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Human pituitary growth hormone: Restoration of full biological activity by noncovalent interaction of two fragments of the hormone*

(growth-promoting activity/prolactin activity/circular dichroism/somatotropin)

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ABSTRACT **The NH_2-terminal 134 amino-acid fragment of the reduced-carbamidomethylated human somatotropin molecule is found to react noncovalently with the COOH-terminal 51 amino-acid fragment in solutions of pH 8.4 at 2° to restore full biological activity as evidenced by the rat tibia and pigeon crop-sac assays. In addition, circular dichroism spectra of the recombinant show the conformation to be completely repaired in comparison with that of the native hormone.**

The use of plasmin for the partial digestion of somatotropins has been described by Ellis *et al.* (1). Chrambach and Yadley (2) reported an 8-fold enhancement of somatotropin activity of human somatotropin (human growth hormone, HGH) produced by plasmin, at the 1970 Endocrine Society annual meeting. Later, Yadley and Chrambach (3) described in detail that digestion of HGH with plasmin leads to a progressive transformation of the hormone to various components of higher electrophoretic mobility and increased prolactin activity. Reagan *et al.* (4) and Mills *et al.* (5) reported that hydrolysis of HGH with plasmin did not impair metabolic effects in the rat or in man. A detailed chemical and physical characterization of the cleaved products of HGH by plasmin digestion was not given in any of these studies.

Li and Gráf (6) reported the isolation and characterization of two biologically active fragments from plasmin digests of HGH. It was shown that these two fragments, $Cys(Cam)^{53}$-HGH-(1-134) and $Cys(Cam)^{165,182,189}$-HGH-(141-191), (Cam is carbamidomethyl) were derived from cleavage of the Arg-Thr (positions 134–135) and the Lys-Gln (positions 140–141) bonds of the hormone molecule (see Fig. 1). The isolation and biological characterization of $Cys(Cam)^{53}$-HGH-(1-134) have also been described by Reagan *et al.* (7). Both peptide fragments possess immunoreactivity in complement fixation and radioimmunoassay experiments (8). In this paper, we report complementation experiments using these two fragments to obtain the recombinant with full growth-promoting and prolactin potency.

MATERIALS AND METHODS

Human somatotropin was isolated from fresh-frozen pituitary glands by the method of Li *et al.* (9). $Cys(Cam)^{53}$-HGH-(1-134) and $Cys(Cam)^{165,182,189}$-HGH-(141-191) were prepared from human plasmin digests of HGH as previously described (6), except that the NH_2-terminal fragment was further purified by gel filtration on Sephadex G-100 in 0.01 M NH_4HCO_3 at pH 8.2. Under these conditions, $Cys(Cam)^{53}$-HGH-(1-134) was soluble but appeared to be largely a trimer with relative elution volume V_e/V_0 of 1.67 and $s_{20,w}$ of 3.81 S (10). $Cys(Cam)^{165,182,189}$-HGH-(141-191) had poor solubility in solutions of pH 6–9.

Exclusion chromatography of the reaction mixture was carried out at room temperature on a 1.5 × 58 cm column of Sephadex G-100 in 0.1 M Tris·HCl buffer (pH 8.2). Elution in the ascending direction was provided at a flow rate of 4.9 ml/hr by an LKB model 10200 peristaltic pump. The protein solution was concentrated to approximately 1 ml by ultrafiltration using an Amicon UM-2 membrane prior to application onto the column. A continuous trace of the elution pattern was obtained with an LKB model Uvicord-II monitor.

Circular dichroism (CD) spectra were obtained on a Cary model 60 spectropolarimeter equipped with a model 6002 circular dichroism attachment according to procedures previously described (11). The concentrations of all solutions were determined spectrophotometrically using the relation $A^{0.1\%}_{1cm,277nm} = 0.931$. For simplicity this procedure was used regardless of whether the sample contained intact HGH modified by limited digestion with human plasmin (PL-HGH) or was a mixture of PL-HGH and its fragments. Spectra were calculated as mean residue ellipticities using 115 as the mean residue molecular weight in all cases. The contents of α-helix were estimated as previously described (12).

