Structural Studies of α -Melanocyte-Stimulating Hormone and a Novel β -Melanocyte-Stimulating Hormone from the Neurointermediate Lobe of the Pituitary of the Dogfish *Squalus acanthias*

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(Received 22 January 1974)

A melanocyte-stimulating hormone (MSH) has been isolated from extracts of the neurointermediate lobe of the pituitary of the dogfish *Squalus acanthias* by gel-filtration and ion-exchange chromatography. It had approximately 1% of the potency of mammalian α -MSH on bioassays *in vitro* on frog skin and dogfish skin. Sequence analysis revealed it to be a hexadecapeptide with the following primary structure: Asp-Gly-Asp-Asp-Tyr-Lys-Phe-Gly-His-Phe-Arg-Trp-Ser-Val-Pro-Leu. It appears to be related to the β -MSH species of mammalian species but has only the sequence -His-Phe-Arg-Trp- in common with the heptapeptide core -Met-Glu-His-Phe-Arg-Trp-Gly- which is characteristic not only of the MSH peptides but also of the adrenocorticotrophins and lipotrophins studied so far. An α -MSH was also isolated, 50% of which was amidated at the C-terminus group. Sequence data from this study taken in conjunction with those from a previous study (Lowry & Chadwick, 1970b) revealed it to be a tridecapeptide which is identical with the N-terminal sequence of dogfish adrenocorticotrophin.

Two types of melanocyte-stimulating hormone in the pituitaries of several mammalian species have been described (Lee & Lerner, 1956; Geschwind, 1966; Lowry & Chadwick, 1970a). One of these, α -MSH,† seems to be the same in all mammalian species studied, although the second type, β -MSH, shows species variability in certain parts of its sequence (Harris, 1960). Recently the neurointermediate lobe of the dogfish pituitary has been shown to contain MSH molecules whose structures were homologous with those of mammalian α -MSH (Lowry & Chadwick, 1970b). No evidence was obtained to indicate the presence of significant amounts of a β -MSH in the extracts of these pituitary glands.

We have reinvestigated the MSH peptides of the neurointermediate lobe concurrently with the isolation and sequence analyses of adrenocorticotrophin and corticotrophin-like intermediate-lobe peptide from the same species of dogfish, *Squalus acanthias*, as described by Lowry *et al.* (1974). We now confirm the presence of dogfish α -MSH with an unacetylated N-terminus group and partly amidated C-terminus group and also report details of the purification and

primary structure of a new MSH possibly related to the mammalian β -MSH species with unusual amino acid substitutions in the MSH heptapeptide core.

Materials and Methods

All materials and methods except those listed below have been described by Lowry *et al.* (1974).

Dogfish skin assay

The dogfish skin assays were performed in a similar manner to the frog skin assays except that only three pieces of skin were used at each dose and the response was measured by microscopic determination of the melanophore index (Hogben & Slome, 1936). Squares of skin, peeled from the dorsal surface of the pectoral fins of the spotted dogfish, *Scyliorhinus caniculus* (with intact pituitaries), were cut into small squares (2mm²) and the melanophores allowed to contract in elasmobranch Ringer solution (Novales & Novales, 1966) containing 0.1% albumin, over a period of 10h before being added to the solutions to be assayed. Synthetic mammalian α -MSH was used as a standard.

These assays were performed in the Zoology Department laboratories of Professor J. M. Dodd at the University College of North Wales, Bangor. *Scyliorhinus caniculus* was used because live

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† Abbreviation: MSH, melanocyte-stimulating hormone.

specimens of *Squalus acanthias* were not available at the time.

Purification of MSH peptides

Acetone-dried neurointermediate lobes were extracted with acetic acid and the extract was freeze-dried. This material was subjected to gel-filtration chromatography on Bio-Gel P-2 and P-6. The two columns were connected in series such that the desalted material from the Bio-Gel P-2 column flowed directly on to the Bio-Gel P-6 column. Fractions with MSH activity were freeze-dried and the MSH-active components were separated by ion-exchange chromatography on CM-cellulose.

