Characterization of pro-opiocortin-converting activity from rat pituitary neurointermediate lobe
(prohormone processing/thiol protease/corticotropin/β-lipotropin/α-melanotropin)

Y. Peng Loh and Harold Gainer

Section on Functional Neurochemistry, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT Lysates of secretory granules from rat pituitary neurointermediate lobes were incubated with [3H]arginine- or [3H]phenylalanine-labeled toad pro-opiocortin. The processed products formed were identified by immunoprecipitation with adrenocorticotropin (ACTH) and endorphin antisera and by migration behavior on acid/urea/polyacrylamide gels. Pro-opiocortin was cleaved by the proteolytic activity in the secretory granule fraction to ≈21,000 Mr ACTH, ≈13,000 Mr ACTH, α-melanotropin, 16,000 Mr NH₂-terminal glycopeptide, β-lipotropin, and an endorphin-related peptide. Characterization of this pro-opiocortin-converting activity shows that it (i) is present in membrane and soluble fractions of the granule lysates, (ii) has a pH optimum of 5.0, (iii) appears to cleave at pairs of basic amino acid residues in the precursor, and (iv) is inhibited by leupeptin, pepstatin A, and p-chloromercuribenzoate but not diisopropyl fluorophosphate, N\(^{\text{N}}\)-p-tosyl-L-lysine chloromethyl ketone hydrochloride, chloroquine, or EDTA. These inhibitor studies suggest that the converting-enzyme activity is due to an acid thiol, arginyl protease, distinct from any known cathepsin B-like activity.

Recent studies (1–6) have shown that several forms of corticotropin (ACTH), α-melanotropin (α-MSH), β-lipotropin (β-LPH), and β-endorphin are derived from a common prohormone (i.e., the ACTH/endorphin precursor, Mr 32,000, or pro-opiocortin). The amino acid sequence of this prohormone contains pairs of basic amino acid residues interposed between the hormone sequences (7). Various investigators (7–9) have proposed that, as for other prohormones with such pairs of basic residues separating the processed products, the conversion of pro-opiocortin occurs via the sequential action of trypsin-like and carboxypeptidase B-like enzymes. Although there have been reports of such enzyme activities in pituitary tissues (10–12), none of these studies has utilized pro-opiocortin as the substrate to determine whether the correct products are formed from the prohormone itself.

Several studies have implicated the secretory granule as a major site of processing of pro-opiocortin (13–16). Therefore, the presence of pro-opiocortin-converting activity in these organelles has been specifically assessed in our study. Pro-opiocortin from the neurointermediate lobe of dark-adapted toads was used because it was relatively easy to isolate and purify labeled prohormone of high specific activity from this tissue. Labeled toad pro-opiocortin was incubated with extracts of purified secretory granules prepared from rat neurointermediate lobes. The labeled peptides that were generated were assayed by immunoprecipitation with specific antiserum to ACTH and β-endorphin, followed by acid/urea or NaDodSO₄ gel electrophoresis.

By using this procedure, a unique thiol, arginyl protease activity was detected in the purified secretory granule fraction. This enzyme activity cleaved pro-opiocortin to products similar to those synthesized by the intact toad neurointermediate lobe, was present in both membrane-bound and soluble forms, and had a pH optimum of 5.0.

MATERIALS AND METHODS

Animals. Female rats (Osborne-Mendel strain) weighing 200–250 g were obtained from that National Institutes of Health animal facilities (Bethesda, MD). Adult toads (Xenopus laevis) weighing 40–70 g were purchased from NASCO Biological Supplies (Fort Atkinson, WI) and were maintained at 22°C in a black plastic aquarium with constant light for 14–21 days prior to use.

