

Isolation and characterization of melanotropins from lamprey pituitary glands

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Dedicated to the memory of Johannes Meienhofer

Three peptides containing the melanotropin-core amino-acid sequence, YXMXHFRWG, were isolated from the pituitary glands of a modern representative of the most primitive vertebrates, the sea lamprey, *Petromyzon marinus*. MSH-A, a nonadecapeptide (NPELYQMNHFRWGQPPTHF), is free at both ends. MSH-B, an eicosapeptide (VQESADGYRMQHFRWGQPLP), is free at the *N*-terminus and amidated at the *C*-terminus. They differ strikingly from gnathostome MSHs in structure. The third peptide, with an apparent molecular weight of 15 kDa, was tentatively designated lamprey ACTH, based on a structural feature: the *N*-terminal 22-residue-MSH (SVSSPKYAMGHFRWGSPDKATI) is followed by four consecutive basic amino acids (RKRR) and a ACTH-like sequence (PVRPNTSDSPEIPDYAF--). MSH-B is 10 and 100 times more potent than α -MSH and MSH-A, respectively, in a frog skin assay *in vitro*, whereas the lamprey ACTH showed no melanotropic activity. Lamprey ACTH did, however, show corticotropic activity on the lamprey pronephric and mesonephric tissue. © Munksgaard 1995.

Key words: amino-acid sequence; corticotropic activity; corticotropin; lamprey; melanotropic activity; melanotropin; pituitary hormone

The Agnatha, or jawless fishes, represent the first vertebrates that diverged from the main lineage of vertebrate evolution leading to gnathostomes over 550 million years ago. Lamprey and hagfish are the only two extant agnathan representatives (1). The adeno-hypophysis of the lamprey is morphologically similar to those of the gnathostome fishes (2), which suggests early establishment of endocrine function of the tissue (3). Recently, molecular characterization of adeno-hypophysial hormones has been significantly advanced in lower vertebrates, especially teleost fishes (4). Thus, the lampreys have become an intriguing group on which to base a rational scenario of the molecular evolution of the adeno-hypophysial hormones.

Of the classical vertebrate adeno-hypophysial functions, melanotropic, corticotropic and gonadotropic activities have been demonstrated in the pituitary of lampreys (5-9), although considerable doubt still exists as to the presence of any other adeno-hypophysial hormones homologous to those of higher vertebrates. In-

deed, corticotropin (ACTH)-like and melanotropin (MSH)-like substances have been demonstrated immunohistochemically in the rostral pars distalis and pars intermedia of lamprey pituitaries using antisera against mammalian hormones (10-13). However, we could detect no immunoblot reactivities in an extract of sea lamprey pituitaries tested with antisera against gonadotropin, growth hormone, prolactin or somatotactin of higher vertebrates.

In gnathostomes, MSH and ACTH are produced from a common precursor protein, proopiomelanocortin (POMC), by post-translational cleavage at a pair of basic amino acids in a tissue-dependent manner (14-16). The melanotropin sequence is repeated three times (α -, β -, and γ -MSH) within the POMC of tetrapods (17, 18) and probably of elasmobranch fish (19-21), while γ -MSH is missing in teleost POMCs (22-24). This suggests that the POMC gene may have evolved by intragenic duplication of a primordial MSH gene during early vertebrate evolution.

The present paper describes the isolation and structural characterization of the classical adenylohypophysial hormones, MSHs and ACTH, from an extant species of the most primitive vertebrates.

EXPERIMENTAL PROCEDURES

Materials. Pituitary glands were dissected from mature sea lampreys, *Petromyzon marinus*, after capture during their anadromous migration from Lake Huron, Michigan. They were stored at -80°C until extracted. Salmon α -MSH, being identical to the mammalian counterpart, was isolated from chum salmon pituitaries by the method reported previously (25). Enzymes used for fragmentation were trypsin and chymotrypsin from Boehringer Mannheim (Mannheim). All chemicals used for structural determination were sequential grade from Wako Pure Chemical (Tokyo). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Tentagel-resin, reagents and solvents for peptide synthesis were purchased from Shimadzu (Kyoto).

