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Characterization of Multiple Forms of Porcine Anterior Pituitary Proopiomelanocortin Amino-Terminal Glycopeptide†

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ABSTRACT: Chromatography on a molecular sieve column of a preparation of porcine proopiomelanocortin N-terminal glycopeptide purified from anterior pituitary resulted in the isolation of three forms of the peptide with respective apparent M_r 21 000, 17 500, and 13 500 on polyacrylamide/sodium dodecyl sulfate gel. Determination of the amino acid composition of each peptide revealed that the form with a molecular weight of 17 500 corresponds to the 80 amino acid residue porcine N-terminal glycopeptide (PNT 1–80) previously characterized [Larivière, N., Seidah, N. G., & Chrétien, M. (1981) *Int. J. Pept. Protein Res. 18*, 487–491]. The forms with molecular weight of 21 000 and 13 500 correspond respectively to longer and shorter forms of the N-terminal glycopeptide. The high molecular weight form contains 107 amino acid residues. Sequencing of the fragments obtained after cleavage of the molecule with cyanogen bromide and *Myxobacter* Lys-C protease indicated that an extension of 27 amino acid residues is linked to PNT 1–80 through a -Lys-Arg- sequence. The sequence of the extension is reported. The low molecular weight form corresponds to the first 61 residues of PNT 1–80. Pronase digestion of the peptide and dansylation of the digest revealed the presence of a residue of phenylalanine amide at position 61. A general model for the maturation of the N-terminal glycopeptide of proopiomelanocortin in porcine anterior pituitary is presented.

Adrenocorticotropic hormone (ACTH), β-lipotropic hormone (β-LPH), α- and β-melanotropic hormones (α- and β-MSH), and β-endorphin (β-END) are synthesized in the pituitary gland from a common glycoprotein precursor (Roberts & Herbert, 1977; Mains et al., 1977; Crine et al., 1978) called proopiomelanocortin (POMC) (Chrétien et al., 1979). These hormones are located on the central and C-terminal portions of the POMC structure leaving a large N-terminal fragment. Using recombinant DNA technology, Nakanishi et al. (1979) determined the nucleotide sequence of the mRNA coding for pre-POMC in bovine pars intermedia. This study revealed an amino acid sequence in the N-terminal portion of POMC homologous to the sequence of α- and β-MSH in ACTH and β-LPH, respectively. This new sequence was called γ-MSH (Nakanishi et al., 1979). More recently, investigations on the genomic DNA structure of human (Chang et al., 1980), bovine (Nakanishi et al., 1980), and rat (Drouin & Goodman 1980) POMC were published confirming the finding of Nakanishi et al. (1979). Pulse and pulse–chase experiments in rat pars intermedia (Crine et al., 1979, 1980a,b; Gossard et al., 1980) have shown that during maturation of POMC, the N-terminal portion of the molecule is released as two glycosylated peptides with apparent molecular weights (M_r) on polyacrylamide/sodium dodecyl sulfate ($NaDodSO_4$) gel of 17 000 and 19 000.

Recently, we purified the N-terminal fragment of POMC from the anterior lobe of porcine pituitary glands and from whole human pituitary glands and reported their complete amino acid sequence (Larivière et al., 1981; Seidah & Chrétien, 1981). The peptide from porcine anterior lobe was found to be 80 amino acids long while its human homologue was 76 amino acids long. However, during the purification procedure, a certain heterogeneity of the preparations could be detected by gel filtration and two-dimensional polyacrylamide gel electrophoresis.

In this paper we present the chemical characterization of two additional forms of the N-terminal fragment of porcine POMC differing in their length from the peptide of 80 amino acids. One has 107 amino acid residues and represents probably the N-terminal portion of POMC up to the putative -Lys-Arg- sequence preceding the ACTH sequence (Nakanishi et al., 1979, 1980; Chang et al., 1980; Drouin & Goodman 1980); the other has 61 amino acids, it contains the γ-MSH sequence, and its C-terminal phenylalanine is amidated. This latter form is possibly a maturation product of the 80 amino acid peptide.

Materials and Methods

Isolation of Porcine N-Terminal Peptides and Two-Dimensional Polyacrylamide Gel Electrophoresis. The methods for the isolation and purification of the peptides by high-performance liquid chromatography (HPLC) have been previously reported (Larivière et al., 1981). One subsequent step of gel filtration on a column (125 × 1.5 cm) packed with Sephadex G-75 superfine (Pharmacia Fine Chemicals) eluted

† From the Clinical Research Institute of Montreal, Montreal, Canada H2W 1R7. *Received February 19, 1982.* This work was supported by a program grant from the Medical Research Council of Canada and by the National Institutes of Health (NS-16315-02). G.B. is a MRC fellow.

