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Research Article

Proteolysis of Human Growth Hormone by Rat Thyroid Gland *in Vitro:* Application of Electrospray Mass Spectrometry and N-Terminal Sequencing to Elucidate a Metabolic Pathway

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The present studies were designed to provide structural characterization of peptide metabolites of biosynthetic human growth hormone (hGH) formed by rat thyroid gland proteases *in vitro*. Electrospray ionization mass/spectrometry (ESI-MS) and N-terminal sequencing were used to characterize the peptide metabolites. The predominant enzyme in the thyroid gland preparations was a chymotrypsin-like serine protease which was biochemically similar to rat mast cell protease-I. Metabolic intermediates were formed by cleavage of hGH exclusively at Tyr/Phe/Leu-Xaa bonds. After a 5- or 45-min incubation of hGH with thyroid gland S9 pellet fraction, the majority of metabolites formed were two-chain variants of hGH having masses ranging from 16,002 to 22,143 Da. These metabolites were formed as a result of proteolysis in the large disulfide loop region of hGH in combination with processing at Tyr^{42}–Ser^{43}. Based upon the time-related appearance and structural characterization of these intermediates, a sequence of metabolic events is proposed. The initial event appears to be cleavage by the chymotrypsin-like protease between Tyr^{143}–Ser^{144} to form a two-chain hGH. This intermediate is then cleaved between Tyr^{42}–Ser^{43}, liberating the N-terminal peptide, Phe^{1}–Tyr^{42}. Two other metabolites were generated as a result of the deletion of the peptides Lys^{140}–Tyr^{143} and Ser^{144}–Phe^{146} from the large loop region. The identification of similar metabolites truncated by a single amino acid at the carboxyl terminus indicated the action of a carboxypeptidase on these metabolic products. After a 4.5-hr incubation, the protease isolated from the S9 pellet fraction degraded hGH to >20 small peptides, having masses ≤2300 Da. The data illustrate the utility of combining ESI-MS and N-terminal sequencing in the study of protein metabolism and the enzymatic pathways involved.

KEY WORDS: human growth hormone; protease; mass spectrometry; metabolism.

INTRODUCTION

In the pituitary gland, human growth hormone (hGH)[5] has been recognized to exist as a family of related proteins. These different "forms" of hGH appear to arise from the processing of the primary hGH transcript and posttranslational processing events (1,2). Variants of hGH which have been described in the pituitary include the "20K variant," lacking amino acids 22–36 from the N-terminal region of the molecule (3), proteolytically clipped two-chain forms (4,5), deamidated forms (6), and hGH aggregates (7). These modifications have been suggested to be related to the diverse physiological activities of hGH (8).

Our laboratory has been investigating the metabolic processing of hGH by rat tissues *in vitro* and *in vivo*. Although exhibiting stability to degradation in rat liver and kidney homogenates, hGH was proteolytically processed by a chymotrypsin-like serine protease in rat thyroid gland preparations (9). We identified the sites in hGH sensitive to cleavage by this proteolytic activity and provided evidence for the formation of several two-chain forms of hGH. These findings were consistent with other studies providing evidence for the formation of "clipped" two-chain forms of hGH and prolactin by membrane or subcellular fractions *in vitro* (10–12). The exact structural identity of these forms has not been elucidated. The characterization of such metabolic intermediates may have biological relevance related to the activation, alteration, or termination of the function of these hormones in target or nontarget tissues. Modifications in the

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[5] *Abbreviations used:* hGH, human growth hormone; ESI-MS, electrospray ionization mass spectrometry; RMCP, rat mast cell protease; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; CNBr, cyanogen bromide.

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N-terminal and large disulfide loop regions of hGH have been shown to alter the specificity of hGH for the growth hormone receptor (13,14). *In vivo,* variants of hGH have altered pharmacokinetic properties compared to hGH (9,15,16) and also possess pharmacological activity (17,18). The present study was undertaken to identify the metabolites of hGH formed by rat thyroid gland *in vitro,* using a combination of N-terminal sequencing and electrospray mass spectrometry. The results demonstrate the utility of this approach in defining metabolic pathways of protein metabolism.

MATERIALS AND METHODS

Materials. Biosynthetic human growth hormone (hGH; lot No. 222HG6) was produced as a recombinant protein by Eli Lilly and Co. hGH was iodinated by the lactoperoxidase technique (19) and purified by gel filtration chromatography. Rat thyroid glands were purchased from Pel-Freez biologicals (Rogers, AK). Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia (Piscataway, NJ). Potato chymotrypsin inhibitor was from Cal Biochem (La Jolla, CA).

Tissue Preparation. Rat thyroid glands (10–20 glands) were polytron homogenized in 50 m*M* sodium phosphate, 1.15% KCl (pH 7.2, buffer A). Homogenates were sedimented at 700*g* for 2 min, and the resulting supernatant was sedimented at 9000*g* for 30 min at 4°C in an Eppendorf microfuge. The pellet fraction (S9 pellet) was then resuspended with a polytron in 50 m*M* sodium phosphate, 0.75 *M* KCl (pH 7.2; buffer B). Protein concentrations were determined by the method of Bradford (20), using BSA as standard.

