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ENZYMATIC MATURATION OF PRO-OPIOMELANOCORTIN BY ANTERIOR PITUITARY GRANULES

METHODOLOGICAL APPROACH LEADING TO DEFINITE CHARACTERIZATION OF CLEAVAGE SITES BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MICROSEQUENCING

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SUMMARY

The coupling of high-performance liquid chromatography, gel-permeation, and reversed-phase chromatography with microsequencing proved to be advantageous for the unambiguous determination of specific sites in pro-opiomelanocortin, cleaved by secretory granule lysates of pig anterior pituitary. This system allows the unambiguous identification of a major chymotrypsin-like enzyme activity, optimal at pH 8.0, in the granule preparation, with a specificity directed towards some Phe ↓ X and Tyr ↓ X cleavage sites. The approach used emphasizes the necessity to use methodologies leading to the unambiguous determination of conversion activities.

INTRODUCTION

The polypeptide pro-opiomelanocortin (POMC), the pluripotent common precursor of adrenocorticotropin (ACTH), β-endorphin, α-melanotropin (MSH) and β-lipotropin (LPH), is synthesized in the pars distalis and pars intermedia of the pituitary and in the hypothalamus of all species studied[1–5]. Sequential proteolysis of POMC converts seemingly identical precursor molecules into a tissue-specific collection of maturation products of varied biological activities[2,5–9]. In the anterior lobe of the pituitary, POMC is converted into a N-terminal glycopeptide (NT)[1,5,9], a joining peptide (JP)[6], ACTH, β-LPH, and to a lesser extent into γ-LPH, β-endorphin and possibly β-MSH[1,2,8]. In contrast, the major maturation products in the pars intermedia require a possible further maturation of the NT[12], the production of α-MSH and corticotropin-like intermediary lobe peptide (CLIP) from ACTH and the formation of γ-LPH and a collection of β-endorphin-like peptides[1–3,7,8,10]. In all cases, the sites of cleavage are represented by a pair of basic residues mostly Lys-Arg or Arg-Arg[1,4,5,8,10]. This signal of a pair of basic residues required for proteolytic con-

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version was originally observed for the β-LPH to γ-LPH conversion[13], and is now known to be common to most pro-hormone conversion processes[8,14,15].

Recent evidence has been accumulated that the secretory granules are one of the major subcellular organelles responsible for cleavage at such a pair of basic residues. Accordingly, conversion of pro-insulin into an insulin-like material was achieved by incubation of lysates of pancreatic secretory granules with pro-insulin[16]. Similarly, pituitary secretory granules isolated from rat pars intermedia or rat and bovine pars nervosa were reported to convert toad POMC into a series of products[17,18] which resemble the expected *in vivo* maturation products, thus indicating the species generality of such a process. Furthermore, post-translational modifications such as acetylation[19] and amidation[20] were also reported to occur within the secretory granules.

However, the methods used for the identification of peptide conversion products most often relied solely on immunoprecipitation and electrophoretic separation as the only criteria for determining the specificity of the observed enzymatic cleavage. Other methods, based on electrophoretic separations followed by tryptic peptide mapping, have also been used in search of the conversion enzyme[22]. Although a relatively important body of information has now been reported on this subject, information pertaining to the exact nature of the conversion enzyme is still scarce. It was, therefore, imperative to develop a methodology which would permit an unambiguous identification of the exact site of *in vitro* cleavage in order to correlate the enzymatic activity in secretory granule lysates with the known *in vivo* pro-hormone conversion products.

This report represents a step in this direction, where a combination of purification by reversed-phase and molecular-sieving high-performance liquid chromatography (HPLC) is coupled with the microsequencing identification of conversion products. Here POMC was used as a model, since it is one of the best studied prohormone molecules and its fate in the pituitary has been well established over the past few years. Moreover, it is possible to prepare *de novo* biosynthetic material as a substrate from rat pars intermedia cells, as previously described[1,10,11]. The enzymatic source chosen was secretory granule lysates of porcine anterior pituitary. From the results obtained it becomes evident that a methodology involving the combination of HPLC and microsequencing is indeed useful for the clarification of some of the leading questions confronting biochemists nowadays.