0006-2960/82/0421-5341$01.25/0

with 1 M acetic acid was added to obtain the high molecular weight form (HM_r) and fractions enriched in low molecular weight form (LM_r) of N-terminal peptide.

Two-dimensional polyacrylamide gel electrophoresis was performed essentially according to the technique of O'Farrell (1975) with a 3–10 pH gradient in the first dimension and a 10–20% acrylamide gradient in the second.

Cyanogen Bromide Cleavage and Peptide Purification. The peptide to be cleaved was reacted with cyanogen bromide (CNBr) in 70% formic acid and in a molar ratio of 1000:1 (CNBr:peptide). The reaction proceeded in the dark at room temperature for 24 h, and the products were lyophilized.

Purification of the resulting peptides was achieved by high-performance liquid chromatography on a Waters μBondapak C_{18} column (0.39 × 30 cm) using a linear gradient of acetonitrile (5–65% in 60 min) or 2-propanol (5–95% in 90 min) in 0.02 triethylamine formate, pH 3.0. The peptides were detected by continuous monitoring of the column eluate at 235 nm. The purified peptides were lyophilized and subjected to amino acid analysis.

Digestion with Myxobacter Lys-C Protease. The lyophilized peptide (500 μg) was resuspended in 1 mL of 20 mM Tris-HCl, pH 8.8, and *Myxobacter* Lys-C protease (Boehringer Mannheim) was added in a ratio of substrate to enzyme of 50:1 (w/w). The digestion was carried out at 40 °C for 6 h. The resulting peptides were purified as described above and subjected to amino acid analysis.

Amino Acid Composition and Sequence Analysis. The amino acid composition of the peptides was performed on an updated Beckman 120C amino acid analyzer after acid hydrolysis of the sample for 24 h at 105 °C in 5.6 N HCl. The determination of glucosamine (GlcN) and galactosamine (GalN) ratio was not corrected for hydrolysis loss. All amino acid determinations were done on triplicate hydrolysates of the peptides. Automatic sequence analysis and identification of the phenylthiohydantoin derivatives were performed as previously described (Seidah et al., 1981a).

Digestion with Pronase. An aliquot (100 μg) of the peptide to be digested was dissolved in 400 μL of 0.1 M ammonium acetate, pH 7.5, containing 10 mM $CaCl_2$. Pronase (Calbiochem-Behring Corp.) was added (ratio enzyme:substrate of 1:10) and the digestion performed at 37 °C for 48 h. The digest was evaporated to dryness. The dry residue was dansylated and the dansylated products analyzed on polyamide thin layer plates (Tatemoto & Mutt, 1978). As control, phenylalanine amide was digested with Pronase under the same conditions.

Results

Porcine N-terminal glycopeptide was purified from pituitary pars distalis by chromatography on a carboxymethylcellulose column and HPLC as described previously (Larivière et al., 1981). Analysis of an aliquot (100 μg) of porcine N-terminal peptide by two-dimensional polyacrylamide gel electrophoresis revealed a very high degree of heterogeneity of the preparation (Figure 1A). Two main rows of spots with apparent M_r 21 000 and 17 500 were detected on the electrophoretogram. A third one with an apparent M_r 13 500 could also be seen and became more evident in the enriched fraction (Figure 1D). Each row contains a number of species different in their net charge. This charge heterogeneity may be caused by differences in the degree of glycosylation and phosphorylation of the molecule or due to deamidation occurring during the purification procedures. Chromatography of the preparation of porcine N-terminal peptide on Sephadex G-75 superfine resulted in the purification of the species with apparent M_r