Extended Incubation Studies. hGH was dissolved in buffer A (1 μg/μL) and incubated with protease activity isolated from the S9 pellet fraction (see below) at a final concentration of 2.25×10^{-5} *M* for 4.5 hr at 37°C, in a total volume of 200 μL. hGH peptides in the reaction mixture were separated using reversed-phase HPLC system I. Peptides were collected in 0.5-mL fractions, divided in half, concentrated to dryness in a Speed-Vac, and stored at −20°C until analysis by N-terminal sequencing and ESI-MS.

Limited Digestion Studies. hGH was dissolved in buffer A to give a final concentration of 2 μg/μL. One hundred fifty micrograms of hGH (2.25×10^{-5} *M*) was incubated with the S9 pellet fraction (0.43 mg/mL) for 5 or 45 min at 37°C, in a total volume of 300 μL. The reaction mixture was sedimented at 16,000*g* for 5 min and the entire volume was injected onto RP-HPLC system II for separation of hGH peptides. Peptides, detected at 220 nm, were collected in 0.5-mL fractions, concentrated to dryness in a Speed-Vac, and stored at −20°C. The peptides were resuspended in 400 μL of 10% acetonitrile/water/0.5% TFA, and 200-μL samples were fractionated using RP-HPLC system III (twice). Peptides were collected in 0.5-mL fractions, aliquoted into three portions, and concentrated to dryness in a Speed-Vac. Peptide fractions were stored at −20°C until analysis by N-terminal sequencing and ESI-MS.

Reduction and Carboxymethylation of Peptides. The structures of several of the putative two-chain hGH metabolites were confirmed by ESI-MS after reduction and carboxymethylation of the individual chains (21). Isolated peptides were resuspended in 20 μL of 50 m*M* Tris–HCl (pH 8.0) and 170 μL of 7 *M* deionized urea and 20 μg of dithiothreitol was added. The reaction was allowed to proceed at room temperature for 60 min. Iodoacetic acid (20 μg) was then added and the reaction was incubated at room temperature in the dark for 15 min. Carboxymethylated peptides were then separated using RP-HPLC system III, collected, and concentrated to dryness in the Speed-Vac. Peptides were stored at −20°C until ESI-MS analysis.

Reversed-Phase HPLC System I. Peptides formed during the extended incubation studies were applied to an Applied Biosystems Aquapore RP-300 (4.6 × 250 mm) column equipped with a guard column of the same packing material. Peptides, detected at 220 nm, were eluted at 1.1 mL/min with a linear gradient of 0–50% B in 50 min. Solvent A was water/0.1% TFA; solvent B was acetonitrile/0.1% TFA.

Reversed-Phase HPLC System II. Peptides formed during the limited digestion studies were applied to an Aquapore butyl (7.0 × 250 mm) column equipped with a guard column of the same packing material. Peptides, detected at 220 nm, were eluted with a two-step gradient of 15–55% B in 35 min and 55–60% B in 5 min at 1.5 mL/min. Solvent A was acetonitrile/30 m*M* NH_4HCO_3 (pH 7.8)/H_2O (10/55/35; v/v/v); solvent B was acetonitrile/30 m*M* NH_4HCO_3 (pH 7.8)/H_2O (70/20/10; v/v/v).

Reversed-Phase HPLC System III. Metabolic products isolated by RP-HPLC system II were further purified on an Aquapore RP-300 (4.6 × 250 mm) column equipped with a guard column of the same packing material. Peptides, detected at 220 nm, were eluted with a linear gradient of 5–75% B in 60 min at 1.1 mL/min. Solvent A was H_2O/0.1% TFA; solvent B was acetonitrile/0.1% TFA.

Electrospray Ionization/Mass Spectrometry (ESI-MS). ESI-MS was performed on a Finnigan MAT TSQ700 quadrupole mass spectrometer (22). Peptide samples were dissolved in 50 μL of acetonitrile/water (50:50; v/v) containing 2.5% acetic acid. Dissolved peptides were infused into the ESI source at 1 μL/min using a syringe infusion pump (Harvard Apparatus). An optimum voltage of −3800 V was maintained on the ESI-MS electrode in order to form the multiply protonated ions of the peptides. The mass spectrometer was scanned from 500 to 2000 mass/charge units in 5 sec, and 12 scans were averaged to obtain the mass spectra. Finnigan's deconvolution algorithm (23) was utilized to interpret the ESI-mass spectra to yield a mass spectrum indicating the molecular weight(s) of the protein sample. Experimental mass values of the peptides are expressed to reflect the variation in mass values determined from different charge states of the molecule.

N-Terminal Sequencing. N-terminal sequences of the isolated peptides were determined by automated sequencing on an Applied Biosystems Model 477A pulsed-liquid sequencer. In those cases where two amino acids were detected in approximately equal yields at each cycle, the sequence assignments were facilitated by the knowledge of the primary structure of hGH.