MATERIALS

HPLC-grade 1-propanol (Burdick & Jackson) was used in all reversed-phase separations. Sequenator-grade heptafluorobutyric acid (HFBA) (Beckman) and trifluoroacetic acid (TFA) (Pierce) were used as ion-pairing reagents in reversed-phase chromatography. Guanidine–HCl obtained from Schwartz/Mann, redistilled triethylamine (Fisher) and phosphoric acid (BDH) were used for molecular-sieving HPLC as described[22]. All buffers were filtered through 0.22-μm filters before use on the HPLC columns.

Sephadex G-75, Percoll and density-marker beads were obtained from Pharmacia. All sequenator reagents and solvents were from Beckman. Aquasol II, [^{3}H]Leu and [^{3}H]Phe were obtained from New England Nuclear.

METHODS

Reversed-phase and gel permeation HPLC

The HPLC apparatus used consisted of a Waters Assoc. Model 204 liquid chromatograph, including Model U6K injector, two Model 6000A pumps, Model 730 data module and Model 720 system controller. An Altex 5-μm ODS (25 × 0.39 cm I.D.) analytical column was used for reversed-phase separations.

Two buffer systems were used. In the first, solvent A consisted of 0.13% (v/v) HFBA in water, and solvent B consisted of 0.13% (v/v) HFBA in 1-propanol. The column was initially equilibrated with 100% A. The sample was applied in this eluent and the column allowed to equilibrate for 10 min at a flow-rate of 1.5 ml/min. The flow-rate was then lowered to 0.5 ml/min and the proportion of B was changed to 25% in 0.1 min. The elution was carried out with a linear gradient of 25% B to 55%B in 150 min at 0.5 ml/min. Collection was made with a Pharmacia FRAC-100 fraction collector, 0.5 ml/tube being collected. Aliquots of 25 μl were counted in Aquasol II scintillation cocktail on a Beckman Model LS-8100 liquid scintillation counter for 5 min. Pooled fractions were stored at −20°C.

In the second buffer system, solvent A consisted of 0.1% (v/v) TFA in water, and solvent B contained 0.1% (v/v) TFA in 1-propanol. The 5-μm ODS column was equilibrated with 100% A. The sample collected from pooled fractions of the first chromatogram, after appropriate dilution in this eluent, was then applied the column. Elution was carried out by a linear gradient from 0% B to 70% B in 140 min at a flow-rate of 0.5 ml/min. Collection and counting were as described above.

Waters protein analysis columns (30 × 0.78 cm I.D.) were used for gel permeation chromatography. These consisted of one I-60, two I-125 and one I-250 columns connected in series in that order. The isocratic eluent buffer used for molecular sieving was 0.2 *M* triethylamine phosphate plus 6 *M* guanidine–HCl at pH 3.0 at a flow-rate of 1 ml/min as previously described[22].

To samples dissolved in this buffer were added thyroglobulin and 2,4-dinitrophenyl (DNP) glycine as internal markers for accurate void volume (V_0) and total volume (V_t) determinations. The sample volume injected never exceeded 500 μl. Collections of 0.5 ml/tube were made and counting was performed as described above.

Purification of [^{3}H]Leu labeled POMC-related peptides from the pars intermedia of rat pituitary

The partial purification of POMC-related peptides from the pars intermedia obtained after either 1-h or 4-h pulse labeling of rat neurointermediate lobes with [^{3}H]Leu was achieved as described[22]. The complete purification of these peptides was achieved using the HFBA–1-propanol system described above, and their identity was confirmed by microsequencing (results not shown).

Isolation and purification of secretory granules of pig anterior pituitary

From 100 freshly dissected pig anterior pituitary lobes (20 g wet weight), a homogenate was prepared in 60 ml of 0.25 *M* sucrose–10 m*M* N-(2-hydroxyethyl)-1-piperazine-N′-ethanesulfonic acid (HEPES), pH 7.4, using a PTFE Potter-Elvehjem tissue grinder (Wheaton) at 1000 rpm with six strokes. Subcellular division was effected by differential centrifugation at 2000 rpm (484 g_{av}) for 10 min using a

