"Prohormone Thiol Protease" (PTP) Processing of Recombinant Proenkephalin†

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ABSTRACT: The "prohormone thiol protease" (PTP) from adrenal medullary chromaffin granules has been demonstrated as a novel cysteine protease that converts the model enkephalin precursor, ([^{35}S]Met)-preproenkephalin, to appropriate enkephalin related peptide products [Krieger, T. J., & Hook, V. Y. H. (1991) *J. Biol. Chem. 266*, 8376−8383; Kreiger, T. J., Mende-Mueller, L., & Hook, V. Y. H. (1992) *J. Neurochem. 59*, 26-31; Azaryan, A. V., & Hook, V. Y. H. (1994) *FEBS Lett. 341*, 197−202]. In this report, PTP processing of authentic proenkephalin (PE) was examined with respect to production of appropriate intermediate products, and kinetics of PE processing were assessed. Recombinant PE was obtained by high level expression in *Escherichia coli*, with the pET3c expression vector; PE was then purified from *E. coli* by DEAE-Sepharose chromatography, preparative gel electrophoresis, and reverse-phase HPLC. Authentic purified PE was confirmed by amino acid composition analyses and peptide microsequencing. In time course studies, PTP converted PE (12 μM) to intermediates of 22.5, 21.7, 12.5, and 11.0 kDa that represented NH_2-terminal fragments of PE, as assessed by peptide microsequencing. Differences in molecular masses of the 22.5, 21.7, 12.5, and 11.0 kDa products reflect PTP processing of PE within the COOH-terminal region of PE, which resembles PE processing *in vivo* [Liston, D. L., Patey, G., Rossier, J., Verbanck, P., & Vanderhaeghen, J. (1983) *Science 225*, 734−737; Udenfriend, S., & Kilpatrick, D. L. (1983) *Arch. Biochem. Biophys. 221*, 309−314]. Products of 12.5, 11.0, and 8.5 kDa were generated by PTP cleavage between Lys-Arg at the COOH-terminus of (Met)enkephalin-Arg^6-Gly^7-Leu^8. The 8.5 kDa product may represent peptide I, which is present in adrenal medulla [Udenfriend, S., & Kilpatrick, D. L. (1983) *Arch. Biochem. Biophys. 221*, 309−314]; the 12.5 and 11.0 kDa fragments most likely contain peptide I and peptide E. Kinetic studies indicated that PTP has a $K_{m(app)}$ value of 18.6 μM PE and $V_{max(app)}$ of 1.98 mmol/(h·mg). These kinetic constants are consistent with estimated intragranular levels of PE and PE-derived products. These results demonstrating PTP conversion of PE to intermediates resembling those *in vivo*, and kinetics that are compatible with *in vivo* processing of PE, implicate a role for PTP in PE processing.

Peptide hormones and neurotransmitters are synthesized as inactive protein precursors that require proteolytic processing at paired basic residues to generate the smaller active neuropeptides (Docherty & Steiner, 1982; Gainer et al., 1985; Hook et al., 1994). Chromaffin granules of bovine adrenal medulla have been used as a model neurosecretory vesicle system for characterization of prohormone processing enzymes (Carmichael & Winkler, 1985; Hook et al., 1994). These chromaffin granules contain high levels of enkephalin precursor and peptides (Udenfriend & Kilpatrick, 1983; Birch & Christie, 1986; Spruce et al., 1988; Hook et al., 1990), indicating that these vesicles contain the corresponding proenkephalin processing enzymes needed to generate active opioid peptides.

In our studies of proenkephalin processing in chromaffin granules, the "prohormone thiol protease" (PTP)[1] was found as an important processing activity for the enkephalin precursor (Krieger & Hook, 1991; Krieger et al., 1992; Hook et al., 1994). PTP cleaves paired basic and monobasic sites within the enkephalin-containing peptide intermediates BAM-22P, peptide E, and peptide F to generate the final peptide product (Met)enkephalin (Krieger et al., 1992). Assessment with fluorogenic peptide-MCA (MCA = methylcoumarinamide) substrates indicates PTP cleavage of paired basic residues at the NH_2-terminal side of the pair and between the two basic residues; PTP also cleaves monobasic sites with preference for cleavage at the NH_2-terminal side of single arginine residues (Azaryan & Hook, 1994a). Importantly, PTP is distinguished from cysteine cathepsins B, L, and H on the basis of PTP binding to concanavalin A (cathepsin B does not bind to concanavalin A) (Krieger & Hook, 1991), selectivity for peptide-MCA substrates, cleavage specificity, potency of active-site directed protease inhibitors, and differences in characteristic proteolytic activity (Azaryan & Hook, 1994b). It is hypothesized that PTP

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[1] Abbreviations: PE, proenkephalin; PTP, prohormone thiol protease; IPTG, isopropyl β-D-thiogalactopyranoside; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid.

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represents a novel cysteine protease involved in prohormone processing.

Chromaffin granules also contain the prohormone convertases PC1/3 and PC2 enzymes (Christie et al., 1991; Kirchmair et al., 1991; Azaryan & Hook, 1992) that belong to a family of subtilisin-like processing enzymes related to the yeast Kex2 gene product that processes the yeast pro-α-mating factor [reviewed in Seidah et al. (1991), Steiner et al. (1992), Smeekens (1993), and Hook et al. (1994)]. Comparison of PTP with PC1/3 and PC2 in chromaffin granules demonstrated that PTP represents approximately 60%, and PC1/3 and PC2 together represented 20%, of total enkephalin precursor cleaving activity in chromaffin granules (Azaryan & Hook, 1995). These results indicate that the cysteine and subtilisin classes of processing enzymes—PTP, PC1/3, and PC2—can account for the majority of enkephalin precursor cleaving activity in chromaffin granules.

In this study, evaluation of the role of PTP as a PE processing enzyme was conducted by assessing conversion of authentic PE to appropriate PE-derived peptide products and by assessing the kinetics of PE processing. Expression of high levels of authentic full-length PE in *Escherichia coli* was achieved to develop an *in vitro* prohormone processing assay with purified PE near *in vivo* levels. PTP converted recombinant PE to appropriate peptide products and showed kinetic constants consistent with *in vivo* levels of precursor and enkephalin products. These results provide evidence supporting a role for PTP in proenkephalin processing.