Characterization of Proteolytic Activity in Rat Thyroid Glands. Thyroid glands (100 glands) were processed to obtain the S9 pellet fraction as described above. The S9 pellet fraction was extracted overnight at 4°C with 3 mL of buffer B. The sample was resedimented at 9000*g* for 30 min and the

supernatant, containing solubilized activity, was applied to an affinity column made by linking potato chymotrypsin inhibitor to CNBr-activated Sepharose 4B (24). The column (0.5 mL resin) was washed with 5 mL of buffer B and enzymatic activity was eluted with 1 *M* acetic acid, 0.75 *M* KCl (pH 2.1), directly into tubes containing an equal volume of 2 *M* sodium phosphate, 0.75 *M* KCl (pH 8.2).

Proteolytic activity in the column fractions was monitored by measuring the conversion of ^{125}I-hGH to peptides soluble in 15% TCA. Twenty-five microliter aliquots of column fractions were incubated at 37°C with ^{125}I-hGH (9×10^{-11} *M*) in a total volume of 0.5 mL, for 180 min before termination with the addition of ice-cold TCA.

SDS-PAGE. Thyroid gland proteins and activity fractions from the affinity column were separated by SDS-PAGE using a 12% acrylamide gel. Protein was detected using silver stain.

Electroblotting and N-Terminal Sequencing. The KCl solubilized fraction from 100 thyroid glands was precipitated with 10% TCA. The precipitated protein pellet was washed twice with ice-cold acetone and resuspended in SDS-PAGE sample buffer. Proteins were separated on a 12% acrylamide gel, electroblotted onto a PVDF membrane (25), and stained with Coomassie blue. The major protein band of approximately 29 kDa was cut out and sequenced. Alternatively, the gel was stained with Coomassie blue, and the 29-kDa band excised. The gel slice was crushed in 200 μL of 70% formic acid, cyanogen bromide was added to 25 μg/mL, and the reaction was allowed to proceed for 20 hrs in the dark at room temperature. CNBr-generated peptides were separated on an 18% acrylamide gel, electroblotted to PVDF, stained with Coomassie blue, and sequenced.

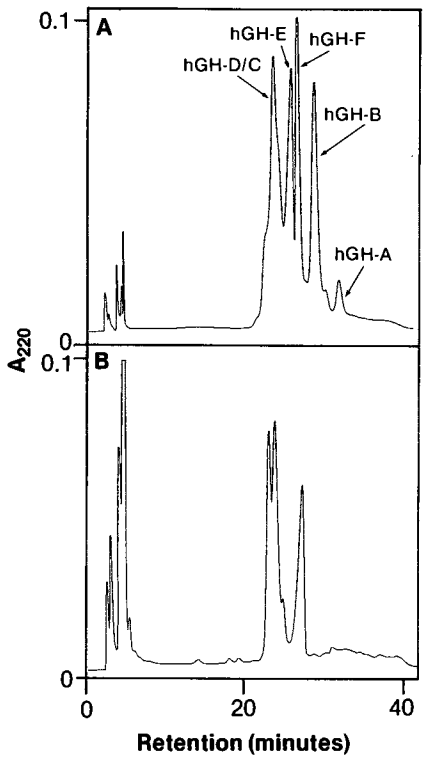

Fig. 1. RP-HPLC profile of hGH metabolite intermediates. hGH was incubated with rat thyroid gland S9 pellet fraction for 5 min (A) or 45 min (B). Metabolites were fractionated on an Aquapore butyl column (7 × 250 mm) using RP-HPLC system II (see Materials and Methods for gradient and solvent composition). Peaks (hGH-A to hGH-E) were collected and further purified on an Aquapore RP-300 column as under Materials and Methods.

RESULTS

Affinity Isolation of Proteolytic Activity. The major eluted protein had a M_r of approximately 29 kDa by SDS-PAGE (data not shown) and was estimated to be approximately 85–90% pure. The three N-terminal amino acids of the purified protein indicated a sequence of Ile–Ile–Gly, suggestive of a serine protease. Further sequence data were obtained from KCl extracts which are enriched in the 29-kDa protein. After cyanogen bromide digestion of the 29-kDa band, three major Coomassie-staining bands were observed after SDS-PAGE and electrophorectic transfer to PVDF. The sequence data from these fragments allowed definition of the N-terminal 29 amino acids and an internal peptide sequence in the protein. Both data indicated 100% sequence homology with that of rat mast cell protease-1 (26) (data not shown). As a result we refer to the thyroid gland proteolytic activity as a RMCP-like enzyme for purposes of discussion in this manuscript.

