Formation of the COOH-terminal amide group of thyrotropin-releasing-factor

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The precursors of peptide hormones that possess a COOH-terminal α-amide group contain a glycine residue following the amino acid which is amidated in the hormone. <Glu–His–Pro–Gly was synthesized as a putative precursor of thyroliberin. Bovine pituitary neurosecretory granules were shown to contain an amide group-forming activity associated with an M_r of about 62000 protein(s) which converts the tetrapeptide to thyroliberin.

Thyroliberin *Carboxy terminal amide* *Amidation* *Pituitary*

1. INTRODUCTION

Many peptide hormones contain a COOH-terminal amino acid α-amide residue. Thus, the terminal residue in oxytocin, vasopressin, and luliberin is glycinamide; in calcitonin and thyroliberin it is prolinamide; secretin and α-melanotropin terminate in valinamide; and gastrin terminates in phenylalanine amide. The details of COOH-terminal amidation of the peptide hormones are not fully understood. Recently, it was shown [2] that porcine pituitary neurosecretory granules contain an enzyme that converts the synthetic tripeptide, D-Tyr–Val–Gly, to D-Tyr–Val–$CONH_2$. The mechanism involved dehydrogenation and hydrolysis of the glycine-containing peptide. Amidation also occurred when valine in position 2 was replaced by either phenylalanine or glycine. A COOH-terminal glycine was, however, mandatory. The authors proposed that a similar mechanism may be involved in the amidation of peptide hormones in tissues and that the COOH-terminal glycine may serve as a signal for amidation of the residue preceding it. Consistent with this proposal is the finding that in several of the hormone precursor molecules, the residue which is amidated in the hormone is usually followed by glycine (thus, for example, the precursors of α-melanotropin [3] and the honeybee hormone, mellitin [4], and the putative precursors of vasopressin [5] and calcitonin [6,7]).

Our report describes the partial purification of an enzyme activity from bovine pituitary neurosecretory granules that converts <Glu–His–Pro–Gly to <Glu–His–Pro–$CONH_2$ (thyrotropin-releasing factor or thyroliberin). The tetrapeptide was synthesized and used with the expectation that thyrotropin precursors molecules, in analogy to the examples cited above, will contain a glycine residue following the hormone sequence.

Abbreviations: The abbreviations used for amino acids and peptide hormones follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1]

2. MATERIALS AND METHODS

Thyroliberin and glycine ethyl ester were purchased from Sigma. Deamido-thyroliberin (<Glu–His–Pro) was obtained from Bachem, CA. [Pro-2,3-^{3}H]Thyroliberin (21.8 Ci/mmol) was purchased from New England Nuclear. Bovine brain thyroliberin deamidase (spec. act. 440 units/mg) was purified as in [8].

<Glu–His–Pro–Gly was synthesized by coupl-

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ing deamido-thyroliberin with glycine ethyl ester using the *N,N'*-dicyclohexylcarbodiimide method [8,9]. The peptide was purified by subjecting aliquots of the synthetic material to high-performance liquid chromatography (HPLC) on a Supelcosil LC-8 column (15 × 0.46 cm) eluted with 0.1% trifluoroacetic acid–0.2% acetonitrile. Appropriate fractions (see fig.3A) were collected, lyophilized, and dissolved in water. The purity and concentration of the tetrapeptide was determined by hydrolysis of an aliquot in vacuo in 6 N HCl at 105°C for 20 h followed by amino acid analysis using a Durrum model D-500 analyzer. Glu, His, Pro, and Gly were the only amino acids detected; the relative amounts of each were: 0.99, 1.0, 0.97 and 0.99, respectively.

[^{3}H]Deamido-thyroliberin was made as follows: [^{3}H]thyroliberin, 50 μmol (stock [^{3}H]thyroliberin was mixed with unlabeled thyroliberin to give a final spec. act. of about 2000 counts.min^{-1}.nmol^{-1}), was incubated at 37°C for 20 h in a solution (2 ml) containing 0.01 M K-phosphate buffer (pH 7.5), 1 mM EDTA, 5 mM dithiothreitol, and 20 μg of thyroliberin deamidase. The solution was then applied to a Whatman 3 MM paper (20 × 60 cm) and subjected to high-voltage paper electrophoresis in pyridine/acetic acid/water (0.5:5:94.5), pH 3.5 [8]. The area corresponding to deamido-thyroliberin (detected by spraying a 0.5 cm wide strip of the paper with Pauly reagent) was cut out and eluted in water. An aliquot was taken for hydrolysis and amino acid analysis. The remaining solution was lyophilized and used for the synthesis of [^{3}H]<Glu–His–Pro–Gly. The product was purified by HPLC as described above (yield, about 12 μmol of the tetrapeptide; 1400 counts.min^{-1}.nmol^{-1}).

