C-terminal amidation of neuropeptides

Gly-Lys-Arg extension an efficient precursor of C-terminal amide

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Biosynthesis of the C-terminal carboxamide group of peptide hormones was studied using comparatively pGlu-His-Pro-Gly and Glu-His-Pro-Gly-Lys-Arg as putative precursors of the tripeptide, thyroliberin (TRH). Rat hypothalamus granules were found to contain an amide group forming activity which converts both peptide substrates into TRH. Comparison of the rate of conversion of the two substrates indicated that the C-terminal dibasic extension favored a 10-fold increase in the production of amidated peptide. It is suggested that this type of structure may be present in the putative biosynthetic precursor of TRH and that it may provide a better substrate for the enzyme(s) involved in C-terminal amidation.

Thyroliberin *Hypothalamic granule* *TRH-Gly* *TRH-Gly-Lys-Arg*

1. INTRODUCTION

Several biologically active peptides with a C-terminal carboxamide group usually appear to be followed by the sequence Gly-X-X (where X is either Lys or Arg) when still included in their precursor [1–4]. This observation suggested that the biosynthesis of the C-terminal amide might involve specific enzyme(s) processing a vicinal C-terminal glycine. Recent in vitro studies, using synthetic substrates derived from D-Tyr-Val-Gly, demonstrated that the mechanism of amidation involves dehydrogenation and hydrolysis of the glycine extended peptide by pituitary granule enzyme(s) [5–7]. In addition, these studies suggested that the carboxyl group of Gly must be free in order to be recognized by the amide group forming enzyme [5,6]. It was thus proposed that a similar mechanism could be involved in the C-terminal carboxamidation of hormones and neuropeptides in various tissues [8].

To assess the validity of the above proposed hypothesis, these studies have however to be extended to other substrates, particularly those containing either a C-terminal Gly extension or a Gly-X-X extension of a naturally occurring sequence. The possibility that a dibasic extension could serve as an additional recognition signal for the enzyme(s) into the sequence of the biosynthetic precursor has to be tested. Studies of the enzymatic amidation processes of TRH were initiated by using both TRH-Gly and Glu-His-Pro-Gly-Lys-Arg as substrates and purified rat hypothalamus granules as a source for amidation activity. This choice reflects:

(i) the fact that TRH is a natural, C-terminal carboxamidated, hormone which is synthesized at its highest level by the hypothalamus, and

Abbreviations: TRH, pGlu-His-Pro(NH_2) (thyroliberin); TRH-Gly, pGlu-His-Pro-Gly; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; CMC, carboxymethylcellulose; CRF, corticotropin releasing factor

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(ii) the proposal that TRH may derive from the processing of a larger peptide precursor [9].

We report here an analysis of the ability of hypothalamic granule extracts to convert TRH-Gly and Glu-His-Pro-Gly-Lys-Arg into TRH. The data give support to the hypothesis that both amidation and processing of active peptides might be concerted mechanisms.

2. MATERIALS AND METHODS

TRH was provided by CRB, Cambridge. Depyrolyzed TRH was obtained from Penninsula Lab., San Carlos, CA. TRH-Gly and Glu-His-Pro-Gly-Lys-Arg were synthesized by an improved procedure of the solid phase method. Crude peptides were cleaved from the resin by liquid HF at 0°C and passed through Sephadex G-10 in 0.5 N acetic acid. Final purification was effected by chromatography on CMC followed by HPLC on a C-18 Bondapack column (250 × 4.6 mm) eluted by 5% acetonitrile, hexanesulfonate (1 g/l) in 20 mM acetic acid. The purity of the peptides was assessed by amino acid analysis on a Carlo Erba 3A28 automatic analyzer, TLC on cellulose plate run in two different solvent systems and HPLC as described above. Preparation and purification of highly labelled [^{3}H]TRH-Gly (25 Ci/mmol) and [^{3}H]Glu-His-Pro-Gly-Lys-Arg (22 Ci/mmol) was achieved by the same methodology as previously used for tritiation of TRH [10].

