Human Somatotropin

SELECTIVE REMOVAL WITH TRYPsin OF RESIDUES 135-145 FROM THE HORMONE MOLECULE WITH NO LOSS OF BIOLOGICAL ACTIVITIES

Laszlo Graff, Choh Hao Li, and Michael D. Jibson

From the Hormone Research Laboratory, University of California, San Francisco, California 94143

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A 11-residue segment, sequence positions 135-145, has been cleaved selectively from the human somatotropin molecule by limited digestion of the hormone with trypsin at pH 6.0 in 90% (v/v) glycerol. Differences between the tertiary structures of the native and trypsin-modified hormones have been demonstrated by circular dichroism spectroscopy. Despite these conformational differences, the biological properties of intact hormone and its modified form appeared to be very similar, as tested by two receptor-binding assays, radioimmunoassay, and the rat tibia test.

The exclusive specificity of thrombin toward the Arg134-Thr135 peptide bond of HGH (1) as first reported by Graf et al. (1) has directed our interest to the possibility that thrombin or trypsin may be applied to reconstitute the intact polypeptide chain of the hormone from the 134- and 57-residue thrombin fragments of reduced carbamidomethylated HGH. Using the noncovalent recombinant of the latter fragments (2) as a substrate and conditions known to shift the peptide bond hydrolysis equilibrium toward syntheses (3), significant resynthesis could be achieved with thrombin (5). Under the same conditions, however, trypsin has been found to split some peptide bond(s) of the COOH-terminal segment of residues 135-191. This observation initiated further studies on the limited hydrolysis of HGH with trypsin at pH 6.0 in 90% (v/v) glycerol, conditions generally used for enzymic resynthesis of some peptide bonds rather than proteolytic cleavages of a polypeptide chain. Results of these investigations are described herein.

MATERIALS AND METHODS

HGH was isolated from fresh frozen pituitary glands as described previously (6). Trypsin was obtained from Calbiochem. The digestion of HGH with trypsin was carried out as follows. HGH (25 mg) was dissolved in 0.5 M ammonium acetate of pH 6.0 (1.0 ml) and glycerol (11.8 ml) was slowly added with stirring. Trypsin (0.2 ml of a 6 mg/ml solution) was then added. After the solution was kept at room temperature for 10 days, 1 ml of glacial acetic acid was added and the mixture dialyzed extensively against 5% acetic acid. The dialyzed solution was lyophilized and yielded 20.5 mg of protein which was subjected to gel filtration on a Sephadex G-50 column (0.9 × 72 cm) in 10% acetic acid. The fractions eluted were assayed for trypsin-like enzyme activity with a synthetic substrate, Z-lys-Pro-Arg-pNA (7). The small amount of contaminating trypsin determined in the main fraction was completely blocked by adding 2% (w/v) lima bean trypsin inhibitor ( Worthington), before studying the fraction for conformational and biological properties.

The disulfide bonds of trypsin-modified HGH were reduced with 20-fold molar excess of dithiothreitol over the cysteine content in 0.05 M NH4HCO3 of pH 8.3 containing 8 M urea under N2 for 1 h. Alkylation was carried out by adding iodosaceticamide (Calbiochem-Behring) in 20-fold molar excess to dithiothreitol. Excess reagents and urea were removed from the mixture by chromatography on Sephadex G-16 in 0.05 M NH4HCO3 of pH 8.7. The tryptic fragments of reduced-carbamidomethylated HGH were separated by gel filtration on a Sephadex G-50 column (0.9 × 72 cm) in 10% acetic acid.

Sodium dodecyl sulfate gel electrophoresis was performed according to Swank and Munkres (8) with the following modifications. The acrylicamide concentration of the gel was 10% (w/v) and the acrylamide to N,N'-methylenebisacrylamide ratio was 29:1 (w/w). Reduction of the disulfide containing proteins was performed in the presence of 2% (w/v) dithiothreitol (pH 7) at 37 °C for 1 h prior to application to the gels. Digestion with carboxypeptidase A ( Worthington) was carried out in 0.1 M NaHCO3 with an enzyme to substrate ratio of 1:10 (w/w) at 37 °C for 6 h. Ammoniacal compositions of the carboxypeptidase and acid (6 M HCl, 110 °C, 22 h) hydrolysates of the peptides were determined in an automatic amino acid analyzer (Model 119A, Beckman Instruments) by the procedure of Spackman et al. (9). NH2-terminal residue analysis was performed by the dansyl method (10, 11).

