# A NOVEL N-TERMINAL FRAGMENT OF PRO-γ-MELANOTROPIN, NOT CONTAINING γ-MELANOTROPIN AND GENERATED FROM A CLEAVAGE SITE LACKING THE TRADITIONAL TWO BASIC RESIDUES

Rolf EKMAN, Rolf HÅKANSON* and Hans JÖRNVALL†

*Department of Nuclear Medicine, Malmö General Hospital, 214 01 Malmö, *Department of Pharmacology, University of Lund, 223 62 Lund* and *†Department of Chemistry I, Karolinska Institutet, 104 01 Stockholm, Sweden*

Received 7 August 1981

## 1. Introduction

A corticotropin-β-lipotropin (ACTH-β-LPH) precursor consists of 239 amino acid residues [1] and its amino acid sequence contains three repetitive His–Phe–Arg–Trp sequences, in the α-, β- and γ-MSH regions, corresponding to positions 111–114, 197–200 and 55–58, respectively. Proteolytic processing of this precursor generates several biologically active peptides with corticotropic, lipolytic or melanotropic activity [2,3]. The N-terminal part of the precursor molecule (pro-γ-MSH), remaining after the cleavages that remove ACTH and β-LPH, consists of 103 amino acid residues [1,4] and contains the γ-MSH sequence.

Little is known about the processing of pro-γ-MSH [5,6]. The same is true for the biological significance of this molecule or for that of γ-MSH. We now describe the purification and primary structure of a pro-γ-MSH fragment, which appears to comprise the first 30 residues of the precursor. This fragment results from proteolysis at an unexpected site in the promolecule. The sequence shows a substrate specificity for the protease involved that suggests the enzyme to resemble enzymes cleaving nascent pre-promolecules rather than those cleaving at dibasic structures in promolecules [7,8].

On the whole, the sequence results confirm the partial N-terminal amino acid sequence for porcine pro-γ-MSH proposed in [4]. In addition, they establish two positions with previously unidentified residues, correct a misidentification and show the occurrence of different partial desamidations.

† To whom correspondence should be addressed

## 2. Materials and methods

The starting material was 1 kg frozen pig pituitaries, extracted and purified by chromatography on an anion-exchange column (DEAE-Sephadex) as described in [4]. The fractions were analyzed fluorimetrically for peptides with N-terminal tryptophan [9]. These were eluted from the column by raising the ionic strength, as in [4]. The material was then dialyzed and freeze-dried. Portions of 1 g were passed through a carboxymethyl-cellulose column (2.6 × 7 cm), equilibrated with 0.02 M sodium acetate buffer (pH 3.5). After elution of non-adsorbed material, the pH was raised to 8.5 (0.02 M phosphate buffer). The material recovered at this pH was rich in peptides with N-terminal tryptophan. Following dialysis, it was freeze-dried and passed through a Sephadex G-75 column (0.9 × 6 cm). The material thus obtained was purified to homogeneity by preparative high-performance liquid chromatography (Waters instrument, 6600 A solvent delivery system, UK injector, model 450 variable wavelength detector) using a 30 × 0.78 cm μBondapak $C_{18}$ column. Acetonitrile/0.1 M ammonium acetate (pH 4.5) (35:65, v/v) was used as eluant and the absorbance was monitored at 280/206 nm. The homogeneity of the final product was established by analytical high-performance liquid chromatography using a 30 × 0.39 cm μBondapak $C_{18}$ column and the same procedure as above.

Amino acid compositions were determined with a Beckman 121 M instrument after hydrolysis at 110°C for 22 h with 6 M HCl/0.5% phenol, in evacuated tubes. Hydrazinolysis was performed for 6 h at 110°C, and the result was determined both by direct applica-

*Published by Elsevier/North-Holland Biomedical Press*
00145793/81/0000–0000/$02.50 

tion to the amino acid analyzer and by dansylation and subsequent identification of dansyl amino acids [10].