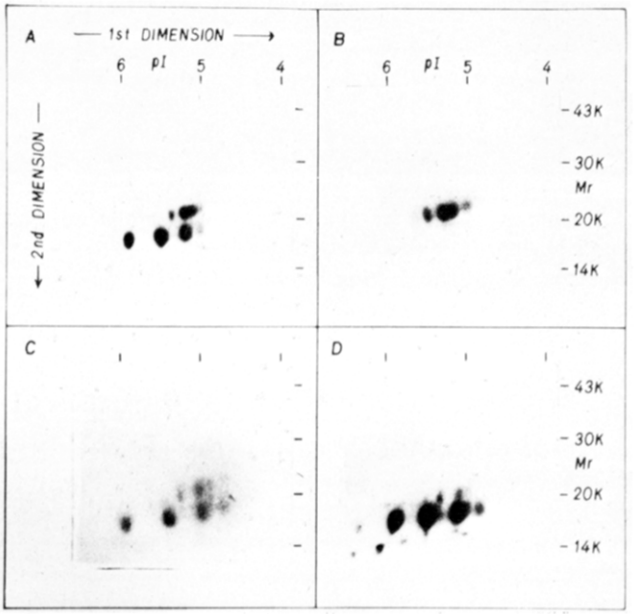

FIGURE 1: Two-dimensional polyacrylamide gel electrophoresis of a preparation of porcine N-terminal peptides (A) and of the three fractions obtained after chromatography of the preparation on Sephadex G-75 superfine (B–D).

21 000 and 17 500 (Figure 1B,C) and in the isolation of a fraction enriched with M_r 13 500 species (Figure 1D). Previous work done in our laboratory (Larivière et al., 1981) has shown that the major form of porcine N-terminal peptide has an apparent molecular weight on polyacrylamide/NaDodSO$_4$ gel electrophoresis of 17 000 and contains 80 amino acid residues (PNT 1–80). Determination of the amino acid composition of the species with an apparent molecular weight of 17 500 identified them as PNT 1–80 (results not shown). We investigated the possibility that the high molecular weight (HMr) form and the low molecular weight (LMr) form of the N-terminal peptide correspond to molecules with different lengths. Since the N-terminal sequence of the preparation proved to be homogeneous (Larivière et al., 1981) and DNA sequencing showed the presence of a peptide between the N-terminal portion of POMC and the ACTH/β-LPH region (Nakanishi et al., 1980; Drouin & Goodman, 1980; Chang et al., 1980), we proceeded to study the C-terminal portion of the molecule.

Chemical Characterization of the C-Terminal Fragment of the HMr Form of Porcine N-Terminal Peptide. The previous sequence study of the N-terminal peptide of porcine POMC has shown the presence of a methionine residue at position 53 of the molecule (Larivière et al., 1981). Therefore, CNBr was chosen to cleave it. A sample containing 3 mg of the HMr form of porcine N-terminal peptide was reacted with CNBr in the conditions described under Materials and Methods, and the reaction mixture was purified by HPLC with a gradient of acetonitrile (Figure 2A). Two peptides called CHMrI and CHMrII were collected. The amino acid compositions of each peptide indicated that peptide CHMrI is composed of the first 53 amino acid residues of PNT 1–80 (data not shown). Fragment CHMrII contained 54 amino acid residues (Table I), 27 more than the homologous fragment obtained after cleavage of PNT 1–80 with CNBr (PNT 54–80). This result strongly suggested that the HMr form of the porcine N-terminal peptide is longer than the previously characterized PNT 1–80 form (Larivière et al., 1981).

Direct sequencing of CHMrII allowed the unambiguous identification of the first 51 residues with the exception of the residues in positions 44 and 46 (corresponding to residues 97