Repurification of the acidic MSH-active peptide (i.e. peak A in Fig. 2) was achieved by ion-exchange chromatography on DEAE-cellulose. The column (0.4 cm internal diam. $\times$ 12 cm length) was eluted with a concave gradient of trimethylamine acetate, pH 6, running from 10 mm to 200 mm (total vol. approx. 50 ml) and 0.5 ml fractions were collected every 20 min.

Repurification of the two other peaks of MSH activity was achieved by combining the fractions from both peaks, freeze-drying them and applying this material to ion-exchange chromatography on CM-cellulose. The column (0.4 cm internal diam. $\times$ 20 cm length) was eluted with a concave gradient of trimethylamine acetate, pH 6, running from 300 mm to 500 mm (total vol. approx. 50 ml) and 0.5 ml fractions were collected every 20 min.

Peptide fragmentation

Fragmentation of the acidic MSH was achieved by tryptic hydrolysis. The peptide (300 μ g) was

Fig. 1. Bio-Gel P-2 and P-6 gel-filtration chromatography of an acetic acid extract of approx. 1000 dogfish neurointermediate lobes

—, E_{280} ; MSH activity is shown by the hatched area. The column (2.5 cm internal diam. $\times$ 25 cm length) of Bio-Gel P-2 and that (2.5 cm internal diam. $\times$ 120 cm length) of Bio-Gel P-6 were eluted with 1 M-acetic acid and 5 ml fractions collected every 20 min. The fractions marked — were pooled, freeze-dried and used in the isolation of dogfish corticotrophin-like neurointermediate-lobe peptide described by Lowry *et al.* (1974).

incubated with trypsin at 37°C in 0.2 ml of 0.05 M-ammonium bicarbonate buffer (pH 8.5) with a peptide/enzyme ratio of 50:1 (w/w) for 4 h. The resulting fragments were separated on a column (0.4 cm internal diam. $\times$ 21 cm length) of CM-cellulose, which was eluted with a concave gradient of trimethylamine acetate, pH 5, running from 10 mm to 600 mm (total vol. approx. 20 ml) and 0.5 ml fractions were collected every 20 min.

Exopeptidase digestions

Aminopeptidase M (treated with di-isopropyl phosphorofluoridate) digestions were performed in 0.03 M-sodium phosphate buffer (pH 7.5) at 37°C with a peptide/enzyme ratio of 100:1 (w/w) for various times. Carboxypeptidase A and B digestions were carried out in 0.5 M-Tris buffer, containing 0.2 M-NaCl (pH 8.5) at 25°C with a peptide/enzyme ratio of 50:1 for carboxypeptidase A and 100:1 for carboxypeptidase B for various times.

Results

The results from the two column chromatographic steps involved in the isolation of MSH-active peptides are shown in Figs. 1 and 2. The fractions with MSH activity in the Bio-Gel P-2/P-6 system were freeze-dried and ion-exchange chromatography on CM-cellulose of this material revealed three peaks of MSH activity A and B and C (Fig. 2). Fractions under peak A containing the more acidic MSH were combined, freeze-dried and repurified by DEAE-cellulose ion-exchange chromatography, and the amino acid analysis of this material after both acid and Sepharose-bound-enzyme hydrolysis is shown in Table 1. The molar ratios indicated that the MSH was a hexadecapeptide. Its potency in both frog skin and dogfish skin bioassays *in vitro* was about 1% of that of mammalian α -MSH.

Exopeptidase digestion of the acidic MSH by carboxypeptidase A revealed leucine to be at the C-terminus (Table 2). Subtractive Edman degradation revealed aspartic acid to be at the N-terminus (Table 1).

The amino acid molar ratios of the tryptic fragments of this acidic MSH peptide and of the residual peptides resulting from their stepwise subtractive Edman degradation are shown in Table 3. Results from exopeptidase digestion of these peptides are summarized in Table 2. As aspartic acid is at the N-terminus, the tryptic peptide TP₁ must constitute the N-terminal hexapeptide sequence. Similarly, as leucine is at the C-terminus, tryptic peptide TP₃ must be the C-terminal pentapeptide. Tryptic pentapeptide TP₂ must therefore lie between peptides TP₁ and TP₃. This is confirmed by the isolation of tryptic peptide

Fig. 2. CM-cellulose ion-exchange chromatography of material with MSH activity from approx. 2000 dogfish neurointermediate lobes after purification on Bio-Gel P-2 and P-6

●, E_{280} ; MSH activity is shown by the hatched areas. The column (0.6cm internal diam. $\times$ 25 cm length) was developed with a concave gradient running from 20mm- to 500mm-trimethylamine acetate, pH 5 (total vol. 150ml), and 1 ml fractions were collected every 20min. A, B and C denote peaks of MSH activity.