Sequential degradations were carried out in a Beckman 890 C liquid-phase sequencer, using a 0.1 M Quadrol program in the presence of Polybrene, added together with glycine and precycled as in [11]. Phenylthiohydantoin derivatives were identified by high-performance liquid chromatography in a Hewlett-Packard 1084B instrument on a Nucleosil $C_{18}$ column using an acetate/acetonitrile system, and by thin-layer chromatography and subsequent ninhydrin staining [11].

Cysteine/half-cystine residues were identified by $^{14}C$-measurements during degradation of a $^{14}C$-carboxymethylated sample that had been prepared by submitting the peptide (40 nmol) in 6 M guanidine–HCl, 0.1 M Tris–HCl, 2 mM EDTA (pH 8.2) to reduction with dithiothreitol (2.5 μmol) for 2 h at 37°C, treatment with iodo[2-$^{14}C$]acetate (7.5 μmol; 3000 dpm/nmol) for 1 h at 37°C, and removal of reagents by dialysis against distilled water in Spectrapor 3 tubing (Spectrum Medical Co., Los Angeles CA).

## 3. Results

### 3.1. *Purity and composition*

A peptide fraction was prepared from porcine pituitaries by two ion-exchange chromatography steps followed by gel chromatography. The material from the latter step was still heterogeneous but was purified to a homogeneous product by high performance liquid chromatography as illustrated in fig.1. Identification of tryptophan as the only N-terminal residue, the composition (below), and the results of amino acid sequence analysis (below) further show the purity. The amino acid composition, given in table 1, fits residues 1–30 of the pro-γ-MSH molecule [1,4] and suggests that the peptide constitutes that fragment.

### 3.2. *Amino acid sequence*

The amino acid sequence was elucidated by degradations in a liquid-phase sequencer. The results are shown in fig.2. On the whole, earlier data [4] regarding the first 30 residues of the pro-γ-MSH molecule were confirmed. Two residues previously missing (positions 15 and 25) were established (cf. fig.2). Furthermore, a misidentified position was also corrected: Glu in position 4. Partial desamidations were detected (cf. fig.2), in particular in position 10. The high yield of aspartic acid, and the identification of that residue

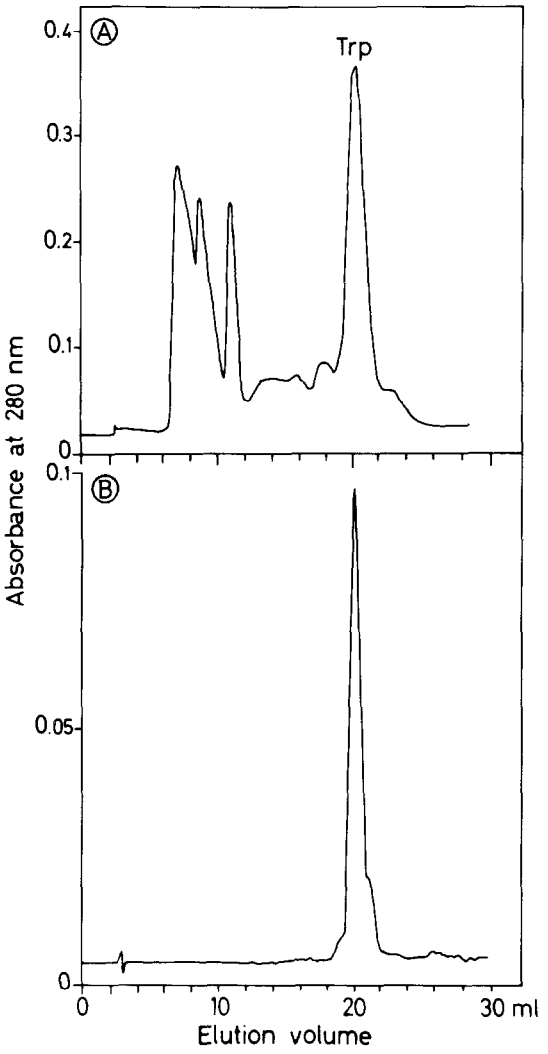


Fig.1. High-performance liquid chromatography of material obtained from Sephadex gel chromatography (cf. text). (A) Elution profile on a preparative μBondapak $C_{18}$ column: flow rate 2 ml/min, with collection of 2 ml fractions. (B) Rechromatography on an analytical μBondapak $C_{18}$ column of the peptide containing N-terminal tryptophan, corresponding to the peak designated Trp in (A).

