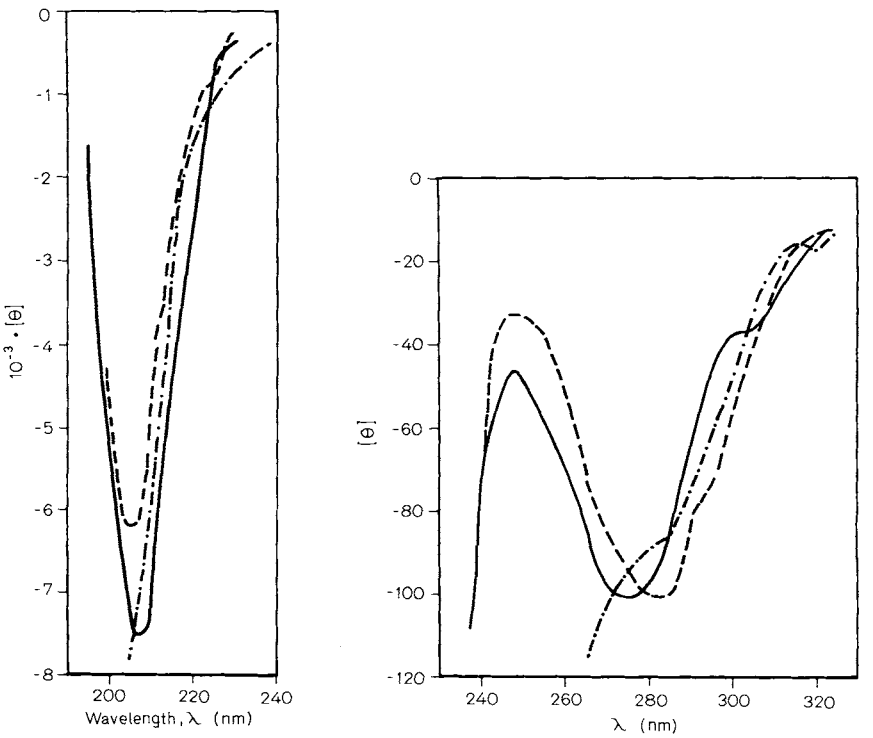

Fig. 7. *CD spectra of lutropin β.* (———) 50 mM Tris-Cl, pH 7.5; (– – – –) 50 mM Tris-Cl, pH 9.5; (–·–·) 50 mM NaOH, pH 12.8

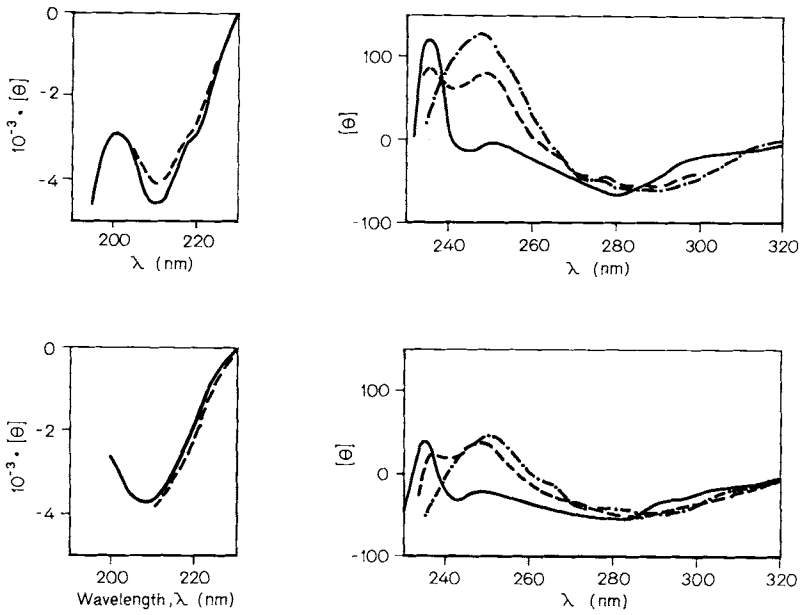

Fig. 8. *CD spectra of the lutropin α + lutropin β recombinant (upper panels) and the des-(92–96)-lutropin β recombinant (lower panels).* (——·——) 50 mM Tris-Cl, pH 7.5; (– – – –) 50 mM Tris-Cl, pH 9.5; (–·–·–) 50 mM triethylamine-Cl, pH 11.6

higher apparent p*K* of the tyrosyl residues of lutropin α [28]. The intensity of the 250-nm band (at pH 12.8) of des-(92–96)-lutropin α is about 60% of that of lutropin α. This finding supports our conclusion that the ionization of tyrosines α92 and α93 is partially responsible for the appearance of the 250-nm band observed at alkaline pH.