Table 1. Amino acid compositions (molar ratios) of peptides contained in peaks A, B and C of Fig. 2 and of the residual peptides resulting from stepwise subtractive Edman degradation

For experimental details see the text. At each step an N-terminal residue is removed and the identification of the amino acid depleted between each successive step (indicated by *) therefore gives the sequence of the peptide. N.D., Not determined owing to breakdown during acid hydrolysis.

Fraction in	Amino acid	...	Asp	Ser	Glu	Pro	Gly	Val	Met	Leu	Tyr	Phe	Lys	His	Arg	Trp
Peak A			3.3	0.8	—	0.8	2.0	0.9	—	0.9	1.0	1.8	0.9	0.8	1.1	0.5
Peak A†			1.6	1.0	—	1.0	1.5	1.0	—	1.0	1.0	2.1	1.0	1.0	1.0	1.0
1st step			2.3*	0.9	—	1.0	2.0	1.0	—	1.0	1.0	1.9	0.8	1.2	1.0	N.D.
Peak B			—	1.5	0.9	0.8	1.0	—	1.9	—	1.1	1.1	1.2	1.1	1.1	0.7
1st step			—	0.8*	1.0	1.0	1.0	—	2.0	—	1.0	1.0	1.0	0.9	1.1	N.D.
2nd step			—	0.9	1.0	1.0	1.0	—	2.0	—	0.1*	1.1	0.9	0.9	1.0	N.D.
Peak C			—	1.5	0.9	0.7	1.1	—	1.9	—	1.0	1.0	1.3	1.3	0.9	0.6
1st step			—	0.7*	0.8	0.9	1.0	—	1.7	—	1.0	1.1	1.2	1.2	1.1	N.D.
2nd step			—	0.8	1.0	0.9	1.0	—	1.8	—	0.3*	1.1	1.1	1.2	1.1	N.D.

† Enzyme hydrolysis; all other results are from acid hydrolysis.

Table 2. Amino acids released from dogfish β -MSH and three of its tryptic fragments after digestion with carboxypeptidase A and B and aminopeptidase M

Times of incubation are shown in parentheses (h). For peptide identification see Fig. 3.

	Amino acids released and incubation times			
	Peptide A	Peptide TP ₁	Peptide TP ₂	Peptide TP ₃
Carboxypeptidase A	Leu (4)	—	—	Leu (4)
Carboxypeptidase B	—	Lys > Tyr (0.5)	Arg > Phe (0.5)	—
Aminopeptidase M	—	—	—	Trp > Ser (0.5)

Table 3. *Amino acid compositions (molar ratios) of dogfish β -MSH tryptic fragments and of the residual peptides resulting from their stepwise subtractive Edman degradation*

At each step an *N*-terminal residue is removed and identification of the amino acid depleted between each successive step (indicated by *) therefore gives the sequence of the peptide. For peptide identification see Fig. 3; peptide TP_{3APM} is a fragment remaining after treatment with aminopeptidase M. Tryptic fragments (170 nmol) of dogfish β -MSH were separated on the CM-cellulose column; order of elution and recoveries were: (1) TP_1 , 46 nmol; (2) TP_3 , 105 nmol; (3) TP_4 , 45 nmol; (4) TP_2 , 36 nmol.