Preparation of Labeled Toad Pro-opiocortin (ACTH/Endorphin Precursor). Neurointermediate lobes were dissected from the toads and preincubated at 22°C in amphibian saline (NaCl, 112 mM; KCl, 2 mM; CaCl₂, 2 mM; Hesper, 15 mM; glucose, 5 mg/ml; bovine serum albumin, 1 mg/ml; pH 7.4) for 1 hr (3, 15). The lobes were then pulse-incubated for 1.5 hr in the presence of 18.2 μM [3H]arginine or 19.5 μM [3H]phenylalanine [New England Nuclear; [3H]arginine specific activity, 14.4 Ci/mmol; [3H]phenylalanine specific activity, 13.7 Ci/mmol (1 Ci = 3.7 × 10¹² becquerels)]. After the pulse, the neurointermediate lobes were homogenized in 0.1 M HCl and were then precipitated with cold 10% trichloroacetic acid (Cl₃CCOOH) for 2 hr. The acid-precipitated pellets were washed with 5% Cl₃CCOOH and then air-dried. The dried pellets were washed twice with ether to remove the Cl₃CCOOH and then dissolved in 0.9 M acetic acid/10% glycerol.

Samples were electrophoresed on acid gels without urea (15, 17). Each gel run was stopped when the cytochrome c marker in a parallel gel had migrated 6 cm from the origin. The polyacrylamide gel was cut into 1.5-mm slices and each slice was eluted with 1 ml of 0.02 M HCl/bovine serum albumin (0.25 mg/ml) for 16–24 hr at 4°C. A 5-μl aliquot taken from the eluant of each gel slice was assayed in a liquid scintillation counter to determine the location of the labeled 32,000 Mr, pro-opiocortin on the gel. The eluants containing the pro-opiocortin were pooled and lyophilized. The labeled pro-opiocortin, extracted and purified in this manner, was evaluated by immunoprecipitation with ACTH antiserum. More than 80–85% of the radioactive in these extracts was found to be associated with pro-opiocortin.

Abbreviations: ACTH, corticotropin (adrenocorticotropic); β-LPH, β-lipotropin; α-MSH, α-melanotropin; PCMB, p-chloromercuribenzoate; iPF₂F-P, diisopropyl fluorophosphatase; PhMeSO₄F, phenylmethylsulfonyl fluoride; TLCK, N\(^{\text{N}}\)-p-tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, L-tosylamide-2-phenylethyl chloromethyl ketone; Con A, concanavalin A.

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Preparation of Rat Neurointermediate Lobe Secretory Granules. Fifty neurointermediate lobes were pooled and homogenized at 0°C (using a Potter–Elvehjem-type homogenizer with eight up-down strokes at 1000 rpm) in 2 ml of 0.25 M sucrose/10 mM Tris-HCl, pH 7.4. The homogenate was then made up to 5 ml with homogenizing buffer and was subjected to differential centrifugation (18). The homogenate was initially spun at 5000 × g (30 sec), yielding the P₁ pellet. The P₁ supernatant was spun at 4000 × g (15 min), yielding a P₂ (mitochondrial) fraction. The P₂ supernatant was then centrifuged at 26,000 × g (15 min), yielding a P₃ (crude granule) fraction. The P₄ (microsome) fraction was obtained by centrifugation at 100,000 × g. The supernatant of the P₄ fraction (S) was also collected for analysis.

Each of the fractions was resuspended in 0.25 M sucrose/10 mM Tris-HCl, pH 7.4, and aliquots were taken for protein (19), monoamine oxidase (20), β-glucuronidase (21), and immunoreactive α-MSH (22) determinations. The analyses indicated that 65% of the tissue’s immunoreactive α-MSH was localized in the P₄ fraction, although 13.5% of total monoamine oxidase (mitochondrial marker) and 37% of total β-glucuronidase (lysosomal marker) were also found in this fraction. The immunoreactive α-MSH content in the P₄ fraction indicated that it was a granule-enriched fraction, and it was taken for further purification by discontinuous sucrose density gradient centrifugation (18). The P₄ fraction, suspended in 1.4 ml of 0.25 M sucrose/10 mM Tris-HCl, pH 7.4, was applied on top of a discontinuous sucrose gradient (2 M sucrose, 2 ml; 1.4 M sucrose, 1 ml; 1.35 M sucrose, 0.5 ml; and 1.3 M sucrose, 0.5 ml) prepared the night before use and spun for 90 min at 100,000 × g. The tube was then pierced with a 19-gauge needle and 25-drop fractions were collected for analysis of monoamine oxidase, β-glucuronidase, α-MSH, β-endorphin, and ACTH. Endorphin was radioimmunoassayed by using a kit purchased from New England Nuclear, with β-endorphin antiserum RB100 substituted for the supplied antiserum. ACTH was radioimmunoassayed by using a kit from Amersham Searle. These analyses indicated that fraction 6 from the gradient contained highly purified secretory granules (see Fig. 1); this fraction was used throughout this study as the source of converting-enzyme activity.

