Evidence for the Proenkephalin Processing Enzyme Prohormone Thiol Protease (PTP) as a Multicatalytic Cysteine Protease Complex: Activation by Glutathione Localized to Secretory Vesicles†

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ABSTRACT: The cysteine protease known as "prohormone thiol protease" (PTP) has been identified as a major proenkephalin processing enzyme in secretory vesicles of adrenal medulla (known as chromaffin granules). This study provides the first demonstration that PTP exists as a multicatalytic cysteine protease complex that can be activated by endogenous glutathione present in chromaffin granules. The high molecular mass nature of PTP, of approximately 185 kDa, was demonstrated by elution of a single peak of ^{35}S-enkephalin precursor cleaving activity by Sephacryl S200 gel filtration chromatography and by a single band of ^{35}S-enkephalin precursor cleaving activity detected on radiozymogram gels under native buffer conditions. Importantly, when 0.1% SDS was included in radiozymogram gels, PTP activity was resolved into three bands of proteolytic activity with apparent molecular masses of 88, 81, and 61 kDa. These activities were all cysteine proteases, since they were inhibited by the cysteine protease inhibitor E-64c but not by pepstatin A or EDTA that inhibit aspartyl protease and metalloprotease, respectively. Purification of native PTP by preparative gel electrophoresis indicated that PTP was composed of four polypeptides of 66, 60, 33, and 29 kDa detected on SDS−PAGE gels. These four protein subunits accounted for the three catalytic activities of PTP, as demonstrated on ^{35}S-enkephalin precursor radiozymogram gels. Results also indicated that the electrophoretic mobilities of the four subunits differed under reducing compared to nonreducing conditions. The multicatalytic activities of the PTP complex all require reducing conditions for activity, which can be provided by endogenous reduced glutathione in chromaffin granules. These novel findings provide the first evidence for a role of a multicatalytic cysteine protease complex, PTP, in chromaffin granules that may be involved in the proteolytic processing of proenkephalin and perhaps other precursors into active neuropeptides.

Peptide hormones and neurotransmitters are synthesized as large polypeptide precursors that require proteolytic processing by endoproteases and exoproteases to generate the smaller, physiologically active neuropeptides (*1*−*3*). Processing of proneuropeptides occurs primarily within the secretory pathway, especially within secretory vesicles that synthesize, store, and secrete bioactive neuropeptides.

Secretory vesicles of bovine adrenal medulla (also known as chromaffin granules) have been studied as a model neuroendocrine system for characterizing proneuropeptide and prohormone processing enzymes. Chromaffin granules contain high levels of enkephalin opioid peptides (*4*−*6*), neuropeptide Y (NPY) (*7*), vasoactive intestinal polypeptide (VIP) (*8*), and several other peptide hormones (*9*). The precursors of these neuropeptides require proteolytic processing at dibasic residues, and sometimes at monobasic residues, to generate active peptides (*1*−*3*). Importantly, chromaffin granules contain the corresponding endoproteases that participate in proneuropeptide processing, including the cysteine protease known as "prohormone thiol protease" (PTP)[1] (*10*−*13*), the subtilisin-like PC1/3 and PC2 enzymes (*14*, *15*), and a yapsin-like aspartyl protease (*16*). In addition, the exoproteases carboxypeptidase E/H (*17*, *18*) and aminopeptidase (*19*, *20*) are required to remove basic residues from COOH and NH_2 termini of peptide intermediates, respectively, to generate the final, active neuropeptides. Some neuropeptides also require COOH-terminal amidation by peptidylglycine α-amidating monooxygenase (*21*) for biological activity.

Our studies of proenkephalin processing enzymes in chromaffin granules have identified a novel cysteine protease known as prohormone thiol protease (PTP) as the primary proteolytic activity for converting proenkephalin into active enkephalin-related opioid peptides (*10*−*13*). PTP, in vitro, converts proenkephalin to intermediates and enkephalin peptide products that are present in chromaffin granules in

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[1] Abbreviations: AMC, aminomethylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; MCA, methylcoumarinamide; NEM, *N*-ethylmaleimide; PC, prohormone convertase; PTP, prohormone thiol protease; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TNB, 5-thio-2-nitrobenzoic acid.