Isolation. Frozen pituitary glands ($n = 1040$, 4.0 g wet weight) were extracted at 4°C for 2 h with 10 mM ammonium acetate, pH 6.1/ethanol (7/13, v/v) containing 1.5 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. After centrifugation (10 000g, 4°C , 15 min), the extract was poured into three volumes of prechilled ethanol and kept at 4°C for 2 h. The resulting supernatant was concentrated by use of a rotary evaporator under vacuum and subjected to a Sep-Pak C18 cartridge (Waters Associates). MSHs were recovered by elution with 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) and further fractionated by ion-exchange HPLC on a TSKgel CM-2SW column (4.6×25 mm, Tosoh) with a linear gradient of 0.01–1 M ammonium acetate (pH 6.8) in 10% acetonitrile for 60 min at 40°C and flow rate of 1 mL/min, and monitored by absorbance at 280 nm. For isolation of ACTH, the ethanol precipitate was fractionated by gel filtration on a Sephadex G-100 column (2.64×85 cm) in 50 mM ammonium bicarbonate, pH 9.0 at flow rate of 24 mL/h. All fractions were finally purified by reversed-phase (RP) HPLC on a TSKgel ODS-120T column (4.6×250 mm, Tosoh) with a linear gradient of acetonitrile in 0.1% TFA for 50 or 60 min at 40°C and flow rate of 1 mL/min, and monitored by absorbance at 220 nm.

SDS-PAGE. Fractions from gel filtration and RPHPLC were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (26). The gel was stained with 0.25% Coomassie brilliant blue, and protein bands were visualized by destaining in acetic acid/methanol/water (1/2.5/6.5, v/v).

Sequence analysis. Purified peptide was digested with trypsin (E/S = 1/30, w/w) in 200 mM ammonium

acetate, pH 8.3 at 37°C for 2 h or with chymotrypsin (E/S = 1/200, w/w) in 200 mM ammonium acetate, pH 8.0 at 37°C for 30 min. Peptide fragments were separated by RPHPLC on a column of TSKgel ODS-120T with a linear gradient of 2–62% acetonitrile in 0.1% TFA for 120 min at 40°C and flow rate of 1 mL/min, and monitored by absorbance at 215 nm.

Amino-acid composition was determined by use of an automated amino-acid analyzer (Hitachi, model L8500) following acid hydrolysis in 6 N HCl at 110°C for 18 or 22 h. Sequence analysis was performed by an automated gas-phase amino-acid sequencer (Shimadzu, model PSQ-1/C-R4A).

Peptide synthesis. Peptides were synthesized using an automated solid-phase peptide synthesizer (Shimadzu PSSM-8). A series of reactions was performed according to the instruction manual as follows. MSH-A was synthesized on TG-AC-Fmoc-Phe-resin (20 mg/vessel, capacity 210 $\mu\text{mol/g}$). MSH-B was synthesized on TG-RAM 95% TFA-resin (21 mg/vessel, capacity 200 $\mu\text{mol/g}$) to introduce an amide group onto the carboxyl terminus. Fmoc-amino acid (42 μmol) was introduced with benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (42 μmol) in the presence of 1-hydroxybenzotriazole (42 μmol) and *N*-methylmorpholine (63 μmol) in dimethylformamide (DMF) (147 μL) for 30 min. The manipulations in each synthesizing cycle consisted of (i) deprotection of the Fmoc group with 30% piperidine/DMF (500 μL , 6 min, 2 times), (ii) washing with DMF (600 μL , 5 times), (iii) coupling of each amino acid in DMF, (iv) washing with DMF (600 μL , 5 times). After completion of all amino-acid couplings, cleavage of the peptides and deprotection of the side chain were performed in TFA/water/thioanisole/ethylmethyl sulfide/ethandiol/thiophenol = 165/10/10/6/5/4 (v/v) supplemented with 0.5% 2-methylindole (<200 μL) for 6 h at room temperature. The peptides were precipitated by the addition of prechilled ether (10 mL) and lyophilized from 0.1% TFA. Peptides were purified by RPHPLC on a TSKgel ODS-120T column. The amino-acid sequences of the synthetic peptides were confirmed by sequence analysis.