Incubation of Toad Pro-opiocortin with Rat Neurointermediate Lobe Secretory Granules. The secretory granules (sucrose gradient, fraction 6; see Fig. 1) were osmotically lysed and adjusted to pH 5 with ammonium acetate buffer (final buffer concentration, 0.1 M). For the assay of pro-opiocortin converting-activity in the membrane and soluble fractions of the secretory granules, the above lysate was centrifuged at 100,000 × g for 60 min in a Beckman Airfuge (Beckman, Palo Alto, CA). The pelleted membranes were resuspended in the same volume of 0.1 M NH₄OAc buffer (pH 5.0) as was the soluble fraction. The labeled pro-opiocortin substrate was dissolved in 0.1 M ammonium acetate (pH 5) buffer so that 90 μl of the substrate solution contained ca. 5000–8000 cpm.

The incubation of secretory granule protein (20–25 μg) with pro-opiocortin (5000–8000 cpm) was carried out at 37°C in a total volume of 180 μl. For determination of the pH optimum of the enzyme activity, buffers used for lysing the granules and incubations were 10 mM Tris-HCl adjusted to pH 7.4 or 8, 0.1 M ammonium acetate adjusted to pH 5, 5.5, or 6, and 0.1 M sodium citrate adjusted to pH 4 or 4.5. The incubation was terminated by addition of HCl to a final concentration of 0.2 M. Aliquots of the incubated reaction mixture were analyzed by acid/urea gel electrophoresis (15, 17) or by immunoprecipitation with specific antiserum to ACTH and β-endorphin followed by electrophoresis.

Identification of the Processed Products. For immunological identification of the processed products, an aliquot of the acidified incubation solution was neutralized to pH 7.0 with NaOH. The aliquot was then incubated with excess antiserum to ACTH (NH₂-terminal-specific Kendall antiserum) or endorphin (COOH-terminal-specific RB100 antiserum) as described (3), except that leupentin (1 mM) also was included in the incubation mixture. Labeled peptide-antiserum complexes were precipitated with Staphylococcus aureus Cowan I cells (IgG, The Enzyme Center, Boston). The immunoprecipitates were then washed with 0.9 M acetic acid/10 M urea to release the bound peptides from the antibodies for analysis on acid/urea gels. An aliquot was also taken for NaDodSO₄ gel electrophoresis. The resultant supernatant—after sequential immunoprecipitation with ACTH and endorphin antisera—was passed through a concanavalin A (Con A) column (23). The Con A-bound peptides were analyzed by acid/urea and NaDodSO₄ gel electrophoresis (24) to identify the 16,000 M₆, glycopeptide product.

Inhibitor Studies. Proteolytic inhibitors, chloroquine, p-chloromercuribenzoate (PCMB), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), N₆-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), phenylmethylsulfonyl fluoride (PhMeSO₄F), and diisopropyl fluorophosphate (iPr₂F-P) were purchased from Sigma. Leupentin and pepstatin A were gifts from W. Troll and H. Umezawa (New York). Inhibitor concentrations are reported in the text.