The growth-promoting activity was determined by the rat tibia test (13) and the prolactin activity by the local crop-sac assay in the pigeon (14, 15).

RESULTS

A mixture was prepared by adding 13.4 mg (0.87 μmol) of $Cys(Cam)^{53}$-HGH-(1-134) to 5.4 mg (0.89 μmol) of $Cys(Cam)^{165,182,189}$-HGH-(141-191) in 10 ml of Tris·HCl buffer at pH 8.4 [0.1 M, 2% (vol/vol) butanol]. The turbid solution was kept at room temperature (23°) for 5 hr and then transferred to the refrigerator (2°). After 240 hr, the solution appeared to have become clear.

The fragment mixture was submitted to exclusion chromatography on Sephadex G-100 and the elution pattern is shown in Fig. 2. Approximately 25% of the protein appeared in a distinct peak (designated fraction IV), with a V_e/V_0 ratio of 2.12. This is precisely the elution position of intact PL-HGH on this same column (18). It is especially noteworthy that no detectable protein was found at a V_e/V_0 ratio of 2.9–3.1, the elution position expected for the free COOH-terminal fragment (16).

Abbreviations: HGH, human somatotropin (growth hormone); PL-HGH, HGH modified by limited digestion with human plasmin; Cam, carbamidomethyl; CD, circular dichroism.

* Paper 48 of the Human Pituitary Growth Hormone Series. Paper 47 is ref. 16.