JS 7.5 rotor on a Beckman J21B centrifuge at 4°C. The pellet (P1) was washed twice, rehomogenized and recentrifuged under the same conditions, and the resulting supernatants (S1) were pooled to give a final ratio of 1 g/10 ml (*i.e.* 200 ml total). S1 was then centrifuged at 5800 rpm (4100 g_{av}) for 15 min, using the same rotor. The resultant pellet (P2) was then aliquoted and frozen. The supernatant (S2) was then centrifuged at 17,200 rpm (26,832 g_{av}) on a 50.2 Ti rotor on a Beckman L8-80 ultracentrifuge, giving a pellet (P3) and a supernate (S3). The supernatant was then centrifuged at 40,700 rpm (150,000 g_{av}) on the 50.2 rotor for 60 min giving a pellet (P4) and a final supernatant (S4) which were aliquoted and frozen. The P3 pellet was suspended in the homogenization buffer to give a final volume of 20 ml. This suspension was then applied on eight Beckman quick-seal tubes (89 × 25 mm I.D.) containing 36.5 ml of Percoll at an initial density of 1.075 g/ml. Ultracentrifugation was done for 45 min at 23,500 rpm (50,000 g_{av}) using a 50.2 Ti rotor. The tubes were then drained from the bottom, using an 18-gauge needle and collecting 0.5-ml fractions. The collected fractions were then frozen at −20°C. The major portion of POMC in the secretory granules was found by a β-endorphin radioimmunoassay to be in P3 and accordingly in the Percoll fraction 10, denoted as P3A10, as described elsewhere[23]. This fraction provided the enzymatic preparation used in this study.

pH dependence of rat [³H]Leu-POMC conversion by secretory granule lysates

Secretory granules obtained from the Percoll fraction 10 (P3A10) were osmotically lysed for 1 h at 37°C by incubation with stirring of 50 μl of P3A10 (1 mg protein) in 200 μl of various buffers. The buffers used were 0.1 *M* sodium formate for pH 3.0, 0.1 *M* sodium acetate at pH 4, 5, 6 and 7, 0.1 *M* ammonium hydrogen carbonate at pH 8, and 0.1 *M* ammonium carbonate at pH 9.

The lysed granule preparation was then added to an Eppendorf tube containing dried 20,000 cpm of [³H]Leu-POMC (purified by Sephadex G-75 and by HPLC as described above, and in ref. 22). Incubation was allowed to proceed with stirring for 5 h at 37°C, and stopped by addition of glacial acetic acid and guanidine–HCl to give a final concentration of 1 *M* acetic acid and 1.5 *M* guanidine–HCl. This was then centrifuged at 100,000 g_{av} for 1 h to remove the Percoll and membrane components, and the supernatant was applied to the protein analysis column for molecular-sieving separation.

Characterization of [³H]Leu- and [³H]Phe-POMC conversion by secretory granule lysates

In order to obtain sufficient counts for microsequence determinations of the products of the conversion of radiolabeled POMC by secretory granule lysates, two large-scale incubations were performed: 200 μl of P3A10 were osmotically lysed with 800 μl of 0.1 *M* ammonium hydrogen carbonate pH 8.0 for 1 h as described above. This preparation was then added to 1 · 10^6 cpm of either [³H]Leu- or [³H]Phe-POMC, purified by HPLC and previously dried on a Speed-Vac concentrator (Savant). The incubation was performed for 8 h at 37°C, with stirring, and the reaction was stopped as described above. The resulting supernatant was applied to a G-75-SF column (100 × 0.9 cm I.D.) eluted with 1 *M* acetic acid at 4°C, as described[22]. Aliquots of the resultant fractions were counted, the material under each peak was then repurified by reversed-phase HPLC using both eluting systems de-

scribed above. Microsequencing was then performed on each HPLC-purified peptide as described below under *Microsequence determinations*.

Microsequence determinations

Amino acid sequence analysis of radiolabeled peptides was performed on a Beckman 890C sequenator using a 0.3 *M* Quadrol program in the presence of 3 mg of polybrene and 2.5 mg of sperm whale apomyoglobin as carrier, as described previously[9]. The resulting thiazoline residues at each sequenator cycle were then counted in Aquasol II as described[11].

