Selective Modification of Tryptophan-149 in Ovine Pituitary Lactogenic Hormone†

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ABSTRACT: The amino acid sequence of ovine pituitary lactogenic hormone contains two tryptophan residues at positions 90 and 149. One of these two residues was selectively modified by reaction with *o*-nitrophenylsulfenyl chloride in sodium acetate buffer (pH 3.5–4.0). The derivative was digested with trypsin and a single NPS-chromophore containing peptide was isolated by exclusion chromatography and low voltage electrophoresis. The amino acid analysis of this peptide established the modified residue as Trp-149. In contrast, when 50% acetic acid was used as the reaction medium, both Trp-149 and Trp-90 were modified. Furthermore, while the singly modified derivative could be obtained in good yield as a monomer, the doubly modified derivative appeared only as a dimer of the hormone. Both derivatives have been characterized by exclusion chromatography, fluorescence, absorption, and circular dichroism spectra, relative rates of digestion by trypsin, and biological activity. In addition to the remarkable change in its quaternary structure, the derivative in which both tryptophan residues had been modified showed evidence of other conformational changes from the native hormone and was found to be completely devoid of biological activity. Behavior on exclusion chromatography, circular dichroism spectra, and tryptic digestion rates indicated that there were no extensive conformational changes between the native protein, the singly modified derivative, and the controls prepared in the same solvents that had been used as reaction media. However, despite their apparent conformational equivalence to the native hormone, the singly modified derivative and the control preparations all showed somewhat diminished lactogenic potency, although none of them was totally devoid of this activity. The significance of the differing chemical reactivities of Trp-149 and Trp-90 and its relationship to the maintenance or loss of biological activity are discussed.

The isolation of highly purified O-LTH,[1] secreted by the ovine pituitary gland, has been previously described (Cole and Li, 1955) and the primary structure of the protein has been reported (Li *et al.*, 1970). The molecule consists of a single polypeptide chain of 198 amino acid residues (mol wt 22,550) containing three disulfide bridges. The two tryptophan residues in this protein occupy positions 90 and 149 in the amino acid sequence. A recent comparison (Bewley and Li, 1971b) of the primary structure of O-LTH with the primary structures of two other proteins which exhibit lactogenic activity (HGH, secreted by the human pituitary gland, and HCS, secreted by the human trophoblast) indicates that Trp-90 in the O-LTH sequence is closely homologous with the single tryptophan residue found in both HGH and HCS. The tryptophan in HGH and HCS has now been shown to occur at position 86 in these sequences (Li, 1972; Li *et al.*, 1972). In contrast, Trp-149 in the O-LTH structure has no homologous counterpart in the sequences of the two human hormones.

We have previously described (Bewley *et al.*, 1972) the use of the tryptophan-specific reagent *o*-nitrophenylsulfenyl chloride (Scoffone *et al.*, 1968) in probing the chemical reactivity of Trp-86 in HGH and HCS. In both human proteins, selective and quantitative modification of the tryptophan residue could be produced in 50% acetic acid. However, no covalent modification of either protein could be achieved in 0.1 M sodium acetate (pH 4.0). These results were shown to be in agreement with evidence from circular dichroism (CD) spectra and perturbation spectra. These spectroscopic studies (Bewley and Li, 1971a, 1972; Bewley *et al.*, 1972) indicated that until the conformation of either protein is perturbed significantly from that of the native molecule, the tryptophan residues will remain largely unexposed to the external solvent. However, in 50% acetic acid, a significant structural transition does occur, resulting in the reversible exposure of these residues. No gross conformational change was observed in either NPS-HGH or NPS-HCS when compared with the native hormones. While NPS-HGH retained full potency as a growth promoting and lactogenic agent, NPS-HCS was completely devoid of lactogenic activity. We have proposed that this difference in biological activity is related to small conformational differences noted when comparing the CD spectra of the two derivatives (Bewley *et al.*, 1972). These spectra suggest that incomplete refolding of NPS-HCS leaves its modified tryptophan residue in a more exposed and/or less sterically hindered environment than is true of NPS-HGH. The retention of full biological activity in NPS-HGH proves that absolute molecular integrity of the tryptophan residue itself is not required. However, the placement of Trp-86 (or any modified form of it) into its proper place *within* these folded polypeptides may be crucial, if only to allow the proper placement of other, more intrinsically necessary surface residues. In order to further investigate the possibility of such a structure–function relationship we have now turned our attention to the closely related lactogenic hormone isolated from ovine pituitaries.

† From the Hormone Research Laboratory, University of California, San Francisco, California 94122. *Received January 11, 1973.* This work was supported in part by the American Cancer Society, the Geffen Foundation, and the Allen Foundation; paper XXXIII in the Pituitary Lactogenic Hormone series.

[1] Abbreviations used are: O-LTH, ovine pituitary lactogenic hormone; NPS-LTH, a nitrophenylsulfenyl derivative of O-LTH which has not been defined as to whether it is monosubstituted or disubstituted: 1·NPS-LTH, $(NPS\text{-}Trp)^{149}$-O-LTH (monosubstituted); 2·NPS-LTH, $(NPS\text{-}Trp)^{149}$-$(NPS\text{-}Trp)^{90}$-O-LTH (disubstituted); NPS-Cl, *o*-nitrophenylsulfenyl chloride; HGH, human pituitary growth hormone; HCS, human chorionic somatomammotropin; NPS-HGH and NPS-HCS, the *o*-nitrophenylsulfenyl derivatives of HGH and HCS; V_e/V_0 the ratio of elution volume to void volume in exclusion chromatography.