EXPERIMENTAL PROCEDURES

Proenkephalin Expression Construct. Expression of recombinant PE utilized the T7 expression system developed by Studier et al. (1990). In this system, the rat proenkephalin cDNA (Yoshikawa et al., 1984) was subcloned into the pET3c expression vector and expressed in BL21(DE3) cells. For subcloning, the preproenkephalin cDNA in the pSP65 vector (Promega) was linearized with *Sma*I, ligated with *Bam*HI linkers with T4 DNA ligase, and digested with *Bam*HI. *Hpa*II digestion then generated a partial PE *Hpa*II/*Bam*HI fragment that was ligated to a 51-base pair synthetic fragment corresponding to the 5′-sequence of proenkephalin, produced by annealing two complementary oligonucleotides (kinased by T4 polynucleotide kinase) to generate a *Nde*I/*Hpa*II DNA fragment. Annealed oligonucleotides were 5′-TATGGACTGCAGCCAGGACTGCGCTAAAT-GCAGCTACCGCCTGGTACGTGG-3′ and 5′-CGGGAC-GTACCAGGCGGTAGCTGCATTTAGCG-CAGTCCTGGCTGCAGTCCA-3′. Ligation of the 51-base pair *Nde*I/*Hpa*II fragment, the 0.72 kb *Hpa*II/*Bam*HI partial PE fragment, and *Nde*I/*Bam*HI digested pET3c vector generated the PE/pET3c expression plasmid construct (Figure 2). After transformation into DH5α *E. coli* cells, minipreps [according to Sambrook et al. (1989)] with *Nde*I and *Bam*HI digestions confirmed colonies with the PE/pET3c plasmid. Appropriate insertion of the PE DNA fragment into the *Nde*I site was confirmed by DNA sequencing by the dideoxy chain terminating method (Sanger et al., 1977; Ausbel et al., 1989).

Expression of Recombinant PE in E. coli. The PE/pET3c vector was transformed into BL21(DE3) cells for PE expression. These cells contain the bacteriophage DE3 which is a λ derivative that carries the lacUV5 promoter and the gene for T7 RNA polymerase (Studier et al., 1990). Induction of the lacUV5 promoter by IPTG (isopropyl β-D-galactopyranoside) results in induction of T7 RNA polymerase and expression of PE under the control of the T7 promoter. Expression is conducted in M9ZB media with 100 μg/mL ampicillin as described by Studier et al. (1990).

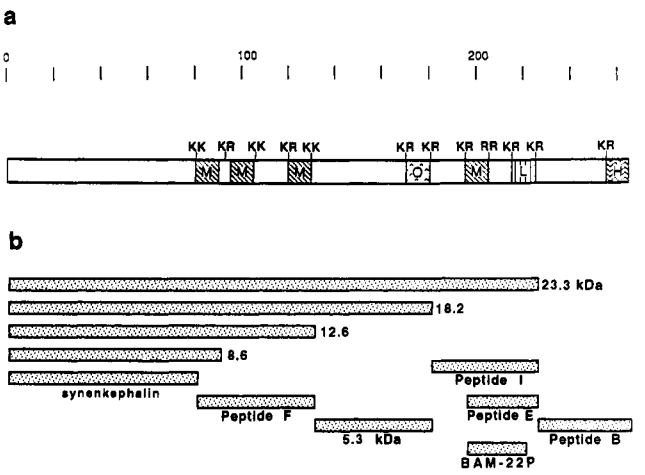

FIGURE 1: Preproenkephalin structure. (a) Proenkephalin is schematically illustrated showing the active peptides (Met)enkephalin (M), (Met)enkephalin-Arg^6-Gly^7-Leu^8 (0), (Leu)enkephalin (L), and (Met)enkephalin-Arg^6-Phe^7 (H). (b) PE products in bovine adrenal medulla. Illustration of many of the PE products identified in bovine adrenal medulla (Fleminger et al., 1983; Birch & Christie, 1986; Spruce et al., 1988; Hook et al., 1990).

To test PE expression in small scale cultures, aliquots from 5 mL overnight cultures were diluted to an optical density at 600 nm (A_{600}) of 0.075−0.10 and incubated at 37 °C until the A_{600} reached 0.15−0.20. Induction of expression with IPTG (0.5 mM) was conducted at 37 °C for 1−2 h. Cells were then centrifuged at 15000*g* for 20 min at 4 °C, and cell pellets were resuspended in 100 μL of sample buffer (5 mM Tris-glycine, 1% β-mercaptoethanol, 10% glycerol, and 2% SDS) per 1 mL cell aliquot of 0.4 optical density at 600 nm for SDS−PAGE gel electrophoresis as described previously (Krieger & Hook, 1991). Expression of PE was detected by Coomassie staining and anti-PE immunoblots utilizing the PE-18 monoclonal antibody (1:1000 dilution) directed against the mid-region of PE (Spruce et al., 1988; B. Spruce, personal communication). Immunoblots were performed with the Bio-Rad immunoblot kit, with alkaline phosphatase-conjugated anti-rabbit IgG, as described previously (Hook et al., 1993).

For large scale production of PE, expression was conducted in 5 L cultures (10 × 0.5 L). After inoculation of 500 mL of M9ZB media to A_{600} of 0.1, cells were incubated for 1 h at 37 °C, incubated with rifampicin at 100 μg/mL for 2 h, and then induced with IPTG at 0.2 mM for 30 min. Cells were harvested by centrifugation at 2600*g* at 4 °C for 20 min, and cell pellets were stored at −70 °C.

PE expressed in *E. coli* was analyzed by two-dimensional gel electrophoresis performed by isoelectric focusing using ampholytes at pH 3.5 to pH 10.0 (Birad), followed by SDS−PAGE (12% polyacrylamide) gel electrophoresis and immunoblotting with PE-18 monoclonal antibody. Isoelectric focusing was performed according to the Hoefer gel apparatus protocol, and SDS−PAGE gels were performed as described previously (Krieger & Hook, 1991).

