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THE ISOLATION AND CHARACTERIZATION OF γ_3-MELANOTROPIN FROM THE NEUROINTERMEDIARY LOBE OF THE RAT PITUITARY

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Summary

γ_3-Melanotropin has been isolated from the neurointermediary lobe of the rat pituitary by reversed-phase high performance liquid chromatography. Peptide mapping and amino acid analysis have demonstrated that γ_3-melanotropin is a glycopeptide of 25 amino acids, which corresponds exactly to the γ_3-melanotropin sequence predicted by DNA sequencing, with the addition of an extra lysine residue at the amino terminus. The carbohydrate chain is linked to the peptide between asparagine 16 and the carboxyl terminal glutamine 25, and contains 4 moles of glucosamine per mole of peptide but no galactosamine.

Introduction

Adrenocorticotropin (ACTH) and β-lipotropin (β-LPH) have been shown to originate from a common precursor molecule (1-3). When the sequence for the bovine precursor protein was determined by complementary DNA methods (4), the hitherto cryptic amino terminal portion of the molecule was found to contain a third copy of the melanotropin core sequence found in α-melanotropin (α-MSH) and β-melanotropin (β-MSH). This peptide was called γ-melanotropin (γ-MSH) and in the bovine was expected to be a dodecapeptide bounded by two pairs of basic amino acids. This bovine γ-MSH (called γ_1-MSH) and two other bovine γ-MSH analogues, called γ_2-MSH and γ_3-MSH were synthesized (5) and used for biological assays (5,6) and for raising antisera (7). Bovine γ_3-MSH consisted of the

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Abbreviations: ACTH, adrenocorticotropin; β-LPH, β-lipotropin; α, β + γ-MSH, α, β + γ melanotropin; NIL, neurointermediary lobe; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid.

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sequence of γ_1-MSH with the addition of a carboxyl terminal extension comprising the the next fifteen amino acids of the bovine precursor molecule. Recently a DNA sequence for the ACTH/β-LPH precursor molecule in the rat has been published (1). In this molecule, the arginine adjacent to the carboxyl-terminal glycine of bovine γ_1-MSH has been replaced by a proline, and thus the recognition site for the enzymic cleavage of γ_1-MSH from the precursor does not occur in the rat. Therefore only γ_3-MSH-related peptides might be expected to be found in the rat pituitary. It has been reported that immunoreactive γ-MSH-related peptides isolated from the bovine pituitary bind to concanavalin A-Sepharose (8), and thus may be glycopeptides. This paper reports the isolation and characterization of a γ_3-MSH glycopeptide from the neurointermediary lobe of the rat pituitary.

Materials and Methods

Extraction and Isolation of γ_3-MSH:

Rats (male, Sprague-Dawley, 200-400 g) were sacrificed by decapitation and the pituitaries were removed and dissected within 30 seconds of death. The neurointermediary lobes (NIL) of the pituitaries were immediately homogenized, centrifuged and extracted in batches of 20-30 by a modification (9) of the previously described octadecylsilyl-silica method (10). The combined extract from 190 rat NILs was chromatographed as previously described (11) by reversed-phase high performance liquid chromatography (RP-HPLC), using a linear gradient of aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) throughout. The material in the partially purified peak of γ_3-MSH was completely purified by rechromatography on the same RP-HPLC column, this time using a linear gradient of aqueous acetonitrile containing 0.13% heptafluorobutyric acid (HFBA) throughout (12). The rat γ-MSH was then rechromatographed on the same RP-HPLC column, this time using a gradient of aqueous acetonitrile containing 0.1% TFA.

Characterization of rat γ_3-MSH

Amino acid analysis and exopeptidase digestions were performed as described elsewhere (9). Trypsin and chymotrypsin digestions and the isolation and analysis of the resultant tryptic and chymotryptic peptides was performed as described elsewhere (11). Amino sugar determination was done by the method of DeLuca et al (13)

Incorporation of N-acetyl [^{3}H] glucosamine

28 rat NILs were incubated for 18 hours in 3 mls of Dulbecco's Modified Eagles Medium (Gibco) to which had been added 2% horse serum (Flow) and 1 mCi of N-acetyl [^{3}H] glucosamine (Amersham, 2.94 Ci/mmol). The tissue and medium were extracted as previously described (14) and the resultant peptides were separated by RP-HPLC as described above. The ^{3}H-labeled γ_3-MSH was isolated by exactly the same protocol as in the preparative experiment, except that ali-