Materials and Methods

Ovine pituitary lactogenic hormone was prepared as previously described (Cole and Li, 1955). The monomeric form of the protein was obtained by exclusion chromatography on Sephadex G-100 in 0.1 M Tris-HCl buffer (pH 8.2) as described by Bewley and Li (1971a). Performic acid oxidized O-LTH was prepared by the method of Li (1957). The *o*-nitrophenylsulfenyl chloride (Eastman Organic Chemicals, Rochester, N. Y.) was recrystallized from chloroform–ether (mp 73°). Trypsin was obtained from Calbiochem. All other chemicals were of analytical grade and used without further purification.

Preparation of NPS Derivatives. Several different solvents were used to test the reaction of O-LTH with NPS-Cl.

ACETIC ACID (50%). NPS-Cl (100 μmol) in 8.0 ml of glacial acetic acid was added to a stirred solution of 111 mg (5 μmol) of O-LTH monomer in 16 ml of 25% acetic acid. The temperature was maintained at 20° by an external thermostated bath. After 1 hr, the protein was separated from the excess reagent by exclusion chromatography on Sephadex G-25 in 20% acetic acid. The protein fraction was dialyzed overnight against sufficient deionized H_2O to provide an equilibrium concentration of 0.1 N acetic acid. The protein in 0.1 N acetic acid was then further dialyzed against a large volume of 0.1 M Tris-HCl buffer (pH 8.2), concentrated by ultrafiltration (Amicon Corp., Lexington Mass., PM-10 membrane), and fractionated by exclusion chromatography on Sephadex G-100 in 0.1 M Tris-HCl buffer (pH 8.2). The dimer fraction was rechromatographed on Sephadex G-200 in the cold (4°) using the same Tris buffer. The purified dimer was either used directly or recovered by lyophilization after thorough dialysis against 0.01 M NH_4HCO_3 buffer (pH 8.4). A control was prepared by treating O-LTH monomer with 50% acetic acid in the absence of NPS-Cl, followed by purification on Sephadex G-100 as described above.

SODIUM ACETATE (0.1, 1, and 2 M). Samples of O-LTH monomer (1 μmol each) were dissolved in the following solvents: 7 ml of 0.1 N acetic acid and 3 ml each of 1 and 2 N acetic acid. The protein solutions were titrated with concentrated NaOH to pH 4.0 in the case of the 0.1 N solution, and to pH 3.5 for the 1 and 2 N solutions. Ten milligrams (50 μmol) of finely powdered NPS-Cl was added and the reaction was allowed to proceed for 1 hr at 20° with stirring. The pH of each solution was maintained by the addition of dilute NaOH from a pH-Stat (Radiometer, Model TTT 11b). The excess insoluble reagent was removed by centrifugation and the supernatants were submitted to exclusion chromatography on Sephadex G-25, eluting with the corresponding concentration of acetic acid. Each preparation was dialyzed overnight against sufficient deionized H_2O to provide an equilibrium concentration of 0.1 N acetic acid. Following extensive dialysis against 0.1 M Tris-HCl buffer (pH 8.2) the solutions were submitted to exclusion chromatography on Sephadex G-100 in the Tris buffer. The purified monomer fractions were either used directly or recovered by lyophilization. A control sample was prepared by treatment of the native monomer with 0.1 M sodium acetate (pH 4.0) in the absence of NPS-Cl, followed by the purification procedure described above.

Protein Concentration. Unless otherwise stated, protein concentrations in all solutions were determined spectrophotometrically. Spectra of the unmodified protein were taken against an appropriate reference, from 360 to 260 nm on a Beckman DK-2A spectrophotometer. Corrections for light scattering were made as described by Beaven and Holiday (1952). Concentrations of the native protein were calculated using the value $E^{1\%}_{1\,cm,\,277\,nm} = 9.09$ (Li *et al.*, 1970). For the NPS derivatives, spectra were taken from 500 to 260 nm. Corrected absorptivities of $E^{1\%}_{1\,cm,\,280\,nm} = 13.3$ for the 1·NPS-LTH and $E^{1\%}_{1\,cm,\,280\,nm} = 17.4$ for the 2·NPS-LTH were computed as described by Scoffone *et al.* (1968). In these calculations the molecular weights of the native and NPS proteins were taken as 22,550 for the native, 22,700 for the monosubstituted, and 22,860 for the disubstituted NPS derivatives.

Estimation of the Extent of NPS Reaction. The amount of modification in each NPS derivative was determined spectrophotometrically from solutions in 80% acetic acid, 50% acetic acid, and 0.1 M Tris-HCl buffer (pH 8.2). Protein concentrations were determined as described above, while the amount of NPS chromophore was determined from the peak OD at or near 365 nm, assuming a molar extinction coefficient of 4000 (Scoffone *et al.*, 1968). Fluorescence spectra of the purified derivatives were also used to detect small amounts of unreacted tryptophan (Parikh and Omenn, 1971).