Bioassay. Melanin-dispersing activities of the synthetic peptides were evaluated by use of frog skins, *Rhacophorus shlegelii*, *in vitro* according to Eberle (27). The frogs weighed about 7 g, and were kept under white background light overnight before assay. The lateral skin was removed from freshly decapitated frogs, washed in Ringer solution for 60 min, and incubated in Ringer containing MSH for 60 min. The response was assessed visually using a microscope. The melanophore index was determined (28). Statistical difference was assessed by Duncan's new multiple-range test.

Stimulation of steroidogenic activity in the adrenal by the lamprey ACTH was evaluated by incubating

pronephric and mesonephric tissue, taken from adult male *P. marinus*, with radiolabeled 11-deoxycortisol, with and without the ACTH. Other labeled substrates (testosterone and progesterone) were incubated at the same time, but the results from these will be reported elsewhere. The lamprey kidney tissues (0.2–0.4 g) were collected from freshly killed animals, minced and incubated in 2 mL Leibovitz medium containing 10 μ Ci labeled substrate at 15 °C for 3 h. Of the four replicates of mesonephric tissue and the two replicates of pronephric tissue, half were incubated with 1000 ng/mL lamprey ACTH and the other half without the ACTH.

RESULTS

Lamprey melanotropins

MSHs were recovered from an ethanol extract of the pituitaries by fractionation with ethanol and by desalting with Sep-Pak cartridges. MSHs were successively purified by ion-exchange HPLC (Fig. 1A) and by RPHPLC (Figs. 2A and 2B). Peptides containing amino acids with a MSH core were subjected to sequence analysis. Two peptides from E1 and F1 were named MSH-A and MSH-B, respectively. The total yields were 20 μ g for MSH-A and 50 μ g for MSH-B.

The sequence analysis of intact MSH-A allowed the

assignment of 13 *N*-terminal residues. RPHPLC of tryptic digests of this peptide resulted in the separation of two peptide fragments (T1 and T2) (Fig. 3A). The T1 sequence of the 11 *N*-terminal residues was determined. T2 overlapped with 12–13 residues of the native peptide and had an additional six residues. Amino-acid compositions of MSH-A and its fragments are listed in Table 1. The synthetic nonadecapeptide corresponding to this sequence was chromatographically identical to the natural peptide. Thus, MSH-A was determined to be a nonadecapeptide with the amino-acid sequence proposed in Fig. 4.

Sequence analysis of intact MSH-B allowed assignment of the 19 *N*-terminal residues. RPHPLC of a chymotryptic digest of this peptide resulted in the separation of three fragments, C1, C2 and C3 (Fig. 3B). Analysis confirmed the sequence of the residues 1–8, 9–13 and 14–19, respectively. Analysis of amino-acid composition suggested that the C3 might be a heptapeptide including Pro (Table 1), although no amino-acid residues in sequence analysis of C3 was detected at the seventh cycle. Because the natural peptide was not available for further analysis such as carboxypeptidase Y digestion, three peptides were synthesized; one was a nonadecapeptide corresponding to the sequence up to the 19th residue; the second peptide was an eicos-

