Kinetic Studies on the Processing of Human \( \beta \)-Lipotropin by Bovine Pituitary Intermediate Lobe Pro-opiomelanocortin-converting Enzyme*

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The kinetics of the previously reported paired basic residue-specific pro-opiomelanocortin-converting enzyme from bovine pituitary intermediate lobe secretory vesicles (Loh, Y. P., Parish, D. C., and Tuteja, R. (1985) J. Biol. Chem. 260, 7194–7205) were studied using human \( ^{125}I \)-\( \beta \)-lipotropin as substrate. The enzyme, at a concentration of 20 ng/100 \( \mu \)l cleaved human \( \beta \)-lipotropin to yield \( \gamma \)-lipotropin, a \( \beta \)-melanotropin linked to \( \beta \)-endorphin intermediate and \( \beta \)-endorphin, whereas at an enzyme concentration of 40 ng/100 \( \mu \)l, the substrate was completely cleaved to yield \( \beta \)-endorphin and \( \beta \)-melanotropin. These products were identified by their immunological properties and size on sodium dodecyl sulfate-polyacrylamide gels. The \( ^{125}I \)-\( \beta \)-endorphin product was further shown by high pressure liquid chromatography to contain two forms; the major form co-eluted with \( ^{125}I \)-Arg\(^2\)–\( \beta \)-endorphin and the minor form with \( ^{125}I \)-\( \beta \)-endorphin\(^{-1} \). No COOH-terminal shortened forms of \( \beta \)-endorphin\(^{-1} \) were detected. The products formed indicate cleavages at two of the three pairs of basic residues of human \( \beta \)-lipotropin, at Lys\(^{57}\)–Lys\(^{58}\) and Lys\(^{57}\)–Arg\(^{58} \), but not at Lys\(^{57}\)–Lys\(^{57} \). The cleavage at Lys\(^{57}\)–Arg\(^{58} \) occurred primarily in these basic residues. The \( K_m \) values for the cleavage of the Lys\(^{57}\)–Lys\(^{58} \) and Lys\(^{57}\)–Arg\(^{58} \) pairs were 1.9 and 2.5 \( \mu \)M, respectively. The \( V_{max} \) values for the cleavage of the Lys\(^{57}\)–Lys\(^{58} \) and Lys\(^{57}\)–Arg\(^{58} \) pairs were 4.8 nmol/\( \mu \)g of enzyme/h and 9.1 nmol/\( \mu \)g of enzyme/h, respectively.

Peptide hormones are synthesized from larger precursors via a number of sequential enzymatic post-translational processing steps which appear to occur within secretory vesicles (2–9). In general, the first processing step involves cleavages at paired basic residues, which flank the biologically active peptides within the precursors (8–15). This step is then followed by the removal of the NH\(_2\)- and COOH-terminal extended basic residues from the cleaved peptides. Processing of a prohormone, therefore, requires both endopeptidases, for example, those that are specific for the paired basic residues and exopeptidases (carboxypeptidase B-like and aminopeptidase B-like enzymes) for the removal of NH\(_2\)- and COOH-terminal basic residues.

The quest for such prohormone processing enzymes has been a focus in many laboratories. Several endopeptidase activities which show specificity for paired basic residues in prohormones and peptides (16–26) and exopeptidases which are specific for NH\(_2\)-terminal (27) or COOH-terminal basic residues (29–32) of peptides have been described. Of these, a carboxypeptidase B-like enzyme has been purified to homogeneity from secretory vesicles of pituitary and adrenal medulla and characterized (28). More recently, we have purified an endopeptidase, named pro-opiomelanocortin-converting enzyme, from bovine pituitary intermediate lobe secretory vesicles, which fulfilled several criteria for a physiologically relevant enzyme (1). This enzyme has an acidic pH optimum, is an M\(_r\) 70,000 glycoprotein, and cleaves specifically at the paired basic residues of the prohormone pro-opiomelanocortin (POMC) to yield ACTH, \( \beta \)-endorphin, and a 16-kDa NH\(_2\)-terminal glycopeptide. This enzyme has not been characterized with respect to its kinetics due to the lack of a rapid quantitative assay for enzyme activity when POMC was used as the substrate.