Purification of Recombinant PE. Cells from 5 L of IPTG-induced *E. coli* cell cultures were resuspended in 300 mL of 50 mM Tris-HCl, pH 7.5 (buffer A), lysed by sonication (Ultrasonics model W185F sonicator at 50% power), and

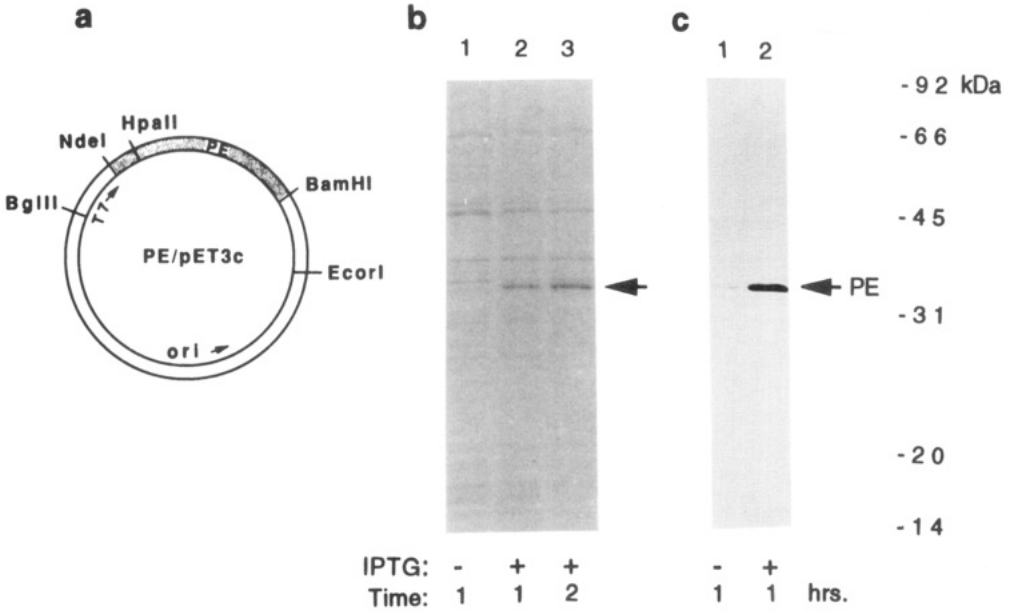

FIGURE 2: Expression of PE in *E. coli*. (a) pET3c/PE expression construct. PE was subcloned into *Nde*I and *Bam*HI sites of the pET3c vector by ligation of a *Hpa*II/*Bam*HI fragment with annealed complementary oligonucleotides corresponding to the *Nde*I/*Hpa*II fragment as described under Experimental Procedures. (b) Induction of PE as detected by Coomassie Blue staining of SDS−PAGE gels. Induction by IPTG of PE expression in BL21(DE3) cells is evident at 1 and 2 h incubation. The arrow indicates induction of a 35 kDa PE band. (c) Anti-PE immunoblots of PE expressed in *E. coli*. Immunoblots with the PE-18 monoclonal antibody (gift from Dr. Barbara Spruce) indicate IPTG induction of PE (indicated by arrow).

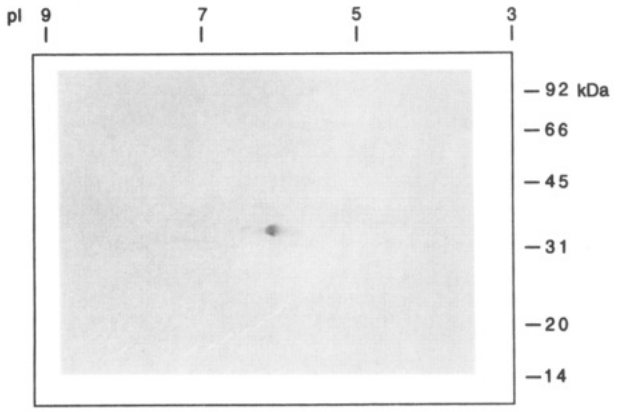

FIGURE 3: Two-dimensional gel electrophoresis of recombinant PE. PE was expressed by IPTG induction (1 h) in 10 mL of BL21-(DE3) *E. coli* culture. Cell pellets were then resuspended in 100 μL of sample buffer (for SDS−PAGE gels, as described under Experimental Procedures) per 1 mL of *E. coli* cells of 0.4 optical densitry at 600 nm. Twenty to 25 μl of this sample of PE from *E. coli* was analyzed on a 2-D gel by isoelectric focusing with ampholytes from pH 3.5 to 10 in the first dimension and 12% polyacrylamide SDS−PAGE gel electrophoresis in the second dimension. After electrophoretic transfer from the SDS−PAGE gel to nitrocellulose, PE was detected by anti-PE immunoblot, as described under Experimental Procedures.

centrifuged at 27000*g* at 4 °C for 30 min. Intact PE was found mostly in the particulate fraction of the cells, representing 70−80% of cellular PE. PE in the soluble fraction (20−30%) was partially degraded. The pellet was washed twice in 35 mL of buffer A and centrifuged at 27000*g* at 4 °C for 30 min. The pellet was then resuspended in 20 mL of 50 mM Tris-HCl, pH 7.5, 6.0 M urea (buffer B), rocked at 4 °C for 1 h, and centrifuged for 30 min at 27000*g* at 4 °C, and the resultant supernatant contained solubilized PE.

Soluble PE was applied to a DEAE-Sepharose column (1.5 × 35 cm) equilibrated in buffer B at a flow rate of 18 mL/h. After washing with buffer B, PE was eluted with a 0−500 mM NaCl gradient in buffer B. PE in column fractions (3.5 mL) was screened by anti-PE immunoblots (PE-18 antibody), and relative protein levels were monitored by absorbance at 280 nm. PE positive fractions were pooled.

Preparative SDS−PAGE gel electrophoresis of PE from the DEAE-Sepharose step utilized the Bio-Rad model 491 preparative electrophoresis cell (37 mm cell) for SDS−PAGE (Laemmli, 1970), performed at 40 mA constant current with elution at a flow rate of 1 mL/min. Fractions (5.0 mL) were analyzed by anti-PE immunoblots. Fractions containing PE were pooled, dialyzed against 0.25 mM Tris-HCl, pH 7.5, and lyophilized.

For reverse-phase HPLC (high-pressure liquid chromatography), lyophilized PE was resuspended in 500 μL of 0.1% TFA (trifluoroacetic acid) and chromatographed on a C8 column (4.6 × 250 mm, Vydac) with precolumn (4.6 × 20 mm, NEST group, MA.), using a Waters HPLC system (model 510 pumps and 680 controller). PE was eluted with an acetonitrile gradient of 10−80% buffer B (buffer A = 0.1% TFA; buffer B = 80% acetonitrile in 0.1% TFA) at a flow rate of 1.0 mL/min with detection of peptides at 215 nm with a Kratos SF-769-Z monochrometer. PE positive fractions (determined by anti-PE immunoblots) were pooled, lyophilized to remove TFA, brought to 5 mM citrate-NaOH, pH 6.0 (10 mL), and lyophilized to less than 0.5 mL. Protein content of purified PE was determined by the method of Lowry et al. (1951). Purity of PE was confirmed by peptide microsequencing and by amino acid composition analyses, performed as described previously (Krieger & Hook 1991; Krieger et al., 1992).

