ISOLATION AND CHARACTERIZATION OF A γ_1-MELANOTROPIN-LIKE PEPTIDE FROM BOVINE NEUROINTERMEDIATE PITUITARY

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1. Introduction

A part of the N-terminal cryptic region of pro-opiomelanocortin (POMC) from bovine intermediate lobe pituitary shares the common sequence, His–Phe–Arg–Trp, with α- and β-melanotropin (MSH) and was therefore named γ-MSH [1]. Since this fragment is located between pairs of basic amino acids at Arg^{-57}–Lys^{-56} and Arg^{-43}–Arg^{-42} or Lys^{-28}–Arg^{-27}, it has been suggested [1] that POMC may be enzymatically processed in vivo at these basic amino acid pairs to yield γ-MSH-like peptides in analogy with the biosyntheses of several peptide hormones [2–4]. To detect γ-MSH-like peptides, radioimmunoassays (RIA) for the possible fragments, γ_1-MSH (Tyr^{-55}–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe^{-45}–NH_2) [5], γ_2-MSH (Tyr^{-55}–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Phe–Gly^{-44}) [5] and γ_3-MSH (Tyr^{-55}–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe–Gly–Arg–Arg–Asn–Gly–Ser–Ser–Ser–Ser–Gly–Val–Gly–Gly–Ala–Ala–Gln^{-29}) [6,7] were developed. The presence of γ-MSH-like peptides in extracts from a human pituitary adenoma [8] and from anterior and intermediate lobes of bovine pituitary [5,9] has been reported. We have now completed the isolation and structural characterization of a native bovine γ_1-MSH-like peptide. The primary structure of the isolated γ_1-MSH was established as H-Lys–Tyr–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe-NH_2. The amino acid sequence of this peptide corresponds to that of the fragment from Lys^{-56} to Phe^{-45} of POMC from bovine pituitary; this γ_1-MSH is probably another naturally occuring peptide arising from specific enzymatic processing of POMC in the intermediate lobe of the bovine pituitary.

2. Materials and methods

For peptide isolation 42 bovine pituitaries were obtained in a local abattoir within 10–30 min after death. The neurointermediate lobes were immediately dissected and frozen in liquid nitrogen. The tissue (11.5 g) was extracted by homogenization in 230 ml of a mixture of 5% (v/v) formic acid, 1% (v/v) trifluoroacetic acid, 1% (w/v) NaCl in 1 M HCl [10] first in a small blender for 2 min and then in a Polytron homogenizer for 1 min. The homogenate was centrifuged (3600 rev./min, 30 min) and the pellet re-extracted with 50 ml of the same solvent and centrifuged again. The supernatants from the two centrifugations were combined and adjusted to pH 2.4 with 5 M NaOH. Lipid material was removed from the solution by extraction with petroleum ether (twice with equal volumes). After degassing with a water aspirator for removal of residual organic solvent the defatted extract was subjected to reverse-phase peptide extraction as in [11,12]. Briefly, the peptide containing solution was pumped through a 20 × 2.5 cm column filled with octadecasilyl-silica (ODS; LRP-2, 37–60 μm particle size, Whatman, Clifton NJ). The column was then washed free of salt with 0.2 M acetic acid. The peptide material retained on the ODS column was eluted with 0.36 M pyridine formate (pH 3.0) in 60% (v/v) *n*-propanol and lyophilized (yield: 173 mg). This crude peptide fraction was subjected to two steps of reverse-phase high-performance liquid chromatography (RPLC) on an Altex Model 332 gradient liquid chromatograph (Berkeley CA) using Ultrasphere C18 columns (5 μm particle size, Altex) in conjunction with a mobile phase consisting of 0.36 M pyridine formate (pH 3) and a *n*-propanol gradient [13]. Column eluates were monitored with the fluorescamine stream-sam-

pling detection system [14] which diverted 2–5% of the effluent for detection. Aliquots of chromatography fractions were subjected to γ-MSH RIA using an antibody which recognizes the antigenic determinant –His–Phe–Arg–Trp–Asp–Arg–Phe-NH_2 [5].

Amino acid analysis was performed on a Liquimat III amino acid analyzer (Kontron, Zürich) equipped with an *o*-phthalaldehyde fluorescence detection system and a proline conversion accessory [15]. Peptide (50–100 pmol) was hydrolyzed and analyzed as in [16]. N-Terminal amino acids were determined with 1 nmol peptide by the dansyl method [17]. Sequence determination was done by automatic Edman degradation on the Beckman 890 C sequencer using the procedures in [18]. For tryptic peptide mapping the peptide was digested with 0.1 μg trypsin (TPCK-treated) in 50 μl of 2% (w/v) ammonium bicarbonate (pH 8), containing 8% (v/v) *n*-propanol (to prevent peptide adsorption to polypropylene surfaces).