Procedures for Characterization. Difference absorption spectra of unmodified O-LTH were taken on a Beckman DK-2A spectrophotometer using a tandem arrangement of four matched silica cuvets. The procedures for sample preparation have been previously described (Bewley *et al.*, 1972).

Fluorescence measurements were carried out on a Hitachi Perkin-Elmer spectrophotometer, Model MPF-2A. Emission spectra were taken with the excitation monochrometer at 285 nm. The emission spectrum of each tube from the final purification on Sephadex was taken without dilution. All fractions were further tested for the presence of unreacted tryptophan by taking the emission spectrum of an aliquot in which the protein disulfide bonds had been reduced by the addition of dithiothreitol.[2]

Circular dichroism measurements were performed on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment. Details of all procedures and methods of calculation have been described previously (Bewley *et al.*, 1969).

Amino acid analyses were performed according to the method of Spackman *et al.* (1958) on a Beckman amino acid analyzer (Model 120-C) following hydrolysis in constant boiling HCl for 22 hr at 110° *in vacuo*.

Lactogenic activity was measured by the local crop-sac assay in the pigeon. The response was determined both as described by Lyons (1937) and by the quantitative method of Nicoll (1967).

Isolation of NPS-(Trp-149) Peptide. A sample of 1·NPS-LTH (24 mg), which had been prepared in 1 M sodium acetate as described under Methods, was dissolved in 6 ml of 0.2 M ammonium acetate buffer (pH 8.5) and digested with trypsin at 37° for 48 hr. The enzyme was added in several aliquots to a final enzyme–substrate ratio of 1:25. The digestion mixture was recovered by lyophilization, redissolved in 1 N NH_4OH, and submitted to exclusion chromatography on a Sephadex G-25 column (2 × 43 cm) using 0.1 N NH_4OH as eluent. The major fraction which showed absorption at 365 nm was lyophilized and resubmitted to exclusion chromatography on a Sephadex G-50 column (2 × 100 cm), again eluting with 0.1 N NH_4OH. Again the major yellow fraction was lyophilized and

[2] O-LTH undergoes a significant denaturation when its disulfide bonds are reduced with dithiothreitol, even in the absence of an external denaturing agent. This denaturation appears to be accompanied by the exposure of both tryptophan residues to the external solvent (Bewley, T. A., and Li, C. H., unpublished results).

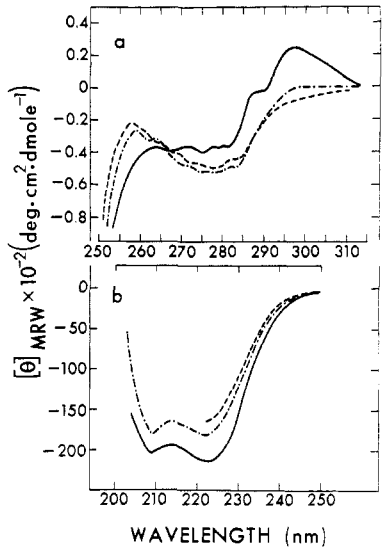

FIGURE 1: Circular dichroism spectra in the region of side-chain absorption (a) and amide bond absorption (b) of O-LTH in: 0.1 M sodium acetate, pH 4.0 (— - - —); 1.0 M sodium acetate, pH 3.5 (- - -); and 0.1 M Tris-Cl buffer, pH 8.2 (—). The ordinate is calculated as mean residue molecular ellipticity (θ_{MRW}).

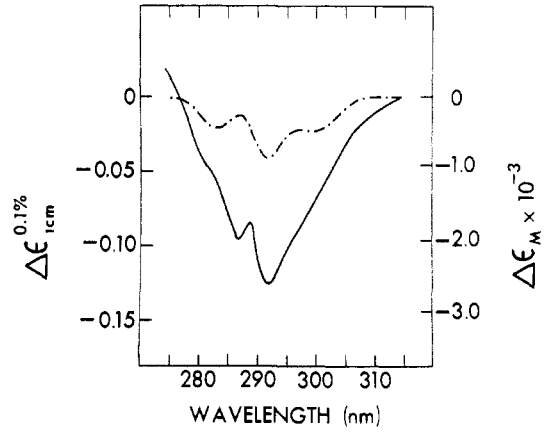

FIGURE 2: Difference absorption spectra of O-LTH in: 0.1 N acetic acid, pH 3.6 (— - - —), and 50% acetic acid (—). In both spectra the reference protein is in 0.1 M Tris-Cl buffer, pH 8.2.

then further purified by electrophoresis on paper (Whatman No. 3MM) at a potential gradient of 8 V/cm in 0.5 M pyridine–acetate buffer (pH 3.7) for 3 hr. The yellow band was cut out and eluted from the paper with 20% acetic acid and recovered by lyophilization.