In this study, the kinetics of this purified pro-opiomelanocortin-converting enzyme were examined using a simpler substrate, \( ^{125}I \)-\( \beta \)-lipotropin, i.e. the COOH-terminal part of POMC (12). Human \( \beta \)-LPH is very similar to bovine \( \beta \)-LPH in structure (12, 33), both having the same three pairs of basic residues (Fig. 1), and is unlike mouse (34) or rat (35) \( \beta \)-LPH, which are missing one pair of basic residues, Lys\(^{57}\)–Lys\(^{57} \). This substrate provided the opportunity to assess if the enzyme, which appears to cleave Lys–Arg and Arg–Arg pairs in POMC and pro-insulin, respectively, is also able to cleave at Lys–Lys paired basic residues in human \( \beta \)-LPH. The inhibitor profile of pro-opiomelanocortin-converted enzyme activity using \( ^{125}I \)-\( \beta \)-lipotropin as substrate was also examined.

EXPERIMENTAL PROCEDURES

Materials—Bovine pituitaries were obtained from a local slaughterhouse and transported to the laboratory on ice. Human \( \beta \)-lipotropin was obtained from the National Pituitary Agency (Baltimore, MD). Human \( ^{125}I \)-\( \beta \)-lipotropin (specific activity, 187 \( \mu \)Ci/mg) was obtained from Du Pont-New England Nuclear. Data from Pronase digestion of the \( ^{125}I \)-\( \beta \)-LPH followed by amino acid analysis showed that \( \approx \)70% of the \( ^{125}I \) label exists as monoiodinated tyrosines. \( \approx \)9% of the label was on histidine residues and \( \approx \)20% of the tyrosines were di-iodinated (Du Pont-New England Nuclear, technical information). Analysis of tryptic peptides derived from \( ^{125}I \)-\( \beta \)-LPH showed the average distribution of \( ^{125}I \) label on the three tyrosines (see Fig. 1) to be: 33.6% on Tyr\(^{34} \), 28.7% on Tyr\(^{38} \), and 37.6% on Tyr\(^{39} \). Carboxypeptidase B (diisopropyl fluorophosphate treated) was purchased from Boehringer Mannheim. Pepstatin A and leupeptin were gifts from Drs. W. Troll and H. Uneszews (United States-Japan Cancer Program, New York University Medical Center, NY). All other chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

1. The abbreviations used are: POMC, pro-opiomelanocortin; \( \beta \)LPH, human \( \beta \)-lipotropin; \( \beta \)-END, \( \beta \)-endorphin; \( \beta \)-MSH, human \( \beta \)-melanotropin; ACTH, adrenocorticotropic; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.

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including protease inhibitors, were purchased from Sigma. Staphylo-
coccus aureus Cowan I cells were purchased from Boehringer Mann-
heim. HPLC reagents were from Waters. Polycarboxamide gel reagents
and the C1 reversed-phase column (RP 304) were from Bio-Rad.
Antisera against β-MSH was a gift from Drs. G. Chrousos and L.
Loriaux (National Institute of Child Health and Human Develop-
ment). Two antisera against β-endorphin were used, Melinda and
DP3. Melinda, a NH2-terminal specific antisera was a gift from
Drs. R. E. Mains and B. A. Epper (Johns Hopkins University,
Baltimore, MD). DP3, a COOH-terminal-specific antisera was gen-
generated by immunizing rabbits with synthetic human β-endorphin
coupled to bovine serum albumin, thyroglobulin, or hemocyanin. Both
β-endorphin antisera recognized β-lipotropin and β-endorphin and
gave identical results in this study. β-MSH, β-endorphin, β-
endorphin-11, and Argβ-endorphin were purchased from Peninsula
Laboratories. These peptides were iodinated, purified by HPLC, and
used as markers for gels and columns.