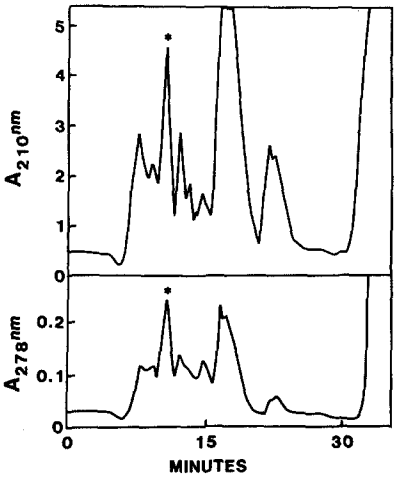

Figure 1. Initial RP-HPLC chromatogram of peptides extracted from 190 rat NILs. The peptides were eluted with a linear gradient from 20 to 30% (v/v) acetonitrile containing 0.1% TFA throughout over 30 minutes. The U.V. peak eluting at 10 minutes (*) was selected as a likely candidate for γ_3-MSH and was purified further (Figure 2).

quots were removed from all fractions for tritium determination by counting in Ready-Solv MP (Beckman) in a Beckman 8000 Liquid Scintillation counter.

Results

The initial RP-HPLC chromatogram obtained from the extraction of 190 rat NILs shows the presence of a large number of U.V. absorbing components (Fig. 1). It is possible to estimate the approximate RP-HPLC elution position of a peptide of known composition by calculating the sum of the retention coefficients of the component amino acids (15). For the predicted rat γ_3-MSH sequence (1) the calculated retention coefficient was 42, which would predict a retention time between 9 and 12 minutes under the conditions used in Figure 1. Furthermore rat γ_3-MSH should contain one tryptophan and one tyrosine residue (1). In figure 1 the U.V. peak at 10 minutes appeared to be a likely candidate for rat γ_3-MSH, based on both its elution position and its U.V. absorbance at 210 and 278 nm. The material in this peak was rechromatographed (Figure 2) to yield a large symetrical U.V. absorbing peak which was well resolved from several minor peaks. The material in this large peak was rechro-

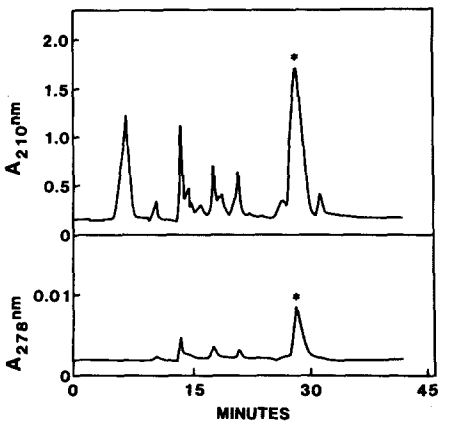

Figure 2. Purification of rat γ_3-MSH. The material indicated in Figure 1 (*) was rechromatographed on the same RP-HPLC column using a linear gradient from 20 to 45.6% (v/v) acetonitrile containing 0.13% HFBA throughout over 1 hour. The U.V. peak eluting at about 28 minutes (*) was the main component, which by the ratio of U.V. absorbance at 210 nm to that at 278 nm contained tryptophan. The material in this peak was rechromatographed using a linear gradient from 16-36% (v/v) acetonitrile containing 0.1% TFA throughout over 1 hour (not shown). A single, symmetrical peak eluting at 22 minutes (23% acetonitrile) was obtained.

matographed using conditions similar to those described in Figure 1. A single, symmetrical U.V. peak resulted, which could not be resolved into any further components (not shown).

Amino acid analysis (Table 1) of the isolated material gave the analysis expected from the reported DNA sequence of rat γ_3-MSH, except that there was an unexpected lysine residue. The analysis also indicated the presence of an amino sugar peak. Tryptic and chymotryptic peptides were prepared from this rat γ_3-MSH-like peptide and were separated by RP-HPLC (Figs. 3&4) and identified by amino acid analysis (Table 1). All of the tryptic and chymotryptic peptides corresponded in composition to those expected for rat γ_3-MSH. Furthermore, the extra lysine residue is clearly located in the amino terminal tryptic and chymotryptic peptides (TP_5, TP_6 and CP_1 respectively). This is consistent with the hypothesis that the amino terminus of rat γ_3-MSH is lysine and not tyrosine. Amino sugar analysis showed the presence of 3.9 moles of glucosamine per mole of peptide. There was no detectable galactosamine. In

