Isolation, structure, biological characterization, and synthesis of β -[Tyr⁹]melanotropin-(9-18) decapeptide from pig hypothalami

(α - and β -melanotropin/ β -lipotropin/corticotropin precursor)

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ABSTRACT A decapeptide with the amino acid sequence H-Tyr-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH was isolated from acid extracts of porcine hypothalami, structurally and biologically characterized, and synthesized. Except for the NH₂-terminal tyrosine, this decapeptide corresponds to the amino acid sequences 9-18 of porcine β -melanotropin (β -MSH) and 49-58 of porcine β -lipotropin (β -LPH); it also has a tetrapeptide sequence of amino acids (Phe-Arg-Trp-Gly) common to the 7-10 sequences in corticotropin (ACTH) and α -MSH. β -MSH, β -LPH, α -MSH, and ACTH from various species all have a histidine residue in the position immediately preceding the common sequence, and the occurrence of a natural peptide with the tyrosine residue in the corresponding site has not been previously reported. This suggests that the β -[Tyr⁹]MSH(9-18) decapeptide might be a fragment of a still larger precursor (prohormone) possibly related to β -LPH.

During our ongoing project on characterization and systematic classification of peptides from the diencephalon (1-5), we isolated a decapeptide from porcine hypothalami which has a COOH-terminal nonapeptide portion identical with amino acids 10-18 of porcine β -melanotropin (β -MSH) and 50-58 of β -lipotropin (β -LPH) but which, in contrast to these two polypeptides, has tyrosine instead of histidine directly preceding the common sequence. This paper describes the isolation, elucidation of structure, synthesis, and biological characterization of this decapeptide, which could possibly be a cleavage fragment of a larger precursor.

MATERIALS AND METHODS

Purification. The extract corresponding to 470,000 hypothalami was the same as that used for the isolation of porcine somatostatin (3) and pro-[Met]enkephalin (4). This extract was purified first by gel filtration on Sephadex G-25 (3). After extraction with phenol, the material was purified by chromatography on CM-cellulose, counter current distribution in solvent system I (0.1% acetic acid/1-butanol/pyridine, 11:5:3, vol/vol) in the A-4 apparatus, chromatography and rechromatography on SE-Sephadex, analytical gel filtration on Sephadex G-15, and finally partition chromatography in solvent system II (1-butanol/1-propanol/acetic acid/water, 7:1:2:10, vol/vol). In all steps, the separation pattern was followed by the Folin-Lowry reaction (6) or by absorbance at 278 nm.

Edman Dansyl Procedure. The Edman degradation was carried out according to the procedure described by Hartley (7). Dansyl amino acids were identified by thin-layer chromatography (TLC) on polyamide sheets (7). Purification of tryptic fragments and comparison of the natural and synthetic materials were carried out by high-pressure liquid chromatography (HPLC). All the extraction and purification proce-

dures and methods used for homogeneity and composition determinations and for the manual Edman dansyl degradation have been described in detail (1-5).

Aminopeptidase M Digestion. The peptide (6.6 μ g) was dissolved in 50 μ l of 0.2 N ethylmorpholine acetate (pH 8.0), and 5 μ g of aminopeptidase M (Pierce) was added. This was incubated at 37°C for 2 hr before amino acid identification.

Carboxypeptidase Y Digestion. Peptide (5.0 μ g) was dissolved in 50 μ l of 0.1 N pyridine acetate (pH 5.5) and 5 μ g of carboxypeptidase Y (Pierce) was added. This was incubated at 37°C for 5 hr. Amino acids were identified in the Beckman analyzer.

Assays for Biological Activity. MSH activity was assayed *in vitro* as described (8). The stimulation and inhibition of the release of ACTH, growth hormone (GH), thyroid-stimulating hormone (TSH), prolactin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were tested by using dispersed rat pituitary cells in monolayer cultures (9) or isolated rat pituitary quarters (2, 3, 10, 11). Each sample was assayed in quadruplicate. The concentration of pituitary hormones in the medium was measured by specific radioimmunoassays using the National Institute of Arthritis, Metabolism, and Digestive Disease kits (2, 3, 10, 11). The opiate activity was evaluated by the use of a mouse vas deferens assay (12). Lipolytic activity was determined by an increase in glycerol production with dispersed cells from rat epididymal adipose tissue.