Purification of Prohormone Thiol Protease (PTP). PTP was purified from chromaffin granules of bovine adrenal medulla, as previously described (Krieger & Hook, 1991). Briefly, the soluble component of the chromaffin granules was chromatographed on concanavalin A-Sepharose, followed by Sephacryl S200 gel filtration, chromatofocusing, and thiopropyl-Sepharose 6B.

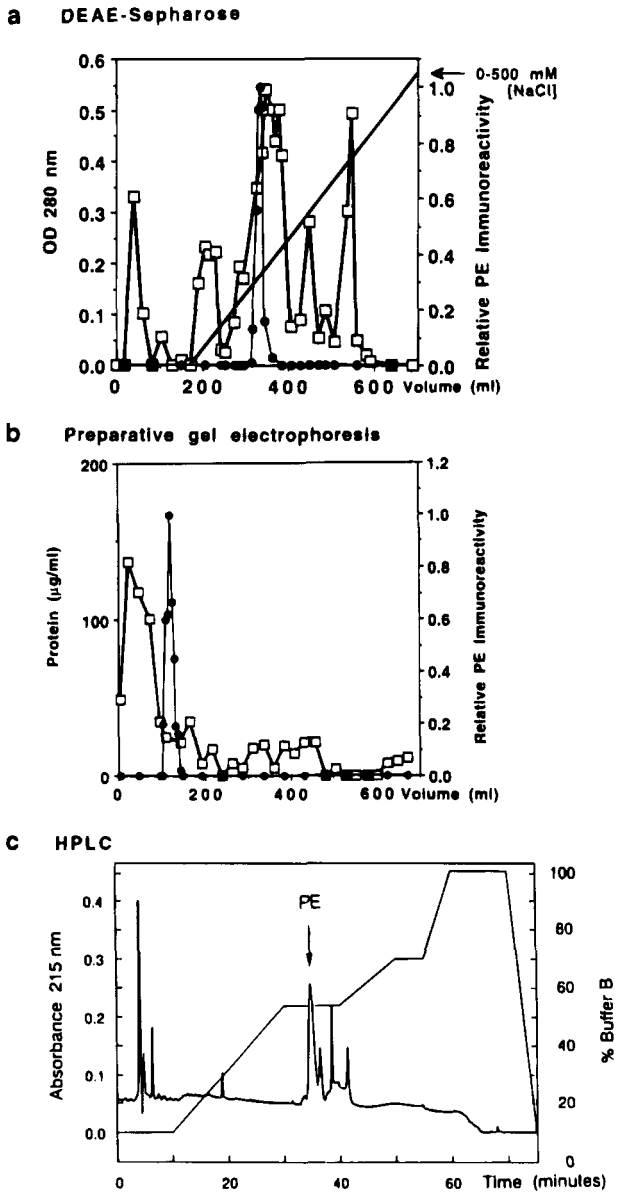

FIGURE 4: Purification of PE from *E. coli*. Intact PE solubilized from *E. coli* was subjected to anion exchange chromatography on DEAE-Sepharose (a), preparative gel electrophoresis (b), and reverse-phase HPLC (c) as described under Experimental Procedures. In panels a and b, PE immunoreactivity (●) was monitored by anti-PE immunoblots with quantitation by densitometry, and relative protein levels were assessed by absorbance at 280 nm (□). In panel c, the arrow indicates the HPLC peak that was positive for PE.

PTP Cleavage of PE and Product Analysis. PTP was incubated with purified recombinant PE in a time-course study. PE (8 µg, equivalent to 12 µM) was incubated with PTP (1 ng) at 37 °C for 0−24 h in PTP reaction buffer consisting of 100 mM citrate-NaOH, pH 5.0, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 10 mM CHAPS in a total volume of 20 µL. A portion of each reaction (17 µL) was subjected to SDS−PAGE gel electrophoresis (12% polyacrylamide) and electrophoretically transferred to PVDF membranes, and amido black stained bands were subjected to peptide microsequencing, as described previously (Krieger & Hook, 1991; Krieger et al., 1992). The remaining part of each reaction (3 µL) was subjected to anti-PE immunoblots using the monoclonal PE-18 antibody (1:1000 dilution) that recognizes an epitope corresponding to the mid-region of PE (residues 130−207 of preproenkephalin).

PTP Kinetic Studies. K_m and V_{max} (apparent) constants for PTP and PE were determined by incubating PTP (0.5 ng) with 1.2−20.0 µM PE in PTP reaction buffer for 10 min at 37 °C. All reactions were in the linear range of the assay (less than 30% PE cleaved). Reactions were subjected to SDS−PAGE gel electrophoresis (12% polyacrylamide), electrophoretic transfer to PVDF membranes, and staining with amido black to indicate PE and product bands. PE in reaction mixtures and standard amounts of PE were quantitated by densitometric scanning of amido black stained bands on nitrocellulose membranes, using the Adobe Photoshop 2.5.1 and NIH-Image 1.5.4 software. Kinetic constants were determined by Lineweaver−Burk plots (Dixon & Webb, 1979); kinetic constants were assessed in three experiments.

RESULTS

Recombinant Proenkephalin (PE). Proenkephalin contains four copies of (Met)enkephalin (M), one copy of (Leu)-enkephalin, and the (Met)enkephalin-related peptides M-Arg^6-Gly^7-Leu^8 and M-Arg^6-Phe^7 (Figure 1) (Yoshikawa et a., 1984). PE in bovine adrenal medulla is converted to multiple high molecular mass intermediates of 8−24 kDa that possess the NH_2-terminus of PE (Liston et al., 1983; Spruce et al., 1988; Hook et al., 1990) and to several smaller enkephalin-containing peptides including peptide I and peptide E related PE products (Udenfriend & Kilpatrick, 1983).