Thin-layer chromatography (TLC) of the peptides was carried out on silica gel-coated plastic sheets (Brinkman) developed in chloroform/methanol/30% ammonia (125:75:25) [8]. The peptides were detected by spraying with Pauly reagent. The R_f-values for thyroliberin, deamido-thyroliberin, and <Glu–His–Pro–Gly in this system were 0.60, 0.40 and 0.28, respectively (fig.1).

Fresh, iced bovine pituitaries were obtained from a local supplier. A particulate fraction containing the neurosecretory granules was prepared as follows [10]: the pituitaries were homogenized (at 4°C) in 8 volumes (w/v) of 0.01 M sodium phosphate buffer (pH 7) containing 0.3 M sucrose using a Potter-Elvehjem homogenizer (Teflon pestle). The homogenate was centrifuged at 750 × g for 3 min; the rotor speed was then increased to 10000 × g and the centrifugation continued for 10 min. The supernatant thus obtained was centrifuged at 25000 × g for 20 min. The pellet was

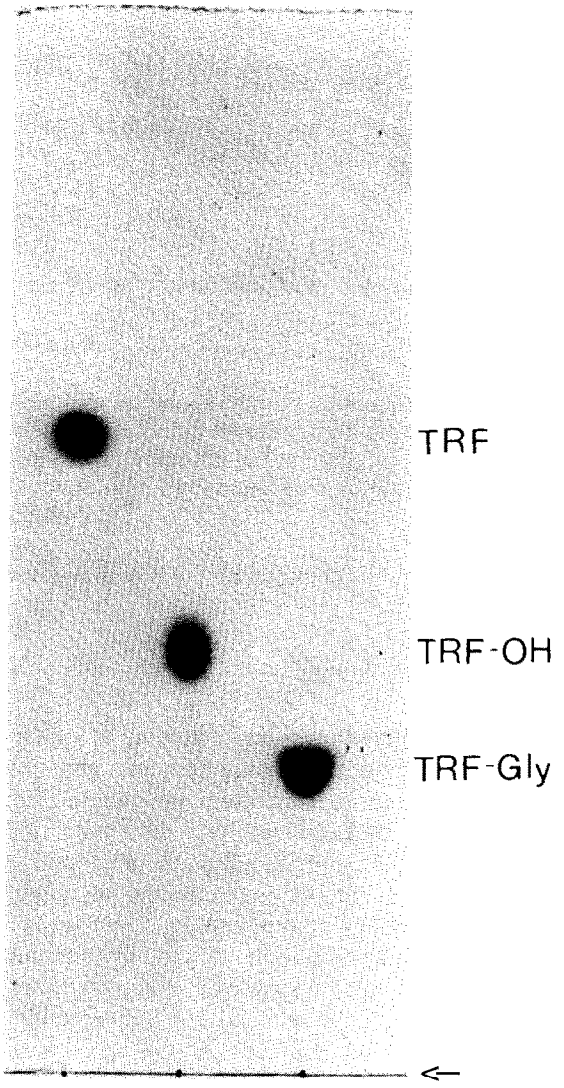

Fig.1. Thin-layer chromatography of thyroliberin (TRF), deamido-thyroliberin (TRF-OH), and <Glu–His–Pro–Gly (TRF-Gly) on a silica-coated plastic sheet. The arrow indicates point of sample application (5 nmol each).

suspended in 0.02 M sodium phosphate buffer (pH 7) containing 0.15 M NaCl (phosphate–NaCl) and recentrifuged at 25000 × *g* for 20 min. The pellet was finally suspended in phosphate–NaCl buffer (1 ml/4 g of tissue) (granule fraction). Acetone powder of the granule fraction was made as follows: acetone (precooled to −15°C; 10 volumes) was added dropwise to the stirred granule fraction. The precipitate was filtered on a Buchner funnel and washed with several volumes of acetone. The dried powder was stored at −15°C. An extract of the acetone powder was prepared by homogenizing the powder in 10 volumes of phosphate–NaCl. The mixture was stirred at 4°C for 1 h and then centrifuged at 10000 × *g* for 20 min (supernatant = acetone powder extract).

