Comparative Studies of the Single Tryptophan Residue in Human Chorionic Somatomammotropin and Human Pituitary Growth Hormone†

Thomas A. Bewley, Hiroshi Kawauchi, and Choh Hao Li*

ABSTRACT: The single tryptophan residue occurring at homologous positions in the polypeptide chains of both human chorionic somatomammotropin and human pituitary growth hormone have been compared by studying their reactivity to the tryptophan specific reagent *o*-nitrophenylsulfenyl chloride. No covalent modification of either protein could be achieved in 0.1 M sodium acetate (pH 4.0) although both reacted quantitatively in 50% acetic acid. Evidence obtained from absorption and circular dichroism spectra of the unmodified hormones is presented, which directly relates this difference in chemical reactivity to conformational changes, clearly indicating the exposure of tryptophan residues in 50% acetic acid which are normally "buried" in the native molecules. The nitrophenylsulfenyl derivatives have been characterized and compared to the native hormones by exclusion chromatography, fluorescence, absorption, and circular dichroism spectra, relative rates of digestion by trypsin, and biological activity. While the nitrophenylsulfenyl derivative of human pituitary growth hormone retained full potency as a growth-promoting and lactogenic agent, the derivative of human chorionic somatomammotropin was completely devoid of lactogenic activity. Exclusion chromatography and tryptic digestion rates both indicate that there are no gross conformational differences between either protein and its nitrophenylsulfenyl derivative. Circular dichroism spectra also suggest that neither derivative has undergone a pronounced alteration in secondary structure. However, from a comparison of circular dichroism spectra in the region of side-chain absorption to those of model compounds, it would appear that the *o*-nitrophenylsulfenyltryptophan residue in human chorionic somatomammotropin is in a more "exposed" and/or less sterically hindered environment than the corresponding modified residue in human pituitary growth hormone. The significance of this structural difference and its potential relationship to the loss or retention of biological activity is discussed.

The acceptance of a definite relationship between three-dimensional structure and biological function in protein molecules has led to a number of important observations which have led in turn to a detailed understanding of the intrinsic role of the molecular architecture in effecting the binding, catalytic, and species specificity characteristics. While this concept is now a firmly established axiom within enzymology, it is not fully accepted as a basic principle governing the behavior of protein hormones.

A comparison (Bewley and Li, 1971a) of the amino acid sequence (Li *et al.*, 1971) of HCS,[1] secreted during pregnancy by the human trophoblast, with a revised sequence (Li and Dixon, 1971) for HGH, secreted by the human pituitary gland, has shown that 160 of the 190 residue positions in either polypeptide chain are occupied by identical amino acids. These identities include a single tryptophan residue at position 85 in both molecules. Of the 30 positions showing differences, 23 may be classified as homologous,[2] with only 7 positions (<4%) being considered nonhomologous. As might be expected, this extensive homology in primary structure is strongly reflected in similarities of secondary structure and some, but not all, aspects of tertiary structure (Bewley and Li, 1971a, 1972; Aloj and Edelhoch, 1971, 1972). The close resemblance in biological activities[3] and immunochemical behavior (Josimovich and MacLaren, 1962; Grant *et al.*, 1971) of these hormonal molecules strongly suggests that there is a directional relationship leading from *sequence* to *conformation* and finally to *activity*. This type of relationship would be further supported if it could be shown that the relatively minor differences in sequence are responsible for the conformational dissimilarities observed in the tertiary structures, and that it is one or more of these conformational differences that are largely responsible for the *quantitative* disparity in biological potencies.

In a previous study (Brovetto-Cruz and Li, 1969) it was found that under mildly acidic conditions (0.2 M sodium acetate, pH 4.0) the single tryptophan residue in HGH was unreactive toward the tryptophan specific reagent *o*-nitrophenylsulfenyl chloride (Scoffone *et al.*, 1968) although quantitative modification could be achieved in 50% acetic acid. These results are consistent with a structural transition observed in HGH by circular dichroism measurements made under similar

† From the Hormone Research Laboratory, University of California, San Francisco, California 94122. *Received June 13, 1972.* This work was supported in part by the American Cancer Society, the Geffen Foundation and the Allen Foundation; Paper XXXIII in the Human Pituitary Growth Hormone Series.

[1] Abbreviations used are: HCS, human chorionic somatomammotropin; HGH, human pituitary growth hormone; NPS-Cl, *o*-nitrophenylsulfenyl chloride; NPS-HCS and NPS-HGH, the *o*-nitrophenylsulfenyl derivatives of HCS and HGH; NPS-Gln$_5$-α-MSH, the NPS derivative of a synthetic analog of α-melanocyte stimulating hormone; CD, circular dichroism; v_e/v_0, the ratio of elution volume to void volume in exclusion chromatography.

[2] When comparing two or more polypeptide sequences, homologous residue positions are those occupied either by identical amino acids, or amino acids regarded as acceptable replacements, as described by Dayhoff (1969). Homologous and nonhomologous positions correspond closely to the conservative and nonconservative replacements of Perutz *et al.* (1965).

[3] A recent comparison (Li, 1970) of the biological activities of these two proteins indicates that HCS is 85% as potent as HGH with regard to lactogenic activity and 10–15% as potent with regard to growth-promoting activity.

conditions (Bewley and Li, 1972), which suggest the exposure of the normally buried tryptophan residue in 50% acetic acid. The NPS-HGH derivative was found to be fully active as a growth-promoting agent. Although there is considerable sequence homology around the tryptophan residues in HCS and HGH, fluorescence and circular dichroism measurements indicate that their microenvironments are not identical (Bewley and Li, 1971b; Aloj and Edelhoch, 1972). Nevertheless, an equivalent transition of the tryptophan residue in HCS has been observed (Bewley and Li, 1971b). We report herein the results of an investigation into the microenvironments of the tryptophan residues in these two hormones, and the reactivity of the tryptophan in HCS. The preparation, physical properties, and biological activity of NPS-HCS, and a comparison to NPS-HGH are described.

Materials and Methods

The isolation and purification of HGH (Li *et al.*, 1962) and HCS (Li, 1970) have been previously described. The syntheses of Gln_5-α-MSH (Blake *et al.*, 1970) and its NPS derivative (Ramachandran, 1970), as well as the criteria of purity for these products, have also been previously described. The *o*-nitrophenylsulfenyl chloride (mp 73°) was purchased from Eastman Organic Chemicals and recrystallized once from chloroform–ether. NPS-L-tryptophan (mp 220° dec) and NPS-*N*-acetyl-D,L-tryptophanamide (mp 140–142°) were prepared by the method of Scoffone *et al.* (1968) and recrystallized from ethanol–H_2O. The purity of these products was confirmed by thin-layer chromatography in three solvent systems: 1-butanol–acetic acid–H_2O (4:1:2, v/v); 1-butanol–benzene–acetic acid (3:3:1, v/v); 1-butanol–benzene (1:1, v/v). Performic acid oxidation of the native proteins was performed as described by Li (1957). Trypsin was obtained from Calbiochem. All other chemicals were of reagent grade and were used without further purification.