RESULTS

The quality of the secretory granule preparations used in this study is illustrated in Fig. 1. Distributions of the hormones (α-MSH, β-endorphin, and ACTH) on the sucrose gradient (Fig. 1A) were taken to reflect the secretory granule content of each

![Fig. 1](image-url)
fraction. Fig. 1A shows a bimodal distribution with a maximum hormone content in fraction 6. In contrast, the mitochondrial (monoamine oxidase) and lysosomal (β-glucuronidase) marker enzymes were maximally concentrated in fraction 10 (Fig. 1B). Fraction 6 appeared to be the most purified secretory granule fraction because it contained 17% of the hormone (i.e., immunoreactive α-MSH) content, virtually no monoamine oxidase activity (mitochondrial marker), and <1% of the β-glucuronidase activity (lysosomal marker) of the original neurointermediate lobe tissue. Hence, this fraction was used as the source of secretory granule enzyme for all the subsequent studies.

Lyased secretory granules from fraction 6 were incubated with toad [3H]arginine-labeled pro-opiocortin (at pH 5, 37°C) for various times (1–18 hr). Incubations in the absence of secretory granule enzymes produced no alteration of the pro-opiocortin substrate, whereas, in the presence of lyased granules, the prohormone was cleaved to identifiable and appropriate peptide products (Fig. 2A).

The peptide products generated from toad pro-opiocortin by these in vitro cleavages are comparable to those produced in pulse-chase in situ studies with intact toad neurointermediate lobes (3, 15, 25). However, the ≈21,000 M₆, ACTH and one of the two forms of the ≈13,000 M₆, ACTH produced by the rat secretory granule lysates migrated somewhat faster (1–2 gel slices) on acid/urea gels than did the analogous peptides synthesized in intact toad lobes. This difference was not observed by using NaDodSO₄ analysis on gels (not illustrated) which is less sensitive to charge differences in proteins than analysis on acid/urea gels.

Fig. 2A Inset shows progressive conversion of the labeled pro-opiocortin to labeled peptide products with time of incubation with granule lysate. A plateau was reached by 5 hr. Especially notable here is that 96% of the [3H]arginine in the pro-opiocortin substrate can be accounted for by the peptide-cleavage products and unconverted substrate, even after 18 hr of incubation. Acid/urea gel patterns of 18-hr incubates are similar to those of Fig. 2A (unpublished data).

Fig. 2B illustrates the labeled peptides shown in Fig. 2A that are immunoprecipitated by ACTH and β-endorphin antibodies. The major immunoprecipitated, labeled peptides that can be identified in this manner are 21,000 M₆, ACTH, two forms of 13,000 M₆, ACTH [these run together as 13,000 M₆, ACTH on NaDodSO₄ gels (unpublished data)], and 11,000 M₆, β-LPH. Note that the acid/urea gel revealed two different anti-endorphin immunoprecipitable peaks [at slice 27 and slice 46 (β-ELP)]. As might be expected for the NH₂-terminal glyccopeptide of pro-opiocortin (5, 7), the Con A-bound 16,000 M₆ peak in Fig. 2A was not immunoprecipitated with either ACTH or β-endorphin antiserum. Given these identifiable peptide products and the known amino acid sequence of pro-opiocortin (7), it is likely that the converting activity in the granules is due to a tryptic-like enzyme that cleaves at lysine or arginine residues.

This is further suggested by results of the inhibitor studies (Table 1). Leupeptin, a known inhibitor of arginyl proteases (26), inhibited converting activity of the granule lysate. PCMB also inhibited the converting activity, indicating that the enzyme is a thiol protease. In addition, the activity was inhibited by pepstatin A, an inhibitor of acid proteases other than cathepsin B (26). Notable in Table 1 are the lack of effects of iPrF-FP (inhibitor of serine proteases) and of chloroquine, EDTA, and TLCK (inhibitors of cathepsin B; see refs. 27–29). TPCK and PhMeSO₄F were also without inhibitory effects. This inhibitor spectrum suggests that the granule-converting activity is due to a thiol, arginyl protease.