The CD spectrum of alkaline solutions of lutropin β is shown in Fig. 7. Since lutropin β does not have positive ellipticity in the near ultraviolet, the results are very different from those obtained for native lutropin or lutropin α. At pH 9.5 a red shift is produced in the 280-nm band, and at higher pH values the ellipticity below 270 becomes more negative.

CD of Refolded Lutropin and Derivatives

Fig. 8 shows the CD spectra of the recombined lutropin and the product obtained by recombination of des-(92–96)-lutropin α and lutropin β. A comparison of the CD spectra of these two products reveals that while the intensity of the 280-nm band of the des-(92–96)-lutropin α + lutropin β recombinant is about 90% of that of the α + β recombinant, the intensity of the 235-nm band is only about 34%. The effect of alkali on both recombinants is qualitatively similar. The ratios of the intensity of the 250-nm band at pH 9.5 and 11.6 of the des-(92–96)-lutropin α + lutropin β recombinant to that of the lutropin α + lutropin β recombinant are 0.66 and 0.35 respectively. These results confirm the involvement of the ionization of tyrosines α92 and α93 in the appearance of the 250-nm band in alkaline solutions of native lutropin.

Table 2. *Estimated content of secondary structure of ovine lutropin, subunits and derivatives*
All samples in 50 mM Tris-Cl, pH 7.5. f_α, f_β are the fractional contents of α-helix and β-structure. See Materials and Methods for details. The average deviation records the differences between the calculated and experimental spectra at 3-nm intervals from 210 nm to 243 nm

Sample	f_α	f_β	Average deviation
			$deg \cdot cm^2 \cdot dmol^{-1}$
Lutropin	0.04	0.31	452
Tri-atyrosyl lutropin	0.05	0.29	396
Tris(aminotyrosyl)-lutropin	0.05	0.29	233
Lutropin α	0.04	0.31	293
Des-(92–96)-lutropin α	0.06	0.27	188
Lutropin β	0.03	0.32	450
α + β	0.05	0.28	237
Des-(92–96)-lutropin α + β	0.06	0.24	251

Far-Ultraviolet CD Spectra of Lutropin, Derivatives and Subunits

The effects of mild alkali on the far-ultraviolet CD spectra of lutropin, derivatives and subunits were minor or absent (Fig. 2, 4–7), indicating that the changes observed in the near-ultraviolet CD spectra occur without significant changes in the main chain conformation of these compounds. Table 2 summarizes the far-ultraviolet CD data of the lutropin derivatives used. The estimated fractional amounts of α-helix and β-structure are very similar or identical for

native lutropin and its two derivatives, as well as the $\alpha + \beta$ recombinant. The only difference detected (although minor) is between des-(92–96)-lutropin α and lutropin α, as well as between the recombinant obtained using this derivative or the 'native subunits'. In both cases the amount of β-structure seems to be lower for the subunit derivative. The estimated values of f_α and f_β (the fractional contents of α-helix and β-structure) shown in Table 2 are in good agreement with those of Garnier *et al.* [28], but higher (especially f_β for the subunits) than those of Holladay and Puett [10], who recorded the spectra in the presence of 6% dioxane. Calculations of f_α and f_β using the reference spectra of Chen *et al.* [30] gave essentially the same results. The uncertainties in the estimation of fractional amounts of secondary structure in such non-helical proteins as lutropin appear to be so large that the f_β values reported in Table 2 have only a relative significance, indicating approximately the same low content of ordered structure in the various preparations used.

DISCUSSION

The effects of alkali on the near-ultraviolet CD spectra of ovine lutropin seem to arise entirely from the ionization of tyrosyl residues, mainly those that are exposed to the solvent (*i.e.*, tyrosines α21, α92 and α93).

Our laboratory reported [31] that phospholipid induced a strong CD effect for lutropin in the 240–250-nm region at pH 9–9.9. The present studies, in part, were planned to extend these observations to interactions with isolated hormone receptors. It soon became apparent that the effects previously described [31] could be ascribed to a pH effect rather than a phospholipid effect, and that we had been misled by an operational artefact in the earlier studies. This resulted from the use of a volatile base in the pH adjustment of our control samples in that study, which introduced an undetected downward shift of approximately 0.5 pH unit prior to measurement of the CD spectra. Thus, we wish herewith to set the record straight with respect to the earlier study.