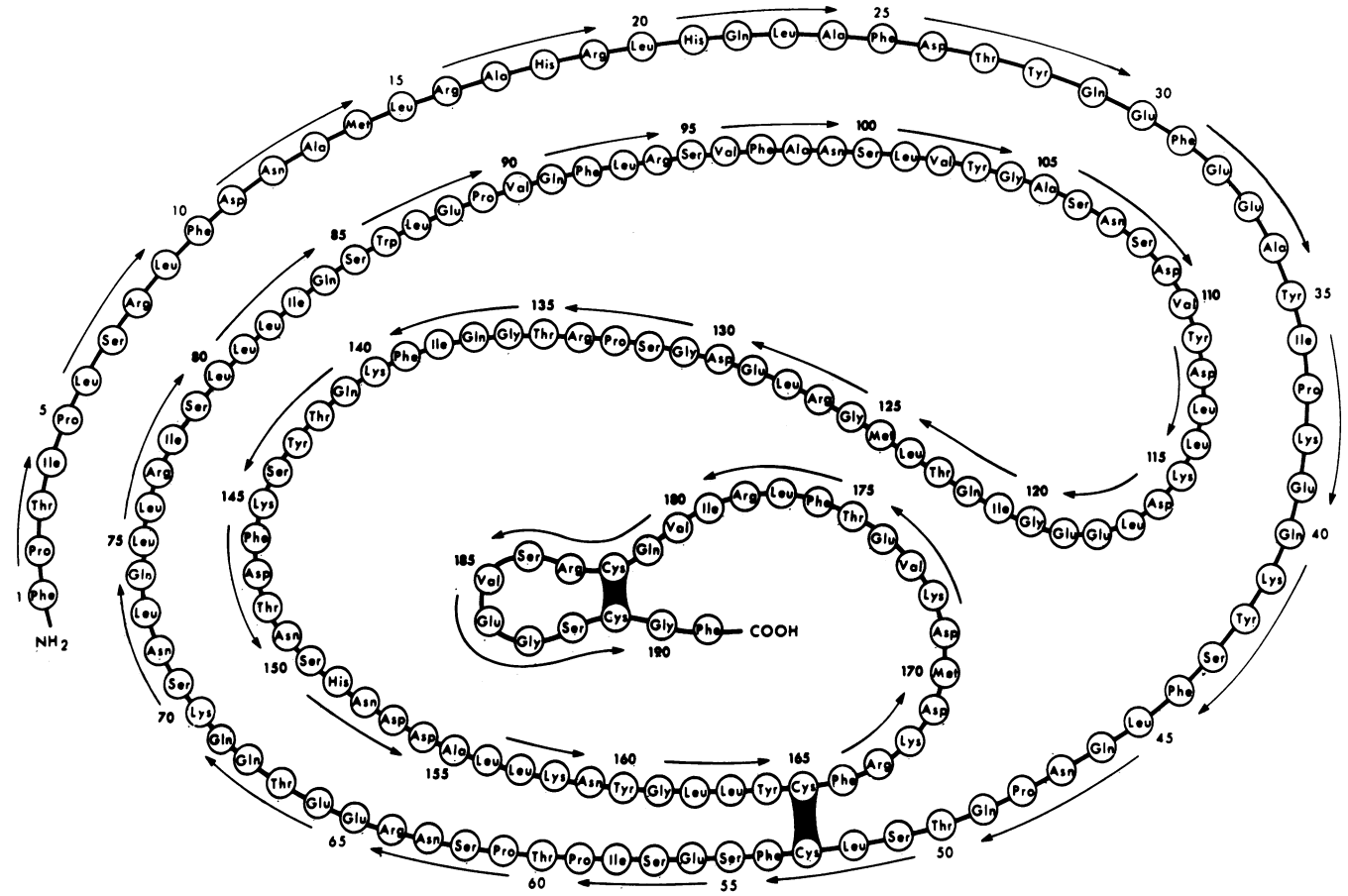

FIG. 1. Amino acid sequence of the HGH molecule.

The CD spectra of the fragment mixture before purification by exclusion chromatography are shown in Fig. 3. The interesting feature of this spectrum is the appearance of a weak negative band around 298 nm. This band has previously been assigned to the Trp-86 residue in the NH_2-terminal fragment (16). The negative sign of this indole dichroism is characteristic of the conformation of the free fragment and is in sharp contrast to the positive indole dichroism at 292 nm, characteristic of the conformation of intact reduced-alkylated PL-HGH or native HGH (16). However, it can be seen (Fig. 3) that in the spectrum of this incubation mixture, the negative band is not as intense as it would be in a pure sample of the NH_2-terminal fragment, suggesting that the mixture contains overlapping contributions from both positively and negatively dichroic indole groups. The CD spectra of this same sample in the far-UV region indicate an average α-helix content of 45–50%.

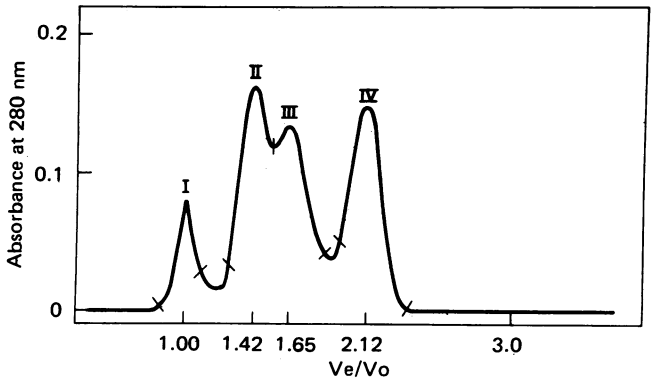

FIG. 2. Exclusion chromatography of the fragment mixture on a Sephadex G-100 column (1.5 × 58 cm) in 0.1 M Tris·HCl buffer (pH 8.2). Fraction IV was immediately concentrated by ultrafiltration and stored in the cold without lyophilization.

The CD spectra of the purified recombinant (fraction IV, Fig. 2) are also shown in Fig. 3 and may be compared with the spectrum of a freshly thawed, undissociated sample of intact, reduced-carbamidomethylated PL-HGH. As can be seen, the spectrum of this material in the region of side-chain absorption is not significantly different from that of intact reduced-carbamidomethylated PL-HGH. The complete equivalence of the positive indole dichroism at 292 nm in these two samples is particularly noteworthy. The far-UV spectrum of fraction IV indicates that this material possibly contains a slightly higher content of α-helix ($\approx$60%) than either reduced-carbamidomethylated PL-HGH or native HGH (16, 12).

It may be seen in Table 1 that the solution containing the two fragments before purification had restored growth-promoting activity from 8 to 60% when compared with the potency of the 134 residue fragment in the tibia test. At the dose tested, the potency of the 51 residue fragment was almost nil. The regeneration of lactogenic activity was also evident in the pigeon crop-sac assay as shown in Table 1.