Preparation of NPS-HCS and NPS-HGH. Two different solvents were used for testing the reaction of HCS and HGH with NPS-Cl.

A. Acetic acid (50%). NPS-Cl (20 μmoles) in 1.0 ml of glacial acetic acid was added to a stirred solution of 22 mg (1 μmole) of HCS or HGH in 2.0 ml of 25% acetic acid. The temperature of the water-jacketed reaction vessel 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 a sufficient volume of 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 0.1 M Tris-Cl buffer (pH 8.2) and purified by exclusion chromatography on Sephadex G-100 using the same buffer as eluent. The monomer fraction was pooled and rechromatographed on the same column. The purified monomer was either used directly or recovered by lyophilization after thorough dialysis against 0.01 M NH_4HCO_3 buffer (pH 8.4). Control preparations of both proteins were prepared by treating the native hormones with 50% acetic acid in the absence of NPS-Cl, followed by purification of the monomer as described above.

B. Sodium acetate (0.1 M, pH 4.0). The protein (1 μmole) was dissolved in 7 ml of 0.1 N acetic acid and the pH adjusted to 4.0 with dilute NaOH. Ten milligrams (50 μmoles) of finely powdered NPS-Cl was added and the reaction was allowed to proceed for 2 hr at 20° with stirring. During this time the pH was maintained at 4.0 by the addition of dilute NaOH in a pH-Stat (Radiometer, Model TTT IIb). After completion of the reaction period, the remaining insoluble reagent was removed by centrifugation and the supernatant was submitted to exclusion chromatography on Sephadex G-25 in 0.1 M sodium acetate (pH 4.0). Following extensive dialysis of the protein fraction against 0.1 M Tris-Cl buffer (pH 8.2) further purification and isolation of the monomer was carried out as described above under step A.

Protein Concentration. Unless otherwise stated, protein concentrations in all solutions were determined spectrophotometrically. Spectra were taken of each solution from 360 to 245 nm on a Beckman DK-2A spectrophotometer, against an appropriate reference. For the NPS derivatives, spectra were taken from 500 to 245 nm. Matched silica cuvets, of 1.0-cm path length were used in all cases. All spectra were corrected for light scattering as described by Beavan and Holiday (1952). Concentrations of the native proteins in all solvents were calculated using the values: $E^{1\%}_{277\,nm} = 9.31$ for HGH (Bewley *et al.*, 1969) and $E^{1\%}_{277\,nm} = 8.22$ for HCS (Bewley and Li, 1971b). Corrected absorptivities ($E^{1\%}_{280\,nm}$), 12.5 for NPS-HCS and 13.5 for NPS-HGH, were computed as described by Scoffone *et al.* (1968). For this purpose the molecular weights of both proteins and their NPS derivatives were taken as 21,700 and 21,900, respectively. A value of $E^{1\%}_{280\,nm} = 90.6$ for NPS-Gln_5-α-MSH was calculated from the molar extinction coefficient of this derivative reported by Ramachandran (1970).

Difference absorption spectra of the native proteins were taken in a Beckman DK-2A spectrophotometer using a tandem arrangement of four matched silica cuvets. Stock solutions of the proteins were prepared in H_2O adjusted to pH 8.5 with dilute ammonia. Aliquots of these stock solutions were then transferred to volumetric flasks and lyophilized. The appropriate solvent was added and the volume adjusted. Clear solutions were obtained in all cases. The proper arrangement of samples and solvent blanks was set up in the spectrophotometer and the spectra were taken from 360 to 250 nm. Peak absorbancies of both reference and sample were never greater than 0.9 ODU.

Fluorescence measurements were made in a Hitachi Perkin-Elmer spectrofluorometer, Model MPF-2A. Emission spectra were taken under excitation at 285 nm. Protein concentrations were determined and in most cases quantitative dilutions were made to bring the absorbance at 277 nm to a value of 0.1 ODU or below. In order to detect small amounts of unreacted tryptophan in the NPS derivatives, samples from the final purification on Sephadex G-100 were measured directly without dilution. Fluorescence data are expressed as relative fluorescence per mole of protein.

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-Cl 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). As mentioned above, fluorescence spectra of the highly purified derivatives were taken in order to detect small amounts of unreacted tryptophan (Parikh and Omenn, 1971).

Rate of Tryptic Digestion. Tryptic digestions were carried out in a water-jacketed cell (25°), at pH 8.5 in the pH-Stat, under a nitrogen atmosphere. The pH was maintained by addition of 0.002 or 0.005 N CO_2-free NaOH and the alkali

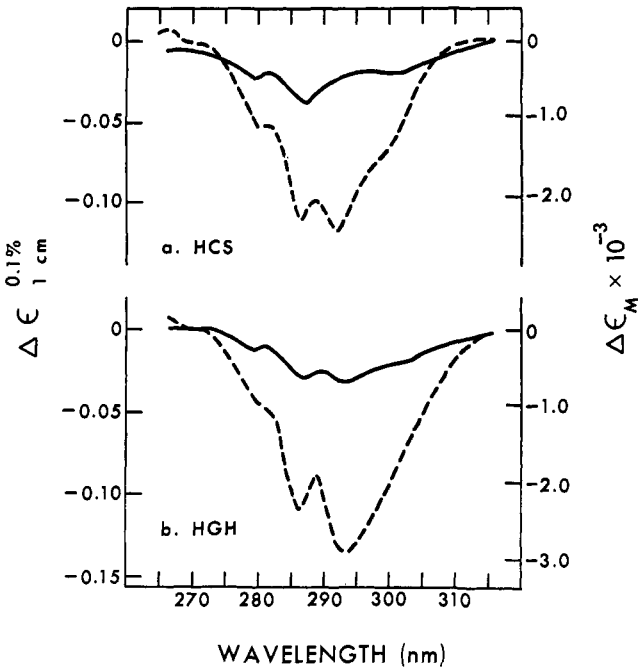

FIGURE 1: Difference absorption spectra of HCS (a) and HGH (b), in 0.1 N acetic acid, pH 3.6 (——), and 50% acetic acid (– –). In both cases the reference protein was in 0.1 M Tris-Cl buffer (pH 8.2). These spectra are shown as they would appear at a protein concentration of 1.0 mg/ml. The actual experiment was done at a protein concentration between 0.8 and 0.9 mg per ml, in order that the maximum absorbance in either reference or sample cell would be less than 0.9 ODU.