3. Results

The isolation of pure γ_1-MSH was achieved in 4 purification steps including tissue extraction, defatting, reverse-phase peptide extraction and RPLC. After the first RPLC step (fig.1A) two broad zones containing γ_1-MSH-like immunoreactive material were obtained. The first zone contained several relatively well defined peaks, aliquots of which were used for amino acid analysis. From the fraction identified with an asterisk (fig.1A) an amino acid analysis was obtained which matched closely that of the POMC fragment Lys^{-56}–Tyr–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe^{-45} (not shown). Rechromatography of this fraction yielded 40 nmol pure γ_1-MSH (fig.1B).

The amino acid composition of the peptide was found to be $Asx_{0.92}$, $Gly_{1.01}$, $Val_{0.97}$, $Met_{1.19}$, $Tyr_{1.04}$, $Phe_{2.00}$, $His_{1.00}$, $Lys_{1.05}$, $Trp_{0.90}$, $Arg_{2.08}$ (means from 5 determinations, corrected for blank contamination). N-Terminal amino acid analysis revealed the presence of lysine and also indicated a high degree of purity of the isolated material. Sequence analysis with 8 nmol showed the peptide sequence as Lys–Tyr–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe. (Identification of all phenylthiohydantoin amino acids by RPLC was unequivocal except for the C-terminal phenylalanine residue.) Because the antiserum that we used recognizes only the amidated synthetic γ_1-MSH and crossreacts negligibly with a synthetic analog having the free carboxyl group at the phenylalanine C-terminus the native material is probably amidated. Further evidence in support of an amidated C-terminal residue was obtained by subject-

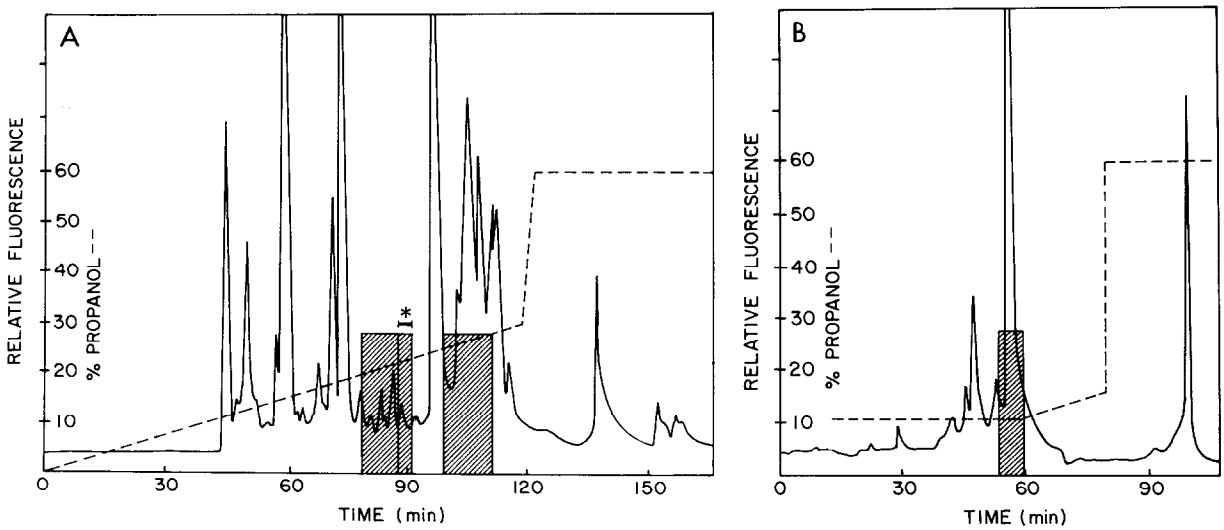

Fig.1. Final purification of γ_1-MSH by RPLC. (A) RPLC of 50 mg peptide extract on a semi-preparative RP-18 column (250 × 10 mm). The sample was dissolved in 10 ml 0.2 M acetic acid and loaded by pumping it directly onto the column. The flow rate was 0.8 ml/min and fractions of 2.4 ml were collected. (B) Rechromatography of the fraction marked with an asterisk (A) on an analytical RP-18 column (250 × 4.6 mm). For loading the fraction was diluted to 5 ml with 0.2 M acetic acid and injected via a 5 ml sample injection loop. The flow rate was 0.6 ml/min and 3 min fractions were collected.