A purified fraction of hypothalamus granule was prepared involving both differential and Percoll gradient centrifugations. Hypothalami from adult male rats (Wistar, 100 g) were homogenized with a Potter Elvejhem in 20 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose. All the experiments were run at 4°C. The homogenate was centrifuged at 10000 × g for 10 min, then at 25000 × g for 20 min. The pellet was resuspended in buffered isotonic sucrose and centrifuged at 25000 × g for 20 min. The final sediment was considered as a crude granular fraction [11] and was resuspended in 1 ml of isotonic buffered sucrose. This preparation was further purified by sedimentation at 60000 × g for 30 min on a continuous 33% Percoll gradient made in 0.25 M sucrose as in [12]. Two major bands appeared under these conditions, the lower (ϱ = 1.08) mainly consisting of secretory granules free from lysosomal or mitochondrial contaminants [12]. Fractions (0.6 ml) were collected from the Percoll gradient (16 ml) and then diluted to 6 ml by 5 mM Tes buffer (pH 7) and gently stirred for 30 min at 4°C. The membrane-bound components were removed by centrifugation at 30000 × g for 30 min and the supernatants were tested for their amidating activity.

The assay system for detecting and estimating amidating activity was based on the ability of enzyme preparations to convert the synthetic substrates TRH-Gly or Glu-His-Pro-Gly-Lys-Arg to TRH or Glu-His-Pro(NH_2), respectively. Assays were carried out at 37°C [in 0.1 M Tes buffer (pH 7) in a total volume of 220 μl] and with 200 μl of each lysed gradient fraction containing 10 μl (0.3 μCi) of either tritiated TRH-Gly or Glu-His-Pro-Gly-Lys-Arg and 10 μl of 0.2 mM bacitracin. After 5 h, the reaction was terminated by the addition of HCl to a final concentration of 1 N. Acidified incubation solutions were centrifuged at 15000 × g for 10 min and the resulting supernatants were evaporated to dryness and resuspended in the appropriate buffer for TLC or HPLC analysis.

TLC was carried out on cellulose plates (10 × 10 cm) developed in chloroform–methanol–ammonia (5:3:1, by vol.) for 30 min at 20°C. The peptides were detected by spraying with Pauli reagent. Areas corresponding to the position of TRH were eluted from the support by pure water and eluates were submitted to HPLC on an apparatus from Beckman Instruments equipped with A110 pumps directed by a 421 controller. A reverse-phase C-18 column (250 × 4.6 mm, Bondapack, Merck) eluted at a flow rate of 1 ml · min^{-1} was used. Elution was performed isocratically by a mixture of 5% acetonitrile, hexanesulfonate (1 g/l) in 20 mM acetic acid. Counting of the radioactivity was done on aliquots of each fraction by means of a liquid scintillation spectrometer (Intertechnique SL 30) using toluene-based medium. Products formed from each substrate were identified by their retention time as compared to synthetic reference peptides.

Under these conditions, a yield of approx. 2% was obtained for the amidation reaction.

Control assays, carried out with omission of the extract, showed that the substrates were stable under the conditions described.

3. RESULTS AND DISCUSSION

Detectable amounts either of labelled TRH or depyrolyzed TRH were formed upon incubation for 5 h at 37°C of pGlu-His-Pro-Gly or Glu-His-Pro-Gly-Lys-Arg with aliquots of hypothalamus granule extracts (fig.1,2). The identity of the tritiated amide peptides generated by the extract was established by comparing their behavior with those of synthetic TRH or depyrolyzed TRH on TLC and/or reverse-phase HPLC (fig.1,2). Fig.2B shows that a small amount of radioactive Glu-His-Pro-Gly was additionally found aside Glu-His-Pro(NH_2) when the hexapeptide was used as primary substrate. Analysis of the Percoll gradient indicated that the amide group forming activity was concentrated into the granule fractions (ϱ = 1.08) where it attained its highest specific activity (not shown). Activity could be readily solubilized by osmotic shock of the granules. Further characterization of the enzyme activity was achieved through filtration on Sephadex G-100 of the granule lysate. The amidating activity emerged in a single peak (fig.1) indicating an apparent molecular mass of approx. 60 kDa. The amounts of amidated peptides generated respectively from TRH-Gly and Glu-His-Pro-Gly-Lys-Arg, under similar experimental conditions, are shown in table 1. The hexapeptide is a 10-times better substrate than TRH-Gly. Cyclisation of the N-terminal Glu took place, to a limited extent, as shown by the formation of TRH from Glu-His-Pro-Gly-Lys-Arg.