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vivo (*10*−*13*). During forskolin-stimulated elevation of enkephalin levels in chromaffin cells, PTP activity is stimulated by cAMP (*13*). In contrast to PTP, the subtilisin-like PC1/3 and PC2 enzymes from chromaffin granules demonstrate lower levels of proenkephalin cleaving activity (*14*). The yapsin-like 70 kDa aspartyl protease has been detected in chromaffin granules, but it possesses lower specific activity than PTP for cleaving the enkephalin precursor (*16*). Thus, the specific activity of PTP for cleaving proenkephalin (*10*−*12*, *22*−*24*) is greater than that of the PC1 and PC2 subtilisin-like (*22*, *25*−*27*) and 70 kDa aspartyl proteases (*16*, *22*).

Our previous biochemical studies demonstrated that purified PTP consisted of a 33 kDa protein (*10*). However, gel filtration chromatography demonstrated PTP activity as a high molecular mass peak of 150−200 kDa. These observations lead to the hypothesis that the 33 kDa PTP protein may be part of a protease complex, consisting of several subunit proteins. Therefore, the goal of this study was to determine the biochemical nature of native PTP.

Results from this investigation provide evidence for native PTP as a multicatalytic protease complex. While PTP demonstrates a single peak of activity under native conditions, PTP was resolved into three cysteine protease activities in the presence of low concentrations of detergent. Moreover, the three cysteine proteases of the PTP complex require reducing conditions for activity, which can be provided by endogenous glutathione present within chromaffin granules. This study provides the first evidence for a multicatalytic cysteine protease complex, activated by endogenous glutathione, that participates in the processing of proenkephalin and perhaps other prohormones. These results also demonstrate the novel finding that glutathione may participate in maintaining the intravesicular redox environment of secretory vesicles.

EXPERIMENTAL PROCEDURES

Isolation of Prohormone Thiol Protease (PTP) from Chromaffin Granules. PTP was purified from bovine chromaffin granules essentially as described previously (*10*). Chromaffin granules were isolated from fresh bovine adrenal medulla (obtained from Shamrock Meats, Los Angeles, CA) by discontinuous sucrose gradient centrifugation as described previously (*10*, *19*). A soluble extract of chromaffin granules was obtained from 3 × 400 adrenal gland preparations as follows. Granules (in 15 mM KCl) were lysed by two cycles of freeze−thawing and centrifuged in a GSA rotor at 10 000 rpm (16000*g* force) at 4 °C for 45 min, and the supernatant was collected. The pellet was resuspended in 150 mL of concanavalin A equilibration buffer (100 mM citric acid−NaOH, pH 6.0, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.5 M NaCl) with a glass/glass homogenizer and centrifuged (GSA rotor at 16 000 rpm). Supernatants from the first and second centrifugations were pooled and subjected to ultracentrifugation (100000*g* at 4° C in a SW28 rotor, Beckman L7-65 ultracentrifuge). The final supernatant was collected as the chromaffin granule extract.

The granule extract was subjected to chromatography on concanavalin A−Sepharose, Sephacryl S-200, chromatofocusing, and Mono S ion-exchange columns. PTP activity was monitored during purification by measuring the conversion of the recombinant ^{35}S-enkephalin precursor into TCA (trichloroacetic acid) soluble radioactivity, as described previously (*10*). Chromatography of PTP activity on concanavalin A−Sepharose, Sephacryl S-200, and the chromatofocusing columns was conducted as described previously (*10*). After the chromatofocusing step, active fractions were pooled and concentrated, with buffer exchange to 100 mM citric acid−NaOH, pH 4.5, using an Amicon ultrafiltration apparatus with a YM 10 membrane. The concentrated sample was loaded onto a Mono S cation-exchange FPLC column (1 mL HiTrap column SP, Pharmacia, Piscataway, NJ) that was equilibrated with 100 mM citric acid−NaOH, pH 4.5 (buffer A). The column was eluted with a NaCl gradient generated with buffer B (consisting of 100 mM citric acid−NaOH, pH 4.5, 2.0 M NaCl), with the gradient consisting of 0% B at 1−15 min, 0−25% B at 15−45 min, 25−100% B at 45−50 min, 100% B at 50−55 min, 100−0% B at 55−60 min, and 0% B at 60−75 min, with a flow rate of 1 mL/min. Fractions (1 mL/fraction) containing PTP activity (fractions 17−28 at 100−200 mM NaCl) were pooled and concentrated by Amicon ultrafiltration with buffer exchange to 100 mM citric acid−NaOH, pH 4.5. Consistent chromatography of PTP activity through these steps was observed with three separate preparations.