Limited Digestion Studies. Several large hGH intermediates were identified from 5- and 45-min incubations of hGH with rat thyroid gland S9 pellet fraction (Fig. 1, Tables I and II). The structures of these molecules were derived from a combination of N-terminal sequence and ESI-MS data (Fig. 2A). The majority of the intermediates were two-chain forms of hGH linked by the disulfide bridge between Cys^{53} and Cys^{165}. The molecular masses of the two-chain molecules ranged from 22,143 Da (hGH-A), representing hydrolysis of a single peptide bond at Tyr^{143}–Ser^{144} with no deletions in the hGH sequence, to a 16,002-Da molecule (hGH-E) resulting from deletions in the large disulfide loop and N-terminal regions of hGH. Identifying the major intermediates (Tables I and II) allowed us to propose a metabolic pathway resulting in the formation of hGH-E from hGH (Fig. 3). The scheme involves the sequential metabolism of hGH by the RMCP-like enzyme and a carboxypeptidase. The first step is the cleavage of hGH by the RMCP-like enzyme between Tyr^{143}–Ser^{144} to form a two-chain hGH (hGH-A). hGH-A is then cleaved between Tyr^{42}–Ser^{43}, liberating the N-terminal peptide, Phe^{1}–Tyr^{42}, forming hGH-B. hGH-C was formed by the deletion of the peptide Lys^{140}–Tyr^{143} from the large loop region. Truncation of hGH-C by a single amino acid (Phe^{139}) at the carboxyl terminus indicated the action of a carboxypeptidase on this intermediate to form hGH-D. The final step we could decipher was the removal of the tripeptide, Ser^{144}–Phe^{146}, from hGH-D to form the major end product hGH-E.

hGH-D is formed by the action of a carboxypeptidase to remove a phenylalanine residue from hGH-C. There were two structures of hGH-D which were consistent with the sequence and mass data. A two-chain molecule with N termini at Ser^{43} and Ser^{144} and having a mass of 16,364 Da could be derived from the linkage of Ser^{43}–Ile^{138} to Ser^{144}–Phe^{191} or Ser^{43}–Phe^{139} to Ser^{144}–Gly^{190}. To resolve this, individual chains of hGH-D were isolated by RP-HPLC after reduction and carboxymethylation and subjected to ESI-MS

Table I. Structural Characterization of Two-Chain hGH Metabolites[a]

hGH metabolite	Determined sequence(s)[b]	Determined mass	Deduced structure	Theoretical mass
hGH-A	FPTIPLS[c] SKFXTNS	22,141.9 ± 2.2	Phe^{1}–Tyr^{143} Ser^{144}–Phe^{191}	22,143.2
hGH-B	SFLQNPQTSLXFS[c] SKFDTNSHNDDAL	17,027.1 ± 1.0	Ser^{43}–Tyr^{143} Ser^{144}–Phe^{191}	17,032.4
hGH-C	SFLQNPQTSLXF[c] SKFXTNSXNXXA	16,510.3 ± 1.0	Ser^{43}–Phe^{139} Ser^{144}–Phe^{191}	16,511.8
hGH-D	SFLQNPQTSL[c] SKFDTNSXN	16,362.1 ± 0.7	Ser^{43}–Ile^{138} Ser^{144}–Phe^{191}	16,364.6
hGH-E	SFLQNP[c] DTNSXN	16,002.9 ± 0.9	Ser^{43}–Ile^{138} Asp^{147}–Phe^{191}	16,002.1
hGH-F	FPTIP	5,127.4 ± 0.4	Phe^{1}–Tyr^{42}	5,128.8

[a] hGH was incubated with rat thyroid gland S9 pellet fraction for 5 or 45 min. Metabolites were isolated using two RP-HPLC systems (Fig. 1) as under Materials and Methods and subjected to N-terminal sequencing and ESI-MS.
[b] X, amino acid designation not made; assignment based on known sequence of hGH.
[c] Two-chain metabolite linked by disulfide bridge (Cys^{53}–Cys^{165}).

(Table II). The same procedure was applied to the unambiguous structure of hGH-C to verify the utility of the approach (Figs. 2B and C). For hGH-C, carboxymethylation increased the mass of the Ser^{43}–Phe^{139} chain by 57.6, consistent with modification of the single free sulfhydryl, and increased the mass of the Ser^{144}–Phe^{191} by 175, consistent with the modification of three free sulfhydryls in this chain (Table II). In addition, a second species with a mass of 5619.0 ± 0.8 was also identified in the mass spectrum (Fig. 2C). This form corresponds to the Ser^{144}–Phe^{191} chain where incomplete carboxymethylation has resulted in the modification of a single cysteine. The results confirmed the proposed two-chain structure of hGH-C. The carboxymethylated individual chains of hGH-D had masses of 10858.4 ± 0.7 and 5737.8 ± 0.4 (Table II), consistent with the masses of the carboxymethylated forms of Ser^{43}–Ile^{138} and Ser^{144}–Phe^{191}, respectively. Hence, hGH-D was formed by removal of Phe^{139}, not the N-terminal amino acid Phe^{191}.