To assess PE products generated by the prohormone thiol protease (PTP), recombinant PE was expressed in *E. coli* with the T7 expression system (Studier et al., 1990). Analysis of PE expression in bacterial lysates showed that after 1−2 h induction with IPTG (isopropyl β-D-galactopyranoside), a new 35 kDa band was detected by Coomassie Blue protein staining (Figure 2). Immunoblots with monoclonal PE-18 antibody, recognizing the mid-region of PE (residues 130−207 of preproenkephalin) (Spruce et al., 1988; B. Spruce, personal communication), indicated expression of recombinant PE (Figure 2). The single 35 kDa PE band agrees with detection of 35 kDa PE *in vivo* (Spruce et al., 1988). Analysis of PE by two-dimensional gel electrophoresis showed a single spot of immunoreactive PE (Figure 3), indicating expression of homogeneous PE.

Purification of PE solubilized from *E. coli* was achieved by DEAE-Sepharose chromatography, preparative gel electrophoresis, and reverse-phase HPLC (Figure 4). Intact PE from sonicated cells was associated primarily with the particulate fraction, whereas PE in the soluble supernatant was somewhat degraded. Therefore, urea solubilization of *E. coli* cells was utilized to obtain intact PE. Solubilized PE was then subjected to DEAE-Sepharose anion exchange chromatography (Figure 4a), with elution of PE by a 0−500 mM NaCl gradient providing full recovery of PE. Preparative gel electrophoresis (Figure 4b) enhanced the purity of PE by 6-fold. On reverse-phase HPLC, PE eluted at 45−50% buffer B (80% acetonitrile in 0.1% TFA) (Figure 4c). Purified PE was achieved after the HPLC step, as shown by a single 35 kDa band on SDS−PAGE (Figure 5) that was recognized by the PE-18 monoclonal antibody. This purification yielded approximately 1 mg of PE (Table 1).

Amino acid analyses (Table 2) indicated that the amino acid composition of PE paralleled its predicted composition based on the known primary sequence of PE (Yoshikawa et al., 1984). Peptide microsequencing also confirmed the

Table 1: Proenkephalin Purification[a]

sample	total PE (mg)	total protein (mg)	concentration of PE (mg PE/mg protein)	fold purification	percent yield
lysed cells	16	553	0.03		100%
urea soluble fraction	4.0	133	0.03	1.0	25
DEAE-Sepharose	3.8	40	0.10	3.3	23
preparative SDS−PAGE	1.0	2.3	0.43	14	6
HPLC	0.9	0.9	1.0	33	5.6

[a] PE was purified from 5 L of IPTG-induced *E. coli*, which corresponds to approximately 17 grams of cells (wet weight). PE (μg) in each sample was quantitated by densitometry of each sample with purified PE as standard.

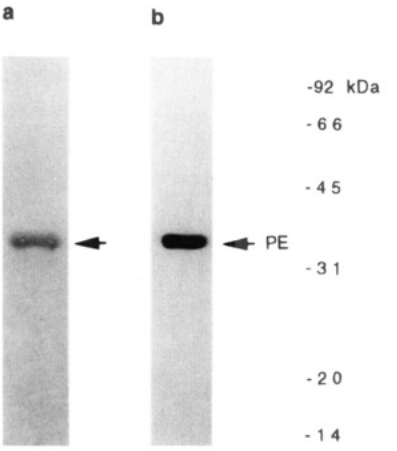

FIGURE 5: Purified PE on SDS−PAGE and anti-PE immunoblot. (a) Purified PE (8 μg), as analyzed by Coomassie Blue staining of an SDS−PAGE gel. (b) Anti-PE immunoblot of purified PE.

Table 2: Amino Acid Composition of Proenkephalin

amino acid	experimental[a] (mol %)	predicted[b] (mol %)
Asx	11.9	11.4
Thr	2.5	2.5
Ser	6.5	7.6
Glx	16.3	14.8
Pro	5.1	4.2
Gly	9.4	10.2
Ala	4.1	4.2
Val	2.6	2.1
Met	4.1	4.7
Ile	1.8	1.7
Leu	8.9	8.5
Tyr	4.5	5.1
Phe	4.8	5.1
His	0.9	0.8
Lys	9.5	10.2
Arg	6.9	6.8
Trp	ND	
Cys	ND	

[a] PE (25.9 pmol) was subjected to amino acid composition analyses as described under Experimental Procedures. [b] Predicted percentages (mol %) of amino acid composition of PEbased on the PE primary sequence deduced from its cDNA. ND indicates not determined, due to the instability of tryptophan and cysteine under acid conditions.

correct NH_2-terminal sequence of a PE fragment generated by PTP (Table 3).

Prohormone Thiol Protease (PTP) and Proenkephalin (PE). The concentration of PE within chromaffin granules is estimated at approximately $10^{-4}-10^{-5}$ M, and enkephalin peptides are present at millimolar levels (Ungar & Phillips, 1983). Time course experiments were performed to examine PTP processing of PE near *in vivo* precursor levels. PTP converted PE (12 μM PE) to high molecular weight PE products of 22.5, 21.7, 12.5, 11.0, and 8.5 kDa (Figure 6). Immunoblots showed that the 22.5 and 21.7 kDa products were recognized by the anti-PE-18 monoclonal antibody that recognizes the mid-region of PE (residues 130−207 of preproenkephalin; B. Spruce, personal communication; Spruce et al., 1988). The smaller 12.5, 11.0, and 8.5 kDa bands were, however, not detected by the PE-18 antibody, suggesting that these products do not contain the mid-region of PE.

Densitometric quantitation of PE cleaved and products formed (Figure 7) indicated cleavage of PE within 10 min incubation, with simultaneous formation of 22.5, 21.7, 12.5, and 11.0 kDa products. PE was nearly completely cleaved by 1−2 h incubation with PTP, as shown by 80% cleavage of PE at 1 h and 90% cleaved by 2 h. However, the 22.5, 21.7, 12.5, and 11.0 kDa intermediates remain up to 4−6 h incubation. The 8.5 kDa product appeared after formation of the 12.5 and 11.0 kDa products, beginning at 1 h and continuing to 24 h incubation, with peak levels of the 8.5 kDa band at 2−6 h. By 12−24 hours, PE and intermediate products of 22.5 to 11.0 kDa were virtually completely cleaved, and some of the 8.5 kDa product was present.