RESULTS

Determination of the pH optimum for POMC conversion by secretory granule lysates

Fig. 1 represents the gel permeation pattern of rat [^{3}H]Leu-POMC cleavage by the granule lysates of pig anterior pituitary at various pH values. The elution positions of the *in vivo* conversion products of POMC obtained from 4-h pulse incubations of rat neurointermediate lobes are also shown. These include POMC, the N-terminal segment of POMC attached to the ACTH sequence (NT-ACTH), the NT, β-lipotropin, γ-lipotropin, β-endorphin and α-MSH —all known (except for α-MSH) to be *in vivo* conversion products of pituitary pars distalis POMC[1,2,24]. Although α-MSH does not contain Leu in its thirteen residues[8,25], and is not expected to be produced by anterior pituitary granules[2,8,24], its position serves as a molecular-weight marker for small peptides.

Based on the observed elution pattern, by comparison with naturally occurring markers, it appears that the optimal specific conversion of POMC into molecules of sizes similar to the natural products occurs *ca.* pH 8. The quantitation of the specific conversion defined as converted POMC (cpm)/unconverted POMC is shown in Table I.

It can be seen that maximal apparent specific conversion occurs at pH 8.0, but that some conversion also occurs at pH 4–5, which appears to be less specific than

TABLE I

CALCULATED pH DEPENDENCE OF SPECIFIC CONVERSION OF POMC BY SECRETORY GRANULE LYSATES

Specific conversion is defined as cpm of converted POMC (sum of cpm from tubes 17–40)/cpm of unconverted POMC (sum of cpm from tubes 10–16) (see Fig. 1).