Extended Degradation Studies. hGH was degraded to approximately 20 peptides during a 4.5-hr incubation with the purified RMCP-like enzyme (Fig. 4). The peptides, isolated by RP-HPLC, were sequenced and their mass was determined by ESI-MS (Table III). As expected for the specificity of this protease, the peptides were generated as a result of cleavages at the carboxyl end of hydrophobic residues (Tyr/Phe/Leu–Xaa bonds). Experimental masses of the peptides were consistent with theoretical masses of hGH peptides containing the determined N-terminal sequences. As a result of the length of the proteolytic digestion, the majority of peptides were ≤20 amino acids in length.

Table II. Confirmation of Two-Chain Metabolite Structures After Reduction and Carboxymethylation[a]

Metabolite	Determined sequence(s)	Determined mass	Deduced structure	Theoretical mass
hGH-C	SFLQNPQTSLXF[b] SKFXTNSXNXXA	16,510.3 ± 1.0	Ser^{43}–Phe^{139} Ser^{144}–Phe^{191}	16,511.8
	ND[e]	11,007.0 ± 1.2	Ser^{43}–Phe^{139c}	11,008.4[c] 10,950.4[d]
	ND	5,737.3 ± 0.8	Ser^{144}–Phe^{191c}	5,739.4[c] 5,565.4[d]
hGH-D	SFLQNPQTSL[b] SKFDTNSXN	16,362.1 ± 0.7	Ser^{43}–Ile^{138} Ser^{144}–Phe^{191}	16,364.6
	ND	10,858.4 ± 0.7	Ser^{43}–Ile^{138c}	10,861.2[c] 10,803.2[d]
	ND	5,737.8 ± 0.4	Ser^{144}–Phe^{191c}	5,739.4[c] 5,565.4[d]

[a] Two-chain hGH metabolites were purified by HPLC and subjected to reduction/carboxymethylation procedure as under Materials and Methods. The masses of intact metabolite and individual carboxymethylated chains were determined for proof of structure.
[b] Two-chain metabolite linked by disulfide bridge (Cys^{53}–Cys^{165}).
[c] Theoretical mass of reduced/carboxymethylated peptide fragment.
[d] Theoretical mass of peptide fragment prior to carboxymethylation.
[e] Not determined.

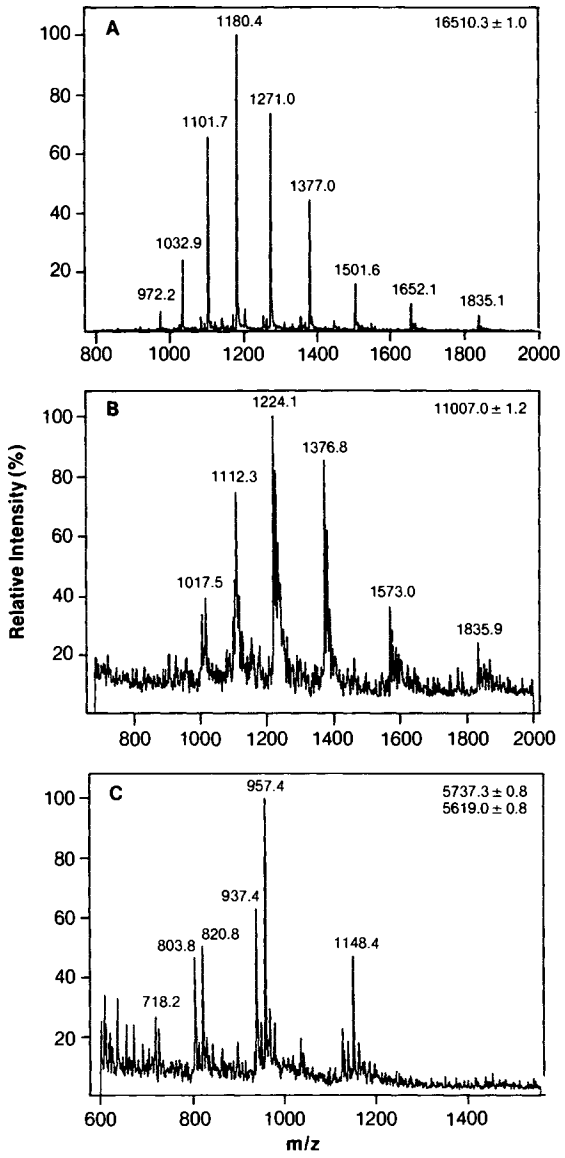

Fig. 2. Electrospray mass spectrum of metabolite hGH-C. hGH was incubated with rat thyroid gland S9 pellet fraction, and metabolites were isolated by RP-HPLC as described under Materials and Methods and subjected to ESI-MS analysis. (A) Mass spectrum of the metabolite referred to as hGH-C (see Fig. 1); (B) mass spectrum of a single chain isolated by RP-HPLC after reduction and carboxymethylation of hGH-C; (C) mass spectrum the second chain isolated after reduction and carboxymethylation of hGH-C. The mass of the peptides derived from the mass spectrum is indicated in each panel.