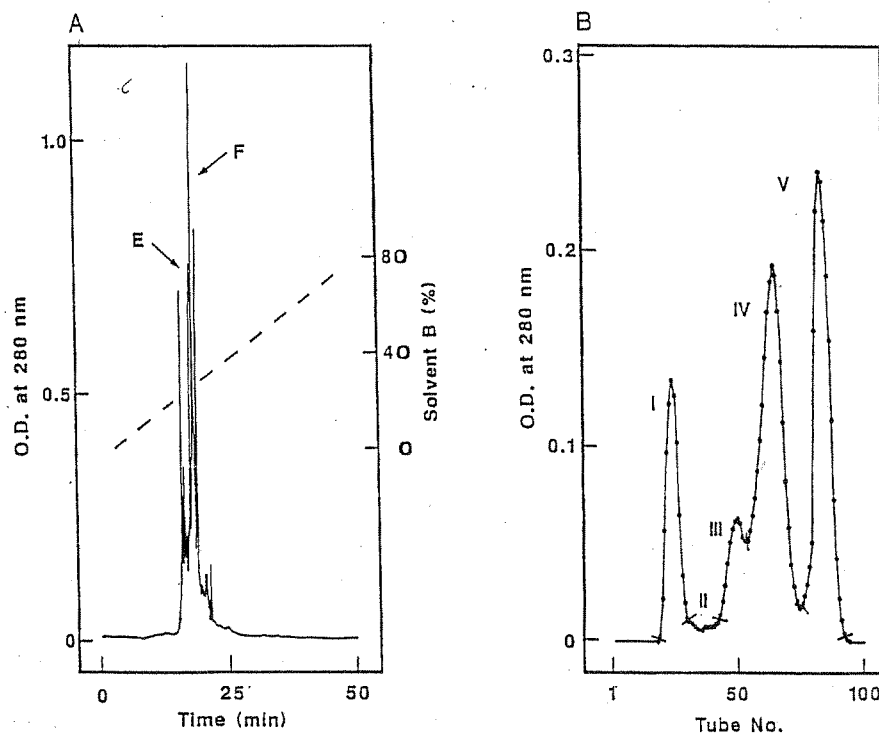


FIGURE 1

(A) Ion-exchange HPLC of an ethanol extract of lamprey pituitaries on a TSKgel CM-25 column (4.6 × 25 mm) with a linear gradient of solvent A and solvent B. Solvent A: 10 mM ammonium acetate, pH 6.8/acetonitrile = 9/1 (v/v). Solvent B: 1 M ammonium acetate, pH 6.8/acetonitrile = 9/1 (v/v). (B) Gel filtration of ethanol precipitate of the lamprey pituitary extract on a Sephadex G-100 column (2.64 × 85 cm) in 50 mM ammonium bicarbonate, pH 9.0. Fraction size: 2.7 mL/tube after forerunning of 50 mL.