Peptide fragment	Amino acid	...	Asp	Ser	Pro	Gly	Val	Leu	Tyr	Phe	Lys	His	Arg	Trp
TP_1 (1-6)			2.9	—	—	1.2	—	—	0.9	—	1.1	—	—	—
1st step			2.3*	—	—	1.0	—	—	1.0	—	0.9	—	—	—
2nd step			2.1	—	—	0.2*	—	—	0.9	—	1.0	—	—	—
3rd step			1.2*	—	—	0.0	—	—	1.0	—	0.8	—	—	—
4th step			0.3*	—	—	0.0	—	—	1.1	—	0.9	—	—	—
5th step			0.0	—	—	0.0	—	—	0.0*	—	1.0	—	—	—
TP_4 (1-11)			3.0	—	—	2.1	—	—	1.0	1.9	1.0	1.0	1.0	—
TP_2 (7-11)			—	—	—	1.3	—	—	—	2.0	—	0.9	0.9	—
1st step			—	—	—	1.3	—	—	—	1.0*	—	1.0	1.0	—
2nd step			—	—	—	0.0*	—	—	—	1.0	—	1.0	1.0	—
3rd step			—	—	—	0.0	—	—	—	0.9	—	0.3*	1.1	—
4th step			—	—	—	0.0	—	—	—	0.0*	—	0.0	1.0	—
$TP_3^\dagger$ (12-16)			—	0.9	0.9	—	1.0	1.0	—	—	—	—	—	0.9
TP_{3APM} (14-16)														
1st step			—	—	1.1	—	0.1*	0.9	—	—	—	—	—	—
2nd step			—	—	0.1*	—	0.0	1.0	—	—	—	—	—	—

† Hydrolysis by Sepharose-bound enzymes (all others are by acid hydrolysis).

TP_4 , which is a product of incomplete trypsin cleavage comprising the sequences of peptides TP_1 and TP_2 .

The position of tryptophan in peptide TP_3 was determined by aminopeptidase M digestion. However, prolonged treatment with this enzyme released only equimolar amounts of tryptophan and serine. The peptide which remained after treatment with aminopeptidase M, TP_{3APM} , and these two free amino acids were then subjected to the subtractive Edman-degradation procedure. Subsequent amino acid analysis of the residual peptide showed that not only tryptophan and serine but also valine had been removed. The next step removed proline and the position of this amino acid explains the resistance of peptide TP_{3APM} to further degradation by aminopeptidase M. This method served to place tryptophan unequivocally in the sequence and also to sequence the rest of peptide TP_3 . From the sequencing evidence presented in Tables 2 and 3 and summarized in Fig. 3, the enzyme hydrolysis of the whole peptide (Table 1) was complete apart from the *N*-terminal tetrapeptide, Asp-Gly-Asp-Asp, which was only partially digested. As there were undetectable amounts of asparagine in this analysis, residues 1, 3 and 4 must be occupied by aspartic acid residues.

These results provide convincing support for the sequence we propose for this new β -MSH shown in Fig. 4.

Table 1 also shows the amino acid analyses from acid hydrolysis of peptides contained in peaks B and C (Fig. 2), and also of the residual peptides remaining after two cycles of the Edman-degradation procedure. The *N*-termini of both peptides were readily digested by di-isopropyl phosphorofluoridate-treated aminopeptidase M releasing 2 mol of serine to 1 mol of tyrosine and 1 mol of methionine. These results, together with previous evidence (Lowry & Chadwick, 1970b), suggest the structures of dogfish α -MSH shown in Fig. 4. The separation of dogfish α -MSH activity into two approximately equal peaks after ion-exchange chromatography on CM-cellulose suggests that half of the molecules are amidated at the C-terminus as proposed by Lowry & Chadwick (1970b).

Discussion

Studies with synthetic analogues of MSH have suggested that the tetrapeptide sequence -His-Phe-Arg-Trp- is the minimum requirement for melanocyte-stimulating activity (Otsuka & Inouye, 1964). However, this tetrapeptide has only 1×10^{-6} of the activity of mammalian α -MSH. Further, the heptapeptide sequence -Met-Glu-His-Phe-Arg-Trp-Gly- has been found in all melanocyte-stimulating hormones, adrenocorticotrophins and lipotrophins so

Fig. 3. Summary of the sequence analysis of dogfish β -MSH

→, Subtractive Edman-degradation steps; → or ←, exopeptidase digestion resulting from treatment with carboxypeptidase A(†), carboxypeptidase B(‡) or amino peptidase M(*).