Circular dichroism spectra were taken with a Cary 60 spectropolarimeter equipped with a Model 6002 attachment. Sample concentration was 0.4 mg/ml, the path length was 0.1 cm or 1.0 cm, the temperature was maintained at 27 °C, and the dynode voltage did not exceed 600 V. The sample was dissolved in 0.1 M Tris-HCl buffer of pH 8.2. Molecular weight and the mean residue weight of modified HGH were determined from the known primary structure. Spectra are reported in terms of molar ellipticity in the near UV region (above 250 nm) and mean residue ellipticity in the far UV region (below 250 nm). Protein concentration was determined spectrophotometrically with a Perkin-Elmer Model 552 spectrophotometer. A correction for light scattering was made according to the method of Leach and Scheraga (12) from optical density values between 360 and 320 nm. Absorption values were estimated as described (13).

The receptor-binding activities were assayed on liver (14), and mammary gland membranes prepared from late pregnant rabbits (15), using iodinated HGH and sheep prolactin ligands, as described (16). For radioimmunoassay, the double antibody technique (17) was applied by using a rabbit antiserum against HGH in a final dilution of 1/50,000. Iodination of HGH was carried out by the lactoperoxidase method (18). The growth-promoting activity was determined by the rat tibia test (19).

RESULTS

The action of trypsin on HGH at pH 6.0 and in 90% (v/v) glycerol was assessed by SDS-gel electrophoresis. While no significant difference was seen between intact and trypsin-treated HGH when the samples were run in the absence of dithiothreitol, SDS-gel electrophoresis of a reduced sample of
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Trypsin-treated HGH clearly indicated that some peptide bond(s), most likely within the large disulfide loop of the molecule, had been hydrolyzed (Fig. 2). After the removal of some aggregated proteins from the digest by gel filtration on a Sephadex G-50 column in 10% acetic acid (Fig. 3), fraction B eluted at the position of native HGH was further characterized. To identify the peptide bonds cleaved by trypsin, fraction B was reduced and carboxamidomethylated and applied to the same Sephadex G-50 column in 10% acetic acid (Fig. 4). The two fractions obtained, N and C, were shown to be homogeneous by SDS-gel electrophoresis and NH₂-terminal residue analyses. Both fragments had only phenylalanine as NH₂-terminal residue. By carboxypeptidase A digestion, COOH-terminal sequence -Gly-Phe-COOH was identified for fragment C, whereas fragment N was resistant to the exopeptidase action. These data together with the amino acid compositions of the two reduced-alkylated fragments and fraction B (Table I) provided evidence that the predominant action of trypsin on HGH is the cleavage of the Arg₁⁴-Thr₁⁵ and Lys₁⁴-Phe₄⁶ peptide bonds (see Fig. 1).

Fig. 1. Amino acid sequence of human somatotropin. Taken from Ref. 30.

Fig. 2. SDS-gel electrophoresis of HGH and trypsin-modified HGH (Tr-HGH) in the absence and presence of dithiothreitol (DTT). The 10-µg protein samples were applied to the gels.

Fig. 3. Chromatography of trypsin-modified HGH (10 mg) on a Sephadex G-50 column (0.9 x 72 cm) in 10% acetic acid. Peaks A and B were pooled as shown, lyophilized, and yielded 1.8 mg and 6.5 mg protein, respectively. SDS-gel electrophoretic patterns (obtained in the absence of dithiothreitol) are also shown.

Fig. 4. Chromatography of reduced-carboxamidomethylated fraction B (4.0 mg) on the same Sephadex G-50 column in 10% acetic acid. Fractions N and C yielded 2.2 mg and 0.8 mg, respectively. SDS-gel electrophoretic patterns of both fractions are also shown.

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<thead>
<tr>
<th>Amino acid compositions of trypsin-modified HGH and the two reduced-carboxamidomethylated fragments</th>
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<tr>
<td>Amino acid</td>
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<tr>
<td>Carboxymethylcysteine</td>
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<td>Arginine</td>
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* Molar ratios.
* See Fig. 3.
* Theoretical values taken from the HGH sequence (30).
* See Fig. 4.
The CD spectrum of this two-chain form of HGH, HGH-(1-134)-(146-191), in the region of side-chain absorption, showed a marked loss of ellipticity centered at 292 nm as compared to the spectra of HGH, thrombin-nicked HGH (Fig. 5) and plasmin-modified HGH (21). The reproducibility of the CD spectra, in the region of side-chain absorption, is clearly indicated by the fact that the curves obtained in different studies for HGH, thrombin-nicked HGH (20), and plasmin-modified HGH (15, 21) match each other. The positive band at 292 nm in the spectra of HGH, thrombin-nicked and plasmin-modified HGH, but missing in that of HGH-(1-134)-(146-191), may be assigned to tryptophan. An increase in intensity in the negative maxima between 265 and 285 nm may also be noted for trypsin-modified HGH. This change may be due to alteration of the local environments of tyrosine residues, changes in disulfide bonds, or may be related to the loss of indole dichroism. It may be recalled that similar differences were noted by Farmer et al. (22) between the CD spectra of the stored and secreted forms of rat prolactin. The far UV CD spectra of native, thrombin- and trypsin-modified HGH are indistinguishable indicating that the loss of residues 135-145 had no effect on the α-helix content (Fig. 5).