Further characterization of PE products was achieved by peptide microsequencing of the 22.5, 21.7, 12.5, 11.0, and 8.5 kDa bands (Table 3). Microsequencing of the 22.5 and 21.7 kDa bands indicated that both possess the NH_2-terminus of PE. These results indicate that the 22.5 and 21.7 kDa bands represent large products that possess the NH_2-terminal region of PE, as well as the mid-region of PE that is recognized by the PE-18 monoclonal antibody. Differences in size of 22.5 and 21.7 kDa bands indicate processing of PE near its COOH-terminal region. Peptide microsequencing of the 12.5 and 11.0 kDa bands indicated that these products also contain the NH_2-terminus of PE. These 22.5, 21.7, 12.5, and 11.0 NH_2-terminal-containing products are consistent with cleavage of PE at paired basic residues sites within the COOH-terminal region of PE. PTP production of several high molecular weight products containing the NH_2-terminal segment of PE resembles the production *in vivo* of high molecular weight PE products of similar size possessing the NH_2-terminal region of PE (Liston et al., 1984).

PTP cleavage at paired basic residues was indicated by microsequencing of 12.5, 11.0, and 8.5 kDa bands (Table 3). These bands result from PTP cleavage between Lys-Arg located at the COOH-terminal side of (Met)enkephalin-Arg^6-Gly^7-Leu^8 within PE. Differences in size among the 12.5, 11.0, and 8.5 kDa bands presumably represent PTP processing of paired basic residues within the COOH-terminal region of PE. The 8.5 kDa band is compatible in position (within PE) and size to peptide I, a PE product *in vivo* (Udenfriend & Kilpatrick 1983). The 12.5 and 11.0 kDa bands may contain peptide I; the size of the 12.5 kDa band corresponds to a COOH-terminal fragment that is predicted to include the heptapeptide (Met)enkephalin-Arg^6-Phe^7. These results provide evidence for PTP cleavage at a

Table 3: Microsequencing of PE Products[a]

PE-derived fragment (kDa)	reactivity to PE-18 antibody	determined sequence	region of PE
22.5 kDa	+	MDXSQDXAKX	NH_2-terminus of PE
21.7 kDa	+	MDXSQDXAKX	NH_2-terminus of PE
12.5 kDa	−	MDXSQDXAK	NH_2-terminus of PE
		RXPQLE	KR site at COOH-terminus of ME-RGL
11.0 kDa	−	MDXXQDXAK	NH_2-terminus of PE
		RSPQLEDEAK	KR site at COOH-terminus of ME-RGL
8.5 kDa	−	RSPQLEDEAK	KR site at COOH-terminus of ME-RGL

[a] Sequencing of the 12.5 and 11.0 kDa bands indicated the presence of two sequences as indicated above. The X residues within MDXSQDXAKX correlate to the cysteine residues known to be present at NH_2-terminal sequence of rat proenkephalin as MDCSQDCAKC. Cysteine was not determined, since the fragment was not carboxymethylated to form carboxymethylcysteine; only modified cysteine, not free cysteine, can be detected by the microsequencing method. ME-AGL is abbreviated for (Met)enkephalin-Arg^6-Gly^7-Leu^8.

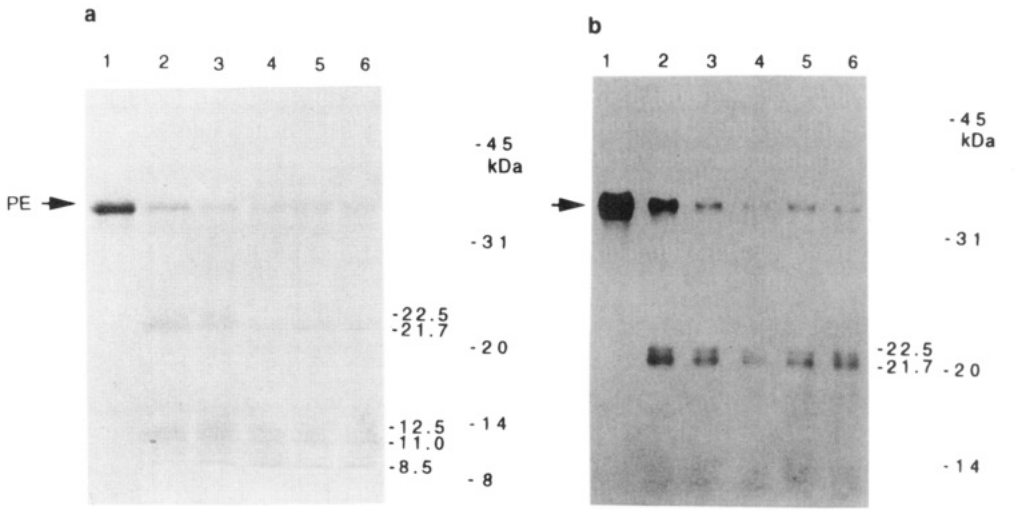

FIGURE 6: PTP incubation with purified PE: time course. (a) Time course of PTP cleavage of PE, assessed by amido black protein staining. PTP (1 ng) was incubated with PE (8 μg) for 0, 1, 2, 3, 4, and 6 h (corresponding to lanes 1−6) at 37 °C as described under Experimental Procedures. Approximately four-fifths of each reaction was analyzed by SDS−PAGE gel electrophoresis, with detection of PE and product bands by amido black. (b) Anti-PE immunoblot of PTP and PE digestions. From the same time course experiment in Figure 6a, one-fifth of each reaction was subjected to immunoblot analysis with the PE-18 antibody, as described under Experimental Procedures.

paired basic residue site within PE as well as PTP processing near the COOH-terminal region of PE.

Kinetics of PTP cleavage of PE were examined to compare kinetic values ($K_{m(app)}$ and $V_{max(app)}$) with intragranular concentrations of PE, estimated at 10^{-4} M, and enkephalin peptide products, estimated at 10^{-3} M (Ungar & Phillips, 1983; Fleminger et al., 1983). Cleavage of PE was assessed at 1−20 μM PE, with PTP incubation for 10 min. The amount of PE cleaved was quantitated by densitometric scanning of PE blotted onto nitrocellulose and stained with amido black. Lineweaver−Burk plots (Figure 8) indicated $K_{m(app)}$ of 18.6 μM and $V_{max(app)}$ of 2 mmol/(h·mg), with $k_{cat(app)}$ of 18 s^{-1} and k_{cat}/K_m of 1×10^6 s^{-1} M^{-1}.

