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Processing of pro-opiomelanocortin-derived amidated joining peptide and glycine-extended precursor in monkey pituitary

Mogens Fenger

Mental Health Research Institute, University of Michigan, Ann Arbor, MI 48109 (U.S.A.) and National Institutes of Health, Bethesda, MD 20892 (U.S.A.)

The molecular forms of proopiomelanocortin (POMC) derived amidated and C-terminal glycine-extended joining peptide from monkey *(Macaca mulatta)* pituitary were determined. The predominant forms of joining peptide found were the low molecular peptides POMC(76–105) and POMC(76–106), respectively. Significant amounts of N-terminally truncated POMC(78–105) and POMC(78–106) were also detected in the posterior-intermediate lobe. No N-terminal extended forms were detected. The relative amount of amidated joining peptide to total joining peptide was 6–35%. It is concluded that not only is the primary sequence of monkey and human POMC extremely conserved, but also the processing patterns are similar. The monkey therefore serves as a suitable model for studying regulation of the processing of POMC and the hypothalamus-pituitary-adrenal axis in man.

Proopiomelanocortin (POMC) has served as an excellent model to study the maturation of precursors of regulatory peptides. Comparative studies across tissues and species point to some of the elements, which determine the pattern and extent of processing [1, 6, 10, 11, 13, 14, 16, 18]. Generally, the precursor is initially cleaved at the C-terminal side of ACTH (Fig. 1) followed by cleavage at the N-terminal side. This universal pattern of cleavage releases ACTH from the precursor along with N-terminal POMC (N-POMC) and β-lipotropin (β-LPH). Successive cleavage of β-LPH to β-endorphin (βEP) and ACTH to αMSH is tissue specific [7, 18].

It is now clear that what has been detected as 16 kDa γ_3MSH-immunoreactivity mainly consists of the most N-terminal part of POMC (POMC(1–76) in human) in which the fragment linking the γ_3MSH sequence with ACTH, i.e. the joining peptide, has been removed. This has been confirmed by studies of the molecular forms of joining (or hinge) peptide in human [2, 11], rat and mouse pituitaries [9], and rat hypothalamus [7]. In these tissues the joining peptide is almost fully processed. Only

the rat anterior lobe appears to store significant amounts of fully N-terminal extended joining peptide [9].

Studies of the amidated joining peptide and its C-terminal glycine-extended precursor have now been extended to the monkey pituitary. The monkey POMC resembles the human POMC closely, in that all the putative α-amidated peptides are present in the precursor [17], which is not the case in any other species. Monkey POMC can therefore serve as a model system for studies of the synthesis and regulation of human POMC in vivo as well as in vitro.

Pituitaries from *Macaca mulatta* ($n = 6$) were dissected into anterior and posterior-intermediate lobes while kept frozen on dry ice. POMC-related peptides were extracted in boiling water and acetic acid as previously described [11]. Combined water and acetic acid extracts were applied to molecular sieve chromatography on 1.6 × 87 cm bioGel P10 columns (Biorad, Richmond, CA) and eluted at room temperature in 0.5 M acetic acid. Peak fractions of joining peptide were applied to an analytical Nucleosil C_{18}-reverse phase HPLC at room temperature and were eluted by a gradient of acetonitril in 10 mM trifluoric acetic acid (TFA). The eluted joining peptide immunoreactivities were subjected to automated amino acid sequence analysis [11].

Radioimmunoassays for amidated joining peptide were performed as previously described [10, 11]. The as-

Correspondence: M. Fenger, Department of Clinical Chemistry KK 3011, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.
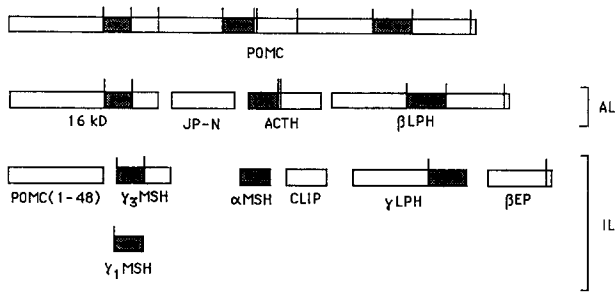
Fig. 1. Processing pattern of proopiomelanocortin (POMC) in the anterior lobe (AL) and intermediate lobe (IL) of the pituitary gland. The vertical lines indicate dibasic amino acids and the hatched areas indicate the MSH-sequences. β-LPH, β-lipotropin; βEP, β-endorphin; JP-N, amidated joining peptide; CLIP, corticotropin-like intermediate lobe peptide. The vertical line on the N-terminal side of γ₃MSH and γ₁MSH indicates that these peptides have been identified also as Lys-γMSH peptides. The C-terminal glycine extended intermediate forms of the amidated peptides (γ₁MSH, JP-N and αMSH) have been omitted from the figure.

say is specific to joining peptide and the cross-reactivity to the glycine-extended precursor is less than 0.02% at half-maximum displacement of the tracer. No other POMC-related peptides were detected by the assay. A new radioimmunoassay for glycine-extended joining peptide was developed essentially as described [10, 11], in which a glycine-extended truncated form of joining peptide (D-Tyr-Ala-Lys-Pro-Gly-Pro-Arg-Glu-Gly) was used as immunogen, tracer and standard [11].