**Purification of Pro-opiomelanocortin-converting Enzyme**—Bovine
intermediate lobe secretory vesicle pro-opiomelanocortin-converting
enzyme was purified to apparent homogeneity exactly as described
previously (1). This purified enzyme was used for all the studies
described below.

**Analysis of Products Cleaved from Human β-LPH by Pro-opome-
lanocortin-converting Enzyme**—[125I-β-LPH (6,000–12,000 cpm)
was incubated with purified pro-opiomelanocortin-converting enzyme
(20–40 ng) and 10 μg of bovine serum albumin in a final volume of
100 μl of 0.1 M sodium citrate buffer (pH 4.0) at 37 °C for 30 min. To
identify the products formed, the incubate was quantitatively immu-
oprecipitated with excess β-MSH or β-endorphin antisera, and
the antigen-antibody complex was precipitated with S. aureus Cowan
I cells as previously described (1). The immunoprecipitates were then
analyzed on the basis of molecular weight by SDS-gel electrophoresis
and after treatment with carboxypeptidase B (10 pg/ml) and 5 pl of
bovine intermediate lobe secretory vesicle membrane extract (8.7
mg/ml), both immunoprecipitated products were further analyzed by HPLC before
and after treatment with carboxypeptidase B (10 μg/ml) containing aminopeptidase B-like activity (27) for 1 h in
0.1 M Tris-HCl buffer, pH 7.4. HPLC was carried out using a C4 reversed-phase column on a Beckman model 332 HPLC. The eluants
were isotonic 30% acetonitrile in 0.1% trifluoroacetic acid or 28% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min.
0.5-ml fractions were collected and the 125I radioactivity counted
on a γ counter (Micromedic Systems).

**Determination of Kinetic Constants**—Pro-opiomelanocortin-con-
verting enzyme (20 or 40 ng) was incubated with different concentra-
tions of unlabeled β-LPH and 15,000 cpm of [125I-β-LPH in a volume
of 100 μl for 15 min at 37 °C. 45 μl of the incubate was then
immunoprecipitated with β-endorphin antisera and another 45 μl with
β-MSH antisera. The immunoprecipitates were counted for
radioactivity and analyzed by SDS-polyacrylamide gel electrophoresis
to determine the β-MSH, β-endorphin, γ-LPH, and β-MSH-β-END
products formed. The pmol of each product formed at different
substrate concentrations was calculated from the cpm (after correct-
ing for the immunoprecipitated product and the specific activity of the product. Kinetic constants were calculated from Line-
weaver-Burk plots by conventional procedures.

**Assay of Pro-opiomelanocortin-converting Enzyme Activity with
γ-LPH**—Pro-opiomelanocortin-converting enzyme (20 ng) was incu-
bated with [125I-β-LPH (7,000–30,000 cpm) and 10 μg of bovine serum
albumin in 0.1 M sodium citrate buffer (pH 4.0) in a final volume of
100 μl, for varying times (0–4 h) at 37 °C. At the end of the incubation
period, 100 μl of 2 × concentrated sample buffer (1% SDS, 10% glycerol, 0.36 M bromphenol blue, and 0.5 M Tris/Cl, pH 6.8) was
added to the incubate. The sample was then applied to a 15% polyacrylamide tube gel for electrophoresis. The gel was sliced into
1.5-mm slices. Each slice was counted for radioactivity in a γ counter. The cpm in cleavage products formed were determined from the gel
profile (see “Results”).