Rate of Tryptic Digestion. The rate of short-term tryptic digestions of native O-LTH, performic acid oxidized O-LTH, the NPS derivatives, and control preparations were followed at pH 8.5 by procedures previously described (Bewley *et al.*, 1972). The concentration of the performic acid oxidized hormone was based on the weight of material used, assuming a 20% moisture content. In all solutions, protein concentrations were adjusted to 25 nmol/ml and the enzyme to substrate ratio was 1:250.

Results

Circular Dichroism and Difference Absorption Spectra of Native O-LTH in Acidic Media. The CD spectra of O-LTH in 0.1 M Tris-HCl buffer (pH 8.2) and 50% acetic acid have been previously described (Bewley and Li, 1972). Spectra of O-LTH in 0.1 M sodium acetate (pH 4.0) and in 1 M sodium acetate (pH 3.5) are presented in Figure 1. Only minor differences can be seen between the two spectra in the acetate media. In both solvents the asymmetric positive band at 298 nm, seen in the spectrum of the native protein in 0.1 M Tris-HCl buffer (pH 8.2), is entirely absent. This positive band (or set of bands) has been previously assigned to the ^1L_a and ^1L_b transitions of the tryptophan residues in O-LTH (Bewley and Li, 1972). In the region of amide bond absorption the CD spectra in the acetate buffers show slightly diminished intensities from that described previously for the protein in the Tris buffer (Bewley and Li, 1972). Due to excessive optical absorption by the solvent it was not possible to get reliable data below 225 nm in the 1 M acetate buffer.

Difference absorption spectra of O-LTH in 0.1 N and 50% acetic acid, taken against the protein in 0.1 M Tris-HCl buffer (pH 8.2) as reference, are shown in Figure 2. A weak difference spectrum is generated by 0.1 N acetic acid, with negative peaks at 298, 291, and 282 nm. In contrast, the difference spectrum generated by 50% acetic acid is quite intense, with negative maxima at 292 and 286 nm and a shoulder at 280 nm.

Exclusion Chromatography of NPS Derivatives. In the case of 2·NPS-LTH (both tryptophans modified in 50% acetic acid) the major fraction, containing 80–90% of the protein, exhibited V_e/V_0 ratios of 1.45 on Sephadex G-100 and 2.05 on Sephadex G-200, in the 0.1 M Tris buffer. These values may be compared with V_e/V_0 ratios of 1.45 (G-100) and 2.08 (G-200) for an unmodified dimer of O-LTH[3] under the same conditions. Highly purified O-LTH monomer was found to give ratios of 1.85 (G-100) and 2.47 (G-200) on the same columns. The derivatives prepared in the various sodium acetate media all gave major fractions with V_e/V_0 ratios of 1.85 (G-100), with smaller amounts of material appearing in more highly aggregated forms. However, the yield of modified monomer was not the same in all three acetate solvents, being greatest (55%) in the case of 1 M acetate and significantly less (35–45%) in the other two. Furthermore, as described below, the material prepared in 0.1 M acetate contained approximately 1 mol of noncovalently bound NPS chromophore in addition to the covalently bound form. Further purification of this product to remove the contaminant caused additional loss of recoverable modified monomer.

Extent of Reaction with NPS-Cl. Spectrophotometric estimates of the amount of NPS chromophore introduced per mole of O-LTH were based on the corrected absorptivities mentioned under Methods. Although modification ratios were calculated from spectra taken in 80% acetic acid, 50% acetic acid, and in the Tris buffer (pH 8.2), no significant differences were found. This behavior was previously observed also for NPS-HGH and NPS-HCS (Bewley *et al.*, 1972). The dimeric 2·NPS-LTH derivative, prepared in 50% acetic acid, gave 2.1 mol of NPS per mol of O-LTH (4.2 mol of NPS per dimer molecule). The derivative was Erlich negative, and exhibited only weak fluorescence at 316 nm due to tyrosine residues, with no indication of fluorescence due to residual unreacted tryptophan. The monomeric 1·NPS-LTH derivatives which were prepared in acetate (1 and 2 M) gave 1.0–1.1 mol of NPS per mol of O-LTH with fluorescence maxima around 336 nm.

[3] The dimer form of O-LTH has been partially characterized by exclusion chromatography and analytical ultracentrifugation (Squire *et al.*, 1963). Further characterization of this fraction is now under investigation in our laboratory.

In sharp contrast, estimations of the extent of reaction on the material prepared in 0.1 M acetate showed 2.0 mol of NPS per mol of O-LTH. In addition, this derivative exhibited no fluorescence at 336 nm, with essentially the same emission spectrum as the 2·NPS-LTH derivative. Following the addition of dithiothreitol to this derivative in the Tris buffer, there was a large increase in the fluorescence emission intensity with the maximum shifting from 316 to 336 nm. The other NPS proteins, prepared in 50% acetic acid and sodium acetate (1 and 2 M), showed no significant changes in fluorescence on treatment with dithiothreitol. This characteristic change in fluorescence spectra could also be produced by "washing" the modified protein with 50% acetic acid. Following this treatment of the derivative with 50% acetic acid and repurification of the "washed" monomer, the modification ratio was found to have been reduced from 2.0 to 1.0–1.1 mol of NPS per mol of LTH. The fluorescence maximum was also shifted from 316 to 336 nm and the absorption maximum from 371 to 365 nm.