TABLE 1

AMINO ACID ANALYSES* OF RAT γ_3-MSH AND ITS CHYMOTRYPTIC (CP) AND TRYPTIC (TP) PEPTIDES

	Asx	Ser	Glx	Pro	Gly	Ala	Val	Met	Tyr	Phe	His	Lys	Arg
γ_3-MSH	2.0	3.4	1.0	1.2	3.5	2.0	0.9	1.0	0.9	1.8	1.0	1.0	3.0
Expected Values	2	4	1	1	4	2	1	1	1	2	1	0	3
CP1	0.1	0.4	0.1	0.1	0.3	0.2	0	0	1.0	0	0	1.0	0.1
CP2	1.0	3.7	1.0	1.0	2.8	1.9	0	0	0	0	0	0	1.0
CP3	1.1	3.6	1.0	1.0	2.8	1.9	0	0	0	0	0	0	0.9
CP4	--	--	--	--	--	--	--	--	--	--	--	--	--
CP5	0	0	0	0	1.1	0	0.9	1.0	0	1.0	1.0	0	0
CP6	1.0	0	0	0	0	0	0	0	0	1.1	0	0	2.0
TP1	1.1	3.8	0.9	0	2.0	1.9	0	0	0	0	0	0	0
TP2	1.1	2.8	1.4	0	2.0	1.8	0	0	0	0	0	0	0
TP3	1.0	0	0	0	0	0	0	0	0	0	0	0	1.0
TP4	0	0	0	1.0	0.9	0	0	0	0	1.0	0	0	1.0
TP5	0	0	0	0	1.1	0	0.8	0.8	1.0	1.0	1.0	1.0	0
TP6	0	0	0	0	1.0	0	0.9	0.9	1.0	1.1	1.0	1.0	1.1

* The values for threonine, cysteine, isoleucine and leucine were zero throughout. Serine values are uncorrected for losses during hydrolysis. Expected values are taken from Ref. 1. There was insufficient CP4 to obtain an analysis, but by U.V. absorbance and elution position, CP4 was identified as Arg-Trp.

order to confirm the presence of glucosamine, ^{3}H-labeled N-acetyl glucosamine was incorporated into rat NILs, and the ^{3}H-labeled rat γ_3-MSH was isolated as described above. At each stage in the isolation, ^{3}H counts corresponded exactly to the appropriate peaks in the chromatogram. Tryptic digestion of this material demonstrated that all the radioactivity was confined to the carboxyl-terminal tryptic peptides TP_1 and TP_2 (Fig. 4). All of the radioactivity from 4N HCL hydrolysates of these labeled tryptic peptides was eluted from the amino acid analyzer in the position corresponding to glucosamine. It was possible to digest the isolated rat γ_3-MSH with leucine aminopeptidase indicating that there was no N-acetyl group present at the amino terminus, and carboxypeptidase Y, indicating the presence of a free carboxylate group at the carboxyl terminus. Limited exopeptidase digestion has confirmed that the amino terminal amino acid was lysine, and that the carboxyl terminal sequence was Ser-Ala-Gln.

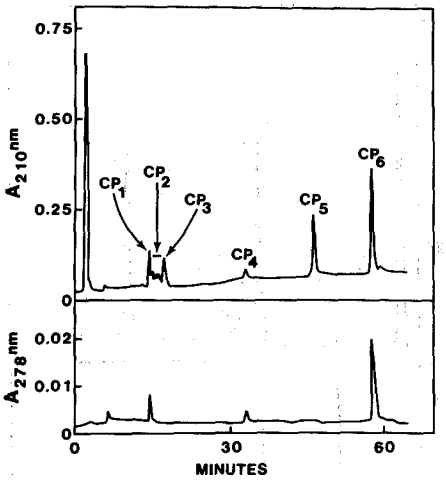

Figure 3. Separation of the chymotryptic peptides derived from rat γ_3-MSH. The products obtained from an 18 hour digestion of rat γ_3-MSH with α-chymotrypsin (enzyme to substrate ratio 1:20), were separated by RP-HPLC with a linear gradient from 1.5 to 24% acetonitrile containing 0.1% TFA throughout over 1 hour. From the amino acid analyses (Table One), the chymotryptic peptides could be identified as follows: CP_1 Lys,Tyr (γ_3-MSH_{1-2}); CP_2 + CP_3 Gly,Pro,Arg,Asx,Ser,Ser,Ser,Ala,Gly,Gly,Ser,Ala,Gln (γ_3-MSH_{13-25}); CP_4 Arg,Trp (γ_3-MSH_{8-9}); CP_5 Val,Met,Gly,His,Phe (γ_3-MSH_{3-7}); CP_6 Arg,Trp,Asp,Arg,Phe (γ_3-MSH_{8-12}). The presence of tryptophan in CP_4 and CP_6 was indicated by the ratio of the U.V. absorbance at 210 nm to that at 278 nm. The heterogeneity of the CP_2 and CP_3 carboxylterminal peptide is probably due to microheterogeneity of the carbohydrate chain.