RESULTS

Isolation. The decapeptide was a by-product from the purification of corticotropin-releasing factor (CRF) after gel filtration of 2 M acetic acid extracts from 470,000 pig hypothalami on a preparative column of Sephadex G-25 (1-3); some CRF activity was eluted in fractions 1157-1256 with R_F = 0.39-0.36 (elution volume/total volume = 0.88) (see figure 1 of ref. 1, 2, or 3). These combined fractions (166 g) were desalted by phenol extraction (1, 2), which reduced the weight to 14.4 g. The phenol extract was purified by ion-exchange chromatography on a CM-cellulose column (Fig. 1). Some CRF activity appeared in several fractions, including tubes 154-235 with a conductivity of 5.7-9.8 mS. Fractions from three such runs were combined (0.385 g) and subjected to 400 transfers (1-5) by countercurrent distribution in solvent system I. The peptide profiles are shown in Fig. 2. Fractions 190-269 (mean partition coefficient K = 0.87), which contained CRF activity (40.5 mg), were repurified by chromatography on SE-Sephadex (Fig. 3). Fractions 189-203, with a conductivity of 11.2-11.8 mS, contained CRF ac-

Abbreviations: ACTH, corticotropin; CRF, corticotropin-releasing factor; TLC, thin-layer chromatography; MSH, melanotropin; β -LPH, β -lipotropin; TSH, thyroid-stimulating hormone; LH, luteinizing hormone; GH, growth hormone (somatotropin); HPLC, high-pressure liquid chromatography.

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FIG. 1. Chromatography of phenol extract on CM-cellulose column, 2.8×80 cm, equilibrated with 2.0 mM ammonium acetate buffer (pH 4.5). Gradient to 0.1 M buffer (pH 7.0) through a 2000-ml mixing flask started at fraction 36 (fraction size, 25 ml). After 328 fractions were collected, 2 M acetic acid was applied to the column to displace the material not eluted with 0.1 M ammonium acetate.

tivity (4.5 mg). After rechromatography on SE-Sephadex, the weight was reduced to 1.9 mg. The next purification step was chromatography on an analytical column of Sephadex G-15 (Fig. 4). CRF activity emerged in fractions 64–80 with $R_F = 0.56$. After lyophilization, 1.2 mg of material was obtained, which was subjected to partition chromatography on a column of Sephadex G-25 using the upper phase of solvent system II. In the separation pattern (Fig. 5), fractions 11–40 (peak 1) showed CRF activity, fractions 85–105 proved to represent lysine vasopressin, and fractions 70–84 (R_F , 0.15; 0.42 mg) showed only one major spot by TLC in several solvent systems.

Composition and Structure Analyses. Aliquots of the composite of fractions 70–84 subjected to amino acid analyses contained 30% amino acids or a total of 126 μ g of peptide material. The amino acid composition is shown in Table 1. Because the amino acid ratios were essentially integral, this also indicated that the fraction was probably homogeneous. Elucidation of the decapeptide's structure included Edman dansyl degradation (7) of the intact peptide and of its tryptic digest, and

digestion with carboxypeptidase Y and aminopeptidase M. The two tryptic fragments were separated by HPLC. The structural work is summarized in the amino acid sequence,

The residues were identified by Edman degradation and two-dimensional TLC of dansyl-amino acids ($\rightarrow$); aminopeptidase M digestion and amino acid analysis ($\rightarrow$); and carboxypeptidase Y digestion and amino acid analysis ($\leftarrow$), $\downarrow$ shows the trypsin cleavage site.

On the basis of these results, the structure of decapeptide was determined to be H-Tyr-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH. This decapeptide was then readily synthesized by solid-phase methods, and the HF-cleaved material was purified by gel filtration on Sephadex G-25 followed by column chromatography on silica gel with eluent n -BuOH/AcOH/ H_2O /EtOAc, 1:1:1:1 (vol/vol). The decapeptide was obtained in 47% yield and was homogeneous by TLC in several solvent systems, by HPLC, and by amino acid analysis. The R_F s of natural and synthetic materials were compared by TLC on silica gel and found to be identical (Fig. 6). The retention times on HPLC of both products in 2-propanol/ammonium acetate with a linear gradient of 2-propanol from 5% to 50% for 25 min at 25°C were also the same (13 min, 20 sec).

Biological Characterization. The decapeptide of natural origin in doses of 0.1 μ g dry wt/ml failed to stimulate ACTH release from rat pituitary quarters or from monolayer cultures of rat pituitaries. However, since the natural preparation of the decapeptide was contaminated with 3% lysine vasopressin, which can stimulate the release of ACTH and influence the release of other hormones (13, 14), most tests were carried out with synthetic material.

The synthetic decapeptide at doses of 0.1 and 1.0 μ g/ml failed to stimulate the release of ACTH, GH, LH, FSH, or TSH but caused some stimulation of the release of prolactin from isolated rat pituitary quarters. However, this stimulation was inconsistent, and there was no clear dose-response relationship. When monolayer cultures of rat pituitary cells were used, the decapeptide in doses of 0.01, 0.033, 0.1, 0.33, 1.0, 3.0, and 10 μ g/ml did not alter the release of ACTH, GH, prolactin, LH, FSH, and TSH. Since thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and somatostatin have significant

FIG. 2. Countercurrent distribution of 385 mg of material from CM-cellulose in solvent system I. Lower phase was 3 ml and upper phase was 5 ml. The number of transfers was 400. Peptide analyses were carried out on 50- μ l aliquots of the upper phase.