Identification of the Modified Tryptophan Residue in 1·NPS-LTH. The monomeric 1·NPS-LTH fraction (1.2 μmol) prepared in 1 M acetate buffer was digested with trypsin. The elution pattern of the digest contained two peaks having absorption at 365 nm as shown in Figure 3a. The small retarded peak was assumed to come from a small amount of NPS chromophore which had been noncovalently bound to the protein. As shown in Figure 3b, the mixture of peptides was further separated into two fractions absorbing at 365 nm. The minor first fraction was assumed to be a small amount of high molecular weight peptides resulting from incomplete digestion. The main fraction was pooled and lyophilized (recovery, 0.6 μmol based on ODU at 365 nm). This lyophilized material was further purified by low voltage paper electrophoresis, the electrophoretogram exhibiting four ninhydrin positive, but Erlich negative bands. One of these exhibited a strong yellow color and was clearly separated from the other three bands. Following extraction from the paper, the amino acid analysis of this NPS peptide was compared to the amino acid compositions of the peptides containing Trp-90 (residues 89–102) and Trp-149 (residues 142–158) which are expected from the tryptic digestion of O-LTH (Li *et al.*, 1970). These amino acid compositions are presented in Table I. A comparison of the amino acid composition of the NPS peptide to the theoretical compositions of the two tryptophan-containing tryptic fragments showed complete correspondence to the fragment from residue 142 to residue 158, containing Trp-149. While there did appear to be a small degree of contamination by aspartic acid, there was no evidence for any contamination by the Trp-90 peptide.

Circular Dichroism of MNPS-LTH and DNPS-LTH. Circular dichroism measurements were performed on the peak tubes from the final Sephadex G-100 purifications for 1·NPS-LTH and from the Sephadex G-200 purification of 2·NPS-LTH. In the region of side-chain absorption, 1·NPS-LTH exhibited two unresolved negative bands at 304 and 298 nm, in addition to a positive peak centered at 278 nm as presented in Figure 4a. In contrast, 2·NPS-LTH showed a weak positive peak at 305 nm, a small negative band around 298 nm, and a broad region of weak negative dichroism between 295 and 260 nm. In the region above 320 nm, both derivatives exhibited very slight negative dichroism. Due to the extremely weak and diffuse nature of these bands, it has been impossible to accurately determine the positions of their maxima. This band (or bands) in 1·NPS-LTH appears to be centered between 370 and 400 nm with an approximate molecular ellipticity of −5000 deg cm² dmol⁻¹. The corresponding band(s) in 2·NPS-LTH is considerably weaker than in 1·NPS-LTH and its characterization will require further investigation. In the region of amide bond absorption, the CD spectra of both 1·NPS-LTH and 2·NPS-LTH show the two negative bands at 209 and 221–223 nm typical of α-helical polypeptides (Figure 4b). The mean residue ellipticity of 1·NPS-LTH was found to be −21,800 deg cm² dmol⁻¹ at 223 nm and −20,500 deg cm² dmol⁻¹ at 209 nm. These values are essentially unchanged from those reported for the native hormone (Bewley and Li, 1972). However, 2·NPS-LTH showed somewhat diminished negative ellipticity compared to native O-LTH, amounting to a loss of about 5000 deg cm² dmol⁻¹ in the 223-nm band and 4000 deg cm² dmol⁻¹ in the 209-nm band.

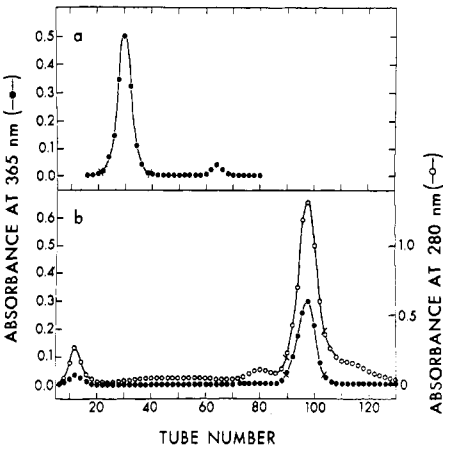

FIGURE 3: Elution patterns of a tryptic digest of 1·NPS-LTH on Sephadex G-25 (a). The main fraction in a was rerun on Sephadex G-50, producing the elution pattern shown in b. Conditions of chromatography are as described in the text.

Rate of Tryptic Digestion. The rates of alkali uptake during

TABLE I: Amino Acid Analysis of NPS Peptide from 1·NPS-LTH.

Amino Acid	Theoretical: Trp-90[a] Peptide	Theoretical: Trp-149[b] Peptide	Found[c]
Lys	0	1	0.70
His	1	0	0.00
Arg	1	0	0.00
Asp	2	0	0.47
Thr	1	2	1.83
Ser	1	3	1.95
Glu	1	3	2.92
Pro	1	3	2.88
Gly	0	1	1.21
Val	2	1	1.00
Leu	2	2	2.00
Tyr	1	1	0.72
Trp	1	1	1[d]

[a] Residues 89–102 according to Li *et al.* (1970). [b] Residues 142–158 according to Li *et al.* (1970). [c] The NPS peptide purified as described under Methods. [d] Determined spectrophotometrically as NPS-Trp.