^{35}S-Enkephalin Precursor Radiozymogram Gel Assay of PTP Activity: Native and Modified Electrophoresis Conditions. PTP obtained from the Mono S column was subjected to polyacrylamide gel electrophoresis under native conditions, and PTP activity was assayed in situ in the gel with the ^{35}S-(Met)-enkephalin precursor substrate embedded in the polyacrylamide slab gels, referred to as radiozymogram gel assays (*29*, *30*). Radiozymogram gels were prepared by copolymerization of the polyacrylamide solution (7 mL of 12% polyacrylamide with 0.16% bisacrylamide and 0.375 M Tris-HCl, pH 8.8) with the ^{35}S-(Met)-enkephalin precursor (3.5 × 10^6 cpm) in a 1 mm thick gel cassette (Novex, San Diego, CA) to result in a resolving gel with dimensions of 8 × 7 × 0.1 cm^3; the stacking gel was 6% polyacrylamide, prepared according to Laemmli (*31*). The PTP enzyme sample (2 μL of the Mono S-purified pool) was prepared in 1× native sample buffer (50 mM Tris-HCl, pH 8.3, 2% glycerol) and was electrophoresed in the radiozymogram gel at 4 °C in 1× running buffer (consisting of 25 mM Tris-HCl, 192 mM glycine, pH 8.3) for 2.5 h at a constant current of 25 mA. The gel was then washed in cold 2.5% Triton X-100 solution for 10 min and with cold sterile water for 10 min. Assay of the ^{35}S-enkephalin precursor cleaving activity in the gel was conducted by incubating the gel at 37 °C for 2 h in 1× PTP assay buffer (100 mM citric acid−NaOH, pH 5.0, 1 mM EDTA, 1 mM DTT, 10 mM CHAPS). The gel was fixed in 50% methanol/7% acetic acid for 10 min and incubated in Amplify solution (Amersham, Cleveland, OH) for 30 min. The gel was dried onto filter paper under vacuum at 65 °C for 1 h and subjected to autoradiography (Biomax film, Kodak, Rochester, NY) overnight. All buffer solutions for radiozymogram gel analyses were filtered (through 0.2 μm membranes, Millipore, Bedford, MA) and boiled before use. In addition, proteins in the gel were stained by Gelcode Color Silver Stain (Pierce, Rockford, IL) according to the manufacturer's protocol.

PTP was also subjected to radiozymogram gels that included 0.1% SDS. PTP (from the Mono S step) was

prepared in sample buffer (50 mM Tris-HCl, pH 6.8, 2% glycerol, and 1.5% SDS) and was electrophoresed at 4 °C in 1× running buffer (containing 25 mM Tris-HCl, 192 mM glycine, pH 8.3, 0.1% SDS) for 1.5 h at a constant current of 25 mA. The gel was then assayed for ^{35}S-enkephalin precursor activity as described for the native radiozymogram gels in the previous paragraph. When the effects of protease inhibitors or stimulators on PTP activity in radiozymogram gels were assessed, radiozymogram gel slices were incubated with inhibitor in 1× PTP assay buffer for 30 min at room temperature and then at 37 °C for 2 h, as described in the previous paragraph.

For radiozymogram gels containing 0.1% SDS, protein molecular mass markers (Bio-Rad, Hercules, CA) were stained with Coomassie Blue and soaked in GelDry solution (Novex, San Diego, CA) for 30 min before being air-dried. Alignment of protein markers and bands of PTP activity detected on the radiozymogram gels was achieved by photographing gels with the Kodak DC120 digital camera and by image analysis with the EDAS120 (Electrophoresis Documentation and Analysis System 120) software system. Radiozymogram gel experiments were repeated at least three times, with reproducible results.