The biological activities of the purified recombinant (fraction IV, Fig. 2) are summarized in Table 2. In comparison with the activities of PL-HGH, the recombinant had full growth-promoting and prolactin potency. It is significant that the prolactin activity of the purified recombinant is re-

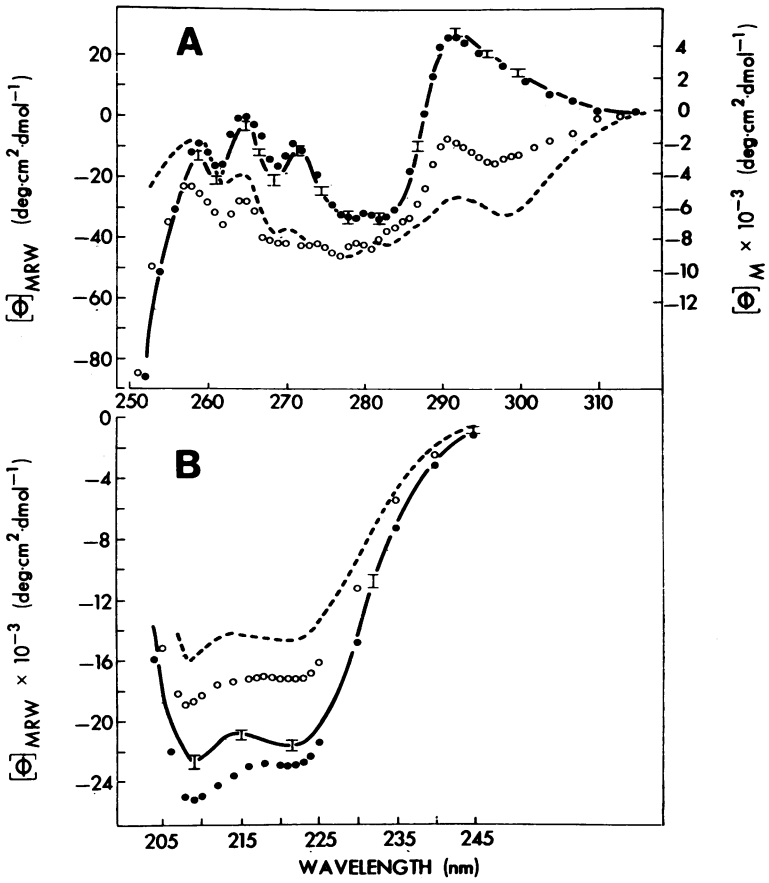

FIG. 3. Circular dichroism spectra in the region of side-chain absorption (A), and amide bond absorption (B) of: the unfractionated fragment mixture after incubation (○), fraction IV (●), 1-134 (- - -). The CD spectra of an undissociated sample of reduced-carbamidomethylated-PL-HGH are included for comparison (——). The vertical bars indicate typical values at selected wavelengths for the standard error of the mean obtained from ten spectra of PL-HGH. Both molar (M) and mean residue weight (MRW) ellipticity (Θ) scales are used.

stored from less than 1 to 100% of PL-HGH potency as seen in Tables 1 and 2.

DISCUSSION

HGH is a globular protein with a molecular weight of 22,000 (17) and α-helical content of 55% (12). Reduction and alkylation of the two disulfide bridges in the presence or absence of denaturants cause no alteration of biological activity (18, 19). Following reduction of the disulfide bridges, the HGH molecule is readily reoxidized to the native hormone (20). Removal of a hexapeptide (residues 135–140; Fig. 1) by human plasmin does not change the biological po-

Table 1. Prolactin and growth-promoting activities of the unpurified recombinant and the fragments of HGH