DISCUSSION

We have structurally characterized multiple two-chain forms of hGH as metabolic products formed during the incubation of hGH with rat thyroid gland S9 pellet fraction. By identifying the metabolites, a pathway by which hGH is processed has been proposed (Fig. 3). Metabolism appears to occur through the interaction of a chymotrypsin-like serine protease (9) and a carboxypeptidase-like activity. The molecular weight (approx. 29 kDa), N-terminal sequence analysis, solubility characteristics, and substrate specificity (chymotrypsin-like) of the rat thyroid gland enzyme were consistent with the known characteristics of rat mast cell protease-I (RMCP-I) (26–28). Characterization of this thyroid gland enzyme as a rat mast cell protease is supported by earlier studies demonstrating the presence of mast cells and proteolytic activity in this tissue (29). Mast cell endoproteases exist in a macromolecular heparin proteoglycan–protein complex with carboxy- and aminopeptidases (30,31). Proteolytic degradation involving this complex has been proposed to be a function of the coordinated activity of endo- and exopeptidases (31,32). The findings of the present study were consistent with the sequential metabolism of hGH by a chymotrypsin-like protease and a carboxypeptidase with a specificity toward removal of C-terminal hydrophobic residues (32). The mast cell proteases from rat thyroid glands provided a useful model by which to characterize the interaction of multiple proteolytic enzymes in the metabolism of hGH.

The use of ESI-MS in combination with N-terminal sequencing allowed us to define a metabolic pathway involved in the metabolism of hGH using a crude system containing multiple enzymatic activities. The general characteristics of the endoprotease(s) involved could be deduced from the N-terminal data, however, the involvement of the carboxypeptidase required comparative information on the C-terminal end of the metabolites. Along with the N-terminal data, the structure of the metabolites could have been deduced by defining the C-terminal sequence of the protein. However, unlike N-terminal sequencing methods, C-terminal sequence analysis is cumbersome and not yet well established or widely accepted (33). As the results indicate, the ESI-MS data was critical to defining the involvement of a carboxypeptidase in the metabolic pathway. In combination with the N-terminal data, the sensitivity of the ESI-MS analysis allowed unambiguous identification of the metabolites. This was particularly valuable in the characterization of metabolites consisting of two disulfide-linked chains (see Table II). ESI-MS also eliminated the need for more labor intensive and material demanding techniques such as comparative tryptic mapping of the metabolites and hGH. Although most of the peptides were highly purified by the HPLC steps, several contained multiple components whose identity could be determined from the mass spectral data (34). The data indicated that hGH was not randomly proteolyzed and that the major two-chain hGH intermediates (hGH-C and hGH-D) and the N-terminal$^{1\text{-}42}$ fragment were stable over a 45-min incubation. Identification of these major metabolites allowed us to speculate on the involvement of specific classes of proteases in the metabolic scheme, even in a crude biological matrix. The proposed sequence of events is also supported by the extended incubation studies in which we identified the tripeptides, Lys^{140}–Tyr^{143} and Ser^{144}–Phe^{146}, removed during the formation of hGH-C and hGH-D, respectively, as well as peptides resulting from the more extensive proteolysis of the larger intermediates (see Table III). The findings are consistent with our biochemical characterization of the chymotrypsin-like protease and knowledge of other proteolytic activities in mast cells of the thyroid gland and other tissues (29–31).

The metabolic fate of endogenous or exogenously administered hGH is not known in any detail. Although the physiological significance of the metabolic sequence de-