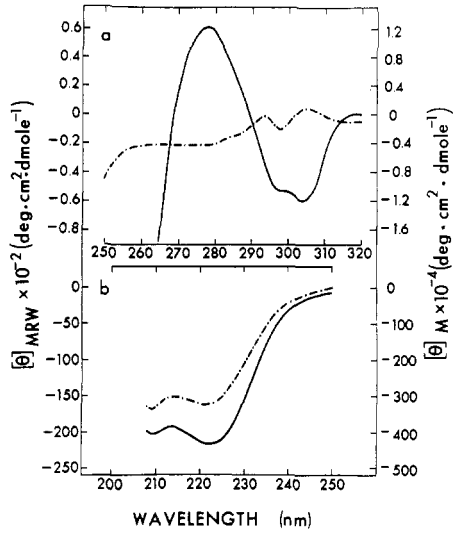

FIGURE 4: Circular dichroism spectra in the region of side-chain absorption (a) and amide bond absorption (b) of: 1·NPS-LTH (—) and 2·NPS-LTH (— - —). Both spectra were taken in 0.1 M Tris-Cl buffer, pH 8.2.

tryptic digestions of native O-LTH, the control prepared in 50% acetic acid, 1·NPS-LTH, 2·NPS-LTH, and performic acid oxidized O-LTH are presented in Figure 5. From the lysine and arginine contents in O-LTH (Li *et al.*, 1970) it can be concluded that about 20 mol of alkali should be consumed upon complete digestion. In the case of native O-LTH, 0.9 mol of alkali is taken up within the first 20 min. The digestion rate of the control prepared in 50% acetic acid is essentially the same as native O-LTH. 1·NPS-LTH is digested slightly more rapidly, with 1.3 mol of alkali being consumed. 2·NPS-LTH is digested much more rapidly, with 2.7 mol of alkali consumed in the same period. These initial digestion rates are in sharp contrast to the very rapid digestion of the performic acid oxidized hormone which consumes 12 mol of alkali in 20 min.

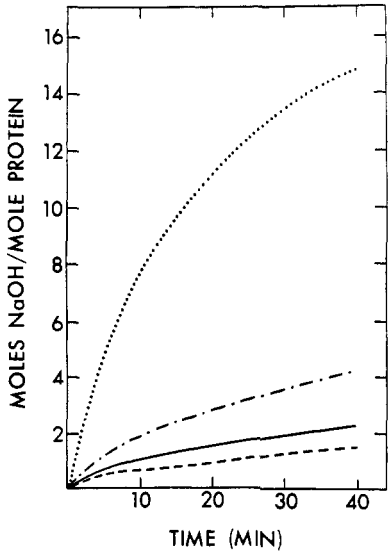

FIGURE 5: Rate of tryptic digestion for: native and 50% acetic acid treated O-LTH (- - -), 1·NPS-LTH (—), 2·NPS-LTH (— - —), and performic acid oxidized O-LTH (· · ·). Conditions of digestion are as described in the text.

TABLE II: Lactogenic Activity of O-LTH, Control Preparation, and NPS Derivatives Measured by the Pigeon Local Crop Sac Assay.

Preparation	Total Dose (μg)	Response[a]
Native O-LTH	1	18.7 ± 1.4
	2	26.1 ± 3.2
	4	36.1 ± 3.0
0.1 M sodium acetate treated O-LTH	2	26.9 ± 3.5
	4	30.5 ± 3.1
1·NPS-LTH	4	21.9 ± 2.8
	20	39.3 ± 11.0
	50	51.4 ± 8.8
50% acetic acid treated O-LTH	2	23.3 ± 2.5
	4	26.1 ± 2.0
2·NPS-LTH	20	13.6 ± 1.3
	50	21.3 ± 2.8
Saline		12.8 ± 1.5

[a] Mean ± standard error of the mean of the dry weight of crop sac mucosal epithelium in milligrams. Each response is calculated from four test animals.

Biological Activity. The lactogenic activities of O-LTH, control preparations, 1·NPS-LTH, and 2·NPS-LTH are presented in Table II. 1·NPS-LTH exhibits about one-fourth of the potency of the native hormone while 2·NPS-LTH is essentially devoid of activity. The 50% acetic acid and 0.1 M sodium acetate treated controls also appear to have lost some activity.

Discussion

Circular Dichroism and Difference Absorption Spectra of Unmodified O-LTH. From a comparison with CD spectra of model compounds (Strickland *et al.*, 1969), the asymmetric positive band at 297–298 nm in the CD spectrum of O-LTH has been assigned to overlapping ^1L_a and ^1L_b electronic transitions of tryptophan (Bewley and Li, 1972). In this regard it corresponds very closely to a similar band seen in the CD spectrum of HGH (Bewley and Li, 1972). HCS, a third closely related hormone, exhibits quite a different pattern, with a single negative band at 291–292 nm having been assigned to the ^1L_b indole transition (Bewley *et al.*, 1972). Although the CD bands assigned to the single tryptophan residues in HGH and HCS are largely conserved in 0.1 N acetic acid, the corresponding dichroism in O-LTH is completely lost. This complete loss of indole dichroism might at first be interpreted as an indication of a substantial structural transition for O-LTH in this solvent,[4] with both tryptophan residues being freed from any steric restrictions to rotation about their α–β carbon bonds. However, the maintenance of most of the amide bond CD would argue against the occurrence of a pronounced denaturation. Evidence for a limited structural transition, involving the tryptophan(s) in O-LTH, occurring between pH 5 and pH 3,