FIG. 3. Chromatography of material from countercurrent distribution on SE-Sephadex column, 1.5 × 140 cm, equilibrated with 0.2 M pyridine acetate buffer (pH 4.5). A linear gradient to 0.85 M buffer (pH 5.3) was applied at fraction 36. A stepwise elution with 1.0 M buffer (pH 5.3) was started at fraction 238 (fraction size, 10 ml).

effects on the *in vitro* release of TSH, LH plus FSH, and GH, respectively, in doses as small as a few nanograms per milliliter, it can be assumed that the decapeptide does not possess hypophysiotropic activities.

When the MSH activity was carefully measured by using an *in vitro* assay method on frog skin (8), the decapeptide was found to have small but definite activity. The mean MSH activity from three assays was calculated to be $1-2 \times 10^5$ units/g, when expressed as suggested by Lande and Lerner (15). This corresponded to 1×10^{-5} of the activity of α -MSH. On a molar basis (16) the melanocyte-stimulating activity was about 0.6×10^{-5} that of α -MSH. At doses as high as 2 μ M, it did not inhibit the electrically stimulated contractions of the vas deferens of mice. The [D-Ala²,Met]enkephalin-NH₂ standard had a median effective dose ranging from 3.0 to 4.8 μ M in the same four assays. Similarly, the α -MSH did not inhibit the contractions of the mouse vas deferens at 1 μ M (12). When assayed for lipolytic

activity, this decapeptide failed to stimulate an increase in glycerol production in doses 4 to 4000 times greater than the effective lipolytic dose of ACTH in our system (17).

DISCUSSION

Much additional work is needed to characterize chemically the additional releasing and inhibiting hypothalamic hypophysiotropic hormones. However, in addition to regulating the secretory activity of the anterior pituitary gland, the hypothalamus is involved in control of various other bodily functions, such as regulation of temperature, heart rate and blood pressure, state of sleep and rage, and gastrointestinal functions and satiety (18). Some of these functions could be mediated by neural, endocrine, or paracrine transmitters (neurohormones), a number of which could be neuropeptides. Therefore, it would not be unexpected if some of the peptides detected in the hypothalamus could have important biological activities and physiological significance. Other polypeptides may lack bio-

FIG. 4. Gel filtration of 1.9 mg of material from SE-Sephadex on Sephadex G-15, 1.5 × 169 cm; hold-up volume, 125 ml; solvent, 0.2 M acetic acid; fraction size, 3.0 ml. Folin-Lowry analyses were carried out on 200- μ l aliquots.

FIG. 5. Partition chromatography of material from Sephadex G-15 on column of Sephadex G-25, 0.9 × 148 cm with solvent system II; fraction size, 2 ml; hold-up volume, 24 ml. Aliquots for Folin-Lowry analyses were 200 μ l.

FIG. 6. TLC of the natural (N) and synthetic (S) decapeptide. (A) Solvent system: 1-butanol/acetic acid/water/ethyl acetate, 1:1:1:1 (vol/vol); $R_F = 0.35$. (B) Solvent system: 1-butanol/pyridine/acetic acid/water, 15:10:3:12 (vol/vol); $R_F = 0.56$.

logical activities but could be the precursors or metabolites of regulatory hormones and of other physiologically active peptides. Thus, systematic characterization and classification of all peptides found in the hypothalamus is desirable (19).

This work reports the isolation and structural elucidation of a decapeptide which, except for its NH_2 terminus, corresponds to amino acids 10–18 of porcine β -MSH and 50–58 of β -LPH and which also shares the sequence Phe-Arg-Trp-Gly of amino acids 7–10 of ACTH and MSH. However, unlike these four

Table 1. Amino acid composition of the decapeptide from pig hypothalamus

Amino acid	nmol/ μg	Amino acid ratio	Integral residues
Aspartic Acid	0.63	1.00	1
Serine	0.63	1.00	1
Proline	1.15	1.76	2
Glycine	0.70	1.10	1
Tyrosine	0.53	0.84	1
Phenylalanine	0.59	0.94	1
Lysine	0.64	1.01	1
Arginine	0.65	1.03	1
Tryptophan*	0.57	0.90	1

Hydrolyzed in 6 M HCl.