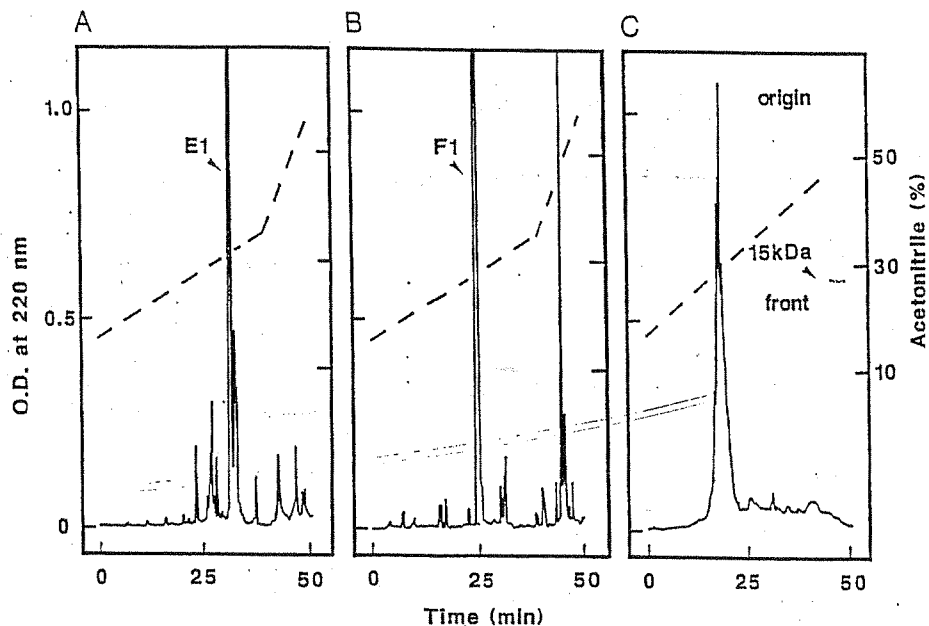


FIGURE 2

Purification of lamprey MSHs and ACTH by RPHPLC on a TSKgel ODS-120T column (4.6 × 25 mm, 5 μm). (A) MSH-A (E1) from fraction E of Fig. 1A. (B) MSH-B (F1) from fraction F of Fig. 1A. (C) ACTH from fraction IV of Fig. 1B.

apeptide, having an additional Pro at the C-terminus of the nonadecapeptide, and the third was an eicosapeptide with Pro amide at the C-terminus of the nonadecapeptide. The third peptide exhibited an identical retention time with the natural peptide. Thus, MSH-B was determined to be an eicosapeptide with the amino-acid sequence proposed in Fig. 4.

Lamprey ACTH

Results of gel filtration of the ethanol precipitate are shown in Fig. 1B. Fraction IV gave a broad peak, with several spikes in RPHPLC (Fig. 2C). The pool of the several peaks showed a single band of 15 kDa on SDS-PAGE (Fig. 2C, inset). Sequence analysis of the pooled fraction proved to have a single amino-acid sequence

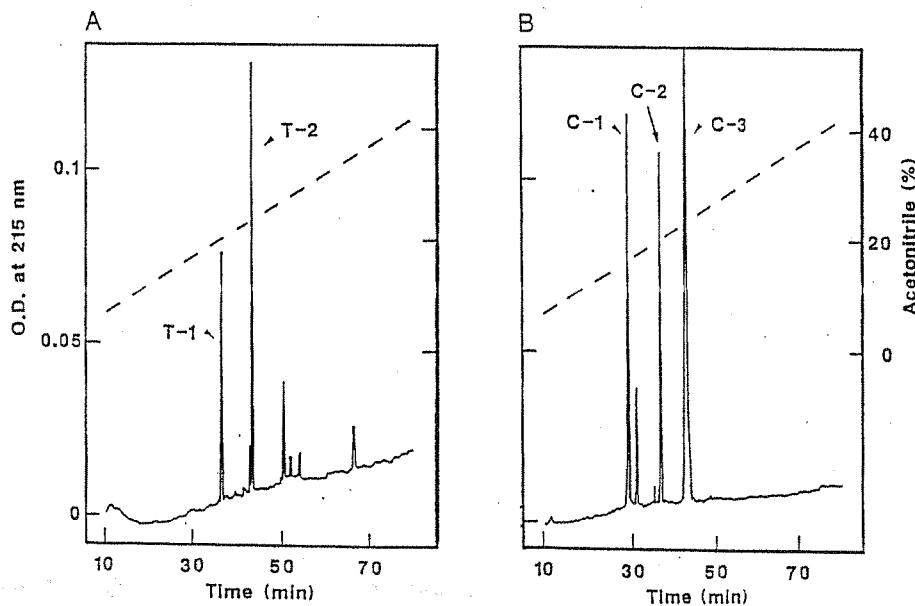


FIGURE 3

Peptide maps of MSHs on a TSKgel ODS-120T RPHPLC column. (A) tryptic digest of MSH-A. (B) chymotryptic digest of MSH-B.