**Inhibitor Studies**—The inhibitors were added in concentrations
indicated in Table II. 16 ng of pro-opiomelanocortin-converting en-
zyme were incubated with ~7,000 cpm of [125I-β-LPH, the inhibitor
(except diazaoxetaxyl-norleucine methyl ester), and 10 μg of bovine
serum albumin in 100 μl of 0.1 M sodium citrate buffer, pH 4.0, for
30 min at 37 °C. The products formed were determined by SDS-
polyacrylamide gel electrophoresis as described for the time study.
Reaction of the pro-opiomelanocortin-converting enzyme with dia-
azaoyetyl DL-norleucine methyl ester, an active site affinity label for
aspartyl proteases (37, 38) was carried out using a procedure modified
from Ref. 38 as follows. 30 ng of enzyme was incubated with 5 μl of
0.01 M cupric acetate and 10 μl of 1.5 mm diazaoyetyl-norleucine
methyl ester (rapidly mixed with cupric acetate before addition to the
incubate) and 5 μl of 0.01 M sodium acetate buffer, pH 5.5, in a total
volume of 15 μl for 30 min at 14 °C. An aliquot (μl) of the reacted
enzyme mixture was then assayed for activity as described for other
inhibitors. For the control, a similar enzyme incubation was carried
out in the absence of the diazo compound and assayed for activity.

## RESULTS

**Processing of β-LPH on Pro-opiomelanocortin-converting Enzyme**—The cleavage of human [125I-β-LPH by pro-opiomel-
anocortin-converting enzyme was assayed at two different enzyme concentrations. Fig. 2A shows that in the absence of
pro-opiomelanocortin-converting enzyme, the substrate 125I-
β-LPH was not processed. In the presence of pro-opiomelan-
ocortin-converting enzyme (20 ng/100 μl), the substrate, 125I-
β-LPH, was cleaved to yield three iodinated products (Fig.
2B). One product was identified as β-endorphin, based on its
coelectrophoresis with synthetic 125I-β-endorphin marker on
SDS gels and by immunoprecipitation with β-endorphin anti-
serum (Fig. 2B and Table I, A). The other two iodinated products co-run with each other and have an apparent mass of ~7 kDa (Fig.
2B). They were identified by sequential immunoprecipitation with β-MSH and β-endorphin antisera. β-MSH antisemum immunoprecipitated essentially all the radioactivity in the ~7-kDa peak (1701 cpm), whereas β-endor-
phin antisemum immunoprecipitated 1280 cpm of radioactivity in the ~7-kDa peak (Fig. 2B and Table I, A). Furthermore, immunoprecipitation with β-endorphin antisemum followed by a second immunoprecipitation with β-MSH anti-
serum yielded a ~7 kDa product containing 481 cpm (Table
I, A, and gel profile not shown). These results suggest that all
the molecules in the ~7-kDa peak contain the β-MSH sequence.

The stoichiometry of the three products is analyzed in Table
I, A. The amount of product formed was normalized against
the number of tyrosines in the molecule. This normalization
is valid since there is approximately equal 125I labeling of the
three tyrosines (see “Experimental Procedures”). The γ-LPH
product contains 481 cpm and one tyrosine (Fig. 1), and hence
the normalized cpm of γ-LPH formed is 481 cpm. The cpm

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**Kinetic Studies on Pro-opiomelanocortin-converting Enzyme**

**Fig. 1. Diagrammatic representation of human β-lipotropin
(β-LPH) showing the paired basic residues. The asterisks indi-
cate the position of the tyrosine residues (residues 43, 59, and 85) in
β-LPH; the first Lys-Lys pair consists of residues 37 and 38, and the
second pair, residues 86 and 87 of β-LPH. The Lys-Arg pair consists of residues 57 and 58.**
in the β-MSH-β-endorphin intermediate is 1280 cpm, and there are three tyrosines in this molecule. The normalized amount of this product formed is, therefore, 427 cpm (Table I, A). The β-endorphin product contained 843 cpm and 2 tyrosines, and hence the normalized product is 421 cpm. From the quantitation of the products after normalizing for the number of tyrosines, there appears to be approximately equal radioactivity in γ-LPH, β-endorphin, and β-MSH-β-endorphin, indicating equal numbers of cleavages by the enzyme at one of the paired basic residues, Lys17-Lys28 or Lys27-Arg28 (see Fig. 1). Since the NH2-terminal peptide liberated by a Lys27-Lys28 cleavage (see Fig. 1) contains no tyrosine, it could not be detected in this assay.