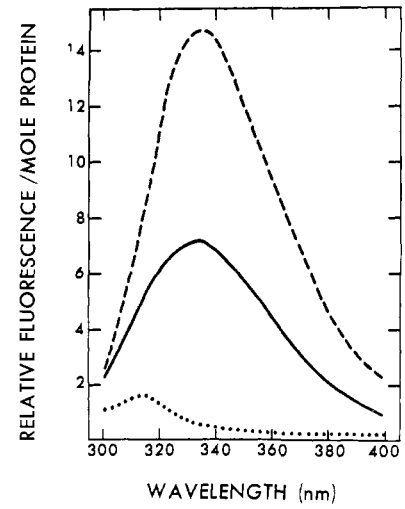

FIGURE 2: Fluorescence emission spectra of HCS (——), HGH (– –), and the purified NPS derivatives of these proteins (· · ·) in 0.1 M Tris-Cl buffer (pH 8.2). Excitation was at 285 nm. In all cases, the absorbance at 277 nm was less than 0.1 ODU.

uptake was recorded as a function of time. The enzyme:substrate ratio was 1:250 in all cases. The lyophilized proteins (free of buffer salts) were dissolved in 0.1 M KCl and the pH was adjusted to 9.0 with dilute CO_2-free NaOH. Any insoluble material was removed by centrifugation. The concentration of the performic acid oxidized hormones was based on the weight of lyophilized material used, assuming a 20% moisture content. The protein concentration in all solutions was adjusted to 30 nmoles/ml.

Circular dichroism (CD) spectra were obtained on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment. The instrument was calibrated with *D*-10-camphorsulfonic acid (Eastman Organic Chemicals) as recommended by the manufacturer. All spectra were taken at 27°. Fused-quartz cells with path lengths of 1, 5, 10, and 25 mm were used. Measurements were made as close to 200 nm as possible, reducing the optical path whenever the dynode voltage exceeded 500 V. Scanning speeds were 1 nm/min or less. Mean residue molecular ellipticities, $[\theta]_{MRW}$, were calculated using a value of 115 for the mean residue weight. Molecular ellipticities, $[\theta]_M$, were obtained using the molecular weights of the native proteins and NPS derivatives mentioned above. Helical contents were estimated as previously described (Bewley *et al.*, 1969). CD spectra of the NPS–protein monomers were measured on a pooled sample consisting of the three peak tubes from the final Sephadex G-100 purification.

Biological Activity. The growth-promoting activity was determined by the rat tibia test (Greenspan *et al.*, 1949); the lactogenic activity, by the local crop assay in the pigeon. The response in the pigeon assay was determined both as described by Lyons (1937) and by the quantitative method of Nicoll (1967). Since a reliable estimation of the growth-promoting activity of HCS requires an inordinate amount of test material, only the lactogenic activity of this hormone was tested in the present investigation.

Results

Native HCS and HGH. CIRCULAR DICHROISM. The CD spectra of both HCS (Bewley and Li, 1971b) and HGH (Bewley and Li, 1972) in the 0.1 M Tris-Cl buffer (pH 8.2), as well as in 50% acetic acid, have been previously described. During the present investigation, CD spectra in 0.1 N acetic acid (pH 3.6) were found to be essentially equivalent to those already reported for these two proteins in 0.1 M glycine-HCl buffer (pH 3.6) (Bewley and Li, 1971b, 1972). Thus it is quite evident that the 0.1 N acetic acid produces only very minor changes in the spectrum of HGH relative to what is seen in the Tris buffer and only slightly greater alterations of the HCS spectrum. In particular, the nearly complete maintenance of the negative shoulder at 291–292 nm in HCS as well as the positive peak at 295 nm in HGH was observed. In sharp contrast, both these features are entirely absent in the spectra taken in 50% acetic acid. Estimated α-helix contents in the 0.1 N acetic acid solvent were 40–45% for HCS and 45–50% for HGH.

DIFFERENCE ABSORPTION SPECTRA of the two hormones in 0.1 N and 50% acetic acid, taken against the protein in the Tris buffer as reference, are shown in Figure 1. Both proteins generate only a weak difference spectrum in 0.1 N acetic acid, with negative peaks at 300–301, 287, and 279 nm for HCS, and 293, 287, and 279 nm for HGH. In addition, HGH exhibits a weak shoulder at 301–302 nm. In contrast, the difference spectra generated by 50% acetic acid are quite intense, with negative maxima at 292, 286, and 280 nm for HCS and at 293, 286, and a shoulder at 280 nm for HGH. HCS also exhibits a shoulder near 300 nm which is not apparent in HGH although the *shape* of the HGH spectrum indicates the presence of negative difference absorption centered above 293 nm.

FLUORESCENCE. The relative fluorescence intensities per mole of protein for HCS, HGH, and their NPS derivatives are presented in Figure 2. The emission maxima of native HCS and HGH were 333 and 336 nm, respectively, with excitation in both cases at 285 nm. In general agreement with results reported by Aloj and Edelhoch (1972) the fluorescence in-

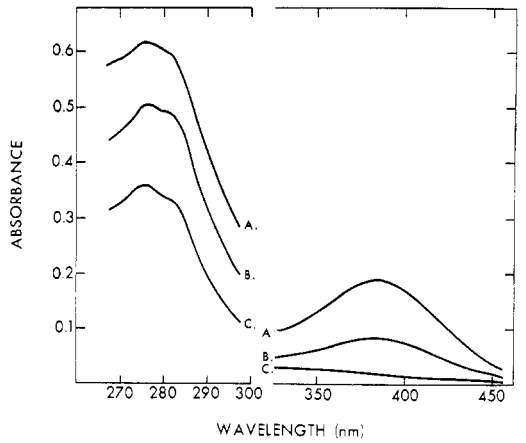

FIGURE 3: Absorption spectra of HGH treated with NPS-Cl in 0.1 M sodium acetate (pH 4.0) during progressive stages of purification. (A) Following desalting of the reaction mixture on Sephadex G-25 in 0.1 M sodium acetate (pH 4.0), NPS/HGH = 2.2. (B) Material from part A, further purified on Sephadex G-100 in 0.1 M Tris-Cl buffer, (pH 8.2), NPS/HGH = 1.2. (C) Material from part B, treated with 50% acetic acid and repurified as described under Materials and Methods. NPS/HGH = 0.0.

tensity maximum of HGH was found to be somewhat more than twice that of HCS.

Characterization of NPS-HCS and NPS-HGH. EXTENT OF REACTION. Spectrophotometric estimates of the amount of NPS chromophore introduced per mole of HCS and HGH were based on the corrected absorptivities described under Materials and Methods. In these particular NPS derivatives, no significant differences were found when modification ratios were obtained from spectra taken in 80% acetic acid, 50% acetic acid, or 0.1 M Tris-Cl buffer of pH 8.2. Both hormone derivatives showed 1.2 moles of NPS/mole of protein when 50% acetic acid was used as the reaction medium. These modification ratios are identical with those previously reported by Brovetto-Cruz and Li (1969) for NPS-HGH. Both NPS derivatives were Erlich negative and exhibited only weak fluorescence due to tyrosine at 316 nm, with no indication of fluorescence due to residual unreacted tryptophan (Figure 2). Estimates of the extent of reaction in 0.1 M sodium acetate (pH 4.0) were at first very confusing. Spectrophotometric analyses of the monomeric products following the final Sephadex G-100 purification indicated highly variable modification ratios between 1 and 2 moles of NPS per mole of protein. This ratio was found to depend on the amount of NPS-Cl used and also on the number of times the entire reaction was repeated on the same sample of protein. However, in all these products the absorption maximum of the NPS chromophore in the Tris buffer was found to be 381 nm rather than 365 nm as found in the NPS-*N*-acetyl-D,L-tryptophanamide model compound and in the NPS derivatives of the hormones prepared in 50% acetic acid. In addition, the purified monomers retained considerable fluorescence at 336 nm. These "variably modified" proteins were then treated with 50% acetic acid (in the absence of NPS-Cl) followed by the same stepwise removal of acetic acid and purification of monomers as described above for the preparation of the NPS derivatives in 50% acetic acid. This simple procedure resulted in purified products for both hormones which showed no spectrophotometric evidence of modification. Absorption spectra for HGH, indicating the removal of noncovalently bound NPS chromophore by this procedure, are shown in Figure 3.