DISCUSSION

Evidence for a role of the candidate processing enzyme prohormone processing enzyme (PTP) in proenkephalin (PE) processing was demonstrated by PTP production of appropriate PE-derived intermediates and by PTP kinetics that are consistent with *in vivo* levels of PE and enkephalin-containing peptide products. These studies were accomplished by high level expression of authentic full-length PE in *E. coli*, which yields adequate amounts of PE to achieve *in vitro* PE levels near estimated *in vivo* concentrations of 10^{-5}−10^{-4} M (Ungar & Phillips, 1983). Analysis of PE products by SDS−PAGE, peptide microsequencing, and reactivity with the PE-18 monoclonal antibody (recognizing the mid-region of PE) indicated production of PE products that resemble those *in vivo* in adrenal medulla (Liston et al., 1983; Udenfriend & Kilpatrick, 1983; Hook et al., 1990) (Figure 9). Importantly, PTP kinetic constants are compatible with *in vivo* levels of PE and its peptide products. These results support a role for PTP as a putative PE processing enzyme.

Recombinant PE of apparent molecular mass of 35 kDa was expresssed in *E. coli* as a homogeneous population of molecules with pI of 6.0, which is consistent with the predicted pI based on the primary sequence of PE (Yoshikawa et al., 1984). High level expression of PE in *E. coli* followed by DEAE-Sepharose, preparative gel electrophoresis, and HPLC results in purified PE as assessed by amino acid composition, NH_2-terminal sequence analyses, and immunoreactivity with the PE-18 monoclonal antibody. Thus, large quantities (milligram) of recombinant PE can be obtained by expression in *E. coli*. This high level expression of proenkephalin in *E. coli* allowed the development of an *in vitro* prohormone processing assay with recombinant PE near estimated *in vivo* levels of 10^{-5} to 10^{-4} M (Ungar & Phillips, 1983).

PTP processing of PE *in vitro* was analyzed by SDS−PAGE, peptide microsequencing, and reactivity to PE-18 monoclonal antibody. Microsequencing indicated that PTP produced high molecular mass intermediates of 22.5, 21.7, 12.5, 11.0, and 8.5 kDa that posssess the NH_2-terminus of PE (Figure 9). Among these intermediates, only the 22.5 and 21.7 kDa were immunoreactive with the PE-18 antibody.

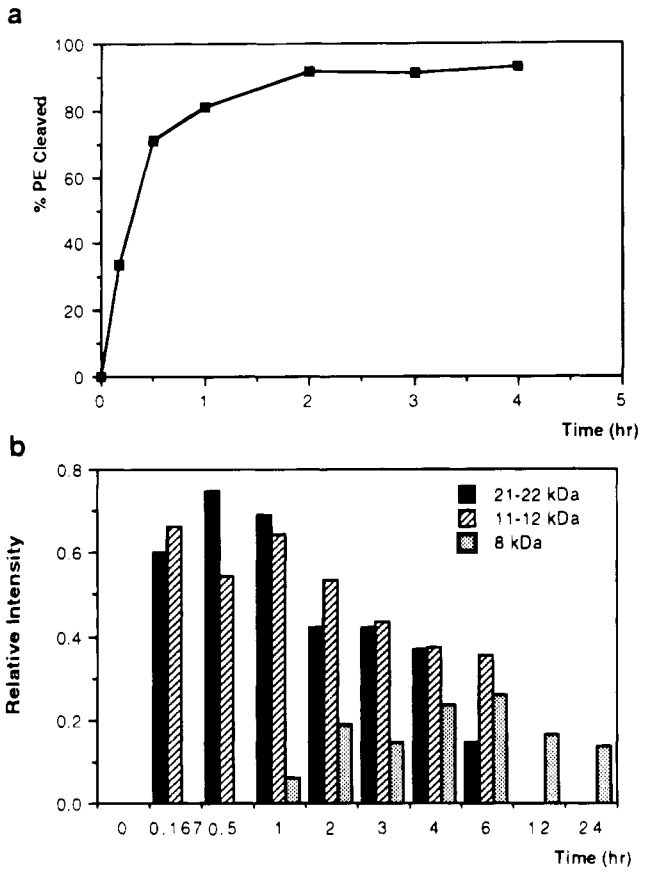

FIGURE 7: Densitometry to assess relative cleavage of PE and production of intermediates in time course study. (a) Relative levels of PE. Densitometry of amido black stained PTP digestions of PE was performed to quantitate percent PE cleaved. (b) Relative levels of the 21−22 kDa (21.7 and 22.5 kDa), 11−12 kDa (11.0 and 12.5 kDa), and 8.5 kDa products. Densitometric scanning of amido black stained membranes estimated relative levels of 21.7 and 22.5 kDa products, and 11.0 and 12.5 kDa products, generated up to 24 h incubation with PTP. Differences in molecular mass of 1−2 kDa were not well resolved by densitometry; therefore, densitometry indicates relative levels of 21−22 kDa and 11−12 kDa products. Relative densitometric values illustrated in the bar graph are taken as the average from at least three determinations; all time points in the experiment were repeated 4−5 times with reproducible production of intermediate products.