Discussion

We have isolated rat γ_3-MSH in high yield without the aid of biological or immunological assays and have shown by peptide mapping and exopeptidase digestion that the tentative sequence for this peptide is Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Pro-Arg-Asn-Ser-Ser-Ser-Ala-Gly-Gly-Ser-Ala-Gln. This sequence is in complete agreement with the sequence predicted by DNA sequencing (1). Rat γ_3-MSH clearly is a glycopeptide which contains glucosamine but no galactosamine, and the carbohydrate moiety is clearly located in the carboxylterminal tryptic peptide γ_3-MSH_{16-25} (TP_1, TP_2). The precise site of attachment of the carbohydrate chain to the peptide is not known. There are two possibilities, either the asparagine at position 16 via an N-glycosidic bond, or one of the four serine residues in the carboxylterminal tryptic peptide via an O-glycosidic bond. The asparagine is an especially strong

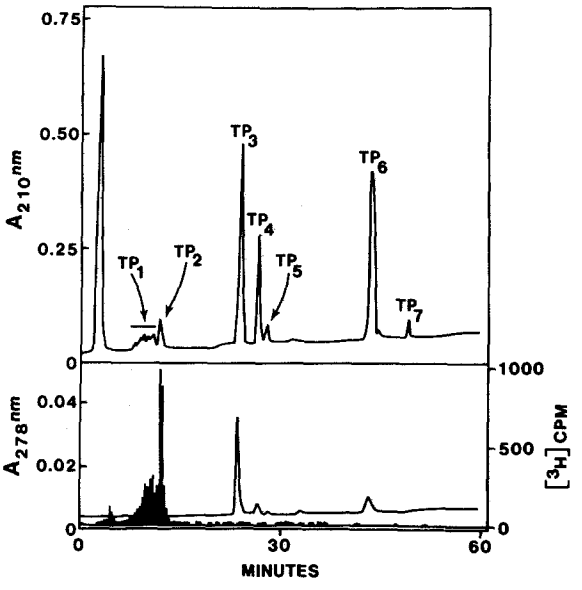

Figure 4. Separation of the tryptic peptides derived from rat γ_3-MSH. The products obtained from an 18 hour digestion of rat γ_3-MSH with trypsin (enzyme to substrate ratio 1:50) were separated by RP-HPLC with a linear gradient from 1.5 to 24% acetonitrile containing 0.1% TFA throughout over 1 hour. From the amino acid analyses (Table One) the tryptic peptides could be identified as follows: TP_1 + TP_2 Asx,Ser,Ser,Ser,Ala,Gly,Gly,Ser,Ala,Gln (γ_3-MSH_{16-25}); TP_3 Trp,Asp,Arg, (γ_3-MSH_{9-11}); TP_4 Phe,Gly,Pro,Arg, (γ_3-MSH_{12-15}); TP_5 Lys,Tyr, Val,Met,Gly,His,Phe (γ_3-MSH_{1-7}); TP_6 Lys,Tyr,Val,Met,Gly,His,Phe,Arg (γ_3-MSH_{1-8}). TP_6 was undigested γ_3-MSH. The presence of tryptophan in TP_3 was indicated by the ratio of the U.V. absorbance at 210 nm to that at 278 nm. The heterogeneity of the carboxylterminal peptides (TP_1 and TP_2) is probably due to microheterogeneity of the carbohydrate chain. This is reinforced by the histogram, which shows the distribution of ^3H through the tryptic peptide chromatogram of rat γ_3-MSH labeled with N-acetyl [^3H] glucosamine.

candidate as it is part of an Asn-X-Ser sequence which has been shown to be a recognition site for the glycosylation of asparagine residues (16). As most serine linked carbohydrates contain galactosamine, the absence of galactosamine from rat γ_3-MSH makes the serine linkage less likely.

The biological role of rat γ_3-MSH has not yet been defined. Synthetic bovine γ_3-MSH has been reported to have weak melanotropic activity (5) and to have a synergistic effect on ACTH-stimulated steroidogenesis in the rat (6,16). However, this synthetic bovine γ_3-MSH differs from the isolated rat γ_3-MSH in three important respects. Firstly, the sequence of the rat γ_3-MSH differs from the bovine in several places in the carboxyl terminal half of the

molecule, where there is a three amino acid deletion and four amino acid substitutions (1,4). Secondly, the amino terminal amino acid is lysine in rat γ_3-MSH not tyrosine, and thirdly, there is a carbohydrate chain in rat γ_3-MSH which is not present in the synthetic bovine γ_3-MSH peptide. As all of these factors may affect the biological activity of γ_3-MSH, biological testing of the natural, isolated rat γ_3-MSH glycopeptide needs to be undertaken. Finally, the yield of rat γ_3-MSH was 0.7 nmoles per NIL compared to approximately 1.5 nmoles per NIL for α-MSH related peptides, γ-LPH related peptides and corticotropin-like intermediary lobe related peptides (17). Thus only half of the amino terminal part of the ACTH/β-LPH precursor is processed to γ_3-MSH. The fate of the other half of the aminoterminal part of the precursor remains unknown.

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