Z-Phe-Arg-MCA Fluorescent Substrate Gel Assay of PTP Activity. Z-Phe-Arg-MCA was also used as substrate to monitor PTP activity in nonreducing SDS−PAGE gels (with 0.1% SDS). The Z-Phe-Arg-MCA substrate was embedded into the gel as described for the preparation of ^{35}S-enkephalin precursor radiozymogram gels, by copolymerization of Z-Phe-Arg-MCA (250 μM) with resolving gel components. After electrophoresis and incubation in PTP assay buffer, proteolytic activity in the gel was visualized under a UV transilluminator to detect fluorescent bands that represented MCA cleaved from the Z-Phe-Arg-MCA substrate. The fluorescent image was photographed with a Kodak DC120 digital camera and analyzed with the EDAS120 image software system. Reproducible results were obtained when experiments were repeated at least three times.

Preparative Native Tube Gel Electrophoresis of PTP: Purification to Apparent Homogeneity. PTP obtained from the Mono S column was subjected to preparative polyacrylamide gel electrophoresis under native conditions (without SDS and without reducing agent) using the MiniPrep Cell system (Bio-Rad, Richmond, CA). Tube gels (7 mm internal diameter) were prepared with the resolving gel (10 cm) consisting of 6% polyacrylamide (with 0.16% bisacrylamide and 0.375 M Tris-HCl, pH 8.8) and a stacking gel (1 cm) of 4% polyacrylamide (with 0.11% bisacrylamide and 0.125 M Tris-HCl, pH 6.8), prepared according to the manufacturer's protocol (Bio-Rad). The PTP enzyme sample (200−300 μL from the Mono S step) was prepared in 1× native sample buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3, 10% glycerol) and was subjected to electrophoresis in the native tube gel at a constant power of 1 W at 4 °C for 48 h (with 1× running buffer consisting of 25 mM Tris-HCl, 192 mM glycine, pH 8.3). During electrophoresis, fractions (0.6 mL/fraction) were eluted in 1× running buffer at a flow rate of 0.02 mL/min; the stability of eluted PTP activity was improved with adjustment of fractions to pH 6.0 (with an equal volume of 0.1 M citric acid−NaOH, pH 4.5). Fractions were immediately assayed for ^{35}S-(Met)-enkephalin precursor cleaving activity as described previously (*10*) or for Z-Phe-Arg-MCA cleaving activity as described previously (*23*). The single peak of PTP activity was reproducible when the preparative native gel was repeated three times. Active fractions were pooled and concentrated (10-fold concentration) and stored at −80 °C. The protein content in the fractions was measured by the method of Lowry (*28*).

Determination of Glutathione Content in Chromaffin Granules. Total glutathione, consisting of reduced GSH and oxidized GSSG forms of glutathione (Calbiochem, La Jolla, CA), in chromaffin granules was determined as described by Adams et al. and others (*32*, *33*). Total glutathione was measured in coupled reactions of 2GSH + DTNB → GSSG + TNB and of GSSG + NADPH + H^+ → 2GSH + $NADP^+$ in the presence of glutathione reductase. After the DTNB reacts with the free thiol of GSH to generate GSSH and TNB, reduction of GSSG by glutathione reductase will recycle to give an increase in TNB formation with time. The rate of TNB formation with time is used as a measure of glutathione. Specifically, determination of total glutathione was assessed by incubating the chromaffin granule lysate (25 μL of granule sample at 15 mg/mL) in 100 mM sodium phosphate, pH 7.5, 4.5 mM EDTA, 0.25 mg/mL NADPH (Calbiochem, La Jolla, CA), and 0.6 mM DTNB (in a total volume of 1.0 mL) at room temperature for 10 min, followed by addition of GSSG reductase (2.7 units/mL final concentration, Boehringer-Mannheim, Indianapolis, IN). The initial rate of TNB formation (at room temperature) was assessed by monitoring absorbance at 412 nm every 10 s for 3 min. Standard curves with known amounts of GSH (0, 0.1, 0.25, 0.5, and 1 nmol) were generated by measuring rates of absorbance at 412 nm.