pH	*Converted/unconverted POMC*
3	0.2
4	1.94
5	1.78
6	1.34
7	3.08
8	3.60
9	0.48

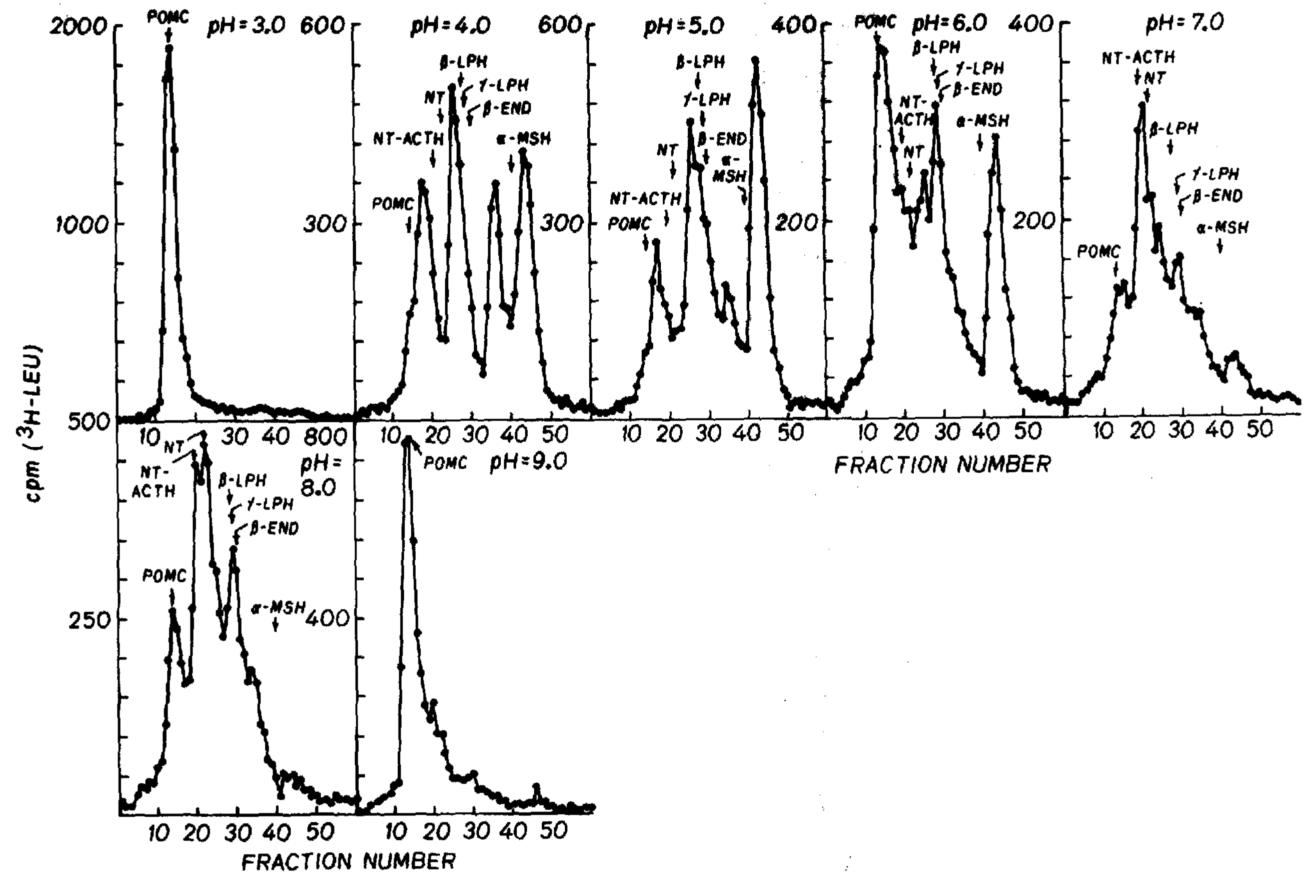

Fig. 1. pH dependence of rat [^{3}H]Leu-POMC cleavage by secretory granule lysates of pig anterior pituitary (fraction P3A10, see Methods). Gel-permeation HPLC was used with protein analysis columns eluted with 0.2 *M* triethylamine phosphate (pH 3.0) plus 6 *M* guanidine-HCl, at a flow-rate of 1 ml/min (see Methods). The elution positions of rat marker peptides such as POMC, N-terminal ACTH (NT-ACTH), NT, β-LPH, γ-LPH, β-endorphin (β-END) and α-MSH are shown for comparison.

at pH 8.0, based on elution together with marker peptides (see also Fig. 1). The radioactivity eluted in tubes 40–50, representing very-small-molecular-weight peptides, are not included in the calculation of the specific conversion, since no similar peptides occur *in vivo*[1–13,22].