The new assay for glycine-extended joining peptide was specific for the glycine-extended form of joining peptide with cross-reactivity of less than 0.01% of the amidated form (not shown). No other POMC-derived peptides were recognized by the assay. The sensitivity of the assay was 1 fmol.

The relative amount of amidated joining peptide varied from 6% to 35%. The elution of anterior pituitary joining peptide immunoreactivities from BioGel P10 columns is shown in Fig. 2. The majority of the joining peptide immunoreactivities eluted as single peaks. A small amount of immunoreactivities eluted approximately in the middle of the column, but no immunoreactivity was detected in the void volume and none eluted after the elution position of ACTH(1–39). A similar elution profile was detected from the posterior-intermediate lobe (not shown).

The major amidated joining peptide immunoreactive peaks (Fig. 2, $K_{av} = 0.36$) from anterior and posterior-intermediate lobes were applied to reversed phase HPLC as shown in Fig. 3. The immunoreactivities eluted essentially in single peaks. However, the amidated joining peptide immunoreactivity from the anterior lobe eluted at a significant higher concentration of acetonitrile (50%, upper figure) compared to the posterior-intermediate

lobe (37%, lower figure). A small amount of immunoreactivity eluted at 20% acetonitril (fraction 20) from both lobes. This coincided with the elution position of the smaller peaks of amidated joining peptide (Fig. 2, $K_{av} = 0.57$), when applied to HPLC (not shown).

The immunoreactive peaks shown in Fig. 3 were subjected to amino acid sequence analyses and proved to be pure enough to determine the sequence of joining peptide unambiguously (Table I). The major peak from the anterior lobe contained only the low molecular form of joining peptide generated by cleavage of N-POMC C-terminal to γ₃MSH. Approximately two thirds of the amidated joining peptide in the major peak from the posterior-intermediate lobe was identical to the joining peptide from the anterior lobe, the remaining one third being low molecular weight amidated joining peptide in which the amino-terminal glutamate and aspartate have been removed. The amino acid sequence analysis of the minor amidated joining peptide peaks in Fig. 2 (and
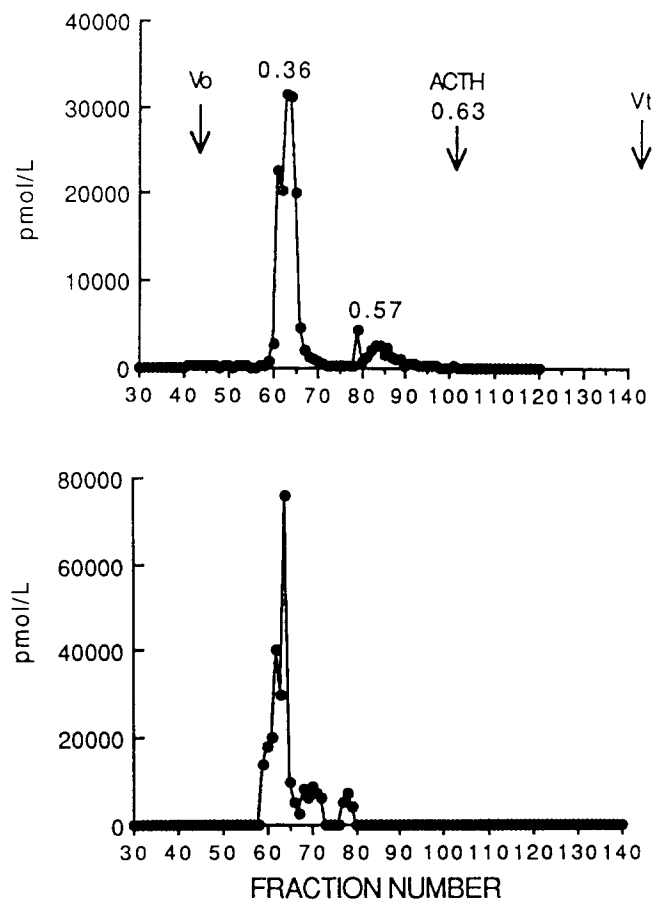