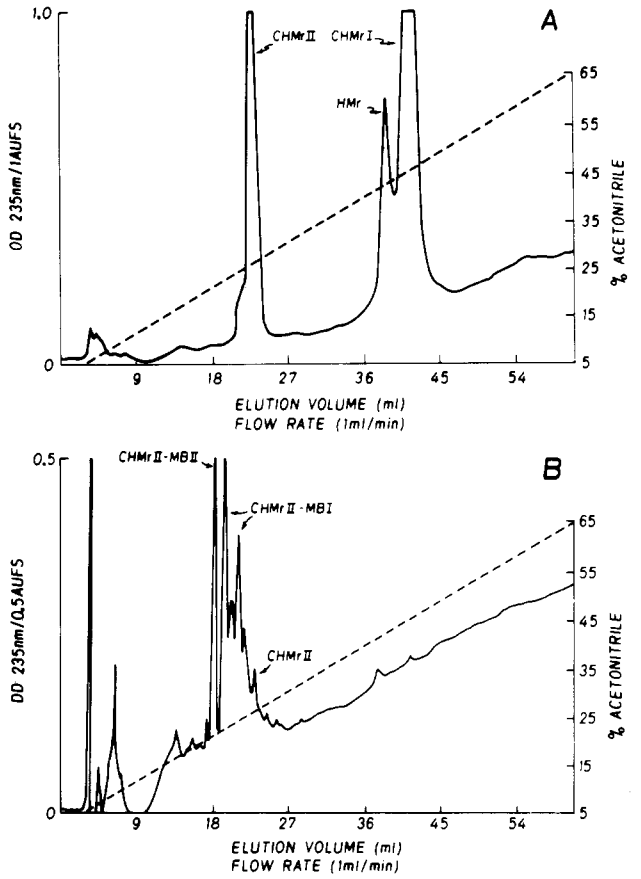

FIGURE 2: High-performance liquid chromatography purification on a μBondapak C_{18} column (Waters) of CNBr digest of the HMr form of porcine N-terminal peptide (panel A) and *Myxobacter* Lys-C protease digest of peptide CHMrII (panel B). The linear gradient of acetonitrile is shown as a dashed line on the chromatograms.

and 99 of HMr) (Figure 3). No residue could be identified at position 12 of CHMrII. This position corresponds to position 65 of PNT 1–80 which is an N-glycosylated asparagine residue (Larivière et al., 1981). The failure to identify this residue comes from the insolubility of the PTH-Asn-sugar in the sequenator PTH extraction solvent (butyl chloride) (Seidah et al., 1981a). This sequence confirmed the identity between the N-terminal portion of CHMrII and PNT 54–80 and the presence of an extension at the C-terminal end of CHMrII.[1]

For completion of the sequence of CHMrII and identification of residues in positions 97 and 99, 500 μg of the peptide was digested with *Myxobacter* Lys-C protease. This enzyme is reported to specifically cleave peptide chains on the amino side of lysine residues (Wingard et al., 1972). The digest was purified by HPLC with a gradient of acetonitrile (Figure 2B). Two predominant peptides called CHMrII-MBI and CHMrII-MBII eluting at respective volumes of 20 and 18.5 mL were collected. The two peptides were found in close to equimolar amounts as can be expected from the unique lysine residue in the structure of CHMrII (Table I and Figure 3). Their amino acid compositions are presented in Table I. CHMrII-MBI has an amino acid composition similar to that of PNT 54–80 except for the presence of an extra lysine residue. This result indicates that CHMrII-MBI corresponds to the N-terminal fragment of CHMrII and that CHMrII-MBII must be the C-terminal fragment. The presence of the lysine residue associated with CHMrII-MBI indicates that in our hands, the *Myxobacter* Lys-C protease cleaved the peptide on the carboxyl side of the lysine residue. A third less abundant peptide eluting at a volume of 22 mL was also collected. Its amino acid composition was similar to that of CHMrII-MBI. The structural difference between the two peptides is not known, but it could represent a glycovariant of CHMrII-MBI.

[1] The yield of recovery of the different amino acids at each cycle of automatic sequencing was presented to the scrutiny of the reviewers and can be obtained by writing directly to the authors.

Table I: Amino Acid Composition of the Fragments Produced by Treatment of HMr and LMr with CNBr and *Myxobacter* Lys-C Protease[a]

amino acid	PNT 54-80[b]	CHMrII	CHMrII-MBI	CHMrII-MBII	CLMrII
Lys	–	1.18 (1)	0.83 (1)	–	–
His	1	1.18 (1)	0.97 (1)	–	1.03 (1)
Trp	1	1[c]	1[c]	–	–
Arg	4	7.19 (7)	3.87 (4)	2.93 (3)	2.00 (2)
Asx	2	4.07 (4)	1.89 (2)	1.87 (2)	1.06 (1)
Thr	–	–	–	–	–
Ser	3	3.32 (3)	2.60 (3)	–	–
Glx	1	6.67 (7)	1.25 (1)	5.77 (6)	–
Pro	–	3.55 (4)	–	3.8 (4)	–
Gly	12	17.63 (18)	11.69 (12)	6.33 (6)	1.10 (1)
Ala	1	4.35 (4)	1.37 (1)	3.4 (3)	–
Val	–	2.3 (2)	–	1.69 (2)	–
Met	–	–	–	–	–
Ile	–	–	–	–	–
Leu	–	–	–	–	–
Tyr	–	–	–	–	–
Phe	2	1.98 (2)	1.85 (2)	–	1.82 (2)
GlcN	++++	++++	++++	–	–
GalN	+	–	–	–	–
total	27	54	28	26	7

[a] Amino acid analysis of the fragments CHMrII, CHMrII-MBI, CHMrII-MBII, and CLMrII. Triplicate samples of the peptides were hydrolyzed for 24 h in 5.6 N HCl at 105 °C and analyzed on a modified Beckman 120C amino acid analyzer. The amino acid composition of PNT 54–80 was calculated from the sequence presented in Larivière et al. (1981). (–) indicates that it is not present in the peptide. (+) indicates the presence of GlcN and GalN; the number of (+) represents the relative amount of each sugar in the peptide. [b] Composition derived from the sequence presented in Larivière et al. (1981). [c] Determined by sequence.