Although it is seen from Fig. 1 and Table I that an important conversion does

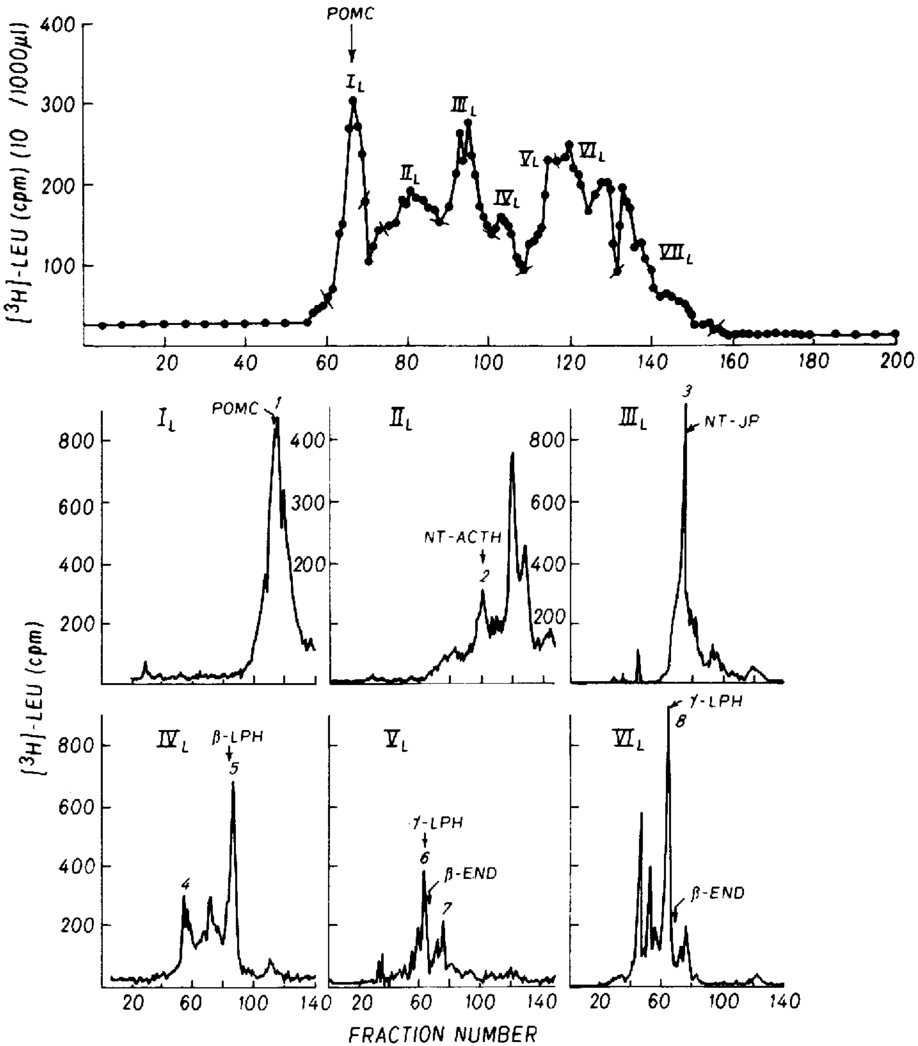

Fig. 2. Upper panel: Preparative Sephadex G-75 Superfine chromatography of cleavage products obtained following incubation of $1 \cdot 10^6$ cpm of [^{3}H]Leu-POMC with P3A10 at pH 8.0 (see Methods). The seven peaks resolved are denoted I_L to VII_L, where the subscript L denotes [^{3}H]Leu-labeled peptides. The fraction size was 1 ml and an aliquot of 10 µl was counted. Lower panels: The repurifications of peak I_L to VII_L by reversed-phase HPLC using the HFBA-1-propanol system (see Methods). The elution positions of rat marker peptides are shown for comparison. The material under peaks 1–8 was repurified using a reversed-phase HPLC TFA-1-propanol system (see Methods) before microsequence determination. The size of each fraction was 0.5 ml and an aliquot of 25 µl was counted.

occur at pH values between 4 and 5, this conversion appears to be less specific than that at pH 8, when one compares the elution position of the conversion products with that of natural markers. Moreover, at acidic pH values (see Fig. 1), a large fraction of the conversion products were eluted after α-MSH, *i.e.* around the salt position of the column, thereby reinforcing the argument presented above on the choice of pH 8 as optimal for apparent specific conversion.

Characterization of POMC conversion products at pH 8.0

The preparative purification by Sephadex G-75 of [^{3}H]Leu-POMC conversion products obtained at pH 8.0 is shown in Fig. 2 (upper panel). This allowed the separation of seven peaks (denoted I_L to VII_L). The material under each pooled peak was then repurified by reversed-phase HPLC in the HFBA–1-propanol system as shown in the lower panels of Fig. 2. In the same figure are also shown the reversed-phase HPLC elution positions of similar-sized rat POMC marker peptides. The material under each peak, which appeared to be eluted together with the rat marker, was subsequently repurified in the TFA–1-propanol system. In this system, apparent elution together with the rat markers was also observed, thereby providing the source of peptides used for microsequence determination.

Fig. 2 shows that incubation of rat POMC with secretory granule lysates from pig anterior pituitary results in conversion products eluting close to the position of

TABLE II

MICROSEQUENCING RESULTS OF [^{3}H]Leu- and [^{3}H]Phe-LABELED PEPTIDES 1–8

The identification of the peptides and the deduction of the cleavage sites are also shown, based on the nucleotide[25] and protein[11,24,26] sequences of rat POMC and related peptides. The numbers in parentheses represent the residue positions of the peptide along the proposed protein sequence[25]. No leucine appeared during the first twenty cycles in the sequence of peptides 4, 6 and 7. For peptides 4 and 6, the (52-) and (164-) mean that no carboxy-terminal identification could be deduced although these peptides start at residues 52 and 164 of POMC, respectively.

Peak	*Sequence positions*		*Peptide starts from POMC residue No.*	*Probable identification*	*Probable cleavage site*
	[^{3}H]Leu	*[^{3}H]Phe*			
1	3, 11, 17, 18	–	1	POMC (1–209)	–
2	3, 11, 17, 18	–	1	POMC (1–136)	136↓137 Phe-Lys
3	3, 11, 17, 18	–	1	POMC (1–99)	99↓100 Tyr-Ser
4	–	5, 10	52	POMC (52–)	51↓52 Tyr-Val
5	4, 12, 16	–	137	POMC (137–209)	136↓137 Phe-Lys
6	–	5	100 (or) 164	POMC (100–136) or (164–)	99↓100 Tyr-Ser or 163↓164 Tyr-Arg
7	–	14	169	POMC (169–209)	168↓169 Phe-Arg
8	4, 12, 16	–	137	POMC (137–168)	136↓137 Phe-Lys

in vivo POMC conversion products in the rat. These include the NT-ACTH, NT-JP, β-LPH, and γ-LPH marker peptide positions (see peaks 2, 3, 5, 6, and 8, respectively). However, no peak appeared to be eluted together with β-endorphin. Moreover, peak 5 is eluted slightly later than authentic rat β-LPH marker (see Fig. 2, panel IV_L).