While the trypsin-modified HGH had practically the same activity as native HGH when tested for binding to rabbit liver membranes (Table II), the binding assay with mammary gland membranes indicated a slightly increased activity for HGH-(1-134)-(146-191) (Table II). In radioimmunoassay, it gave 73% crossreaction with the HGH antibody (Table II). The tibia assay data did not reveal significant difference between the in vivo biological potencies of HGH and its trypsin-modified form (Table III).

**DISCUSSION**

After plasmin was found to split preferentially the Arg'34-Thr'35 peptide bond of HGH-(1-134)-(146-191), it was suggested that other proteinases, like bacterial fibrinolysin (25), thrombin (1, 26) and subtilisin (27) have also been reported to produce selective cleavages between residues 134 and 150 of the HGH structure. As described herein, selective cleavages of this susceptible region can be accomplished with trypsin, using conditions unfavorable for peptide bond hydrolysis. All these proteolytically modified forms of HGH, including a cleaved form isolated from pituitary extracts and designated as α3 by Singh et al. (28), consist of two polypeptide chains connected by a disulfide bond between Cys'20 and Cys'100. They lack, however, different sequence portions of the HGH structure as summarized in Table IV.

None of the two-chain forms of HGH listed in Table IV has been reported to have decreased biological potencies as compared to those of native HGH. This would seem to imply that residues 135-146 do not have an essential role in either interacting with the receptors or maintaining the biologically important features of the HGH conformation. Supporting this view, CD studies on the thrombin-(20) and plasmin-modified forms of HGH (21) have shown that neither the cleavage of the Arg'34-Thr'35 peptide bond nor the loss of the hexapeptide, residues 135-140, from the HGH structure cause any detectable conformational change. Significant changes of the near UV CD spectrum of HGH have been observed, however, in response to the removal of a larger portion, residues 135-145, of the molecule by limited trypptic digestion (Fig. 5). These data indicate that the contribution of residues 135-145 to the surface topography of the HGH molecule is not uniform. While residues 141-145 affect the environment of the single tryptophan residue at position 86, possibly by direct interaction, such as burial of the indole group or simply hindrance of...
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Table IV

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Two-chain forms of HGH</th>
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<tr>
<td>Thrombin-modified</td>
<td>(Gráf et al., Ref. 1) 134 135-Pro-Arg-Thr-Gly-Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe-Asp-</td>
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<tr>
<td>Plasmin-modified</td>
<td>(Li and Gráf, Ref. 23) 134-Pro-Arg-Gln-Thr-Tyr-Ser-Lys-Phe-Asp-</td>
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<tr>
<td>Trypsin-modified</td>
<td>(this study) 134-Pro-Arg-Gly-Asp-</td>
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<tr>
<td>HGH-α2</td>
<td>(Singh et al., Ref. 28) 134-Pro-Arg-Thr-Gly-Asp-</td>
</tr>
<tr>
<td>Subtilisin-modified</td>
<td>(Lewis et al., Ref. 27) 134-Pro-Arg-Thr-Gly-Asp-</td>
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Fig. 6. Schematic representation of the location of residues 133-147 with the folded structure of HGH.

In view of the conformational difference between native HGH and HGH (1-134)-(146-191), it has been of utmost interest to compare their biological properties in different in vitro and in vivo assay systems. The more so, because the structure of HGH (1-134)-(146-191) is very similar to those of HGH-α2 (28) and a subtilisin-modified form of HGH (27) (also see Table IV), two modified HGHs reported to have enhanced growth-promoting activity. The bioassay data (Tables II and III) do not seem to indicate any significant difference between the native hormone and trypsin-modified HGH. The lack of an increase of growth-promoting activity of HGH in response to the removal of residues 135-145 (see Table III) does not necessarily contradict the proposal on the enhancement of the biological activity of HGH by enzymatic modifications (27, 29). Of the two subtilisin-modified forms of HGH, both lacking residues 140-146 (see Table IV), the one containing aspartic acid instead of asparagine at residue position 182 (see Fig. 1) was found to be more active than native HGH (27, 29). Thus, it is possible that in addition to clipping of the HGH molecule at certain positions, the deamidation of Asn152 is also necessary for an enhancement of growth-promoting activity. The exact mechanism of these modifications, their conformational and biological consequences and physiological significance, however, will require further investigations.

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REFERENCES

Selecting Cleavage of HGH by Trypsin

Human somatotropin. Selective removal with trypsin of residues 135-145 from the hormone molecule with no loss of biological activities.
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