The automatic sequencing of CHMrII-MBII was performed as described under Materials and Methods, and it proceeded up to the C-terminal amino acid. Figure 3 shows the complete sequence of CHMrII-MBII. This sequence completes the partial sequence of CHMrII and positions all the amino acid residues found in the amino acid composition of CHMrII-MBII (Table I).[1]

Chemical Characterization of the C-Terminal Fragment of the LMr Form of Porcine N-Terminal Peptide. Since pure LMr form of porcine N-terminal peptide could not be obtained after chromatography on Sephadex G-75 superfine, 1.5 mg of a fraction enriched in the LMr form was treated with CNBr as described above. The reaction mixture was purified by HPLC with a gradient of 2-propanol (Figure 4A). Two peptides called CLMrI and CLMrII′ were collected. Determination of the amino acid composition indicated that peptide CLMrI corresponds to fragment 1–53 of PNT 1–80 (data not shown). Peptide CLMrII′, however, was heterogeneous and was repurified by HPLC under isocratic conditions with 18% 2-propanol (Figure 4B). Two major peptides, CLMrII and CLMrIII, were purified. Amino acid composition of CLMrIII showed that it corresponds to fragment 54–80 of PNT 1–80 (data not shown). The amino acid composition of CLMrII indicated that it contains only seven amino acid residues (Table

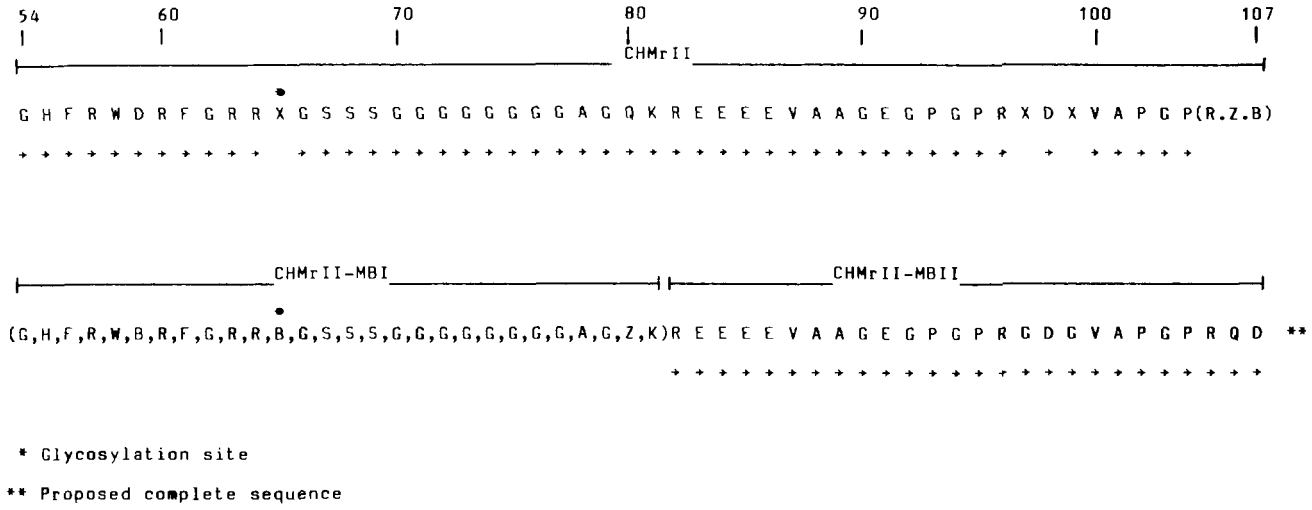

FIGURE 3: Complete sequence of the C-terminal fragment of HMr. Direct sequencing of fragment CHMrII obtained after cleavage of HMr with CNBr gave the identification of residues 54–104 with the exception of residues at positions 65, 97, and 99. Sequencing of fragment CHMrII-MBII obtained after digestion of CHMrII with *Myxobacter* Lys-C protease resulted in the unambiguous identification of residues 82–107.