Fig. 2C shows the products formed from β-LPH using a higher enzyme concentration (40 ng/100 μl). At this enzyme concentration, β-LPH was completely cleaved to yield two products. One product was immunoprecipitated with β-endorphin antiserum and co-migrated with synthetic 125I-β-endorphin. Another product was immunoprecipitated with β-MSH antiserum and co-migrated with synthetic 125I-β-MSH. Analysis of the cpm in the anti-β-endorphin and anti-β-MSH immunoprecipitated peaks shows a ratio of 2:1, respectively (Fig. 2C, Table I, B). After normalizing the cpm of the product for the number of tyrosines present in each of the molecules, there appear to be approximately equal amounts of β-endorphin and β-MSH formed (Table I, B). This is consistent with the enzyme having cleaved the substrate completely at both the Lys27-Lys28 and Lys27-Arg28 pairs of basic residues (see Fig. 1).

Further analysis of the β-endorphin product was carried out by HPLC to determine if the Lys17-Lys28 residues of [125I]β-LPH were cleaved to yield β-endorphin. Fig. 3 shows that the 125I-β-endorphin product cleaved by pro-opiomelanocortin-converting enzyme has the same retention time as human 125I-β-endorphin31. No radioactivity co-eluted with human 125I-β-endorphin31. Since it was previously shown (1) that pro-opiomelanocortin-converting enzyme can cleave between the basic residues, it was possible that NH2- or COOH-terminal extended forms of 125I-β-endorphin31, if formed, may have co-eluted with human 125I-β-endorphin31. As no markers are available for extended forms of β-endorphin31, further analysis of the product was carried out by treatment of the β-endorphin product with pancreatic carboxypeptidase B and an endogenous aminopeptidase B-like enzyme (27) to remove any NH2- or COOH-terminal basic residue extensions. Fig. 3 shows that treatment with these exopeptidases did not change the mobility of the β-endorphin product, indicating that Lys18-Lys28 was not cleaved to yield a truncated form.

The HPLC conditions used in Fig. 3 did not significantly resolve between 125I-Arg0-β-endorphin31 and [125I]-Arg1-β-endorphin31, and, therefore, 28% acetoniトリ isoelic conditions were used to distinguish between these two products. Fig. 4 shows that under these conditions, the β-endorphin product formed by pro-opiomelanocortin-converting enzyme co-eluted primarily with the [125I]-Arg0-β-endorphin31 marker and only a small amount of the product co-eluted with [125I]-Arg1-β-endorphin31. The ratio of Arg1-β-endorphin31 to β-endorphin31 formed was 90:10, averaged from two experiments. These results are consistent with our previous studies (1) showing that pro-opiomelanocortin-converting enzyme preferentially cleaved between Lys45-Arg19 of [3H]POMC to yield NH2-terminal Arg-extended β-endorphin.

Determination of the Kinetic Constants of Pro-opiomelanocortin-converting Enzyme—Fig. 5 (upper panel) shows the velocity of formation of β-endorphin (Fig. 2B) when pro-opiomelanocortin-converting enzyme was incubated with different concentrations of β-LPH. Fig. 5 (lower panel) shows the Lineweaver-Burk plot of the reciprocal of the velocity against the reciprocal of the substrate concentration. The \( K_m \) for the cleavage of the Lys27-Lys28 bond to form β-endorphin was determined from this plot to be 2.5 μM, and the \( V_{max} \), 9.1 nmol/μg of enzyme/h.