To determine the content of oxidized glutathione (GSSG) in chromaffin granules, the sample was incubated with NEM (5 mM NEM, 71 mM sodium phosphate, and 1 mM EDTA) on ice for 5 min to couple NEM to free sulfhydryl groups of GSH. Free NEM was removed by applying the sample to C18 Sep-Pak cartridges (Waters, Milford, MA) that were previously washed with methanol (100%) and equilibrated with water. The eluate (750 μL) obtained from the Sep-Pak column was then assayed for GSSG by the coupled reaction described in the previous paragraph. The GSH content in chromaffin granules was calculated as the difference between total glutathione (GSH + GSSG) and GSSG. Measurements of glutathione content were reproducible in three different samples of isolated chromaffin granules.

RESULTS

Native High Molecular Weight Prohormone Thiol Protease (PTP) Consists of a Multicatalytic Protease Complex. The cysteine protease prohormone thiol protease (PTP) represents the major enkephalin precursor cleaving activity in chromaffin granules (secretory vesicles) (*10*−*13*) of bovine adrenal medulla that produces enkephalin opioid peptides (*4*−*6*). Enkephalin precursor cleaving activity in the soluble chromaffin granule extract represents 80−90% of the total activity in these granules. Molecular sieving chromatography of a concanavalin A-bound fraction of soluble granule extracts demonstrated the high molecular weight nature of PTP, with apparent molecular mass of ∼185 kDa (150−200 kDa) (Figure 1).

Further analyses of PTP utilized radiozymogram gels under native buffer conditions. To prevent overloading of gels with

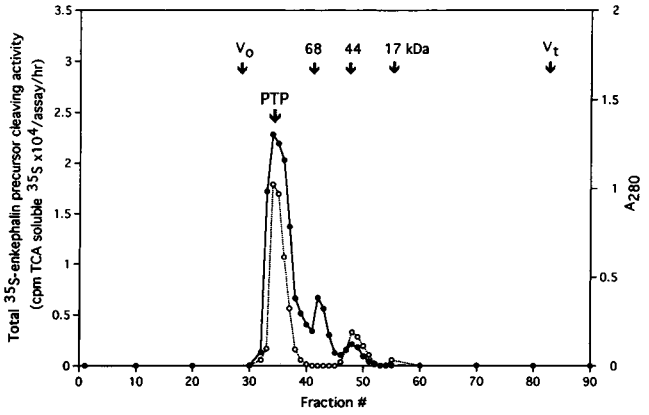

FIGURE 1: High molecular mass native PTP detected by gel filtration chromatography. PTP is represented as the primary peak of ^{35}S-enkephalin precursor cleaving activity (●) from chromaffin granules subjected to Sephacryl S200 gel filtration chromatography. Sephacryl S200 chromatography of the ConA-bound fraction from chromaffin granules (prepared as described in Experimental Procedures) was conducted under native conditions (50 mM dimethylglutaric acid, pH 6.2, 1 mM EDTA). Column fractions were assayed (1 μL aliquots) for PTP activity by monitoring the conversion of the ^{35}S-enkephalin precursor into acid-soluble (TCA-soluble) radioactivity, as described previously (*10*). The relative protein content of the eluted fractions was monitored by absorbance at 280 nm (A_{280}) (○). The S200 column was calibrated with molecular mass markers thyroglobulin (680 kDa, a marker for V_0), bovine serum albumin (BSA, 68 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.3 kDa, a marker for V_t).