Preparation	Pigeon crop-sac assay		Rat tibia assay	
	Total dose (nmol)	Response*	Total dose (nmol)	Response†
PL-HGH	0.093	24.6 ± 0.8	0.93	241.0 ± 2.4
	0.279	28.8 ± 1.7	2.79	267.2 ± 7.7
Recombinant	0.48‡,§	16.8 ± 0.7	2.27**,††	252.5 ± 10.2
	1.44	20.7 ± 1.4	6.81	281.2 ± 6.4
Cys(Cam)^{53}-HGH-(1-134)	0.65¶	9.6 ± 1.8	3.26‡‡	207.2 ± 1.5
	1.95	12.4 ± 2.7	9.78	235.5 ± 1.5
Cys(Cam)^{165,182,189}-HGH-(141-191)	20.3	13.4 ± 1.2	9.78	186.7 ± 4.4
Saline	0	10.5 ± 0.2	0	176.5 ± 4.5

* Dry mucosal weight in milligrams; mean ± SEM; four birds in each group.
† Tibia width in micrometers; mean ± SEM; four animals in each group.
‡ Relative potency to PL-HGH, 23% with a confidence limit of 0.3–62 and λ = 0.27.
§ Relative potency to Cys(Cam)^{53}-HGH-(1-134), approximately 947% with λ = 0.46.
¶ Relative potency to PL-HGH, less than 1%.
** Relative potency to PL-HGH, 60% with a confidence limit of 40–153 and λ = 0.24.
†† Relative potency to Cys(Cam)^{53}-HGH-(1-134), 828% with a confidence limit of 441–3077 and λ = 0.17.
‡‡ Relative potency to PL-HGH, 8% with a confidence limit of 4–12 and λ = 0.12.

Table 2. Prolactin and growth-promoting activities of the recombinant obtained by noncovalent interaction of the two fragments of HGH

Preparation	Pigeon crop-sac assay		Rat tibia assay	
	Total dose (nmol)	Response*	Total dose (nmol)	Response†
PL-HGH	0.093	18.5 ± 0.7	0.47	216.8 ± 2.6
	0.279	25.0 ± 2.1	1.40	261.3 ± 2.2
Recombinant‡	0.093§	19.2 ± 0.8	0.47¶	223.8 ± 10.8
	0.279	24.6 ± 0.8	1.40	257.8 ± 10.4
Saline	0	10.7 ± 0.2	0	155.3 ± 4.7

* Dry mucosal weight in milligrams; mean ± SEM; four birds in each group.
† Tibia width in micrometers; mean ± SEM; four animals in each group.
‡ Fraction IV, see Fig. 2.
§ Relative potency to PL-HGH, 102% with 95% confidence limit of 59–176 and $\lambda = 0.22$.
¶ Relative potency to PL-HGH, 108% with 95% confidence limit of 70–158 and $\lambda = 0.15$.

tency of HGH (6, 7). Reduction and carbamidomethylation of PL-HGH gave rise to two peptide fragments: Cys(Cam)^{53}-HGH-(1-134) and Cys(Cam)^{165,182,189}-HGH-(141-191). Both fragments possess biological activity but only 0.3–14% potency in comparison with HGH (6, 21). A combination of these two fragments in experiments herein described regenerates full biological potency of the hormone as indicated in Table 2. In addition, the recombinant exhibits complete recovery of immunoreactivity by radioimmunoassay system (C. H. Li and T. Hayashida, unpublished observations). It is important to note that the CD spectra (Fig. 3) and elution position (Fig. 2) of the recombinant are identical to those of the native hormone. Apparently the three-dimensional structure of the HGH molecule can be restored to its original conformation by a noncovalent interaction of the NH_2-terminal 134-residue fragment with the COOH-terminal 51-residue fragment.

Preliminary experiments using the synthetic (22) COOH-terminal fragment Cys(Cam)^{165,182,189}-HGH-(141-191) indicate that biological potency can be restored by mixing the synthetic material with the natural NH_2-terminal 134-residue fragment as revealed by both tibia test and pigeon crop-sac assay. The minimal size of each fragment required for the complementation reaction to generate HGH activity remains to be determined.

It is known (23, 24) that peptide fragments derived from native enzymes are capable of recombination by noncovalent interaction to regenerate enzymic activity. As far as we are aware, this report is the first to describe a case in which two peptide fragments from a protein hormone complement to restore full biological potency. It should also be noted that the complementation reaction herein described was performed using two peptide fragments which were derived from reduction and alkylation of disulfide bridges in the plasmin-modified molecule.

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