[4] The circular dichroism (CD) spectra of O-LTH in 0.1 N acetic acid and in the 0.1, 1.0, or 2.0 M sodium acetate buffers (pH 3.5–4.0) are all essentially the same.

has been previously reported (Aloj and Edelhoch, 1970). In agreement with this, the negative shoulder at 295 nm in the difference absorption spectra presented herein indicates that in 0.1 N acetic acid some "blue shift" perturbation of the indole chromophores has occurred. This perturbation results from the transfer of tryptophan side chains from environments of relatively high refractive index to an environment of somewhat lower refractive index (Herskovits and Sorensen, 1968). However, it is clear from the much more intense "blue shift" spectrum produced in 50% acetic acid that the transition in 0.1 N acetic acid is *incomplete* with regard to the "exposure" of all the tryptophan residues from the high refractive index interior of the protein to the low refractive index external medium. In view of this incomplete "exposure," we are unable at the present time to explain the apparent loss of the indole dichroism in the 0.1 N acetic acid and various acetate buffers.

Chemical Reactivity toward NPS-Cl. In spite of the rather low solubility of NPS-Cl in the acetate buffers, exposed tryptophan residues have been shown to react quantitatively under these conditions (Brovetto-Cruz and Li, 1969; Shechter *et al.*, 1972). In the present investigation on O-LTH it was found that in the acetate buffers (pH 3.5–4.0), Trp-149 could be quantitatively modified with no evidence for modification of Trp-90. However, in 50% acetic acid, both Trp-149 and Trp-90 were quantitatively modified. These results are in good agreement with the previously reported chemical reactivities of the homologous Trp-86 residues in HGH and HCS (Bewley *et al.*, 1972). They are also in agreement with the results of difference absorption spectra contained herein, which suggest that in 0.1 N acetic acid, the exposure of *all* the tryptophan residues in O-LTH is incomplete. It is interesting to note that in the 0.1 M acetate buffer, some NPS chromophore was found to be noncovalently bound to the 1·NPS-LTH. This phenomenon was also noted in the case of HGH and HCS in the same solvent (Bewley *et al.*, 1972). However, this noncovalent binding was almost completely absent in the 1·NPS-LTH samples prepared in the 1 and 2 M acetate buffers. Because it was necessary to use denaturing solvents (50% acetic acid) to remove this contaminant, all material used to characterize the 1·NPS-LTH derivative was taken from preparations made in 1 M acetate buffer.

Physical Characterization of the NPS Derivatives. The 1·NPS-LTH derivatives all exhibit V_e/V_0 ratios that are identical with that of the unmodified monomer, indicating that no gross irreversible changes in size or shape occur in this derivative. Similarly, the fact that this derivative is digested by trypsin at a rate only slightly greater than that of the native hormone further implies that it still retains a compact rigid conformation (Markus, 1965). In contrast, the 2·NPS-LTH exhibited V_e/V_0 ratios that indicated it to be a dimer form of the protein on both Sephadex G-100 and G-200. It should be noted that this remarkable change in quaternary structure does not occur when either the unmodified monomer *or* the 1·NPS-LTH derivative is treated with 50% acetic acid in the absence of NPS-Cl. Both these control preparations continue to give V_e/V_0 ratios that are equivalent to the native monomer. It would appear that the modification of Trp-90 is in some way crucial to the abrupt change in quaternary structure. At the present time we cannot evaluate the effect of the simultaneous modification of Trp-149 to this change in molecular form. This is a significant difference between the behavior of O-LTH and the other two related hormones, HGH and HCS. Following modification of Trp-86 in these proteins by NPS-Cl in 50% acetic acid, the NPS derivatives remained as stable monomers (Bewley *et al.*, 1972).