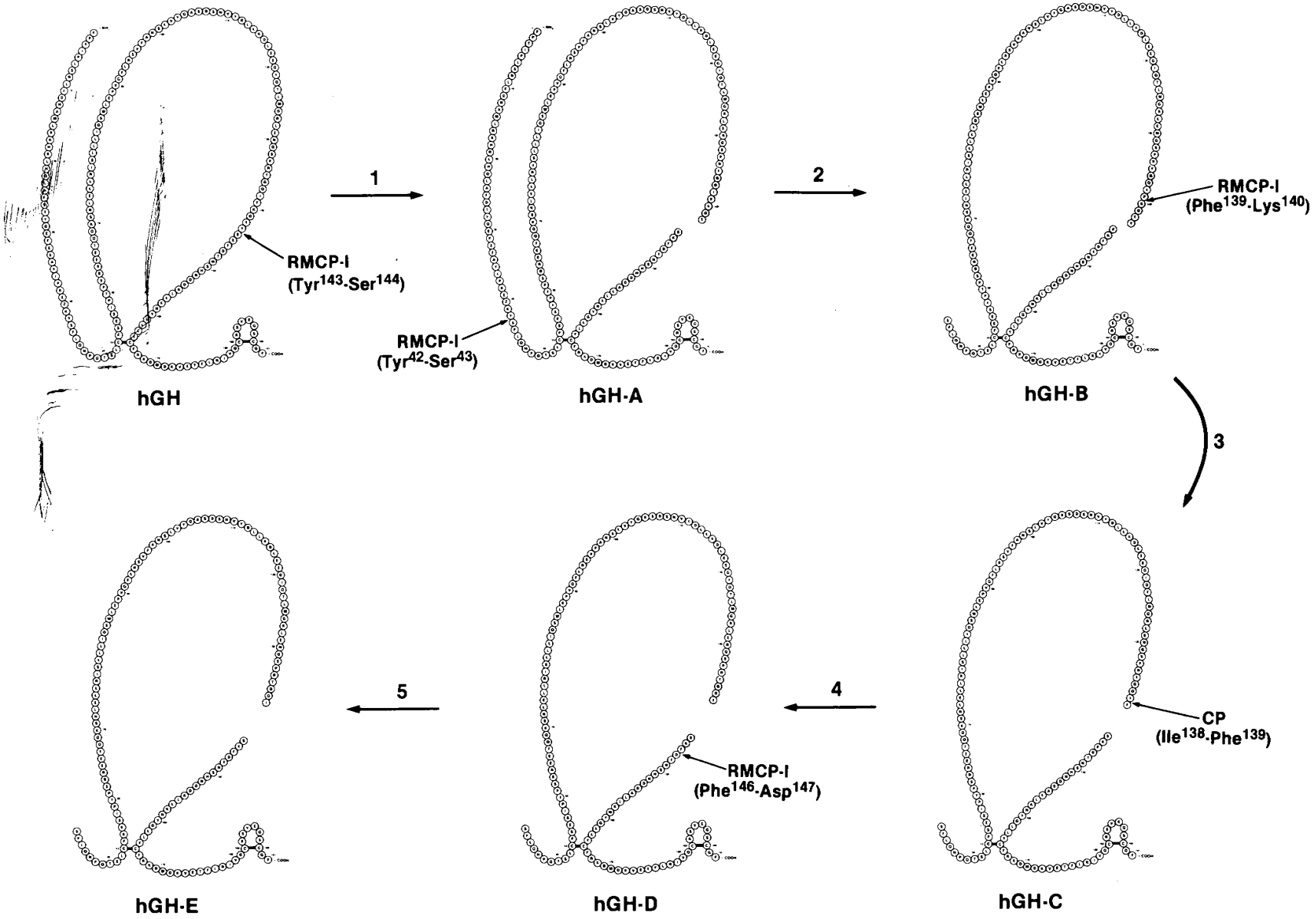

Fig. 3. Proposed sequence by which hGH is metabolized by rat thyroid gland S9 pellet fraction. Based upon identification of *in vitro* metabolites, the sequential formation of hGH metabolites A–E by the action of a rat mast cell protease-like enzyme (RMCP-I) and a carboxypeptidase (CP) is proposed. The peptide bonds sensitive to cleavage are indicated at each step. See Table I for definitive structural information.

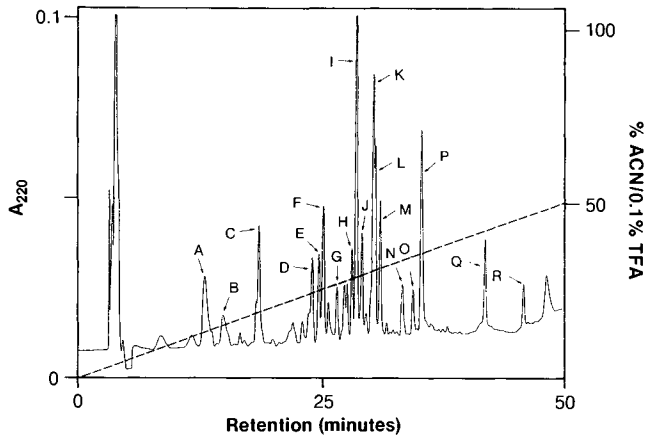

Fig. 4. Peptide map of hGH after incubation with purified RMCP-like enzyme from rat thyroid gland. hGH was incubated with isolated enzyme for 4.5 hr and peptides were separated on an Aquapore RP-300 column (4.6 × 250 mm) using a linear gradient of acetonitrile/0.1% TFA as described under Materials and Methods.