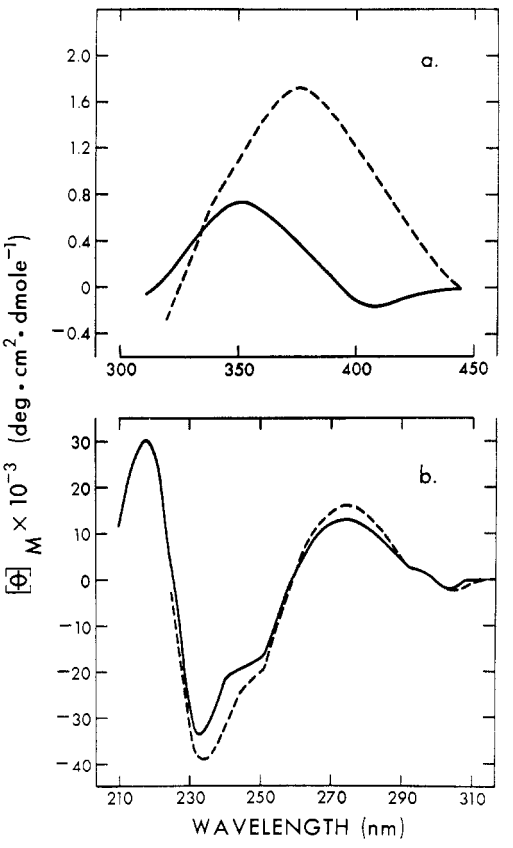

FIGURE 4: Circular dichroism spectra, in the long-wavelength region (a) and the region of amide and side-chain absorption (b) of: NPS-L-tryptophan in methanol (——); and NPS-Gln$_5$-α-MSH in 0.1 M Tris-Cl buffer (pH 8.2) (– –).

EXCLUSION CHROMATOGRAPHY OF NPS-HCS AND NPS-HGH. The monomer fractions obtained from exclusion chromatography on Sephadex G-100 of native HCS, HCS treated with 50% acetic acid, and NPS-HCS prepared in 50% acetic acid, all gave V_e/V_0 ratios[4] of 1.75–1.80. Similar patterns were found for native and modified HGH, with monomer V_e/V_0 ratios around 2.0. Purified monomers of both derivatives were obtained in 40–50% yield.

CIRCULAR DICHROISM BELOW 250 nm. In the region of amide-bond absorption, the CD spectra of NPS-HCS and NPS-HGH in the Tris buffer show the two negative bands typical of α-helical polypeptides. However, in comparison to these same bands in the native molecules (Bewley and Li, 1971b), both derivatives show diminished intensities, amounting to about 3000 (deg cm^2) dmole^{-1} in the 221-nm bands, and 3000–4000 (deg cm^2) dmole^{-1} in the 209-nm bands. These less negative ellipticities result in a lowering of the α-helix content estimates for the modified hormones by 5% as calculated from the 221-nm bands, and 10–15% as calculated from the 209-nm bands. These mean residue molecular ellipticities and the corresponding α-helix content estimates are summarized in Table I. As shown in Figure 4, CD spectra of the two model compounds (NPS-L-tryptophan in methanol and NPS-Gln$_5$-α-MSH in the Tris buffer) exhibit several intense dichroism bands in this region, with negative maxima around 235 nm and negative shoulders near 245–250 nm.

[4] In a previous publication (Bewley and Li, 1971b) the V_e/V_0 ratios of HCS and HGH monomers (Tris buffer, pH 8.2) were reported to be 1.92 and 1.97, respectively. At the present time we are unable to explain the slightly lower V_e/V_0 ratios for HCS reported herein.

TABLE I: Mean Residue Molecular Ellipticities[a] and α-Helix Contents of HCS, HGH, and Their NPS Derivatives.

Preparation	$[\theta]_{(\lambda)}$	% α Helix	$[\theta]_{(\lambda)}$	% α Helix
HCS[b]	−16,700 (220 nm)	45	−18,300 (209 nm)	45
NPS-HCS[c]	−13,500 (221 nm)	40	−14,200 (209 nm)	30
HGH[b]	−19,900 (221 nm)	55	−22,400 (209 nm)	55
NPS-HGH[c]	−17,300 (221 nm)	50	−19,200 (209 nm)	45

[a] Measured in 0.1 M Tris-Cl buffer (pH 8.2). Units are (deg cm^2) dmole^{-1}. [b] Taken from Bewley and Li (1971b). [c] Although these ellipticities are at negative extrema in the CD spectra, the estimated α-helix contents must be considered tentative as described in the Discussion section.

The possibility that these bands may interfere with estimates of the α-helix contents of these derivatives is discussed below. Due to unfavorable signal-to-noise ratios we have not been able to reliably demonstrate a band in the NPS-Gln$_5$-α-MSH corresponding to the positive 217.5-nm band in NPS-L-tryptophan.

CIRCULAR DICHROISM SPECTRA ABOVE 250 nm. The CD spectra of NPS-HCS and NPS-HGH in the region of side-chain absorption are shown in Figure 5. Six negative maxima are found in the spectrum of NPS-HCS at 379, 310, 299, 283, 278, and 269 nm, with a weak shoulder between 260 and 265 nm. In contrast, NPS-HGH exhibits a strong positive envelope between 310 and 260 nm, with two distinct maxima at 290 and 275 nm, and additional shoulders near 307, 297, and 265 nm. The NPS-HGH spectrum also contains a broad negative band with a maximum at 361 nm. This band is similar to the broad negative band centered at 379 nm in NPS-HCS. The long-wavelength absorption maximum of both these derivatives was found to be at 365 nm. As shown in Figure 4, an analogous, broad, long-wavelength band is also seen in the CD spectra of both model compounds although the intensities are only 10–20% of those seen in the two NPS proteins. In addition, both bands in the model compound spectra are positive with maxima at 352 nm for NPS-L-tryptophan in methanol and at 375 nm for NPS-Gln$_5$-α-MSH in the Tris buffer. The NPS-L-tryptophan shows additional negative bands at 408 and 314 nm which are not seen in the other model compound spectrum. The long-wavelength absorption maximum of NPS-L-tryptophan was found to be at 355 nm in methanol, while that of NPS-Gln$_5$-α-MSH in the Tris buffer appeared at 365 nm as was also found for NPS-*N*-acetyl-D,L-tryptophanamide and both NPS proteins in the Tris buffer. Between 260 and 310 nm, the two model compounds exhibit almost identical CD spectra with positive maxima at 275 nm, a positive shoulder around 295 nm and weak negative maxima at 303–305 nm.