Fig. 6 (upper panel) shows the velocity of formation of the
TABLE I
Stoichiometric analysis of the β-endorphin- and β-MSH-related products

<table>
<thead>
<tr>
<th>Product peak*</th>
<th>Total cpm*</th>
<th>1st immuno-</th>
<th>2nd immuno-</th>
<th>3rd immuno-</th>
<th>Theoretical</th>
<th>No. of tyrosines</th>
<th>β-END cpm/wo. of tyrosines</th>
<th>β-MSH cpm/wo. of tyrosines</th>
</tr>
</thead>
<tbody>
<tr>
<td>28-β-END</td>
<td>1701</td>
<td>481</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>427</td>
</tr>
<tr>
<td>28-β-MSH</td>
<td>1673</td>
<td>0</td>
<td>1671</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1671</td>
<td>1673</td>
</tr>
<tr>
<td>28-β-END</td>
<td>1671</td>
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<td>28-β-MSH</td>
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<td>1</td>
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<td>1</td>
<td>1671</td>
<td>1673</td>
</tr>
</tbody>
</table>

* Product peaks for Section A as shown in Fig. 2B and for Section B as shown in Fig. 2C.
* Total cpm in each product peak was determined by running an equal aliquot of the incubate used in the immunoprecipitation studies on a separate gel. Equal aliquots were taken for immunoprecipitation with either β-MSH or β-endorphin antisera.
to form the $\beta$-MSH-$\beta$-endorphin intermediate was determined from this plot to be 1.9 $\mu$M and the $V_{\text{max}}$, 4.8 nmol/µg of enzyme/h.

**Time Course of Processing of $\beta$-$\text{LPH}$—**Analysis of gel profiles after different times of incubation of $^{125}$I-$\beta$-$\text{LPH}$ with enzyme shows that formation of the "intermediate" and "product" peaks occurred with the same time course (Fig. 7). Cleavage products migrating as the "intermediate" and "product" peaks are $\gamma$-$\text{LPH} + \beta$-MSH-$\beta$-END and $\beta$-END, respectively, as identified in Fig. 2. Processing of the $\beta$-$\text{LPH}$ substrate was linear for 30 min and then leveled off by 30 min were not further processed to yield the final products, $\beta$-MSH and $\beta$-endorphin, with extended time.

**Effect of Protease Inhibitors on the Processing of $\beta$-$\text{LPH}$—**Various protease inhibitors were incubated with pro-opiomelanocortin converting enzyme and their effect on $\beta$-$\text{LPH}$ processing is shown in Table II. Pepstatin A (10$^{-8}$ M) and diazocetylornleucine methyl ester (2 × 10$^{-4}$ M), both aspartyl protease inhibitors, inhibited the processing of $\beta$-$\text{LPH}$, whereas the thiol (p-chloromercuribenzoate and dithiodipiridine at 10$^{-5}$ M) and serine (phenylmethanesulfonyl fluoride at 10$^{-5}$ M) protease inhibitors had no effect. Leupeptin at 10$^{-5}$ M concentration showed a partial inhibition of the enzyme activity.

**DISCUSSION**

In this study, the bovine intermediate lobe secretory vesicle pro-opiomelanocortin-converting enzyme (1) was further characterized with respect to its kinetics and inhibitor profile, using human $\beta$-lipotropin, the COOH-terminal part of POMC, as substrate. Results presented in Table II show that the inhibitor profile using $^{125}$I-$\beta$-$\text{LPH}$ as substrate was very similar to that reported previously using POMC as substrate (1). The serine protease inhibitor phenylmethanesulfonyl fluoride (10$^{-5}$ M) did not inhibit the activity, indicating that the enzyme is not a serine protease. Thiol protease inhibitors, p-chloromercuribenzoate and dithiodipiridine at concentrations of 10$^{-4}$ M, and previously tested at 10$^{-5}$ M (1), showed no inhibition. Since these agents typically inhibit thiol proteases in the micromolar range and pro-opiomelanocortin-converting enzyme was not inhibited in that range, it is unlikely to be a thiol protease. The most effective inhibitor is pepstatin A, an aspartyl protease inhibitor, which completely inhibited the activity of the enzyme at 10$^{-4}$ M concentration. In addition, diazocetylornleucine methyl ester, which inactivates aspartyl proteases (37, 38) by affinity labeling one of the aspartic acid residues at the active site (37, 38), inhibited the pro-opiomelanocortin-converting activity. Inhibition by these two reagents is consistent with the classification of pro-opiomelanocortin-converting enzyme as an aspartyl protease.