scribed in the thyroid gland is unclear, studies in our laboratory have indicated the formation of similar metabolites by rat skeletal muscle *in vitro* and mast cells in muscle tissue may also account for this metabolism (9,35). Mast cells proteases, released into physiological fluids within a granule matrix, are insensitive to inactivation by endogenous inhibitors (36). These proteases have been suggested to play a role in the local degradation of neuropeptides (37,38), and can metabolize a wide variety of naturally occurring proteins (36). Although we have no evidence for mast cell-mediated metabolism of hGH *in vivo,* we were able to detect an immunoreactive form of hGH in rat plasma having a molecular weight similar to that of the *in vitro* metabolites (approximately 16 kDa) after intramuscular injection (9). The presence of several immunoreactive forms of hGH in human plasma having molecular sizes of 12, 16, and 30 kDa has also been demonstrated (39). There are numerous sites of cleavage preferred by chymotrypsin- and trypsin-like proteases in the regions of hGH shown to be sensitive to metabolism in this study (40). It seems reasonable that other intracellular serine proteases could metabolize hGH via a pathway similar to that described here, resulting in the formation of comparable intermediates. In this regard, a non-trypsin-like serine protease in the endosomal fraction of rat liver has been suggested to be involved in the processing of bovine growth hormone to a 15 kDa fragment (41). The similarity in the size of hGH fragments found in plasma (9,39) and the present study indicates some predictibility from the *in vitro* to the *in vivo* studies. Since hGH appears to be metabolically stable in plasma (42), the heterogeneity of plasma immunoreactivity may result from retroendocytosis of hGH fragments after receptor-mediated uptake and limited intracellular processing by a combination of endo- and exopeptidases (10,39,41,43). This mechanism has been proposed to be involved in modifying the peripheral activity of other protein hormones including prolactin, parathyroid hormone, proinsulin, and glucagon (12,43–46). Alternatively, limited proteolytic processing at the site of injection may account for heterogeneity of hGH forms after exogenous administration (46,47). The proportion of the various molecular forms of hGH detected in the circulation can be influenced by differential receptor-binding affinity, clearance kinetics, and different immunoreactivities and has not been clearly estab-

Table III. Structural Characterization of hGH Peptides[a]

Peptide	Determined sequence[b]	Determined mass	Deduced sequence	Theoretical mass
A	KQTY	538.4 ± 0.1	Lys^{140}–Tyr^{143}	538.6
B	SKF	380.1 ± 0.0	Ser^{144}–Phe^{146}	380.5
C	GASDSNVY	2165.3 ± 1.8	Gly^{104}–Val^{110}	2167.3
D	AFXTY	616.0 ± 1.1	Ala^{24}–Tyr^{28}	615.8
E	EXAYIPKE	1396.9 ± 0.4	Glu^{32}–Tyr^{42}	1397.6
F	ANSLVY	665.4 ± 0.0	Ala^{98}–Tyr^{103}	665.8
G	SXLF	521.4 ± 0.0	Ser^{7}–Phe^{10}	521.6
H	LXS	620.4 ± 0.1	Leu^{93}–Phe^{97}	620.8
I	QEFEEAYIPKEQKY	1801.5 ± 0.0	Gln^{29}–Tyr^{42}	1802.0
	GLLY	464.3 ± 0.0	Gly^{161}–Tyr^{165}	464.6
J	XQLAFDTY	993.5 ± 0.1	His^{21}–Tyr^{28}	994.1
K	MGXL	1663.3 ± 0.1	Met^{125}–Phe^{139}	1663.9
	LEPV	731.5 ± 0.0	Leu^{87}–Phe^{92}	731.9
L	MGXLEDGSPXTGQ	1663.3 ± 0.1	Met^{125}–Phe^{139}	1663.9
M	LXIVQXXSVEGSXGF	1651.0 ± 0.1	Leu^{177}–Phe^{191}	1652.0
	SXLFDNAML	1221.7 ± 0.1	Ser^{7}–Arg^{16}	1222.4
N	SXLFXNA	1065.4 ± 0.0	Ser^{7}–Leu^{15}	1066.2
O	LLIQS	758.2 ± 0.2	Leu^{81}–Trp^{86}	758.9
P	FPTIPLR	686.3 ± 0.1	Phe^{1}–Leu^{6}	686.9
Q	DLLKDLEEGI	1485.8 ± 0.1	Asp^{112}–Leu^{124}	1486.7
R	FPTIPL	5127.4 ± 1.2	Phe^{1}–Tyr^{42}	5128.9

[a] hGH was incubated with purified RMCP-1 for 4.5 hr as described under Materials and Methods. Peptides were isolated by RP-HPLC (Fig. 3) and subjected to N-terminal sequence analysis and ESI-MS.

[b] X, amino acid designation not made; assignment based on known sequence of hGH.

lished. Due to the analytical difficulties, lack of metabolite standards, and paucity of information on enzyme systems which may be involved, the precise nature of circulating hGH immunoreactivity *in vivo* is not known.

In summary, we have defined an *in vitro* pathway by which hGH is metabolized using a combination of N-terminal sequence and ESI-MS data. By developing chromatographic systems for separating hGH and potential metabolites, and evaluating immunological methods (9) in the relatively simple matrix of the *in vitro* incubations, analytical difficulties associated with studying the degradation of proteins were circumvented. Since the regions in hGH shown to be susceptible to proteolysis in the present study are consistent with the general characteristics of previously described variants of hGH (9,39,41), these methods should be adaptable to the purification and characterization of circulating forms of hGH in plasma. With further application of sensitive methods of analysis such as microbore or capillary HPLC–electrospray–mass spectrometry (48,49), a clearer understanding of the biochemical and physiological implications of hGH metabolism can be established.

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