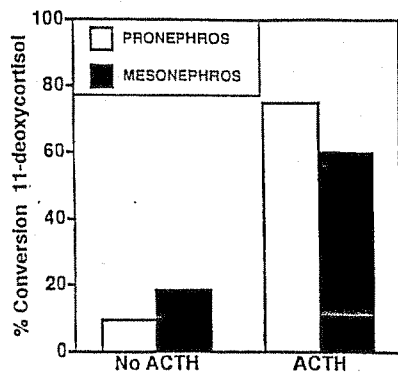


FIGURE 6 Percentage conversion of the corticosteroid precursor, 11-deoxycortisol, in the presence and absence of the lamprey ACTH, after incubation for 3 h with lamprey pronephric or mesonephric tissue.

properties to deoxycorticosterone in the TLC separation system used (29), but has not yet been identified. In the anterior mesonephros, the effect of ACTH was also marked but none of the products of conversion have been identified.

DISCUSSION

This is the first structural identification of the classical adenohypophysial hormones, the MSHs, in a modern representative of the most primitive vertebrates. The lampreys resemble gnathostome fishes in the basic morphology of the adenohypophysis, so that it is conceivable that adenohypophysial hormones homologous to those of gnathostomes may occur in the lamprey. Among them, α -MSH has been believed to be one of the most 'ancient' molecules. Apparently little molecular change occurred over the last few hundred million years, and there has been remarkable conservation of its amino-acid sequence across the different species that

have been studied so far. Indeed, the presence of MSH activity in the lamprey pituitary has long been recognized. Not only does removal of the lamprey pars intermedia cause pallor (30, 31), but also extracts of whole pituitary of adult lampreys cause melanin-dispersion in frogs (32) and lizards (6). However, pituitary extracts of lampreys were so inactive in a radioimmunoassay for α -MSH that lamprey MSH was suggested not to be chemically identical to α -MSH (6). In fact, no lamprey pituitary molecule could readily be assigned as α -MSH, whereas three distinct peptides containing the melanotropin core sequence were identified in this study.

The amino-acid-sequences of these lamprey peptides were compared with those of MSHs and ACTHs from salmon and dogfish by aligning the MSH-core sequence and introducing five gaps for salmon and dogfish ACTHs, to obtain maximal homology (Fig. 7). MSH-A is a nonadecapeptide, being free at both ends. MSH-B is an eicosapeptide, being free at the *N*-terminus and amidated at the *C*-terminus. Both are somewhat larger than β -MSHs. The third peptide, a major component of the pituitary extract, has an apparent molecular weight of 15 kDa (Fig. 2), and shares a similar molecular architecture with gnathostome ACTHs, in which the *N*-terminal MSH sequence is followed by four consecutive basic amino acids and ACTH C-terminal region-like sequence (Fig. 7). The lamprey ACTH resembles dogfish ACTH more closely than salmon ACTH. Thus, the 22 *N*-terminal residues of the lamprey polypeptide may be compared to lamprey α -MSH.

Recently, Heinig *et al.* (33) have cloned a lamprey POMC-cDNA that encoded ACTH and endorphin, but neither MSH-A nor MSH-B. Subsequently, we cloned two distinct POMC-cDNAs from the lamprey pituitary λ gt11 cDNA library, in which the antisera against ACTH(1-16) and MSH-B were used as probes. One POMC encoded ACTH, 'nasohypophysial factor' (34) and endorphin and the other POMC-cDNA encoded MSH-A, MSH-B and another endorphin. The