An identical approach was used for the purification of [^{3}H]Phe-POMC conversion products, and gave similar results (data not shown). This provided [^{3}H]Phe-labeled conversion products for further confirmation of the microsequencing identification of peptides 4, 6 and 7 (see Table II).

Microsequence identification of POMC conversion products

Table II summarizes the [^{3}H]Leu and [^{3}H]Phe microsequencing data of the peptides under peaks 1 to 8 defined in Fig. 2. The [^{3}H]Leu amino acid sequences of peptides 1, 2 and 3 clearly showed that they contain the NH_2-terminal segment of POMC, since leucine appears at residues 3, 11, 17, 18[25,26].

These results, taken with the apparent molecular weights on Sephadex G-75 and the elution position on reversed-phase-HPLC in either HFBA–1-propanol or TFA–1-propanol of these peptides, allowed their possible identification as authentic unconverted POMC (residues 1–209), for peak 1; NT-ACTH for peak 2 (POMC 1–136); and NT-JP-like (1–99) for peak 3 (ref. 25).

Table II also shows that peptides 5 and 8 contain leucine at residues 4, 12 and 16. Based on the known leucine sequence of rat β-LPH, γ-LPH and POMC[11,25,26], it is clear that these two peptides contain the NH_2-terminal sequence of either β-LPH or γ-LPH plus two residues (since the natural form of these peptide sequences contains Leu at 2, 10 and 14). From the reported DNA sequence of rat POMC[25], and the elution positions on either G-75 or reversed-phase-HPLC, it can be inferred that peak 5 represents Lys-Arg-β-LPH (POMC 137–209). This implies a Phe ↓ Lys cleavage site. Using the above arguments, added to the fact that no β-endorphin-like material was observed, peak 8 was identified as a Lys-Arg-γ-LPH-related peptide. Furthermore, the finding of Phe at position 14 in peak 7 (see Table II) suggests that this peptide could start either from position 168 or from position 183 of POMC, both indicating a Phe ↓ X cleavage. However, the absence of leucine in the first twenty residues clearly points to position 169 in POMC as the most probable starting position for peptide 7. This is reinforced by the elution of this peptide in peak V_L of G-75, thus indicating a molecular weight slightly greater than that of β-endorphin. Thus it can be proposed that peak 8 represents a shorter Lys-Arg-γ-LPH-like peptide ending at residue 168 (POMC 137–168), while peak 7 represents an N-terminal extended β-endorphin-like peptide starting at residue 169 (POMC 169–209), implying a Phe ↓ Arg cleavage site (Table II).

With respect to the minor peptide 4, the absence of Leu within the first twenty residues and the presence of Phe at residues 5 and 10 (Table II) allow the identification of this peptide as a γ-MSH-like peptide produced via a Tyr ↓ Val cleavage, thereby starting at residue 52 of the POMC sequence. However, no exact carboxy-terminal length can be deduced from these data, and its presence in minor amounts (see Fig. 2) argues against it representing a major cleavage site.

Finally, in the case of another minor relatively short (Fig. 2) peptide 6, based on the Phe at residue 5, and the absence of Leu within the first twenty residues (Table

II), one cannot decide between two possibilities: the first could be a shorter ACTH--like peptide starting at residue 100 (POMC 100–136), and the second could be a β-MSH-like fragment starting at residue 164 (Table II). Both possibilities clearly indicate a Tyr ↓ Ser or a Tyr ↓ Arg cleavage.