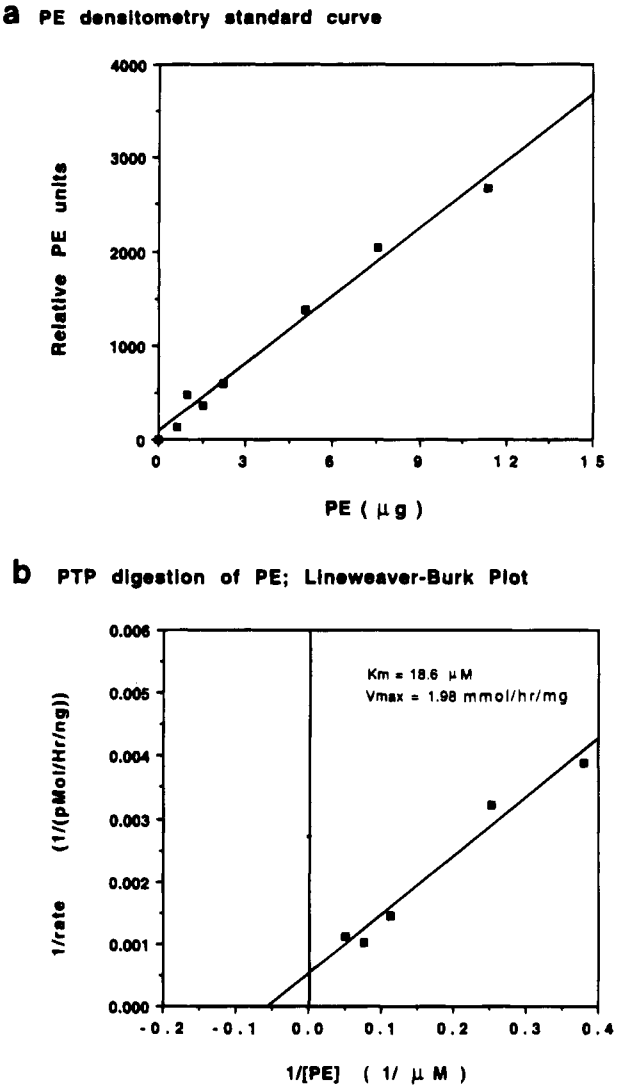

FIGURE 8: Kinetics of PTP endoproteolysis of PE. (a) PE standard curve. Different amounts of PE (0−11.3 μg) were subjected to SDS−PAGE, transferred to nitrocellulose, stained with amido black, and quantitated by densitometric scanning. (b) Lineweaver−Burk plot of PTP endoproteolysis of PE. Kinetic constants, $K_{m(app)}$ and $V_{max(app)}$, for PTP proteolysis of PE were determined by an inverse plot of PTP velocity and PE concentration, as described under Experimental Procedures. Kinetic values were assessed in three experiments.

These results indicate the presence of the mid-region (residues 130−207 of preproenkephalin, Figure 9) within the larger 22.5 and 21.7 kDa products, but not with the shorter 12.5, 11.0, and 8.5 kDa fragments. Additionally, PTP cleavage between the Lys-Arg at the COOH-terminal side of the octapeptide (Met)enkephalin-Arg^6-Gly^7-Leu^8 resulted in production of 8.5, 11.0, and 12.5 kDa fragments that may contain peptide I (Figure 9). These products indicate PE processing from its COOH-terminal region to generate several high molecular weight NH_2-terminal containing intermediates as well as COOH-terminal peptide products that presumably include peptide I. *In vitro* production of PE products similar to those *in vivo* (Liston et al., 1983; Udenfriend & Kilpatrick, 1983; Birch & Christie, 1986; Spruce et al., 1988; Hook et al., 1990) support a role for PTP in PE processing.

Kinetic studies showed that PTP processing of PE was compatible with *in vivo* levels of PE and PE-derived products. The affinity of 18.6 μM PE is consistent with PTP activity at an estimated intragranular PE concentration of about 10−100 μM (Ungar & Phillips, 1983). PTP *in vivo* would be active with saturating levels of PE. The combined parameters of PTP's $V_{max(app)}$ [2 mmol/(h·mg)], concentration of PTP *in vivo* (10^{-5} M), and estimated vesicular volumes (Ungar & Phillips, 1983) predict production of 0.1−0.5 mM levels of high molecular mass intermediates (10−25 kDa approximate size) and peptide products. These values are in agreement with estimated *in vivo* concentrations of enkephalin-containing intermediates and peptides (Ungar & Phillips, 1983).

PTP demonstrated more rapid processing of PE than of PE-derived intermediates. The slower PTP processing of intermediates, compared to PE itself, is consistent with the presence *in vivo* of large amounts of high molecular mass intermediates (Fleminger et al., 1983) that are not rapidly converted to the final product (Met)enkephalin. PTP cleaved PE within 10 min, and by 60 min PE was nearly completely processed. PTP simultaneously generates multiple high molecular mass intermediates (22.5−11.0 kDa), suggesting that several paired basic residue sites may be initially processed. These 22.5−11.0 kDa intermediates remain in the reaction for 4−6 h, indicating that they are processed

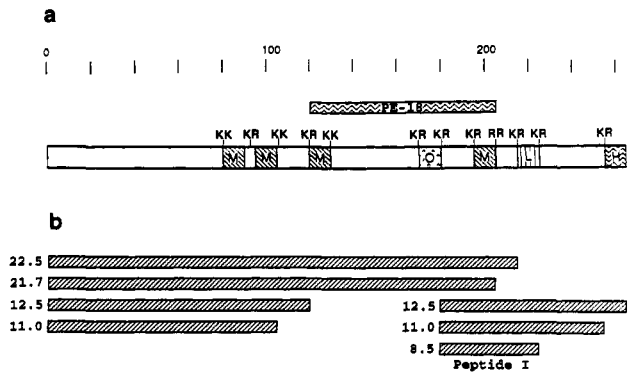

FIGURE 9: PE products generated by PTP. (a) PE-18 monoclonal antibody recognizes the mid-region of PE. The region of PE corresponding approximately to residues 130−207 is recognized by the PE-18 monoclonal antibody (Spruce et al., 1988; personal communication). (b) PE products generated by PTP are shown below PE. Bars with apparent molecular sizes indicate the PTP generated PE products that were identified in this study by SDS−PAGE, peptide microsequencing, and reactivity to PE-18 antibody.

more slowly than PE. The 8.5 kDa product is also slowly processed, as it appears after 1 h incubation, peaks at 4−6 h incubation, and its levels diminish at 12−24 h incubation time.

Comparison of PTP with PC1/3 with regard to *in vitro* processing of PE indicates that PTP cleaves PE more rapidly than PC1/3. Recombinant PC1/3 cleaves recombinant PE at an estimated rate of 0.18 μmol/(h·mg) (Zhou & Lindberg), whereas PTP cleaves PE at 2 mmol/(h·mg) ($V_{max(app)}$). These kinetic results indicate that PTP may cleave PE more rapidly (by 10 000 times) than PC1/3. Although PC1/3 intragranular levels (Christie et al., 1991) are estimated to be several hundred times greater than PTP levels (Krieger & Hook, 1991), total PTP activity for cleaving PE would be predicted to be about 20−100 times greater than that for PC1/3. A greater level of PTP activity compared to PC1/3 activity in chromaffin granules has been confirmed in our recent studies (Azaryan et al., 1995).

Results of this study, combined with previous investigations (Hook et al., 1994), support a role for PTP as a novel cysteine protease involved in PE processing based on (1) localization of PTP to highly purified secretory vesicles, (2) production of appropriate high molecular mass NH_2-terminal-containing intermediates and (Met)enkephalin, with PTP cleavage at paired basic residues, and (3) kinetics consistent with intragranular levels of PE and processing products. It will be of interest in future studies to compare the role of PTP in processing proenkephalin with other prohormones.

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