The finding that Gly-Lys-Arg C-terminal exten-

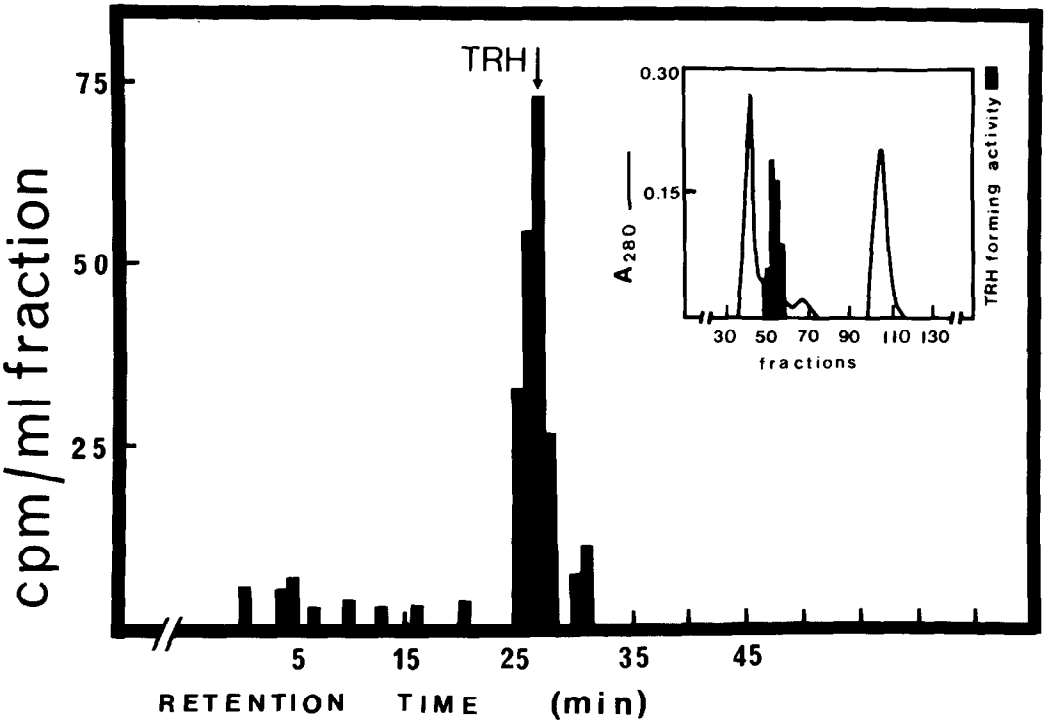

Fig.1. Identification of TRH produced by incubation of TRH-Gly with extract from rat hypothalamus granules. Tritiated TRH-Gly (0.3 μCi) was incubated at 37°C, pH 7, with 200 μl of granule lysate obtained from a Percoll gradient (fractions 7 and 8, d = 1.08). Incubation and analysis of the products were performed as described in section 2. Arrow indicates the retention time of synthetic TRH run under identical conditions. (Inset) Partial purification of the amidating activity from rat hypothalamus granules. An extract prepared from the secretory granule fraction of hypothalamus was filtered on a precalibrated Sephadex G-100 column (1.2 × 100 cm), equilibrated and eluted with 0.1 M ammonium acetate buffer (pH 7) at a flow rate of 4 ml/h. Each fraction (1 ml) was assayed for amidating activity as described above. Amide forming activity elutes like a 60-kDa molecular form.