[4] might even question the conclusion of asparagine in this position, although the amide assignment is the likely alternative (fig.2).

The composition and the sequence degradation suggest that the peptide ends with Ala-30. Analysis by hydrazinolysis confirmed the presence of C-terminal alanine. However, the results also indicated the presence of small amounts of glutamic acid and threonine.

Table 1
Amino acid composition of the investigated fragment of pro-γ-MSH

| Residue | Amount |
|---|---|
| Cys | 1.4 (4) |
| Asx | 3.0 (3) |
| Thr | 1.0 (1) |
| Ser | 4.3 (5) |
| Glx | 4.0 (4) |
| Pro | 1.3 (1) |
| Ala | 2.7 (3) |
| Ile | 0.8 (1) |
| Leu | 4.8 (5) |
| Trp | 0.2 (1) |
| Lys | 0.9 (1) |
| Arg | 0.8 (1) |
| Sum | 30 |

Duplicate samples of the non-modified peptide were hydrolyzed at 110°C for 22 h with 6 M HCl/0.5% phenol in evacuated tubes, explaining the low yield of Cys, Ser and Trp. Analytical values are molar ratios without corrections for destruction, slow release or impurities. The number of residues from the sequence analysis is shown within parentheses

Interestingly, these very residues occur subsequent to Ala-30 in the pro-γ-MSH molecule. Some minor heterogeneity in the C-terminus can therefore not be excluded, but the C-terminus at Ala-30 is consistent with the composition (table 1), the hydrazinolysis and the sequencer degradation (fig.2).

```
 1   2   3   4   5   6   7   8   9   10  11  12  13  14  15
Trp-Cys-Leu-Glu-Ser-Ser-Gln-Cys-Gln-Asn-Leu-Ser-Thr-Glu-Ser-
H,T  R  H,T H,T H,T H,T H,T  R  H,T H,T H,T H,T H,T H,T H,T
          60                                50
                          (Glu)  (Glu)(Asp)
                          (15%)  (30%)(50%)

 16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
-Asn-Leu-Leu-Ala-Cys-Ile-Arg-Ala-Cys-Lys-Pro-Asp-Leu-Ser-Ala.
H,T H,T H,T H,T  R  H,T  H  H,T  R  H,T H,T H,T H,T H,T H,T
     35  40  35      25      25                  15      <5
(Asp)
(20%)
```

Fig.2. Amino acid sequence of the investigated fragment of pro-γ-MSH. The structure was determined by sequencer degradations of the intact peptide (~300 nmol) and of the $^{14}$C-carboxymethylated peptide (~40 nmol). Residue identifications by high-performance liquid chromatography (H), and thin-layer chromatography (T) from analysis of the intact peptide (initial coupling 60%; values give the yield in % for the stable residues; repetitive yield $Leu_{3\rightarrow17}$: 96%), and by $^{14}$C-radioactivity (R) for the analysis of the carboxymethylated peptide. Extensive desamidation was noticed for residue 10, and some amide losses were also encountered for residues 7, 9 and 16, as indicated.

From the structure of pro-γ-MSH, there is no predictable cleavage site explaining its C-terminus. The 1–30 fragment must therefore have been generated by a cleavage at a site different from the traditional prohormone cleavage sites at paired basic residues [7,8].