![Figure 2](image-url)

**Fig. 2.** Conversion of [3H]arginine-labeled pro-opiocortin, at pH 5.0, by lysed rat neurointermediate lobe secretory granules (fraction 6, Fig. 1). (A) Acid/urea gel profiles of labeled proteins and peptides following a 5-hr incubation (pH 5.0, 37°C) of the [3H]-labeled pro-opiocortin substrate in the absence (C) and presence (E) of secretory granule lysates. Note that conversion of the pro-opiocortin to peptide products occurred only in the presence of secretory granule lysate, and that the migration positions on the gel of the major labeled peptide peaks correspond to migration positions of peptides derived from pro-opiocortin (indicated by arrows). The unmarked arrow (on shoulder of 21,000 M₆, ACTH) indicates a Con A-bound glycopeptide (~16,000 M₆). (Inset) Time course of conversion of [3H]arginine-labeled pro-opiocortin (●) to peptide products (○) by lysed secretory granules at pH 5, 37°C. Conversion was complete by 6 hr. (B) Immunological identification of peptide products in A by immunoprecipitation with ACTH (●, without secretory granules; ○, with secretory granules) and endorphin (●, with secretory granules) antisera and electrophoresis on acid/urea gels.

Converting activity appeared to be present in both membrane and soluble fractions prepared from the granule lysates (Fig. 3). Preliminary evidence suggested that the granule membrane contained slightly more activity than did the soluble fraction (note more unconverted 32,000 M₆ pro-opiocortin in Fig.
Table 1. Effect of proteolytic inhibitors on pro-opiocortin conversion

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition, %*</th>
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</thead>
<tbody>
<tr>
<td>Leupeptin, 1 mM</td>
<td>80.3 ± 3.3 (3)</td>
</tr>
<tr>
<td>Chloroquine, 100 μM</td>
<td>4.0 ± 5.7 (5)</td>
</tr>
<tr>
<td>iPr₂F-P₂, 1 mM</td>
<td>2.3 ± 3.3 (3)</td>
</tr>
<tr>
<td>PCMB, 1 mM</td>
<td>100.6 ± 5.9 (5)</td>
</tr>
<tr>
<td>EDTA, 1 mM</td>
<td>9.5 ± 4.5 (2)</td>
</tr>
<tr>
<td>TLCK, 200 μM</td>
<td>3.0 ± 5.0 (2)</td>
</tr>
<tr>
<td>TPCK, 200 μM</td>
<td>2.0 ± 6.0 (2)</td>
</tr>
<tr>
<td>PhMeSO₂F, 1 mM</td>
<td>8.0 (1)</td>
</tr>
<tr>
<td>Pepstatin A, 1 mM</td>
<td>93.4 ± 5.4 (2)</td>
</tr>
</tbody>
</table>

* Values are means ± SEM, with n in parentheses.

3β than in 3α). The immunoprecipitable products produced by the two fractions were identical. Incubations with [³H]phenylalanine-labeled pro-opiocortin, followed by immunoprecipitation with β-endorphin antiserum, revealed the presence of β-LPH but no peak that ran with β-endorphin. Rather, an endorphin-related peptide (β-ELP, slice 46) was observed. This peptide was also present in the toad intermediate lobe after pulse-labeling with [³H]phenylalanine followed by a chase (25). Its amino acid sequence is yet to be elucidated. The membrane and soluble enzyme activities are both inhibited by leupeptin, PCMB, and pepstatin A.

Fig. 3 shows the effects of varying the pH on the pro-opiocortin-converting activity of the granule lysates. The pH optimum of the enzyme activity was around 5.0, with virtually no activity at pH values of 7.4 and above, suggesting that the converting enzyme is an acid protease.

**DISCUSSION**

Some useful criteria for evaluating whether an enzyme activity could be related to the intracellular converting enzyme for a particular prohormone are (i) does the specificity of cleavage of the prohormone substrate in *in vitro* experiments correspond to that seen in *in situ*, (ii) is the subcellular localization of the enzyme activity consistent with the converting site (organelle) in *in vivo*, and (iii) is the pH within an organelle consistent with the pH range of activity of the enzyme.