* Hydrolyzed in 4 M methanesulfonic acid containing 0.2% tryptamine.

polypeptides, this decapeptide has tyrosine instead of histidine as the residue preceding this common sequence. Fig. 7 compares the amino acid sequences of porcine β -MSH (20), known β -MSH of other species (21), α -MSH (22), and parts of porcine β -LPH (23) and porcine ACTH (24). The Phe-Arg-Trp-Gly, common to our decapeptide and to the other hormones, has been preserved in evolution from dogfish to man (21). However, the occurrence of a tyrosine residue in the position preceding this common sequence, where all these hormones (α - and β -MSH, β -LPH, ACTH) have a histidine residue, has not been previously reported. The detection of this decapeptide could offer some basis for conjecture that it is a cleavage fragment of a still larger molecule possibly related to β -LPH and β -MSH, which is indirectly supported by our previous experimental findings of fragments corresponding to residues 33–46 of the porcine hemoglobin α -chain and fragments derived from the NH_2 -terminal sequence 1–10 of the porcine hemoglobin β -chain (4, 10). According to theoretical speculation, such a precursor molecule could possibly contain fragments corresponding to α -neoendorphin (25), dynorphin (26) and [Leu]-enkephalin, or pro-[Met]enkephalin (4) and [Met]enkephalin and thus be the true precursor of some of these neuropeptides.

Recently, β -endorphin-like peptides have been isolated from

β -[Tyr⁹]MSH 9–18:

Human:	H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Macacus:	H-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Porcine:	H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
	1 2 3 4 5 6 7 8 9	10 11 12 13 14 15 16 17 18
Equine:	H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Arg-Lys-Asp-OH
Bovine:	H-Asp-Ser-Gly-Pro-Tyr-Lys-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Camel:	H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Dogfish:	H-Asp-Gly-Ile-Asp-Tyr-Lys-Met-Gly-His	Phe-Arg-Trp-Gly-Ala-Pro-Met-Asp-Lys-OH
Pig		
β -LPH:	-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His	Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-Lys-Arg-
	39 40 41 42 43 44 45 46 47 48 49	50 51 52 53 54 55 56 57 58 59 60
α -MSH:	Ac-Ser-Tyr-Ser-Met-Glu-His	Phe-Arg-Trp-Gly-Lys-Pro-Val-NH ₂
ACTH:	H-Ser-Tyr-Ser-Met-Glu-His	Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-
	1 2 3 4 5 6	7 8 9 10 11 12 13 14 15

FIG. 7. Amino acid sequences of β -[Tyr⁹]MSH (9–18) decapeptide, β -MSH from various species, and porcine β -LPH, α -MSH, and ACTH. Taken in part from Li et al. (21)

beef brains (27). In addition, precursors of β -LPH, β -endorphin, and ACTH have been reported in camel pituitaries (28) and in pars intermedia of rat pituitaries (29, 30). Precursors of [Leu]- and [Met]enkephalin, but different from β -endorphin, have also been reported in the adrenal medulla (31). Two peptides produced by tryptic digestion of a common β -LPH and ACTH precursor contained fragments of our decapeptide, except that histidine was again present instead of tyrosine (32). This precursor was synthesized in an mRNA-dependent reticulocyte cell-free system, under the direction of mRNA (32). The mRNA sequence encoding for the common precursor of bovine ACTH and β -LPH was purified from neuro-intermediate lobes of beef pituitaries (33, 34). cDNA prepared from this template was cloned in *Escherichia coli* and the nucleotide sequence was determined. One of the cloned cDNA inserts corresponded to a new peptide located outside the ACTH/ β -LPH portion and related to α - and β -MSH, which was designated γ -MSH (34). This peptide contained the sequence Phe-Arg-Trp common to α -MSH, β -MSH, and our decapeptide. Four possible γ -MSH peptides corresponding to the hypothetical hormone were synthesized by Ling *et al.* (16); the MSH activities of these four peptides were much lower (10^{-4} or 10^{-7}) than the activity of α -MSH and corresponded to about the same order of magnitude as our decapeptide. The lipolytic activity of the decapeptide could not be detected in our assay system (17). Studies in our laboratory (17, 35) have shown that both α - and β -MSH lacked lipolytic activity when incubated *in vitro* with either human or rat epididymal adipose tissue. Apparently, the occurrence of tyrosine in place of histidine does not potentiate the lipolytic activity of this molecule in rat epididymal adipose tissue. It has been reported that, for melanocyte-stimulating and lipolytic activities, the smallest peptide sequence to show observable activity *in vitro* was His-Phe-Arg-Trp-Gly, corresponding to residues 6–10 of α -MSH or 9–13 of β -MSH (36–38).

Although at this time only a weak MSH activity could be assigned to the decapeptide, its presence in the hypothalamic extracts may provide some insight regarding the potential existence of novel peptides related to β -LPH, α -MSH, and β -MSH with possible biological roles.

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