I). These seven residues match exactly the composition of fragment 54–61 of PNT 1–80 except for the tryptophan residue which was destroyed during acid hydrolysis of the peptide. This result suggests that the LMr form of the porcine N-terminal peptide corresponds to the first 61 amino acids of PNT 1–80. Since a previous immunological study (Shibasaki et al., 1980) suggested the presence of an amidated phenylalanine residue at the C-terminus of γ_1-MSH (residues 51–61 of PNT 1–80), we decided to investigate the possibility that a residue of phenylalanine amide is present at position 61 of PNT 1–61. An aliquot (100 μg) of the fraction enriched in the LMr form of the porcine N-terminal peptide was digested with Pronase, and the resulting digest was dansylated. Chromatography of the dansylated digest on thin-layer polyamide plates revealed the presence of phenylalanine amide in the sample, strongly suggesting the presence of that residue at position 61 of PNT 1–61.[2]

Discussion

Analysis by two-dimensional polyacrylamide gel electrophoresis of a preparation of N-terminal peptide purified from anterior lobes of porcine pituitary glands has revealed a high degree of heterogeneity in the molecular weight of the peptides as well as in their net charge (difference in the isoelectric points). Three forms of N-terminal glycopeptide with respective apparent M_r 21 000, 17 500, and 13 500 were isolated following chromatography on Sephadex G-75 superfine. The major form of the peptides (M_r 17 500) is 80 amino acid residues in length, and we recently reported its complete sequence (Larivière et al., 1981). The heavy (HMr) and light (LMr) forms of the N-terminal glycopeptide were characterized in this work.

The HMr form represents about 20–25% of the N-terminal peptide purified from the porcine glands. Determination of the amino acid composition of different fragments obtained after cleavage of the molecule with CNBr or with *Myxobacter* Lys-C protease and determination of the amino acid sequence of the fragments have shown that HMr is a longer form of the major peptide (PNT 1–80) containing an extension of 27 amino acid residues at its C terminus. This peptide was

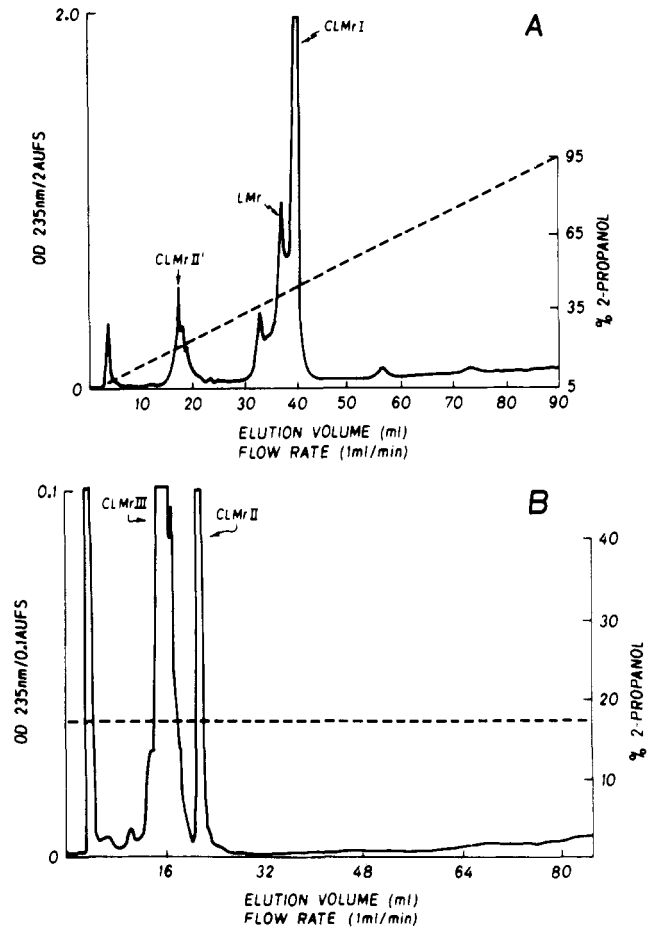

FIGURE 4: High-performance liquid chromatography purification on a μBondapak C_{18} column (Waters) of CNBr digest of the LMr form of porcine N-terminal peptide (panel A). Peak CLMrII' was repurified under isocratic conditions (panel B). The linear gradient of 2-propanol is shown as a dashed line on the chromatograms.

previously named "joining peptide" (Seidah et al., 1981b). This explains the results of our preliminary report (Larivière et al., 1980) in which tryptic digests had revealed the presence, in minor amounts, of peptides related to the C-terminal portion of this molecule. The extension is connected to PNT 1–80 through a pair of basic residues (-Lys-Arg-) at positions 81

[2] The thin-layer chromatograms were presented to the scrutiny of the reviewers and can be obtained by writing directly to the authors.