Fig. 4. Comparison of dogfish and mammalian MSH species

far examined (Lowry & Chadwick, 1970a). The new dogfish MSH described in the present paper with a core sequence -Phe-Gly-His-Phe-Arg-Trp-Ser only fulfils the former criterion, however; its significant biological activity (1% compared with the highly potent mammalian α -MSH) in both dogfish and frog skin assays is surprising considering the lack of the full heptapeptide core. Despite its unusual structure we feel that this MSH should be classified as a β variety on account of the number of homologous residues and the acidic character of the *N*-terminal tryptic fragment of the MSH (Figs. 3 and 4).

Recently, a β -MSH has been isolated and sequenced from a different species of dogfish, *Scyliorhinus caniculus* (G. Love & B. T. Pickering, unpublished observations). In this peptide only glycine has been substituted for glutamic acid in the common heptapeptide core (Fig. 4). Comparison of the mRNA codes (which reflect the DNA sequences) for these two β -MSH peptides with that of a mammal, e.g. pig β -MSH, presents an interesting evolutionary picture. Evidence from palaeontology (Young, 1962) indicates that the two forms of dogfish diverged about 200 million years ago, whereas the elasmobranchs as a whole diverged from the main line of vertebrate evolution about 300 million years ago. Comparison of pig β -MSH with *Squalus acanthias* β -MSH shows that there must be a minimum of 11 base differences in the portion of DNA coding for these peptides, whereas comparison of pig β -MSH with *Scyliorhinus caniculus* β -MSH and *Squalus acanthias* β -MSH with *Scyliorhinus caniculus* β -MSH reveal values of 12 and 8 respectively. These values indicate that for each pair of peptides four base differences have been introduced per 100 million years, a finding which lends support to the belief that the rate of mutation acceptance is constant for each particular protein or peptide (for a detailed review see Dayhoff, 1972).

It has been proposed that the formation of β -MSH in mammals by the enzymic cleavage of β -lipotrophin (Chretien *et al.*, 1973) takes place only in the pars intermedia (Scott & Lowry, 1974). It is also thought that the β -MSH-like immunostaining material in the corticotrophs of the pars distalis of the human pituitary (Phifer *et al.*, 1972) is in fact intact β -lipotrophin (Scott & Lowry, 1974). The slightly shorter length of *Squalus acanthias* β -MSH might simply reflect changes in the amino acid residues surrounding the β -MSH sequence in *Squalus acanthias* β -lipotrophin, leading to a different pattern of enzymic cleavage in the neurointermediate lobe. The isolation and characterization of dogfish β -lipotrophin from the ACTH-containing lobe of the pars distalis (the rostral) would considerably help this hypothesis.

On re-examination of the structure of dogfish α -MSH coupled with a previous study (Lowry &

Chadwick, 1970b) we have found that it has the full tridecapeptide sequence similar to mammalian α -MSH and identical with the 1-13 *N*-terminal sequence of dogfish ACTH (Lowry *et al.*, 1974). We can only conclude that in the previous study the peptide had been subjected to aminopeptidase action either before or during extraction in dilute acetic acid. We have confirmed the findings of Lowry & Chadwick (1970b) that the *N*-terminus of dogfish α -MSH is not acetylated and that 50% of the molecules are amidated at the *C*-terminus. This would suggest that not only is α -MSH formed from ACTH but that amidation of the free carboxyl group probably takes place after cleavage of the ACTH molecule rather than by aminolysis. The acetylation of the *N*-terminus of α -MSH which occurs in the pars intermedia of some mammals could take place at any time after the *N*-terminal sequence of the ACTH molecule has been synthesized.

We thank Mr. B. E. Evans for the amino acid analyses on the Beckman 120C and Mr. E. J. Stevens for his continued assistance in the modification of the Technicon Auto-Analyzer. We also thank Dr. A. M. White, Dr. D. F. Elliott and Dr. R. Wade for discussion and encouragement throughout this work. We appreciate the kind donation of synthetic mammalian α -MSH from Dr. Rittel and also the help and interest shown by Professor J. M. Dodd in the dogfish assay.

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