Thyroliberin synthesis was studied as follows: the reaction mixture (50 μl), in general, contained 2.5 μl of 2 mM ^{3}H-labeled <Glu–His–Pro–Gly (final concentration, 0.1 mM), 7.5 μl of phosphate–NaCl buffer, and 40 μl of either the neurosecretory granule fraction or its acetone powder extract. The incubations were carried out at 37°C (2–24 h) in 1.5 ml stoppered microfuge tubes under an atmosphere of O_2. Controls were simultaneously run in which the peptide and the tissue preparation were separately omitted. The reactants were analyzed either by TLC or by HPLC. For TLC, the reactions were terminated by the addition of 50 μl of 0.1 N HCl and the solution applied to a Dowex 50-H^+ column (200–400 mesh; 0.5 × 1 cm). The column was washed with 5 ml of 0.1 N HCl and then eluted with 3 ml of 2 N HCl. The eluates were evaporated to dryness and the residue dissolved in 50 μl of water containing 50 nmol of unlabeled thyroliberin (added as a carrier). The sample was subjected to TLC as described above. Areas corresponding to thyroliberin were cut out and radioactivity determined in a liquid scintillation counter using toluene-based medium. For HPLC, the reactions were terminated by placing the tubes in a boiling-water bath for 2 min. Denatured proteins were removed by centrifugation and 10–20 μl of the supernatant subjected to HPLC as described above.

3. RESULTS AND DISCUSSION

Thyroliberin is found in several regions of the brain, the highest levels being in the hypothalamus [11]. Thyroliberin-degrading activities are also present in the brain and these have hampered studies on its biosynthesis [8,12,13]. Our preliminary studies showed that bovine pituitary homogenates were significantly less active in degrading thyroliberin than the extracts of other brain regions. Pituitary also synthesizes hormones (e.g., α-melanotropin) which contain COOH-terminal amide groups. Assuming that amidating enzyme(s) exhibit broad specificity towards peptide substrates, we chose pituitary for our studies on the synthesis of the amide group of thyroliberin. The tetrapeptide, <Glu–His–Pro–Gly, was synthesized as a putative precursor for the hormone. Its purity was established by HPLC (see fig.3), thin-layer chromatography (fig.1), and amino acid analysis.

Detectable amounts (by HPLC) of thyroliberin were formed upon incubation of the tetrapeptide with bovine pituitary extracts. Deamido-thyroliberin was also detected (this can be produced from either the tetrapeptide or thyroliberin by the action of post-proline cleaving enzymes [8]). In agreement with previous reports [2], the amide group-forming activity was recovered in the neurosecretory granule fraction. About 75% of the activity was lost upon storage of the granule fraction at 4°C for 48 h. Further studies showed that the amidating activity was retained in acetone powder of the granule fraction. Storage of the acetone powder at −15°C for up to 4 weeks was without significant effect on the activity.

Partial purification of amidating activity was achieved by gel-filtration (fig.2). In the experiment shown, 23 units of thyroliberin-forming activity (see fig.2 legend for definition of the unit) were applied to the column. Comparison of the elution profile of the amidating enzyme with those for proteins of known M_r (M_r calibration kit obtained from Scharz-Mann), indicated that the activity is associated with protein (or proteins) of M_r = 60–65000 (peak fraction volume corresponds to M_r = 62000). About 155 units of amidating activity were recovered in fractions 61–70. This increase in total activity upon gel-filtration is probably due to its separation from thyroliberin-degrading enzymes. Post-proline cleaving activity is still evident in fractions 62 and 64 as seen from the appearance of deamido-thyroliberin (fig.3B and C). The spec. act. in fraction 62 was about 39 (nmol of

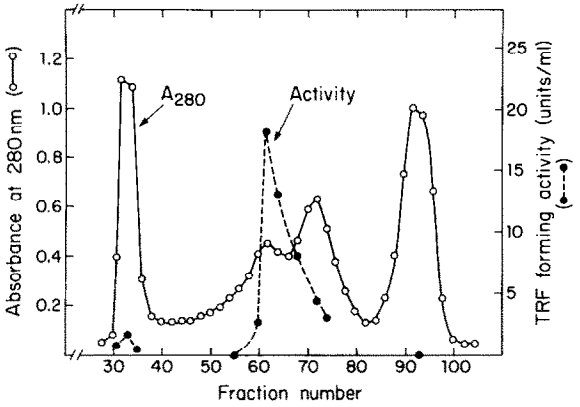

Fig.2. Partial purification of the amidating activity from bovine pituitary. An extract made from 225 mg of acetone powder prepared from the neurosecretory granule fraction of pituitaries was subjected to gel filtration on Ultrogel AcA34 (LKB) column (1.6 × 85 cm), equilibrated and eluted with phosphate–NaCl buffer (1.5 ml/fraction). A unit of amidating enzyme activity is the amount of enzyme that forms 1 nmol of thyroliberin (TRF) from <Glu–His–Pro–Gly per 24 h (see section 2 for assay procedure). Thyroliberin was determined by HPLC (see fig.3).

thyroliberin formed.mg protein^{-1}.24 h^{-1}), a 49-fold increase over that seen in the acetone powder extract.