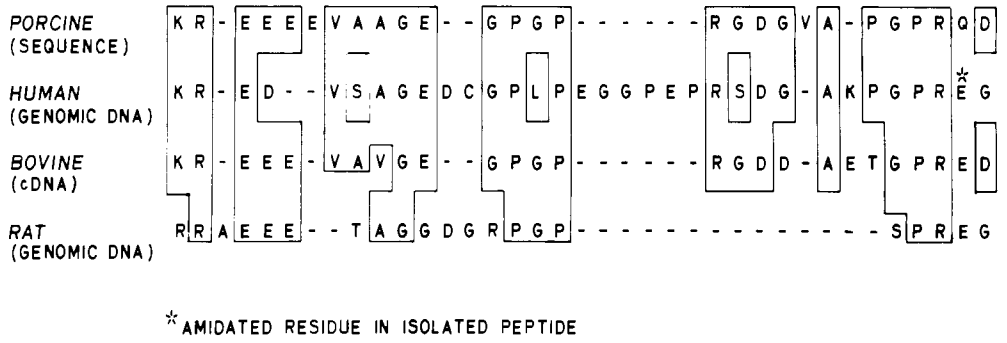

FIGURE 5: Homologies between porcine, human, bovine, and rat joining peptides of POMC. The sequences of the human and rat joining peptides are derived from the sequences of the genomic DNA. The sequence of the bovine peptide is derived from the cDNA sequences. (Asterisk) The human joining peptide was recently isolated in our laboratory, and it was shown to possess a C-terminus amidated glutamic acid residue (Seidah et al., 1981b).

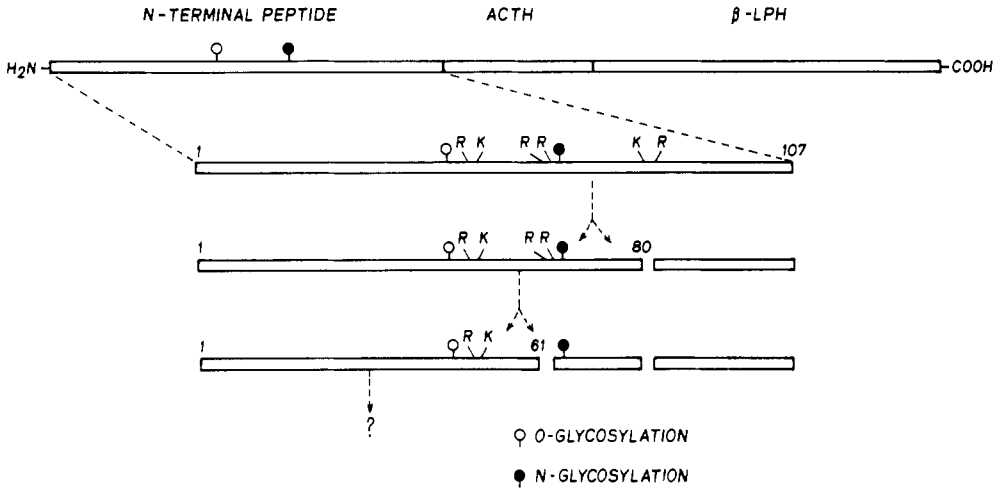

FIGURE 6: Proposed maturation of the N-terminal peptide of POMC in the porcine anterior pituitary.

and 82, respectively. The presence of the pair of basic amino acid residues suggests that the extension peptide can be readily removed by the same maturation enzymes that release ACTH and β-LPH since the -Lys-Arg- sequence is also present at the cleavage sites of these peptides (Nakanishi et al., 1979, 1980; Chang et al., 1980; Drouin & Goodman, 1980).

Figure 5 compares the primary structure of segment 81–107 of porcine N-terminal peptide to the primary structures of homologous peptides from human, bovine, and rat species derived from the genomic DNA or cDNA sequences (Nakanishi et al., 1979; Chang et al., 1980; Drouin & Goodman, 1980). When the sequences are aligned for maximum homology and the corresponding positions are compared, a very high degree of homology (70–90%) exists between the porcine peptide and that of the other species. However, the occurrence of many deletions in the molecule strongly suggests that the peptide has a structural role rather than a biological function.