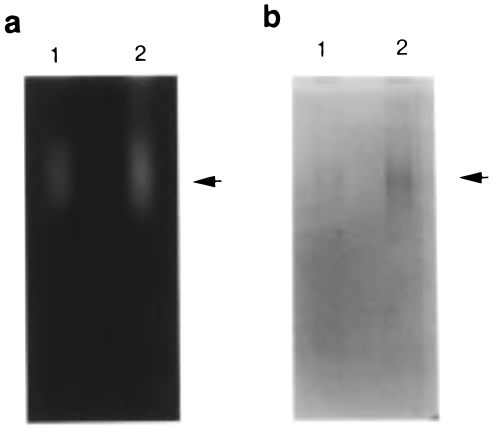

FIGURE 2: Native PTP activity detected in ^{35}S-enkephalin precursor radiozymogram gels. Panel a: ^{35}S-Enkephalin precursor radiozymogram of native PTP activity. PTP was obtained from chromaffin granules by chromatography on ConA−Sepharose, Sephacryl S200, chromatofocusing, and Mono S ion-exchange columns, as described in Experimental Procedures. PTP (2 and 4 μL corresponding to 8 and 16 μg of protein were analyzed in lanes 1 and 2, respectively) was analyzed on radiozymogram gels embedded with the ^{35}S-enkephalin precursor under native conditions (without SDS or reducing agents), as described in Experimental Procedures. Panel b: Native gel electrophoresis of PTP and silver staining. The PTP sample from the Mono S step (2 and 4 μL corresponding to 8 and 16 μg of protein) was subjected to electrophoresis under native gel conditions (identical to gel conditions of panel a), and proteins were assessed by silver staining.

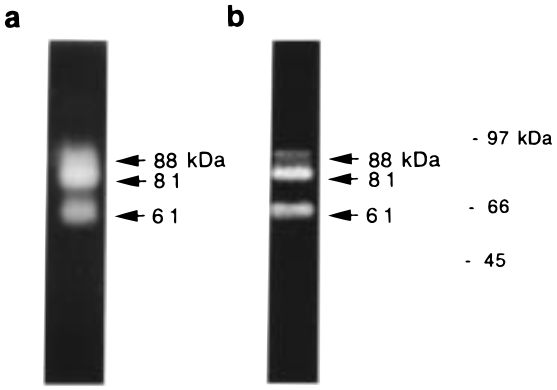

FIGURE 3: Native PTP is composed of multiple protease activities, detected on radiozymogram gels in the presence of SDS. Panel a: ^{35}S-Enkephalin precursor cleaving activity detected in situ in radiozymogram gels (under nonreducing conditions). Native PTP (obtained from the Mono S step, 2 μL corresponding to 8 μg of protein) was subjected to SDS−PAGE (0.1% SDS) under nonreducing conditions, and PTP activity in these ^{35}S-enkephalin precursor radiozymogram gels was detected in situ. ^{35}S-Enkephalin precursor cleaving activities of 88, 81, and 61 kDa were detected (indicated by arrows). Panel b: Z-Phe-Arg-MCA cleaving activity detected in situ in SDS−PAGE gels (under nonreducing conditions). Native PTP (obtained from the Mono S step, 2 μL corresponding to 4 μg of protein) was subjected to electrophoresis in SDS−PAGE gels (0.1% SDS and no reducing agent) embedded with Z-Phe-Arg-MCA. The gel was then incubated in PTP assay buffer to detect Z-Phe-Arg-MCA cleaving activity, monitored by production of fluorescent AMC (corresponds to MCA cleaved from the peptide substrate). Z-Phe-Arg-MCA cleaving activities of 88, 81, and 61 kDa apparent molecular mass were detected (indicated by arrows).

excessive amounts of protein, PTP was further purified as described previously (*10*) by chromatofocusing chromatography and by Mono S ion-exchange FPLC chromatography. For radiozymogram gels, ^{35}S-enkephalin precursor was copolymerized with polyacrylamide (12%) in slab gels under native buffer conditions (without SDS). After electrophoresis of PTP (from the Mono S step) under native gel conditions, ^{35}S-enkephalin precursor radiozymogram gels showed a single band of PTP activity (Figure 2a). PTP activity corresponded to protein detected at the same electrophoretic mobility (Figure 2b); much protein was also detected with slower electrophoretic mobility that did not correspond to ^{35}S-enkephalin precursor cleaving activity. These results indicated that native PTP exists as a single band of enkephalin precursor cleaving activity under native conditions.