Use of [^{3}H]Pro-labeled tetrapeptide followed by TLC of the reaction products confirmed the formation of ^{3}H-labeled thyroliberin. The ^{3}H-labeled product was isolated by HPLC, lyophilized, and incubated under the appropriate conditions with purified thyroliberin deamidase [8]. Both HPLC and TLC (as in fig.1) showed that the major product was deamido-thyroliberin. The synthesis of thyroliberin from <Glu–His–Pro–Gly (using fraction 62) was not affected by the addition of either NH_4Cl or glutamine precluding transamidation as a possible mechanism for the generation of the amide group. Furthermore, no thyroliberin was formed upon incubation of deamido-thyroliberin with either the acetone powder extract of secretory granules or partially purified preparation (fraction 62, fig.2) in the presence of ATP, $MgCl_2$, and either NH_4Cl or glutamine. Thus a direct amidation of the proline residue by mechanisms similar to those for the biosynthesis of glutamine and asparagine is considered unlikely.

The characteristics of the amidating enzyme(s) which converts the tetrapeptide to thyroliberin requires further study. The enzyme is presumably

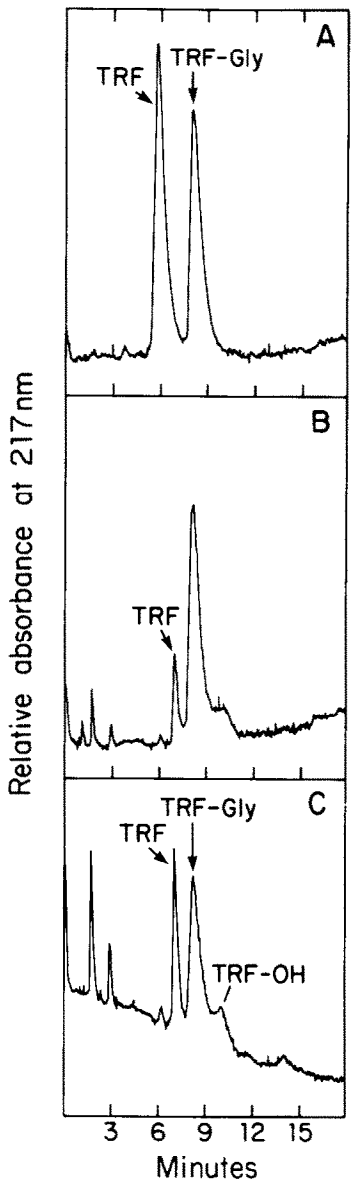

Fig.3. HPLC separation of reaction products: (A) Separation of thyroliberin (TRF) and <Glu–His–Pro–Gly (TRF-Gly), 0.9 nmol each; (B) and (C) are representative HPLC runs of reaction products obtained when TRF-Gly (2.5 μl of a 2 mM solution) was incubated with 50 μl of either fraction 64 (24 h incubation) or fraction 62 (36 h incubation), respectively, from the gel filtration experiment described in fig.2; 10 μl aliquots of the assay mixture were analyzed. TRF-OH, deamido-thyroliberin.

similar to that studied in [2]. At present, there is scanty information regarding the biosynthetic precursors of thyroliberin [14]. In analogy with other peptide hormone precursors and consistent with the mechanism for amidation proposed in [2], we postulate that the thyroliberin precursor will in all likelihood contain a glycine residue subsequent to the tripeptide sequence. The mechanism of biosynthesis of the pyroglutamyl residue of thyroliberin is also not known. Model peptides of the type described here may aid in studies on this group. Our preliminary studies indicate that the post-mitochondrial 100000 × *g* particulate fraction from rat hypothalami can produce thyroliberin from <Glu–His–Pro–Gly. This fraction is, however, very active in degrading thyroliberin. Further studies are required on the characteristics of amidating enzyme(s) in hypothalamus which makes not only thyroliberin and luliberin but also the posterior pituitary hormones, vasopressin and oxytocin.

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