Fig. 2 shows that processing of $\beta$-$\text{LPH}$ is dependent on enzyme concentration. At an enzyme concentration of 20 ng/100 µl, equal amounts of $\gamma$-$\text{LPH}$, $\beta$-MSH-$\beta$-endorphin inter-
mediate, and β-endorphin were formed, indicating cleavages at only one of the paired basic residues (Lys^7^-Lys^10 or Lys^7^-Arg^10), with equal probability of cleavage of each pair (Figs. 1, 2B, and Table I, A). At a concentration of 40 ng/100 μl, both Lys^7^-Lys^10 and Lys^7^-Arg^10 were cleaved to yield the final products, β-MSH and β-endorphin (Fig. 2C, Table I, B).

It is tempting to hypothesize from these observations that by regulating the enzyme to substrate concentration within a given secretory vesicle, the extent of processing of a number of paired basic residues within a multivalent prohormone such as POMC can be regulated. By such a regulatory mechanism, the same pro-opiomelanocortin-converting enzyme can process POMC differentially, for example, to yield β-LPH or β-endorphin or both (47, 48), in vivo. While paired basic residues are a primary requirement for the pro-opiomelanocortin-converting enzyme, other secondary factors, perhaps the conformation of the substrate, may also play a role. This is borne out by the finding that the second Lys^5^-Lys^7^ pair within the β-LPH molecule was not cleaved to yield COOH-terminal truncated forms of β-endorphin. This pair of basic residues may be conformationally less accessible to the enzyme and, therefore, show more resistance to cleavage. Indeed, the studies of Geisow and Smyth (41) showing an intrinsic resistance of the COOH-terminal sequence of β-endorphin (1) to cleavage by carboxypeptidase A also suggest that this region of the molecule is highly stable to proteolysis. Another endopeptidase may be necessary to cleave this pair of basic residues.

It is interesting that at an enzyme concentration of 20 ng/100 μl, the intermediates γ-LPH and β-MSH-β-endorphin, once formed, did not get further processed with time (up to 4 h) to yield β-MSH and β-endorphin (Fig. 7). However, given sufficient enzyme (40 ng/100 μl) both the Lys^7^-Lys^10 and Lys^7^-Arg^10 pairs appear to be simultaneously cleaved. These two pairs of basic residues are probably highly accessible for cleavage within the conformation of the intact β-LPH structure. However, when only one of these pairs within the β-LPH is cleaved, as occur at low enzyme concentrations, the intermediates formed may assume a conformation that is unfavorable for further pro-opiomelanocortin-converting enzyme binding, and hence no further processing occurs. Good conformational matching between pro-opiomelanocortin-converting enzyme and its substrate may be very important for cleavage to occur and is perhaps best achieved with larger substrates such as the pro-hormone (POMC) itself, or the large intermediates β-LPH and 21–23-kDa ACTH (1).

In conclusion, pro-opiomelanocortin-converting enzyme has been further characterized with respect to its specificity, kinetic constants, inhibitor profile, and time and enzyme concentration dependence for activity, using β-LPH as substrate. The study revealed several important features about the enzyme. First, the enzyme has been identified as an aspartyl protease. Second, the enzyme shows a preference for larger substrates such as POMC (1) and β-LPH than for smaller substrates such as γ-LPH. Third, the K_m values for the cleavage of the Lys^7^-Lys^10 and Lys^7^-Arg^10 pairs were very similar, 1.9 and 2.5 μM, respectively. The number of paired basic residues within β-LPH cleaved was dependent on the enzyme concentration. Finally, pro-opiomelanocortin-converting enzyme appears to exhibit little discrimination toward the specific basic amino acid (Lys, Arg) forming the paired basic residues to be cleaved. This latter property raises the possibility that pro-opiomelanocortin-converting enzyme may be a converting enzyme for other prohormones in various endocrine tissues and peptidergic neurons.

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REFERENCES

Kinetic Studies on Pro-opiomelanocortin-converting Enzyme