Native PTP (obtained from the Mono S step) was then analyzed by radiozymogram gels in the presence of SDS detergent (0.1%). Inclusion of SDS resulted in the dissociation of native PTP into three bands of ^{35}S-enkephalin precursor cleaving activities with apparent molecular masses of 88, 81, and 61 kDa (Figure 3a). The 88, 81, and 61 kDa bands corresponded to 21%, 45%, and 34% of the total enkephalin precursor activity in the gel (measured by densitometry). The three activity bands of 88, 81, and 61 kDa were also detected with Z-Phe-Arg-MCA substrate that was copolymerized into the gel (Figure 3b), indicating cleavage at Arg. These activity bands were also detected with the substrate Boc-Val-Leu-Lys-MCA, indicating cleavage at the basic Lys residue (data not shown). Thus, all three activity bands demonstrate cleavage at Arg and Lys basic residues. Overall, these data demonstrate the multicatalytic nature of the PTP protease complex, consisting of three associated proteolytic activities with cleavage specificities for basic residues.

Purification of PTP Complex by Native Preparative Gel Electrophoresis. To determine the protein(s) that represent PTP activity, large-scale preparative native gel electrophore-

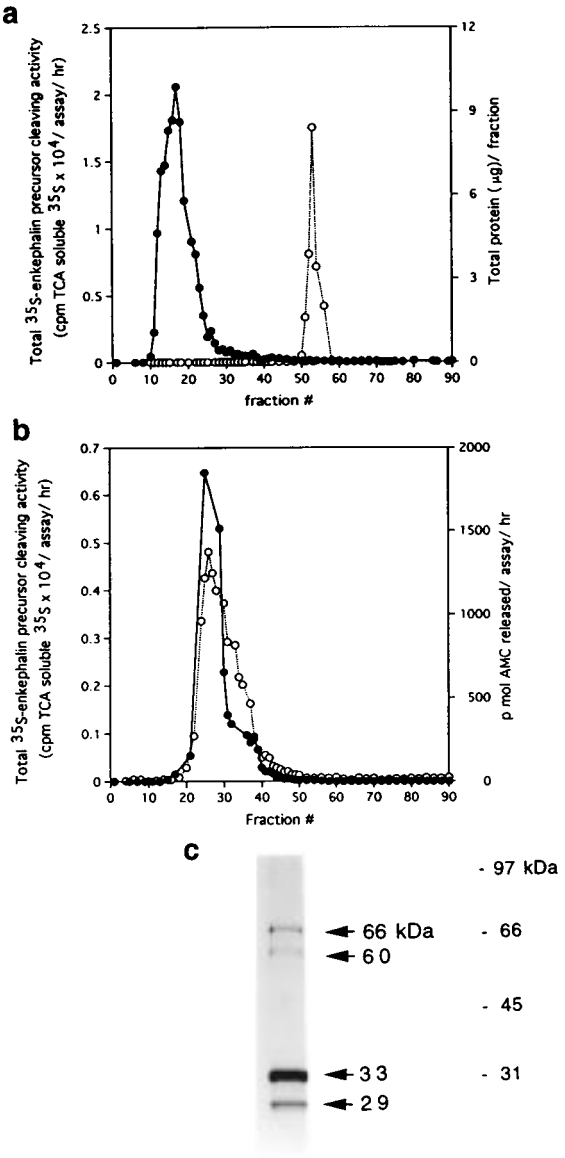

FIGURE 4: PTP activity purified by preparative native gel electrophoresis. Panel a: ^{35}S-Enkephalin precursor cleaving activity of PTP. PTP (200 μL, 800 μg of protein, from the Mono S column) was subjected to preparative tube gel electrophoresis under native conditions (no SDS or reducing agent). Aliquots (10 μL) from eluted fractions (0.6 mL/fraction) were assayed for ^{35}S-enkephalin precursor cleaving activity (●) measured by monitoring the production of TCA-soluble radioactivity. Protein content in each fraction was also determined (○). Panel b: Z-Phe-Arg-MCA cleaving activity of PTP. PTP (50 μL from the Mono S step) was fractionated by preparative tube gel electrophoresis. Aliquots (10 μL) of eluted fractions (0.6 mL/fraction) were assayed for Z-Phe-Arg-MCA cleaving activity (●) and ^{35}S-enkephalin precursor cleaving activity (○) as described previously (*10*, *23*). Panel c: SDS−PAGE and silver staining of native PTP. The peak of PTP activity obtained from the preparative gel (panel a) was pooled and concentrated, and proteins (from one-eighth of the PTP sample) were analyzed on reducing SDS−PAGE gels by silver staining. Protein bands of 66, 60, 33, and 29 kDa were detected (indicated by arrows).