In our preparation, the LMr form of porcine N-terminal peptide represents less than 5% of the N-terminal peptide purified from the anterior lobes. Determination of the amino acid composition of the fragments obtained after cleavage of the molecule with CNBr has shown that the LMr form contained the 61 first amino acid residues of PNT 1–80. An apparent molecular weight of 13 500 as determined by polyacrylamide/NaDodSO$_4$ gel electrophoresis is coherent with the loss of 19 amino acid residues and of the site of glycosylation at Asn$_{65}$. The presence of a residue of phenylalanine amide at position 61 suggests that LMr, although found in low amount, is a normal maturation product of the N-terminal peptide. Amidation of the penultimate residue of a peptide often occurs when a glycine residue is at its C terminus (Harris & Lerner 1957; Suchanek & Kreil, 1977; Amara et al., 1980; Seidah et al., 1981b). Examination of the sequence of porcine N-terminal peptide indicates that the phenylalanine residue at position 61 is followed by the sequence -Gly-Arg-Arg-. It is proposed that the cleavage of the peptide first occurs at the position of the paired basic residues. The C-terminus basic residue(s) is then released by a carboxypeptidase B type enzyme generating a peptide with a glycine residue at its C terminus. Removal of the glycine residue by a specific enzyme results in the amidation of the phenylalanine residue at position 61. Recently, larger amounts of PNT 1–61, representing 20–25% of the total porcine N-terminal peptide, have been purified from the anterior lobe (K.-L. Hsi, N. G. Seidah, and M. Chrétien, unpublished results). This finding indicates that PNT 1–61 is produced in the anterior lobe and that the small amounts detected in this study are not due to a contamination from the intermediate lobe where the maturation of the peptides is known to proceed further and the amidation reaction to occur.

Pulse and pulse–chase experiments in rat pars intermedia (Crine et al., 1979, 1980a,b) showed that the N-terminal peptide of POMC accumulates during maturation as two glycopeptides with apparent M_r 19 000 and 17 000 on polyacrylamide/NaDodSO$_4$ gel. No or very little subsequent maturation of the N-terminal peptide could be observed. By analogy to the present findings in porcine pituitaries, it can be speculated that these two rat N-terminal glycopeptides observed conceivably represent the 1–95 and 1–74 forms, respectively, expected from the nucleotide sequence of rat POMC (Drouin & Goodman, 1980). However, more chemical characterizations of the in vitro biosynthetic products are

needed to confirm this assumption. The discovery in this study of a third smaller porcine N-terminal peptide (PNT 1–61) shows that further maturation occurs.

Recently, a tryptic digest of murine N-terminal peptide (Pedersen & Brownie, 1980) and a bovine γ_3-MSH synthetic peptide (Pedersen et al., 1980) were shown to act in synergism with ACTH to activate the release of glucocorticoids. In other studies, the 80-residue porcine N-terminal peptide (Lis et al., 1981) and the 76-residue human N-terminal peptide (Seidah et al., 1981a) stimulated the release of aldosterone from a human adrenal adenoma in vitro. The biological function(s) of PNT 1–61 and PNT 1–107 remains (remain) to be studied.

Figure 6 presents a tentative model for the maturation of the N-terminal peptide of porcine POMC in the anterior lobe of the pituitary gland. The N-terminal portion of POMC is first released as a glycopeptide of 107 amino acid residues by enzymatic cleavage at the site of the putative paired basic residues preceding the ACTH sequence. The same enzymes subsequently or concomitantly remove the joining peptide and release a glycopeptide of 80 amino acid residues which was identified as the major form of porcine N-terminal peptide. This peptide can be further processed into a glycopeptide of 61 amino acid residues by enzymatic cleavage at the -Arg-Arg- sequence at positions 63 and 64. The glycine residue at position 62 is subsequently removed with concomitant amidation of the phenylalanine residue at position 61. Although the arginine residue is preferred on the carboxyl side of the paired basic residues for enzymatic cleavage, the presence of the sequence -Arg-Lys- at positions 49 and 50 suggests a possible further maturation into a smaller peptide. Purification and chemical characterization of such peptide has recently been reported in rat pars intermedia (Browne et al., 1981). However, the peptide isolated extends beyond the site of amidation (position 61) probably due to the replacement, in the rat POMC, of Arg-64 by a residue of proline (Drouin & Goodman, 1980) which may block the enzymatic cleavage.

Acknowledgments

We thank Jim Rochemont, Gilles De Serres, and Josée Hamelin for their excellent technical assistance. We thank D. Marcil for typing the manuscript.

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