We have attempted to evaluate the conformations of the NPS derivatives from their CD spectra by comparisons with the previously described spectra of NPS-HGH, NPS-HCS, and a small model compound Gln^5-(NPS-Trp)^9-α-MSH (Bewley *et al.*, 1972). In the amide bond region, the CD of 1·NPS-LTH was essentially equivalent to that of the unmodified protein, indicating little or no change from the α-helix content characteristic of the native hormone (Bewley and Li, 1972). It has been suggested that what appears to be a small loss of α helix ($\approx$10%) in NPS-HGH and NPS-HCS may, in part, be due to overlapping of strong far-ultraviolet bands of the NPS chromophore with the normal amide bond spectra of the derivatives. These far-ultraviolet bands are clearly evident in the spectra of several model compounds. It was further suggested that the intensity of these bands could be approximately inferred from the intensity of the long-wavelength NPS chromophore band(s) seen above 320 nm, since both might be expected to increase in strength as rotations within the NPS-Trp residues become more restricted (Bewley *et al.*, 1972). The facts that the long-wavelength band in 1·NPS-LTH is approximately one-third as intense as the corresponding bands in NPS-HGH and NPS-HCS, and that there is little or no effect on the amide bond spectrum in the LTH derivative, lend support to these suggestions. The CD spectrum between 260 and 320 nm would be expected to exhibit ellipticity from both the modified Trp-149 and the unmodified Trp-90 residues, as well as from the tyrosine, phenylalanine, and cystine chromophores. The positive peak at 278 nm can be identified as *predominantly* a contribution of the NPS-indole group by comparison with strong corresponding bands seen in model compounds. The broad negative band with maxima at 298 and 304 nm may be due to overlapping of transitions from both the modified and unmodified tryptophan residues. The CD spectrum of the 2·NPS-LTH derivative is very difficult to interpret since at the present time the spectrum of O-LTH dimer is not available for comparison. The apparent absence of any long-wavelength NPS band in this derivative may arise from the presence of two equally intense bands of opposite sign, each being associated with one of the tryptophan residues. Long-wavelength NPS bands, both positive and negative, have been reported in model compounds (Bewley *et al.*, 1972).

Biological Activity. Interpretation of the bioassay results for the NPS-LTH derivatives is difficult in view of the fact that the control prepared by treating the unmodified hormone with 50% acetic acid lost nearly two-thirds of its biological potency. There may have been a slight loss of activity even in the control treated with 0.1 M acetate buffer, but this will require further investigation. Exclusion chromatography, CD spectra, and the rates of tryptic digestion have failed to show any clear evidence of irreversible conformation changes in these control preparations. A similar situation was found in the case of HCS treated with 50% acetic acid, in which one-half of its lactogenic potency was lost without any evidence of conformational change (Bewley *et al.*, 1972). In contrast, HGH treated with 50% acetic acid retains its original lactogenic potency (Brovetto-Cruz and Li, 1969). Apparently, some very subtle irreversible change occurs on treating HCS or O-LTH with 50% acetic acid. Nevertheless, the retention of about 25% lactogenic activity by 1·NPS-LTH indicates that absolute molecular integrity of Trp-149 is not a requirement. We cannot clearly state whether this is also true for Trp-90 since the complete loss of activity in the 2·NPS-LTH may reflect the fact that the molecule undergoes a change in quaternary structure.

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Estrogen Receptors in the Rat Uterus. Multiple Forms Produced by Concentration-Dependent Aggregation†

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ABSTRACT: The sedimentation coefficient of the estradiol receptor proteins from rat uterine cytosol is highly variable and concentration dependent. In dilute buffer solutions without added salt the value of the sedimentation coefficient varies from 7 to 9 S, in 0.15 M KCl solutions the values vary from 3.8 to 7 S, and in 0.4 M KCl solutions the values vary from 3.8 to 5.2 S. The observed concentration dependence seems to result from a slow aggregation of estradiol binding proteins following tissue homogenization. The aggregation does not appear to be self-association of estrogen binding proteins, but seems rather to represent an interaction of estrogen binding units with other proteins present in uterine cytosol. Protein present in the soluble fraction of nontarget tissues appears to interact with uterine estradiol receptors to a more limited extent. The time-dependent aggregation can be minimized by working with dilute solutions. This suggests that *in vivo* the uterine estradiol receptor may exist as a 3.8–4.8S species, rather than 8 S as previously believed.

It is well established that estrogen responsive tissues contain a macromolecular fraction, at least partially protein, which is capable of binding estrogenic compounds tightly and specifically (Gorski *et al.*, 1968; Jensen *et al.*, 1971). This macromolecular fraction has been extensively studied because it appears to be the initial site at which estrogens interact with target tissues. In the uterus and other target tissues the initial interaction of estrogens with these tissue-specific "receptors" is thought to initiate the sequence of events which produces the characteristic growth response of the uterus to estrogenic hormones.

When estradiol enters the uterine cell, it is initially found in the cytosol as part of a macromolecular complex which sediments in sucrose density gradients at approximately 8 S in dilute buffer solutions (Toft and Gorski, 1966; Toft *et al.*, 1967), and at about 4 S in solutions containing 0.4 M KCl (Korenman and Rao, 1968; Erdos, 1968; Rochefort and Baulieu, 1968). This macromolecular complex, containing the bound hormone, is then transferred to the nucleus by a temperature-dependent process which is not clearly understood (Jensen *et al.*, 1971). This nuclear form of the hormone receptor complex sediments at approximately 5 S (Jensen *et al.*, 1968; Shyamala and Gorski, 1969) or 6 S (Giannopoulos and Gorski, 1971b) in 0.4 M KCl.

Currently, sucrose density gradients provide the most widely used method for distinguishing between the cytoplasmic and nuclear forms of the estrogen receptor. In addition, sucrose gradient centrifugation provides a tool for comparing receptors from various species. Despite widespread use of this technique, reported sedimentation coefficients from different laboratories vary considerably (Chamness and McGuire, 1972). Furthermore, the gradient profiles

† From the Departments of Physiology and Biophysics and Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received December 4, 1972.* Supported by National Institutes of Health Grants HD 4828 and HD-ITI-00181, and Ford Foundation Training Grant 700-0333.

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