RATE OF TRYPTIC DIGESTION. The rate of alkali uptake during tryptic digestions of HCS, 50% acetic acid treated HCS, NPS-HCS, and performic acid oxidized HCS are presented in Figure 6. From the content of lysine and arginine residues in HCS (Li *et al.*, 1971) it can be concluded that about 20 moles of alkali should be consumed upon complete digestion. The native and 50% acetic acid treated HCS are digested at the same rate with 2 moles of alkali being taken up in the first 48 min. The NPS derivative is digested somewhat more rapidly

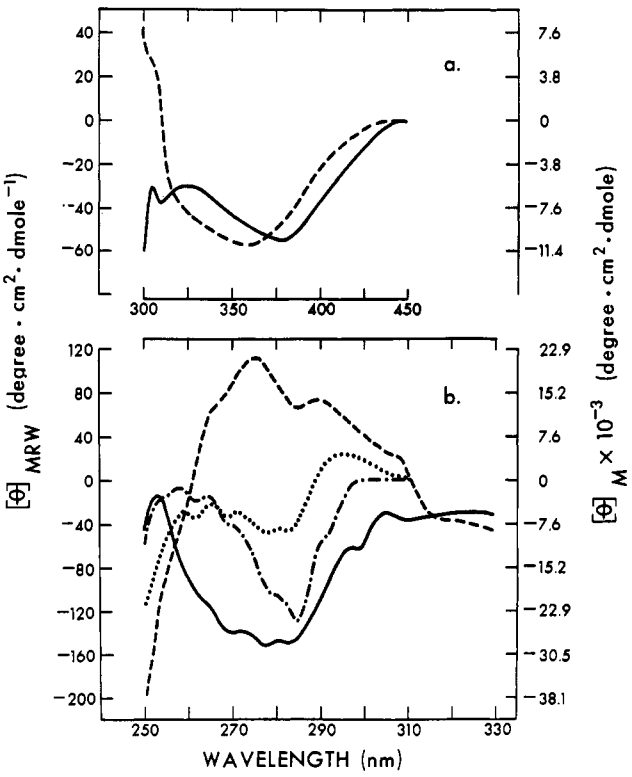

FIGURE 5: Circular dichroism spectra in the long-wavelength region (a) and the region of side-chain absorption (b) of : NPS-HCS (——) and NPS-HGH (– –). The spectra of native HCS (–·–) and native HGH (· · ·) are reproduced for comparison. The solvent in all cases was 0.1 M Tris-Cl buffer (pH 8.2).

with just under 4 moles of alkali consumed in the same time period. However, these digestion rates are in sharp contrast to the very rapid digestion of the performic acid oxidized hormone which is 65–70% complete in 48 min. The tryptic digestion rates of HGH and its derivatives closely paralleled the results shown in Figure 6 for HCS, and were essentially no

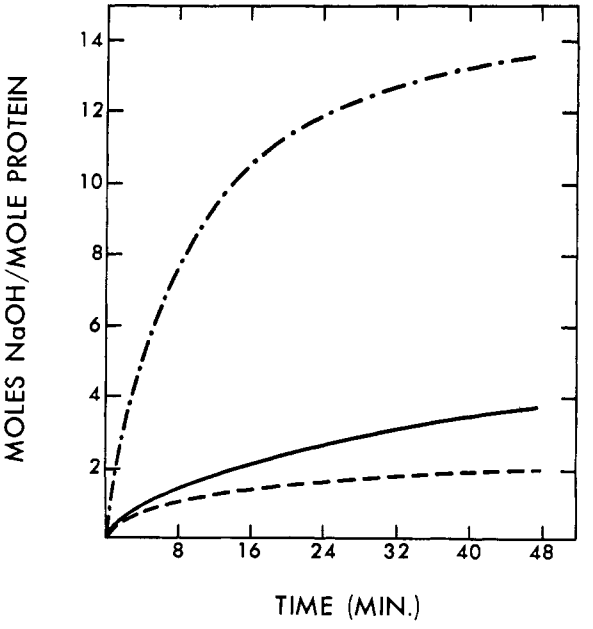

FIGURE 6: Rate of digestion by trypsin for: native and 50% acetic acid treated HCS (— —); NPS-HCS (——); and performic acid oxidized HCS (– · –). Conditions of digestion were as described in the text.

TABLE II: Lactogenic Activity of HCS, HGH, and Their NPS Derivatives Measured by the Pigeon Local Crop Assay.

Preparation	Total Dose (μg)	Response[a]
HCS (untreated)	5	24.2 ± 4.0
	20	32.6 ± 4.1
HCS (50% acetic acid treated)	5	19.5 ± 2.0
	20	23.0 ± 1.0
NPS-HCS	5	13.0 ± 1.1
	20	13.6 ± 0.5
Saline	0	12.8 ± 1.5
HGH (untreated)	4	16.5 ± 1.2
	20	19.3 ± 0.3
NPS-HGH[b]	4	17.9 ± 0.4
	20	23.0 ± 2.8

[a] Mean ± SEM of the dry weight of crop-sac mucosal epithelium in milligrams. [b] The potency of NPS-HGH, in the crop-sac assay, has been demonstrated on three separate preparations.

different from those already reported by Brovetto-Cruz and Li (1969).

BIOLOGICAL ACTIVITY. The lactogenic activity of HCS, HGH, and their NPS derivatives are presented in Table II. It is evident that while NPS-HGH retains full lactogenic potency, NPS-HCS is devoid of this activity. The 50% acetic acid treated HCS also appears to have lost about half of its potency compared to the native molecule. It has been previously shown (Brovetto-Cruz and Li, 1969) that treatment of HGH with 50% acetic acid does not cause a reduction in either lactogenic or growth-promoting activities. Data indicating that NPS-HGH also retains full potency as a growth-promoting agent are presented in Table III.

Discussion

Comparison of the Environments of the Tryptophan Residues in the Native Molecules, and the Structural Transition in 50% Acetic Acid. Despite the remarkable degree of homology in the primary structures of these two hormones, including homology around the identical tryptophan residues at position 85, there is considerable evidence that the microenvironments of the two indole rings are not identical. From a comparison to previously published CD spectra of model compounds (Strickland *et al.*, 1969) the negative shoulder at 291–292 nm in the HCS spectrum may be assigned to the ^1L_b transition of the single tryptophan in this molecule. Similarly, the positive, asymmetric band at 294–295 nm in the spectrum of HGH is almost certainly due to overlapping positive bands of both ^1L_a and ^1L_b indole transitions.[5] The difference in sign of the ^1L_b transitions in these two molecules, as well as the apparent absence of any ^1L_a transition in the CD spectrum of HCS, is consistent with the known sensitivity of this chromophore's CD spectrum to the local environment of the indole ring. Unfortunately, we cannot deduce the exact nature of this environmental difference from these spectra. The maintenance in 0.1 N acetic acid (pH 3.6) of the CD bands characteristic of the indole chromophores in the native molecules, clearly indicates that the native environment of the tryptophan residue in both proteins is largely conserved in this solvent. However, in both molecules the tryptophan dichroism is completely lost in 50% acetic acid. This loss is consistent with a structural transition in both proteins occurring in 50% acetic acid, which exposes the "buried" tryptophan residues to the aqueous medium. These exposed residues are able to rotate more freely about their α–β carbon bond and hence lose most of their dichroic strength (Beychok, 1966).