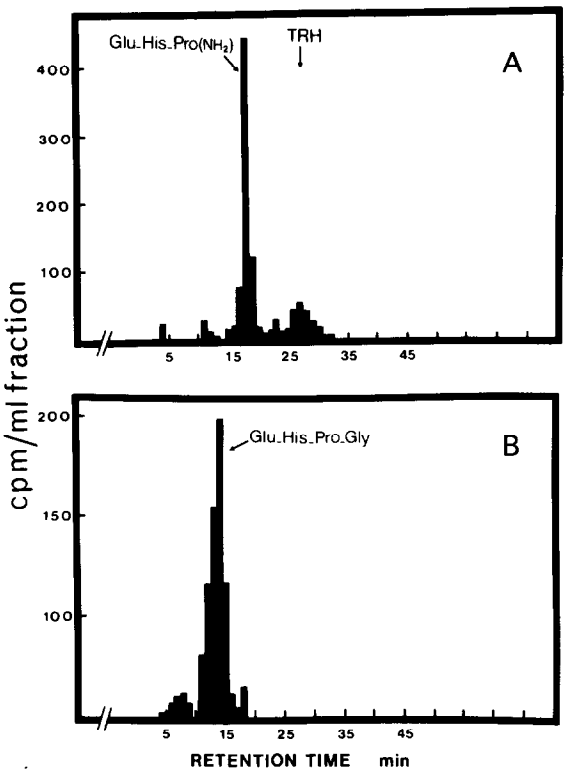

Fig.2. Identification of Glu-His-Pro-NH_2 and TRH produced by incubation of Glu-His-Pro-Gly-Lys-Arg with extract from rat hypothalamus granules. The assay system for detecting and estimating amidating activity using tritiated Glu-His-Pro-Gly-Lys-Arg as primary substrate was conducted as described in fig.1 except that TLC analysis was replaced at first by an HPLC, preparative, run. (A) The fractions corresponding to retention times of 13–15 min, from the first HPLC, were pooled, concentrated, and rerun on HPLC under the same experimental conditions as in fig.1. (B) The fractions corresponding to retention times of 17–19 min and 26–28 min, from the first HPLC, were pooled, concentrated, and rerun under similar conditions as in fig.1. Arrows indicate the respective retention times of synthetic TRH, Glu-His-Pro(NH_2) and Glu-His-Pro-Gly.

sion acts as a better substrate for the carboxamide activity than glycine alone suggests that the latter [5,6] is a minimal requirement. Indeed, if the result we observed could partly be explained by a stepwise action of trypsin-like and carboxypeptidase-like enzymes [13] on Glu-His-Pro-Gly-Lys-Arg followed by dehydrogenation and hydrolysis of the remaining tetrapeptide, we should have observed a higher, or at least the same, amount of amide product by using TRH-Gly as substrate. We thus have to assume that basic residue(s) can be involved in the recognition by specific amidating enzyme(s) and/or participate in the mechanism of amidation in a still unknown manner.

A recent report [14] indicated that the precursor for another C-terminal amide peptide, CRF, presents a Gly-Lys extension at its C-terminus. The differential rates of amidation we observed strongly suggest thus that C-terminal basic residue(s), which potentiate(s) amidation, are involved in a concerted mechanism with Gly, even though these basic amino acids have first to be sequentially removed. In line with this, the finding [6] that no amidation occurred using D-Tyr-Val-Gly-Lys as substrate is perplexing although it might be due to differences between experimental conditions used in that study (nature of substrates and source of enzyme activities), and the present one. In any case, the present data and [14] raise the hypothesis that the presence of a C-terminal basic amino acid residue(s) is required to confer on a substrate the ability to be recognized by amidating enzyme(s) that act on the hormonal sequence either included in its biosynthetic precursor form or after its cleavage.

Table 1

Amounts of amidated products obtained by incubation of either TRH-Gly or Glu-His-Pro-Gly-Lys-Arg ^{3}H-labelled peptides with granule extracts from rat hypothalami

Primary substrate used	Amount of generated products		Amount of amidated products
	[^{3}H]TRH	[^{3}H]Glu-His-Pro-NH_2	
[^{3}H]TRH-Gly	2.7 pg	–	2.7 pg
[^{3}H]Glu-His-Pro-Gly-Lys-Arg	4.8 pg	21.2 pg	26 pg

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