DISCUSSION

Lately HPLC has enjoyed widespread use because of its speed and high resolving power. Nevertheless when one studies a wide array of closely related polypeptides, it becomes apparent that no single chromatographic separation method can achieve the total resolution of the peptide components sought. In such a situation, a combination of two or more separation modes involving different resolution parameters was found to be useful[27]. During the initial phase of this study, we were unable to separate all the known maturation products of rat POMC in one single chromatographic experiment by using either TFA or HFBA as organic modifiers combined with either 1-propanol, 2-propanol or acetonitrile as organic eluents. Since the various maturation products of POMC exhibit a wide array of molecular weights, it was therefore logical to combine molecular sieving with reversed-phase HPLC. Recently[22], we reported a gel permeation method by HPLC using 6 *M* guanidine–HCl as a denaturing agent. It is applicable to a molecular-weight separation range of 500–90,000 daltons. Such a procedure was also used to separate POMC maturation products and was combined with a radioimmunoassay for analysis of the POMC peptides in the anterior and intermediate pituitary of the rat[22].

In this paper, this method permitted the rapid determination of the pH optimum for the specific conversion of POMC by secretory granule lysates (Fig. 1). Although quite useful for analytical separations, such a method suffers from limitation of sample injection volume, which has to be kept to a minimum to obviate peak broadening and decreased resolution of the various peptides. In order to maximize the recovery of radiolabeled peptides, we repeatedly had to wash the membrane components of the lysates which tended to adsorb the conversion products. Furthermore, drying the supernatants by Speed-Vac or by lyophylization did not permit the complete recovery of the radiolabeled maturation products, which tend to "stick" to the walls of either glass or plastic tubes. Therefore, for preparing sufficient amounts of radiolabeled peptides for microsequence characterization, Sephadex G-75 chromatography was preferred.

The material under the seven peaks eluted from the Sephadex column was then fractionated by reversed-phase HPLC with two different modifiers, TFA and HFBA[27], which proved to be sufficient for complete purification of the conversion products. The use of 100% A in both systems as an initial eluent, removed the limitation on sample injection volume. In fact, for Sephadex G-75 chromatography, samples were actually pumped onto the column, thereby diminishing peptide losses by lyophylization and adsorption on the container walls. Therefore the combination of molecular sieving with these two types of reversed-phase HPLC allowed the complete purification in optimum yield of all POMC-related peptides, both from *in vivo* sources (pulse experiments) and from *in vitro* conversion by granule lysates. The proof of the resolving power of such a system was obtained from microsequencing, where all peptides sequenced exhibited minimal contamination and hence permitted their unambiguous N-terminal sequence identification.

In the pH study, using gel permeation by HPLC, it was observed that pH 8.0 was optimal for POMC conversion by secretory granule lysates into products similar in size to known maturation products (Fig. 1). Graf *et al.*[21] and Austen and Smyth[28] reported a similar pH optimum for the conversion of β-LPH into β-endorphin-like and γ-LPH-like peptides by granule lysates obtained from pig or bovine anterior pituitaries. However, other laboratories, using secretory granules from either pituitary intermediate and posterior lobes[17,18] or from pancreatic islets[16,29], reported that POMC- or pro-insulin-converting activity appears optimal at acidic pH between 4 and 6. Fig. 1 shows that, although conversion does occur at acidic pH values, this process does not seem to lead to cleavage products similar in size to endogenous markers, which, however, could be mistakenly recognized by specific antibodies as the correct maturation product.

From the sequence results obtained (Table II), it is evident that the major cleavage activity observed at pH 8.0 is directed towards either Phe ↓ X or Tyr ↓ X bonds. This suggests a chymotrypsin-like proteolytic acitivity.

Indeed a similar major chymotrypsin-like activity has been reported to be present in anterior pituitary lobes using luteinizing hormone-releasing hormone as a substrate[30]. However, the subcellular localization of the reported activity is lacking.

In conclusion, the methodology used in this work allowed the exact definition of major proteolysis sites of POMC by secretory granule lysates from anterior pituitary lobes. It also clearly pointed out the pitfalls and limitations of subcellular fractionation for obtaining sufficiently pure secretory granules. This is especially pronounced when a specific enzymatic activity is sought, which could well be masked by another major contaminating activity, *e.g.* the chymotrypsin-like enzyme reported here. Therefore, although molecular sieving and reversed-phase HPLC indicated similar conversion products as compared to endogenous material, miocrosequencing was the final proof of characterization of the exact enzymatic cleavage site. Finally, this approach used for the characterization of conversion products, coupled with a further purification of the granulate lysates, should lead to a more realistic procedure for the search of pro-hormone maturation enzyme(s).

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