Pflumm and Beychok [26] have reported that the 225-nm band of *N*-acetyltyrosineamide is red shifted (to 240 nm) upon ionization of the phenolic group. The intensity of the 240-nm tyrosinate band is essentially the same as that of the 225-nm tyrosine band. Similar results have been obtained with Gly-Tyr-Gly and cystinyl-bis-tyrosine (Ascoli, Ward and Jirgensons, unpublished). Ionization also produces a red shift in the long-wavelength band of tyrosine-containing compounds [32].

Ionization of the exposed tyrosyl residues of native lutropin results in the appearance of a strong band centered at 250 nm and a red shift in the 280 nm band. The appearance of the 250-nm band seems to arise mainly from the ionization of tyrosyl residues in the α subunit, and the red shift of the 280-nm band from the ionization of the tyrosyl residues in the β subunit.

It was shown in a previous report from this laboratory that isolated lutropin α has four exposed tyrosyl residues (in positions 21, 41, 92 and 93) and one buried (in position 30). The two tyrosyl residues in isolated lutropin β (tyrosines β37 and β59) are also exposed [16]. Several reports from other laboratories [7,10,28] have concluded, using different kinds of spectroscopy, that some tyrosyl residues become buried when the subunits associate to form the active homone. Burleigh *et al.* [16] have shown that in the native hormone, only tyrosines α21, α92, and α93 are exposed to the solvent; tyrosines α41, β37, and β59, which are exposed in the isolated subunits, are buried in native lutropin.

Some of these residues give rise to the 235-nm band of ovine lutropin, which seems to arise mainly from tyrosine transitions [10,28]. From the data of Cheng *et al.* [8], as well as those presented here, it is safe to conclude that tyrosines α92 and α93 contribute to this band. It has also been postulated that tyrosines α21 and α30 contribute to the 235-nm band [10,33]. Based on the data available for model compounds and those presented here, it is concluded that the 250-nm band observed in alkaline solutions of lutropin arises from the red shift of the 235-nm band produced by ionization of the exposed tyrosyl residues. It is not known if the 235-nm band disappears completely upon ionization of the three exposed tyrosyl residues, we can only say that its intensity is greatly reduced. Our results, however, support the involvement of tyrosines α21, α92, and α93 in generating optical activity in this region of the CD spectra of ovine lutropin.

The intensity of the tyrosinate band in this hormone is much greater than that expected from model compounds. This could result from one or more of the following mechanisms: (a) summation of the tyrosinate band with the short-wavelength band of the disulfides which, according to the resolved spectra of Holladay and Puett [10], is positive and centered at 250 nm; (b) alteration in the orientation of the tyrosyl residues and/or alteration of the optical activity of the disulfide bond adjacent to tyrosine α92; and (c) enhancement of the intensity of the optical activity of tyrosinate α21 by the presence of phenylalanine α22. It is worth noting that the first two mechanisms could also explain the increased intensity of the tyrosinate band of oxytocin. The results presented might be helpful in understanding the contribution of specific tyrosyl residues to the 235-nm band of ovine lutropin. First, it is worth noting that we have confirmed the observations of Garnier *et al.* [28] on the variability of the intensity of this band. We have observed that

the intensity of the 235-nm band ranges from 40 -135 deg·cm^2·dmol^{-1} in different lutropin preparations with similar (or identical) biological potencies. The intensity of this band (in native lutropin and lutropin α) is also increased by lowering the temperature or in the presence of guanidine (D. Puett and L. A. Holladay, personal communication) or urea [6]. The increase in intensity of the 232-235-nm extremum of lutropin α produced by denaturants does not seem to be accompanied by a decrease in the amount of secondary structure of the subunit, but it correlates with the exposure of tyrosine 30, which is buried in the 'native subunit' [16]. Therefore, it seems that this band has contributions from exposed (*i.e.*, tyrosines α21, α92, and α93) as well as buried (*i.e.*, tyrosine α30) tyrosyl residues.

It was previously pointed out [28] that it is in this region of the spectra where the only difference between the native hormone and the $\alpha + \beta$ recombinant is observed. We have confirmed this observation (compare Fig. 1 and 8). Since some of the tyrosyl residues that are very important in the expression of the biological activity of lutropin seem to contribute to this band (*i.e.*, tyrosines α92 and α93), it is quite possible that the failure to recover 100% of the biological activity of the native hormone upon recombination of the isolated subunits is due (at least partially) to the incorrect orientation of these residues.

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