Fig. 2. Elution of amidated (upper panel) and glycine-extended (lower panel) joining peptide from an anterior pituitary lobe on a 1.6 × 87 cm BioGel P10 column in 0.5 M acetic acid at room temperatur. Approximately half of the pituitary extracts were applied to the column, and 1.5 ml fractions were collected. The peaks eluting at $K_{av} = 0.36$ and $K_{av} = 0.57$ were further purified by HPLC (Fig. 3). $K_{av} = V_e - V_0/V_t - V_0$ where V_e is the elution position of the peptides.
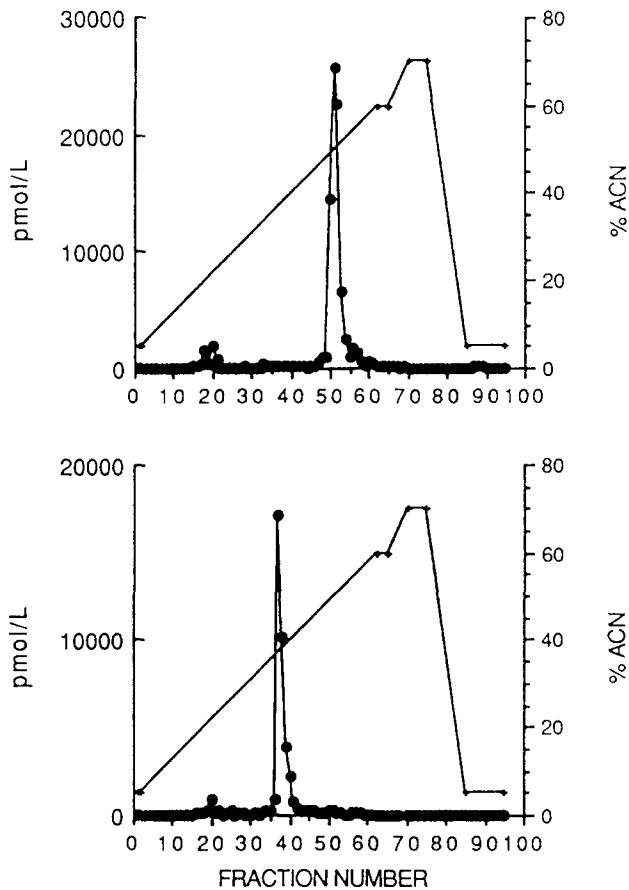
Fig. 3. Purification of the major amidated joining peptide peak indicated in Fig. 2 (upper panel) and a similar peak from posterior-intermediate lobe (lower panel). The peptides were applied to a 4 × 250 mm Nucleosil C_{18} reversed phase HPLC column and eluted at room temperature at a flow rate of 0.5 ml/min. The peptides were eluted by a acetonitrile (ACN) gradient in 10 mM TFA, and 0.5 ml fractions were collected. The major peaks were concentrated and subjected to amino acid sequence analysis.

similar peaks from the posterior-intermediate lobe) indicated that several truncated forms were present, one of which was consistent with the sequence of amidated joining peptide POMC(89–105).

This study shows, that in the monkey pituitary only low molecular forms of POMC-derived amidated and C-terminal glycine-extended joining peptide are present. The joining peptide is cleaved from the N-terminal part of POMC at the Lys-Arg residues between joining peptide and γ_3MSH (Fig. 1), and no N-terminal extended forms are present. The joining peptide derived from the posterior-intermediate lobe is to some extent N-terminally truncated by removal of both the glutamate and aspartate, which is probably the reason for the different elution profiles of the joining peptide on HPLC (Fig. 3). The molecular forms of the amidated and glycine-extended forms of joining peptide are similar, but the relative amount of joining peptide varies. As low as 6%

TABLE I

AUTOMATED EDMAN DEGRADATION OF JOINING PEPTIDE FROM MONKEY PITUITARY

The amino acid sequences are shown for the major joining peptide peaks. The Glu-Asp-truncated joining peptide detected from the intermediate lobe is not shown in the table. Amino acid sequences consistent with POMC(89–105) were determined for the minor peaks in Fig. 2 (not shown). The yields of the last determined amino acids were approximately 2 pmol.