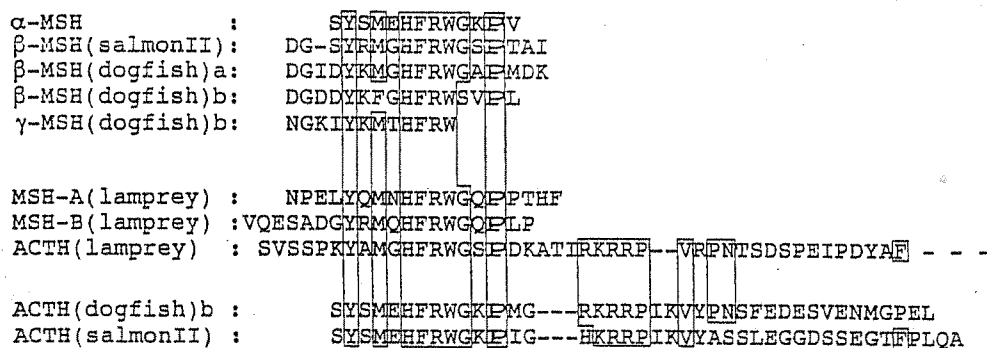


FIGURE 7 Sequence comparison of MSHs and ACTHs. Amino-acid sequences are taken from dogfish β -MSH (19, 36), dogfish γ -MSH (21), dogfish ACTH (20), salmon β -MSH (37), salmon ACTH (23). α -MSH corresponds to ACTH (1-13). a: *Scyliorhinus canicula*. b: *Squalus acanthias*. -: gap.

TABLE 1

Amino-acid composition of lamprey MSH-A and MSH-B, and their trypsin- and chymotrypsin-digested fragments^a

Amino acid	MSH-A	T-1	T-2	MSH-B	C-1	C-2	C-3
Asp	1.8 (2)	2.0 (2)		0.7 (1)	1.0 (1)		
Thr	0.9 (1)		0.8 (1)				
Ser				0.9 (1)	1.1 (1)		
Glu	3.2 (3)	2.2 (2)	1.3 (1)	4.1 (4)	2.1 (2)	1.3 (1)	1.2 (1)
Pro	3.3 (3)	0.8 (1)	1.6 (2)	2.0 (2)			1.7 (2)
Gly	1.1 (1)		1.2 (1)	2.0 (2)	1.2 (1)		1.1 (1)
Ala				1.1 (1)	0.9 (1)		
Val				1.0 (1)	1.0 (1)		
Met	0.9 (1)	0.9 (1)		0.7 (1)		1.0 (1)	
Leu	1.0 (1)	1.1 (1)		1.2 (1)			1.0 (1)
Tyr	0.8 (1)	0.9 (1)		0.9 (1)	0.7 (1)		
Phe	2.4 (2)	0.9 (1)	0.9 (1)	1.0 (1)		0.9 (1)	
His	1.7 (2)	1.2 (1)	1.2 (1)	1.2 (1)		0.8 (1)	
Arg	0.9 (1)	0.9 (1)		2.2 (2)		1.0 (1)	1.0 (1)
Trp	ND ^b (1)		ND ^b (1)	ND ^b (1)			ND ^b (1)
Total	(19)	(11)	(8)	(20)	(8)	(5)	(7)

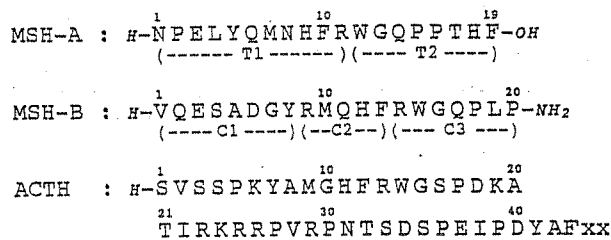
^a Numbers in parentheses are proposed by sequence analyses.^b ND, not detected.

FIGURE 4

Sequence analysis of lamprey MSHs and ACTH.

and allowed assignment of 43 *N*-terminal residues as shown in Fig. 4. Thus, the peptide is characterized by a structure in which the *N*-terminal MSH sequence is followed by four basic amino-acid residues and the C-terminal ACTH-like sequence. Accordingly, the 15 kDa polypeptide was tentatively designated as lamprey ACTH (see Fig. 6 later), although the complete amino-acid sequence remains to be elucidated.

Melanotropic activity

The melanin-dispersing activities of synthetic peptides, MSH-A, MSH-B, and natural peptides, lamprey ACTH and salmon α -MSH by *in vitro* frog skin assay are summarized in Fig. 5. Lamprey ACTH was virtually inactive in the assay. MSH-B was about 10 and 100 times more potent than α -MSH and MSH-A, respectively.