## 4. Discussion

### 4.1. *Structure of pro-γ-MSH*

The complete structure of bovine pro-γ-MSH had been deduced from determination of the mRNA-coded cDNA [1], and a partial structure of porcine pro-γ-MSH obtained from a protein sequence analysis [4]. This partial sequence is now corrected and confirmed by final assignments of residues in positions 4, 15, 25 and possibly 10, as shown in fig.2. Comparisons between the structures in fig.2 and [1], show that the porcine pro-γ-MSH differs from its bovine counterpart in only a few positions. Position 10 has a probable Asp/Asn exchange (the occurrence of extensive desamidations still question the amide), and position 12 has a definite Thr/Ser exchange.

### 4.2. *New type of proteolytic processing*

The structure in fig.2 does not end in a pair of basic residues. Nor are any such residues found in the immediate proximity inside the pro-γ-MSH molecule. The closest base pair is 19 residues further away [1] towards the C-terminus of the pro-form. Since it is unlikely that so many residues are removed by exoprotease processing after an initial endoprotease cleavage, it can be concluded that the fragment now characterized is generated by a direct endoprotease cleavage at a site lacking the traditional dibasic structure. This cleavage site is therefore different from the other known cleavage sites in the ACTH-β-LPH precursor, where 10 dibasic structures occur [1]. In contrast, the substrate specificity of the protease generating the present fragment appears more related to those of the proteases hydrolysing nascent pre-promolecules, like the Gly–Trp cleavage involved in generating the ACTH-β-LPH precursor [1] or the Ala-cleavages that frequently produce the C-terminal ends of signal sequences [12].

The finding of a new small pro-γ-MSH fragment explains other data. Thus, several smaller pro-γ-MSH-related peptides in extracts of porcine pituitaries have been found by the use of a gel permeation system and a radioimmunoassay for pro-γ-MSH [13]. One of these pro-γ-MSH-related peptides eluted at a position

similar to that of β-endorphin, suggesting $M_r \sim 3500$, close to that expected for the present 1–30 fragment ($M_r$ 3281).

Whether the 1–30 peptide is a normally occurring fragment of pro-γ-MSH, and whether it exists in the ACTH or the MSH cells or in both remains to be established. In any event, these results define a new cleavage site, and establish the structure of the N-terminal part of this porcine pro-γ-MSH.

## Acknowledgements

This work was supported by grants from the Swedish Medical Research Council, the Medical Faculty, University of Lund, the Swedish Cancer Society, and the Knut and Alice Wallenberg's Foundation.

## References

[1] Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N. and Numa, S. (1979) Nature 278, 423–427.
[2] Mains, R. E., Eipper, B. A. and Ling, N. (1979) Proc. Natl. Acad. Sci. USA 74, 3014–3018.
[3] Roberts, J. L., Phillips, M., Rosa, P. A. and Herbert, E. (1978) Proc. Natl. Acad. Sci. USA 75, 3609–3618.
[4] Håkanson, R., Ekman, R., Sundler, F. and Nilsson, R. (1980) Nature 283, 789–792.
[5] Shibasaki, T., Ling, N. and Guillemin, R. (1980) Nature 285, 416–417.
[6] Estivariz, F. E., Hope, J., McLean, C. and Lowry, P. J. (1980) Biochem. J. 191, 125–132.
[7] Giesow, M. J. and Smyth, D. G. (1980) in: The Enzymology of Post-Translation Modification of Proteins (Friedman, R. B. and Hawkins, M. C. eds) pp. 260–287, Academic Press, New York.
[8] Pradayrol, L., Jörnvall, H., Mutt, V. and Ribet, A. (1980) FEBS Lett. 109, 55–58.
[9] Håkanson, R. and Sundler, F. (1971) Biochem. Pharmacol. 20, 3223–3225.
[10] Eriksson, U., Lindberg, A. A., Jörnvall, H. and von Bahr-Lindström, H. (1976) FEBS Lett. 72, 15–18.
[11] Jörnvall, H. and Philipson, L. (1980) Eur. J. Biochem. 104, 237–247.
[12] Austen, B. M. (1979) FEBS Lett. 103, 308–313.
[13] Ekman, R., Håkanson, R., Larsson, I., Thorell, J. and Sundler, F. (1981) submitted.