In this study we attempted to address these criteria with respect to the converting activity of pro-opiocortin. Our choice of secretory granules as the site (organelle) in which prohormone conversion occurs in *in situ* was based on evidence from several laboratories (8, 13–16, 30–32). In this regard, it is significant that the converting activity described here is associated with a purified secretory granule fraction from the rat neurointermediate lobe. Furthermore, the pH optimum (and range) of this converting activity is fully consistent with the internal pH of 5.5 found in secretory granules (33–35). At pH 5.5 the converting activities of our granule lysates are close to maximum (Fig. 4). Although we cannot discount the possibility that this enzyme may also be found in other intracellular organelles (e.g., microsomes, Golgi vesicles, etc.), if this is so, the internal pH of these organelles would have to be <7.4 for the enzyme to be operative. To date, lysosomes are the only other organelles known to be internally acidic. Incubation of labeled pro-opiocortin with extracts of fraction 10 (which contains the lysosomes) of the gradient (see Fig. 1) caused rapid and random proteolysis of the prohormone to unidentifiable products (unpublished data).
The specificity of conversion of pro-opiocortin exhibited by the granule lysates appeared to be limited to cleavages at basic amino acid residues, and no further breakdown of the peptide products was detected after 18 hr of incubation. This suggests that the activity was specific for the limited proteolysis of pro-opiocortin to 21,000 M. ACTH, 13,000 M. ACTH, B-LPH, α-MSH, and the 16,000 M. glycopeptide. The apparent inability of the converting-enzyme activity to produce β-endorphin from B-LPH requires further study; possibly we are dealing with enzyme activities within a subpopulation of granules which do not convert B-LPH to β-endorphin (25). Although the granule lysate was quite potent in the cleavage of pro-opiocortin, we found no activity of the lysate by using conventional peptide substrates (e.g., benzoylprolylphenylalanylarginine[14C]-lanilide) for the assay of pancreatic trypsin. Furthermore, synthetic α-MSH, which has one basic amino acid at position 8 and at position 11, was cleaved by trypsin but not by the granule-converting activity.

These findings suggest that the enzyme cleaves at pairs of basic amino acids (e.g., Lys-Arg) and not at single basic amino acid sites that are also found—but not cleaved—in the amino acid sequence of pro-opiocortin (7). The activity appears to be both soluble and membrane-bound. However, the physiological significance of this observation is unclear. Although the granule-converting activity has some overlapping characteristics with cathepsin B (inhibition by PCMB and leupeptin), it is unlike cathepsin B in that chloroquine (27), TLCK (28), and EDTA (29) did not inhibit its activity. In addition, pepstatin A, which inhibits acid proteases (cathepsin D, renin, pepsin [26]) other than cathepsin B, also inhibited the converting activity. This further distinguishes the converting activity from lysosomal cathepsin B. Lack of inhibition by iPrF-F and PhMeSO₂F indicates that the converting activity is not a serine protease or like pancreatic trypsin.

The recent characterization of prohormone converting activity in secretory granules prepared from pancreatic islets of angelfish (30, 31) is of particular interest because the authors reported the presence of an acid thiol-arginyl protease (in these secretory granules) that effectively converts prosomatostatin (30), proinsulin, and proglucagon (31) to correct peptide products. This enzyme activity is similar to the one we have found in rat neurointermediate lobe secretory granules, raising the question of whether the converting enzyme is prohormone-specific.

In our studies toad pro-opiocortin was converted by enzymes derived from rat tissues. Furthermore, purified secretory granules prepared from either rat intermediate or posterior pituitary alone could serve equally well to convert toad pro-opiocortin (unpublished data). Because posterior pituitary lobe granules normally convert pro-oxyphysin and pro-preopressin (32, 36), it is interesting that these converting enzymes can also effectively cleave toad pro-opiocortin. Further work will be necessary to determine whether all of these enzymes, despite their similar characteristics, are in fact distinct members of a related family of tryptic-like converting enzymes. It is possible that these enzymes may contain subtle specificity differences and may exhibit pH optima depending on the microenvironment (i.e., intracellular site) in which the particular enzyme functions within the cell.

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