Corticotropic activity

The corticotropic activity of the lamprey ACTH is summarized in Fig. 6. Conversion of 11-deoxycortisol by the pronephric tissue was greatly enhanced in the presence of the lamprey ACTH, being almost negligible

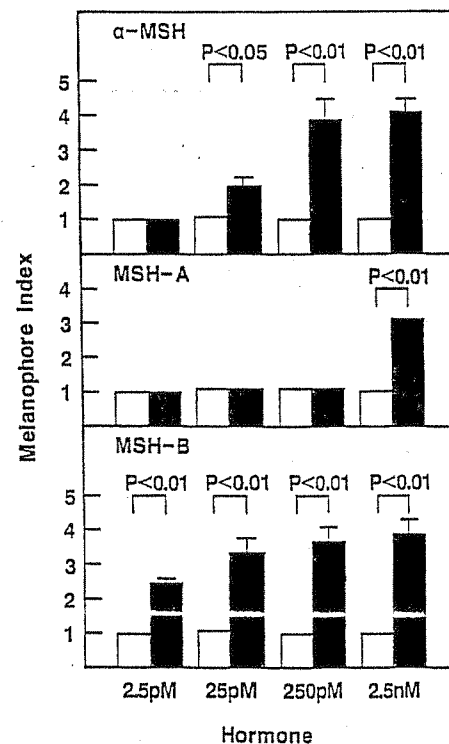


FIGURE 5

Melanotropic activity of synthetic lamprey MSHs and salmon α -MSH in frog skin assay ($n = 4$).

without the ACTH. At least two steroid products were formed, one of which was confirmed by recrystallization as 11-deoxycorticosterone. The other had similar

proposed sequences for ACTH, MSH-A and MSH-B in the present paper were found to be identical to those deduced from the nucleotide sequences. Amidation of MSH-B at the C-terminus was also supported by a consensus sequence for amidation; the C-terminal Pro was followed by Gly as amide donor (manuscript in preparation).

It is well established in the gnathostomes that ACTH and MSHs are produced from a common POMC in the pars distalis and the pars intermedia, respectively (15, 16). Attempts have been made to identify POMC cells of lamprey pituitaries using antisera against mammalian ACTH, α -MSH and Met-enkephalin (10-13). Even in these heterologous systems, these POMC-related peptides were found to be localized in the rostral pars distalis and in the pars intermedia (10, 12). However, the present study has revealed that lamprey ACTH and MSHs are significantly different from other vertebrate counterparts. Therefore, we prepared antisera against synthetic peptides corresponding to the amino-acid sequence of MSH-A, MSH-B and ACTH(1-16) and identified cellular sites of origin by homologous systems (35). The ACTH-positive reaction was found in the pars distalis (strong) and the pars intermedia (weak), but the immunoreactivity of the pars intermedia was abolished by preabsorption of the ACTH antiserum with MSH-A or MSH-B. Specific immunoreactivities for both MSH-A and MSH-B were found in the pars intermedia. These results suggest that one POMC gene is expressed and processed to ACTH in the pars distalis and the other gene is expressed and processed to MSHs in the pars intermedia. The differential expression of two POMC genes was also demonstrated by northern blot analysis (manuscript in preparation).

α -MSH has been recognized as the most potent naturally occurring melanotropic peptide (27). The present study has demonstrated that synthetic lamprey MSH-B is about 10 times more potent than α -MSH. Since hypophysectomy results in melanin concentration and pallor in *Geotria australis* and *Lampetra fluviatilis* (30, 31), it is plausible to speculate that MSHs are physiologically functioning as melanotropic hormones in lampreys. Lamprey ACTH was virtually inactive in the frog skin assay. In preliminary studies, ACTH was biologically active in stimulating steroidogenesis in the adrenocortical cells located in the pronephric kidney and less so in the mesonephric kidney of the male sea lamprey. Further studies will be needed to confirm fully the corticotropic activity of lamprey ACTH.

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A. Takahashi *et al.*

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