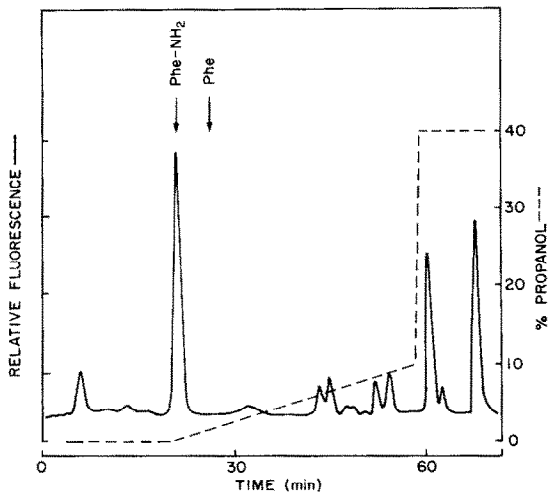

Fig.2. RPLC of a tryptic digest of 5 nmol purified γ_1-MSH peptide on an Ultrasphere RP-18 column (250 × 4.6 mm) in the 0.36 M pyridine formate (pH 3)/*n*-propanol mobile phase. For loading the peptide digest was diluted to 0.5 ml with 0.2 M acetic acid and injected via a 0.5 ml sample loop. The flow rate was 0.6 ml/min. Fractions of 1.8 ml were collected and aliquots of peak fractions subjected to amino acid analysis. Arrows indicate retention times of synthetic standards.

ing the native peptide to tryptic digestion and resolving the fragments by RPLC (fig.2). Amino acid analysis of the major peaks showed that one of the early eluting peaks contained only phenylalanine and therefore corresponded to the C-terminal fragment of γ_1-MSH. Aliquots of the phenylalanine containing fraction were rechromatographed in the same HPLC system with and without the presence of exogenous phenylalanine and phenylalanine amide standards. The isolated fragment coeluted with phenylalanine amide (not shown).

4. Discussion

There is considerable knowledge regarding the enzymatic processing of the corticotropin/β-lipotropin portion of POMC in the bovine intermediate lobe pituitary [4]. However, not much is known with regard to the processing of the N-terminal cryptic region of this precursor. γ_1-MSH isolated here is a fragment from this cryptic region. The γ_1-MSH sequence is located between 2 pairs of consecutive basic amino acid residues at Arg^{-57}–Lys^{-56} and Arg^{-43}–Arg^{-42}. Such double basic sequence regions are potentially important points for enzymatic cleavage of precursor proteins to yield mature peptide hormones, such as insulin [2], parathyroid hormone [3] and corticotropin or β-endorphin [4]. However, our data do not permit us to say definitely that this γ_1-MSH is generated in vivo by enzymatic processing of the precursor protein because we did not perform pulse-chase experiments to determine the precursor/product relationship. Nevertheless, we favor the view that this γ_1-MSH arises from intrinsic enzymatic processing of POMC, because γ_1-MSH-like immunoreactivity (1500 M_r by gel filtration) was found to be released into cell culture medium from dispersed intermediate lobe cells of bovine pituitary (not shown). Interestingly, the structure of the isolated γ_1-MSH has lysine at the N-terminus suggesting that POMC can be processed by cleavage between the two basic residues Arg^{-57} and Lys^{-56}. This finding is in contrast with evidence of a γ-MSH-like peptide with N-terminal tyrosine and the third residue being methionine [18]. Processing at the C-terminus of γ_1-MSH is strikingly similar to that of α-MSH. In both peptides cleavage is before a characteristic sequence $Gly-(^{Arg}_{Lys})-(^{Arg}_{Lys})$ and both peptides have an amidated C-terminus. It remains to be established whether such a cleavage pattern is due to a specific enzymatic process which would produce peptides with an amidated C-terminus that are present in many biologically active peptides in nature. Our data also indicate that more than one γ_1-MSH-like peptide exists in the intermediate lobe of the bovine pituitary (see e.g., fig.1A, which indicates the presence of a γ_1-MSH-like immunoreactive fraction eluting prior to the characterized γ_1-MSH). We do not know the structure of the additional γ_1-MSH-like peptide(s). Further processing of the protein precursor after cleavage between the double basic sequence Arg^{-57}–Lys^{-56} and removal of the N-terminal lysine might occur and thus yield des-Lys^1-γ_1-MSH similar to the γ_1-MSH-like peptide in [8]. In addition, as with α-MSH [19] acetylated forms of γ_1-MSH may be present.

The isolation of this γ_1-MSH-like peptide was based on a novel approach [10–12] which will be detailed elsewhere. Briefly, the goal of the new approach is to eliminate all procedures which are inefficient (such as classical chromatography on soft gels) and, furthermore, may lead to loss of material. Instead, reverse-phase peptide extraction and RPLC are the principal techniques employed in the isolation. Reverse-phase peptide extraction is suitable for the rapid preparation of a crude, but highly enriched peptide fraction

and serves for quick transition from large volumes/masses of the original tissue extract to amounts which can be subjected directly to RPLC. Also typical in this approach is that RPLC is used immediately for the separation of complex mixtures rather than for the final purification of already highly purified materials as has frequently been done. Overall peptide recovery with our new procedure is high (>90%) making it suitable for microisolation as illustrated in our isolation of γ_1-MSH which yielded 2.4 nmol γ_1-MSH/1 bovine pituitary fragment. The whole isolation procedure was completed in 3 days (immunoassays excluded). This approach to peptide isolation should enable one to isolate other peptides from this or other tissues with equal facility.

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