TABLE III: Growth-Promoting Activity of HGH and NPS-HGH, Measured in the Rat Tibia Assay.

Preparation	Total Dose (μg)	Response[a]
HGH	20	228 ± 5
	40	243 ± 4
	60	270 ± 7
NPS-HGH	20	226 ± 5
	40	225 ± 6
	60	261 ± 8
Saline	0	170 ± 2

[a] Mean ± SEM in microns. Each response is calculated from five test animals.

In agreement with the results obtained from CD measurements, difference absorption spectra (Figure 1) indicate that there are only minimal conformational differences between the proteins in the Tris buffer and 0.1 N acetic acid. This is in good agreement with results reported by Aloj and Edelhoch (1971, 1972). However, difference spectra, indicating large perturbation blue shifts relative to the native proteins, are generated in 50% acetic acid. The negative peaks at 291–292 nm, as well as the difference absorbance above 300 nm, is clear evidence (Herskovits and Sorensen, 1968) that 50% acetic acid causes a transfer of tryptophan residues from environments of high refractive index (hydrophobic interior of the protein) to an environment of relatively low refractive index (external medium of 50% acetic acid). This structural transition, occurring in 50% acetic acid, has already been noted above in the CD spectra.

Two other facts may be inferred from Figure 1. The difference spectra generated in 50% acetic acid are, in both cases, essentially the same as corresponding difference spectra generated in 6 M guanidine hydrochloride.[6] In view of the extensive denaturation observed for these molecules in the latter solvent (Bewley and Li, 1971b, 1972) we conclude that the exposure of tryptophan residues in 50% acetic acid is essentially complete for both HCS and HGH. Therefore, assuming that the final state in the structural transition is the same for both proteins as evidenced by the fully and equally exposed tryptophan residues, we may conclude that the differences between the perturbation spectra of HGH and HCS in the region of indole absorbance above 290 nm are *not* due to differences in the microenvironments of the final states, but to differences in the microenvironments of their respective *initial* states. This interpretation is consistent with the nonequivalence of the indole microenvironments mentioned above in connection with the CD spectra of the native molecules. It should be emphasized that in this argument we are concerned only with the initial and final states of the tryptophan

[5] Although the disulfide bonds do contribute a small amount to the CD spectra above 290 nm; in previous publications (Bewley and Li, 1971b, 1972) they have been ruled out as major contributors under the tryptophan bands in both HCS and HGH.

[6] T. A. Bewley and C. H. Li, unpublished results.

residues. We are not implying that the conformations of HCS and HGH are equivalent in 50% acetic acid with regard to other structural aspects. It may also be seen in Figure 1 that when dissolved in acetic acid, the absorptivities of both hormones at 277 nm are only slightly altered from those of the native proteins. Thus, in acetic acid solutions of up to 50%, the concentration of these proteins may be determined spectrophotometrically with an error of only 2–3% by using the extinction coefficients at 277 nm mentioned above.

Finally, the fluorescence emission spectra of these molecules (Figure 2) also indicate a difference in the local conformations around the tryptophan residues. It would appear that the tryptophan in HGH has a much higher quantum yield than the tryptophan in HCS. This relative quenching of the tryptophan fluorescence in HCS again indicates that there are some differences in microenvironments. One potential source of the relative quenching in HCS is the carboxyl group of a glutamic acid residue at position 83 (Li *et al.*, 1971). In HGH, position 83 is occupied by a glutamine residue (Li and Dixon, 1971). Of course, the quenching may also be caused by the proximity of some other group as a result of the three-dimensional folding of the molecule.

Chemical Reactivity toward o-Nitrophenylsulfenyl Chloride. As a more direct means of investigating the structural transition described above, the chemical reactivities of the tryptophan residues in HCS and HGH were evaluated in both 0.1 M sodium acetate (pH 4.0) and 50% acetic acid. The choice of the sodium acetate solvent was based on the need for a reaction medium in which the microenvironment of the tryptophan residues in both proteins would be essentially equivalent to that in their respective native states (defined in 0.1 M Tris-Cl buffer, pH 8.2), and in which NPS-Cl was sufficiently soluble to be effective. The ability of NPS-Cl to react quantitatively with exposed tryptophan residues in this solvent has been demonstrated.[7] The evidence presented herein demonstrates that no covalent modification occurs in either hormone in the acetate buffer. This strongly suggests that in native HCS and HGH the single tryptophan residue is buried within the interior of the molecule. Apparently however, the native molecule is able to bind NPS chromophore noncovalently. This binding can be reversed simply by treating the complex with 50% acetic acid (Figure 3). Similar noncovalent binding may account for the partial reactivity (0.4–0.5 mole of NPS/mole of HGH) seen in the reaction previously reported by Brovetto-Cruz and Li (1969) in 0.2 M sodium acetate (pH 4.0). The quantitative formation of NPS derivatives in 50% acetic acid directly demonstrates that one important aspect of the structural transition brought about in this solvent is the exposure of the previously buried tryptophan residue in both proteins. Thus, the results of chemical reactivity studies are in excellent agreement with the physical measurements.

Physical Characterization of NPS-HCS and NPS-HGH. Elution patterns from exclusion chromatography indicate that the monomer fractions of the 50% acetic acid control preparations and the NPS derivatives exhibit V_e/V_0 ratios that are identical with those of the native monomers. This would imply that no gross, irreversible changes in size or shape are caused by these treatments. Similarly, the fact that the two NPS derivatives are digested by trypsin at rates (Figure 6) only slightly greater than the native and control preparations further implies that these modified proteins still retain compact, rigid conformations (Markus, 1965).