Cycle	AA[a]	Expected amino acid	Anterior lobe	Posterior-intermediate lobe
1	76	Glu	Glu	?
2	77	Asp	Asp	Asp
3	78	Val	Val	Val
4	79	Ala	Ala	Ala
5	80	Ala	Ala	Ala
6	81	Gly	Gly	Gly
7	82	Glu	Glu	Glu
8	83	Asp	Asp	Asp
9	84	Arg	Arg	?
10	85	Gly	Gly	Gly
11	86	Leu	Leu	Leu
12	87	Leu	Leu	Leu
13	88	Pro	Pro	Pro
14	89	Glu	Glu	Glu
15	90	Gly	Gly	Gly
16	91	Gly	Gly	Gly
17	92	Pro	Pro	Pro
18	93	Glu	Glu	Glu
19	94	Pro	Pro	?
20	95	Arg	?	?
21	96	Gly	Gly	?
22	97	Asp	Asp	?
23	98	Gly	?	?
24	99	Ala	Ala	?
25	100	Gly	?	?
26	101	Pro	?	?
27	102	Gly	?	?
28	103	Pro	?	?
29	104	Arg	?	?
30	105	Glu-NH$_2$	?	?

[a] Numbers refer to sequence numbers in monkey POMC [17]. '?' Indicates inconclusive amino acid determination.

of the joining peptide is converted to amidated joining peptide, showing that the amidation reaction is not an efficient process in the case of joining peptide, probably because the penultimate glutamate is a poor substrate for the amidating enzyme [3]. Similar findings have been obtained in human pituitary [11, 12].

The elution position of the joining peptide-immuno-reactivities from the BioGel P10 column are somewhat surprising, considering the lower molecular weight of the joining peptide compared with ACTH(1–39) (30 amino acids versus 39 amino acids). A similar elution profile has been detected in human pituitary extracts [11, 12].

The human amidated joining peptide is conjugated through disulfide bridges to, presumably, another joining peptide. However, no cysteine is detected by the sequence analysis in the monkey joining peptides deduced from the cDNA sequence from another monkey, *Macaca nemestrina* [17]. The explanation for this elution pattern is probably aggregation of the joining peptide, which is also the case for synthetic β-endorphin in the molecular sieve system used in this study (not shown). However, the exact molecular forms of the joining peptide were unambiguously verified by sequence analysis.

The monkey N-terminal domain of the POMC precursor contains three pairs of dibasic amino acid residues (Fig. 1) which are putative sites for endopeptidase cleavage [13, 15, 18]. Four molecular forms of joining peptide can therefore be imagined disregarding additional analogues due to the possible glycosylation of forms extending to the γ_1MSH sequence and beyond [8, 13, 18]. However, only fully processed amidated and glycine-extended forms of joining peptide are detected, i.e. joining peptide is cleaved at dibasic amino acids C-terminal to the γ_3MSH sequence (Fig. 1). The truncated forms are equivalent to what has been found in human pituitary [11], although only the N-terminal glutamate is removed in the human joining peptide. No truncation has been described in the rat or mouse joining peptide [9]. High molecular forms are not found in monkey pituitary, and less than 1% of the amidated hinge peptide in human pituitary is N-terminal extended. This would suggest, that cleavage at the N-terminal side of joining peptide in the primates proceeds rapidly and efficiently following the initial cleavage at the dibasic amino acids flanking ACTH(1–39). At present it cannot be decided if the minor peaks of amidated joining peptide are the result of extensive aminopeptidase activity, a proline endopeptidase activity, or metabolic degradation.

We still do not know whether one or several enzymes are involved in dibasic cleavage, but almost complete cleavage at Lys-Arg residues is the favored pattern across species suggesting one single enzyme to be active. While the low molecular weight forms are also the only forms of joining peptides detected in rat and mouse intermediate pituitary lobe, the AtT20 cell line and rat hypothalamus [7, 9], significant amounts of fully N-terminally extended amidated joining peptide have been detected in the rat anterior lobe [9]. This difference can be explained by the substitution of the lysine to an arginine in the N-terminal dibasic residues, thereby decreasing the efficiency of cleavage by a Lys-Arg-cleaving endopeptidase. However, additional deviations from the preferred Lys-Arg cleavage pattern are obvious. Two other Lys-Arg dibasic residues are present in POMC: in β-LPH at the N-terminal site of β-endorphin and in

ACTH C-terminal to the αMSH. β-LPH is cleaved to β-endorphin in the intermediate lobe cells, but is only cleaved to a minor extent in the anterior lobe. ACTH is processed almost completely to αMSH in the intermediate lobe, but only very small amounts of cleaved ACTH can be detected in the anterior lobe [2, 4, 5, 10, 13]. These cleavage regions are completely conserved in mammals [17]. It can be imagined that only one endopeptidase is present, but regulated differently by e.g. phosphorylation or cofactors such as calcium (Y.P. Loh, personal communication). Alternatively, more than one enzyme is present, which is specifically activated in different tissues.

In summary, the results show that processing of POMC in the monkey pituitary is very similar to the processing of POMC in the human pituitary. The monkey therefore serves as an accurate model for studying regulation of POMC processing in corticotrophic cells in man.

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