We have attempted to evaluate the conformations of the NPS derivatives from their CD spectra, and to compare them with the conformations of the native proteins. This has been quite difficult since no information is presently available concerning the CD of NPS-tryptophan or NPS-proteins. We have approached this problem by investigating the CD spectra of two model compounds containing this chromophore which were synthesized in our laboratory. It is now apparent that considerably more effort must be put into such model compound studies before we can expect any clear understanding of the potential effects which modification by NPS-Cl may have on CD spectra. Due to the insolubility of NPS-L-tryptophan in aqueous media, its CD was measured in methanol. As shown in Table I, both NPS-HCS and NPS-HGH appear to have undergone a small change in secondary structure relative to the untreated proteins. However, a significant part of the apparent loss of α helix in both derivatives may be due to overlapping of their amide bond CD with the very strong, far-uv bands of the NPS-tryptophan chromophores. The presence of such bands is clearly shown in the CD of the model compounds. In addition, we must consider the possibility that these bands might be even stronger in NPS-HCS and NPS-HGH, due to more severely restricted rotation of the NPS-tryptophan chromophores in the large and highly structured proteins. The large ellipticity values exhibited by NPS-L-tryptophan indicate that free rotation must be considerably restricted even in this small molecule. The fact that the molecular ellipticities of the long-wavelength CD band in the NPS-proteins is 5–10 times more intense than the corresponding band in the model compounds, strongly suggests the presence of additional restrictions to rotation in these derivatives. These long-wavelength bands must certainly originate in electronic transitions of the NPS moiety and their behavior may be paralleled by some of the short-wavelength bands. It should be noted that the ellipticities of the model compounds are calculated as *molecular ellipticities* while α-helix contents are estimated from the mean residue ellipticities. Because of the low mole fraction of tryptophan in these proteins, the contribution of the short-wavelength bands of the NPS-indole chromophore, to the mean residue ellipticities of the proteins, would be about 1%. This would not significantly effect estimates of the α-helix contents. However, if these bands were ten times as intense in the protein derivatives as in the model compounds, then any estimate of the α-helix contents would be somewhat questionable. At the present time, we have no clear indication of what this contribution is. However, since the amide bond CD spectra of the protein derivatives show no apparent distortions in *shape* from those of the native hormones, and relatively small changes in intensity, we feel that the secondary structures of the NPS derivatives are not greatly different from those of the native proteins. A most intriguing relationship may be seen in the CD spectra between 300 and 250 nm. NPS-HCS exhibits only negative bands in this region while the bands in NPS-HGH are all positive. These opposite signs are reminiscent of the opposite signs in the tryptophan bands of the unmodified proteins. There is also a much more pronounced change in the spectrum of HGH after modification than is true for HCS. Since both

[7] It has been recently reported (Shechter *et al.*, 1972) that tryptophan-62 in hen egg-white lysozyme may be specifically and quantitatively sulfenylated by NPS-Cl in 0.1 M sodium acetate (pH 3.5). In our own laboratory (H. Kawauchi, T. A. Bewley, and C. H. Li, manuscript in preparation) we have demonstrated the specific and quantitative sulfenylation of one of the two tryptophan residue in ovine pituitary lactogenic hormone in 0.1 M sodium acetate (pH 4.0). This information, in addition to the previously reported reactivity of the tryptophan in α^{1-17}-ACTH (Brovetto-Cruz and Li, 1969) in 0.2 M sodium acetate (pH 4.0) clearly indicates the ability of NPS-Cl to react with exposed tryptophan residues under these conditions.

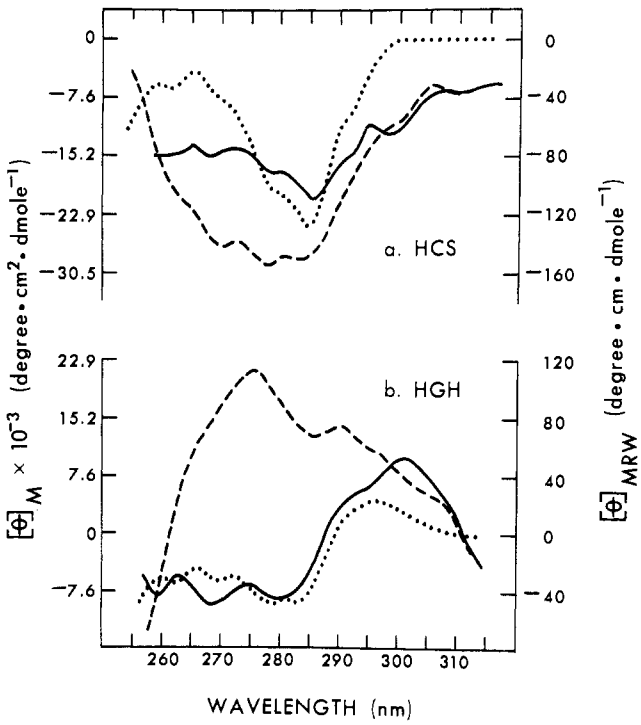

FIGURE 7: Difference circular dichroism spectra (——) in the region of side-chain absorption, produced by subtracting the inverse of the spectrum of NPS-L-tryptophan from the spectrum of NPS-HCS (a), and 2.2 times the spectrum (not inverted) of NPS-L-tryptophan from the spectrum of NPS-HGH (b). The spectra of the native proteins (· · ·) and the NPS derivatives (– –) are included for comparison.

proteins have been modified to the same extent in terms of the number of chromophoric groups added and neither appears to be grossly denatured, it would appear that the two NPS-tryptophan residues might be contributing very different amounts of ellipticity to their respective CD spectra. These differences would include both the sign of the ellipticities and the dichroic strength of the individual bands. If the presence of such differences can be substantiated, then we are justified in stating that the NPS-tryptophan chromophores are in nonequivalent microenvironments in the two derivatives. We have looked for evidence of such differences in the spectral region from 310 to 250 nm, by subtracting the CD of NPS-L-tryptophan from the CD of NPS-HCS and NPS-HGH. NPS-L-tryptophan in methanol may not be the best model for this purpose, but it appears to have nearly the same CD spectrum between 310 and 250 nm as NPS-Gln_5-α-MSH (Figure 4). Our reason for not using the latter compound was possible confusion with dichroism bands from the tyrosine and phenylalanine residues in this model. We have assumed that the NPS-tryptophan in HGH contributes positive bands, while the modified residue in HCS contributes negative bands, (obtained by inverting the CD of the model compound). In this procedure, sufficient ellipticity has been subtracted to regenerate the CD spectrum of the native proteins in the region dominated by the CD of tyrosine and phenylalanine residues. This is based on the assumption that these chromophores are equivalent in both the modified and unmodified proteins. Difference CD spectra, obtained by this method, are presented in Figure 7. By subtracting the inverted CD spectrum of NPS-tryptophan from the spectrum of NPS-HCS, peaks are obtained at 285 and 278 nm which appear to correspond quite closely to the bands attributed to the tyrosine residues in native HCS (Bewley and Li, 1971b). By contrast, the CD of NPS-L-tryptophan must be multiplied by a factor of 2.2 before subtraction from the spectrum of NPS-HGH, in order to properly regenerate the bands between 285 and 275 nm, attributed to tyrosine residues in this molecule (Bewley and Li, 1972). In this region of the spectrum, it would appear that the NPS-tryptophan residue in the HGH derivative contributes more than twice as much dichroism as the modified residue in NPS-HCS. This suggests that the NPS-tryptophan residue in the HGH derivative is confined within a local conformation which imposes a somewhat greater steric hindrance to rotation, thus making its dichroism more intense than the corresponding chromophore in NPS-HCS. The CD properties of additional compounds modified with NPS-Cl are now under investigation in our laboratory in the hope of obtaining better models for comparisons of this type.

Biological Activity. Biological assay data indicate that NPS-HCS is completely devoid of lactogenic activity as measured in the pigeon crop-sac assay. Moreover, it would appear that treatment of the native hormone with 50% acetic acid alone is able to reduce the lactogenic potency by about 50%. This latter result was rather unexpected in view of the demonstration of essentially complete reversability of the entire CD spectrum following perturbation by 50% acetic acid (Bewley and Li, 1971b). To date we have not found a satisfactory explanation for this effect, but we suspect that it is due to incomplete refolding upon reversal of the structural transition in this perturbant. Unfortunately, neither the CD spectrum nor the rate of tryptic digestion of this control preparation show any differences from the native hormone. As mentioned above, there is some evidence that incomplete refolding of NPS-HCS may leave the modified tryptophan residue in a more exposed and/or less sterically hindered condition than is true in the case of NPS-HGH. Perhaps the bulk of the NPS moiety prevents proper refolding in a local area of HCS that is crucial to biological activity. The retention of full lactogenic potency by NPS-HGH indicates that absolute molecular integrity of the tryptophan residue itself is not required for this activity. However, the incomplete "reburying" of the modified tryptophan in NPS-HCS, and the complete loss of lactogenic activity by this derivative may indicate that the *placement* of the tryptophan (or any modified form of it) in its proper place within the folded polypeptide is *crucial*, if only to allow the proper placement of other, more intrinsically necessary residues. The complete reversability of the CD spectra (Bewley and Li, 1972) and the retention of full biological potencies (Brovetto-Cruz and Li, 1969), after treatment of HGH with 50% acetic acid, points out the remarkable efficiency which this molecule displays in regaining its native structure following severe perturbation. The retention of full biological potency by NPS-HGH may be further evidence of this efficiency.

Acknowledgment

The authors thank Dr. P. Neri of the Sclavo Institute, Siena, Italy, for supplying the purified HCS. We also wish to thank J. D. Nelson for the bioassay as well as the National Pituitary Agency in Baltimore for the supply of fresh human pituitary glands.

References

Aloj, S., and Edelhoch, H. (1971), *J. Biol. Chem. 246*, 5047.
Aloj, S., and Edelhoch, H. (1972), *J. Biol. Chem. 247*, 1146.

Beavan, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Bewley, T. A., Brovetto-Cruz, J., and Li, C. H. (1969), *Biochemistry 8*, 4701.
Bewley, T. A., and Li, C. H. (1971a), *Experientia 27*, 1368.
Bewley, T. A., and Li, C. H. (1971b), *Arch. Biochem. Biophys. 144*, 589.
Bewley, T. A., and Li, C. H. (1972), *Biochemistry 11*, 884.
Beychok, S. (1966), *Science 154*, 1288.
Blake, J. J., Crooks, R. W., and Li, C. H. (1970), *Biochemistry 9*, 2071.
Brovetto-Cruz, J., and Li, C. H. (1969), *Biochemistry 8*, 4695.
Dayhoff, M. O. (1969), Atlas of Protein Sequence and Structure, Vol. 4, Silver Spring, Md., National Biomedical Research Foundation.
Grant, D. B., Kaplan, S. L., and Grumbach, M. M. (1971), *J. Clin. Endocrinol. 32*, 88.
Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M. (1949), *Endocrinology 45*, 455.
Herskovits, T. T., and Sorensen, M. (1968), *Biochemistry 7*, 2523.
Josimovich, J. B., and MacLaren, J. S. (1962), *Endocrinology 71*, 209.
Li, C. H. (1957), *J. Biol. Chem. 229*, 157.
Li, C. H. (1970), *Ann. Sclavo Inst. Siena 12*, 651.
Li, C. H., and Dixon, J. S. (1971), *Arch. Biochem. Biophys. 146*, 233.
Li, C. H., Dixon, J. S., and Chung, D. (1971), *Science 173*, 56.
Li, C. H., Liu, W. K., and Dixon, J. S. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 327.
Lyons, W. R. (1937), *Proc. Soc. Exp. Biol. 35*, 645.
Markus, G. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 253.
Nicoll, C. S. (1967), *Endocrinology 80*, 641.
Parikh, I., and Omenn, G. S. (1971), *Biochemistry 10*, 1173.
Perutz, M. F., Kendrew, J. C., and Watson, H. C. (1965), *J. Mol. Biol. 13*, 669.
Ramachandran, J. (1970), *Biochem. Biophys. Res. Commun. 41*, 353.
Scoffone, E., Fontana, A., and Rocchi, R. (1968), *Biochemistry 7*, 971.
Shechter, Y., Burstein, Y., and Patchornik, A. (1972), *Biochemistry 11*, 653.
Strickland, E. H., Horwitz, J., and Billups, C. (1969), *Biochemistry 8*, 3205.

Multiple Forms and Some Properties of Aminoacyltransferase I (Elongation Factor 1) from Rat Liver†

James F. Collins,* Hong Mo Moon,‡ and Elizabeth S. Maxwell

ABSTRACT: Elongation factor 1 (aminoacyltransferase I), the factor involved in binding aminoacyl-tRNA to ribosomes, has been extensively purified from rat liver. On a Sepharose 6B column the factor elutes in two peaks, one of which is larger and one smaller in molecular weight than elongation factor 2 (aminoacyltransferase II). The smaller is relatively unstable, but has been separated from elongation factor 2 and its enzymic activities partially characterized. The material eluting toward the front of the column contains active species of approximately 400,000 and 170,000 molecular weight, as determined on sucrose gradients. After further purification of the larger components through isoelectric focusing, there are two forms differing in isoelectric point, both of which are active in amino acid incorporation, binding of aminoacyl-tRNA to ribosomes, and ribosome-dependent GTP hydrolysis. Each of these can be shown to contain additional multiple forms by phosphocellulose chromatography and disc gel electrophoresis. Each is heterogeneous in size with a range of of 60,000–170,000 molecular weight indicating a decrease in size during purification. The most highly purified enzyme is catalytic in promoting amino acid incorporation and ribosome-dependent GTP hydrolysis, but probably not in binding aminoacyl-tRNA to ribosomes. Amino acid incorporating activity can be stimulated under certain conditions by pyruvate kinase and phosphoenolpyruvate. The binding activity of elongation factor 1 can be inhibited by elongation factor 2.

Polypeptide chain elongation in mammalian cells consists of three steps (Skogerson and Moldave, 1968a): the binding to the ribosome of the aminoacyl-tRNA corresponding to the codon being read, which binding is promoted by elongation factor 1 (aminoacyltransferase I)[1]; the formation of a peptide bond between the peptidyl chain in the donor site on the ribosome and the newly bound aminoacyl-tRNA, promoted by peptidyl transferase, a component of the large ribosomal subunit; and translocation of the newly lengthened peptidyl-

† From the Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received May 26, 1972.* Part of this work was presented in preliminary form at the 1971 meeting of the American Society of Biological Chemists and published in preliminary form; Collins *et al.* (1971a).

‡ Fellow in the Visiting Program, National Institutes of Health. Present Address: Roche Institute of Molecular Biology, Nutley, N. J. 07110.

[1] Abbreviations used are: GDPCP, the β–γ-methylene analog of GTP; EF-1, elongation factor 1 (transferase I, aminoacyltransferase I, T_1); EF-2 elongation factor 2 (transferase II, aminoacyltransferase II, T_2); p*I*, isoelectric point. In the uniform nomenclature recently proposed for the factors involved in protein synthesis, the factor formerly called aminoacyltransferase I was designated EF-1 for elongation factor 1 and aminoacyltransferase II EF-2 for elongation factor 2.