sis was performed (Figure 4). Fractions were eluted from the preparative tube gel and ^{35}S-enkephalin precursor cleaving activity was immediately assayed. One major peak of enkephalin precursor cleaving activity was eluted (fractions 11−26, Figure 4a). This peak was also active with the fluorescent peptide substrate Z-Phe-Arg-MCA (Figure 4b), which has been shown to be an excellent substrate for PTP

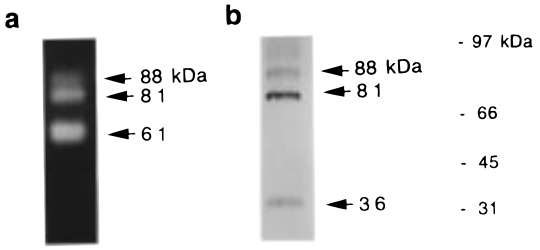

FIGURE 5: Multicatalytic activities of native PTP purified to apparent homogeneity. Panel a: ^{35}S-Enkephalin precursor radiozymogram of purified PTP. PTP purified by preparative native gel electrophoresis (from Figure 4) was subjected to ^{35}S-enkephalin precursor radiozymogram gel electrophoresis with 0.1% SDS detergent (no reducing agent), followed by in situ gel assay of PTP activity. The radiozymogram gel with SDS resolves purified native PTP into three proteolytic activities with apparent molecular masses of 88, 81, and 61 kDa (indicated by arrows). Panel b: Silver staining of purified PTP on SDS−PAGE gel with 0.1% SDS. PTP purified by preparative native gel electrophoresis (from Figure 4) was subjected to SDS−PAGE gel electrophoresis (with 0.1% SDS and without reducing agent) and subjected to silver staining to visualize protein bands (indicated by arrows).

(*23*). Thus, the peak of enkephalin precursor cleaving activity possesses specificity for cleaving at a basic residue which corresponds to the cleavage specificity of PTP (*10*−*13*, *22*, *23*).

The native tube gel step resulted in purification to apparent homogeneity, since the major peak of protein was removed (protein peak in fractions 50−58, Figure 4a) from fractions containing PTP activity. The peak of PTP activity was subjected to SDS−PAGE (under reducing conditions) and silver staining, which indicated four proteins of 66, 60, 33, and 29 kDa (Figure 4c). The 33 kDa band was the most readily stained; this 33 kDa band corresponds to that previously detected with a lower amount of purified PTP [80 glands previously (*10*), compared to 1200 adrenal glands used in this study]. These results indicate that the high molecular mass native PTP consists of four candidate protein subunits of 66, 60, 33, and 29 kDa. It is noted that since detection by silver staining varies among different proteins, it may be inaccurate to estimate molar ratios of these four protein subunits on the basis of silver staining of SDS−PAGE gels.

To determine whether the four protein subunits obtained from purified native PTP (Figure 4) can account for the three protease activities detected by radiozymogram gels (Figure 3), the peak of PTP activity from the preparative tube gel electrophoresis (from Figure 4) was concentrated and subjected to ^{35}S-enkephalin precursor radiozymogram gels containing 0.1% SDS (Figure 5a). The purified native PTP was resolved into two major activity bands of 81 and 61 kDa and one minor band of 88 kDa. These bands of activity corresponded to those originally detected with partially purified PTP from the Mono S column (shown in Figure 3). These results indicate that the four protein subunits of 66, 60, 33, and 29 kDa can account for the three proteolytic activities associated with the native PTP complex.

These four protein subunits of PTP differ in electrophoretic mobilities under nonreducing compared to reducing SDS−PAGE gel conditions. Although these proteins were detected as 66, 60, 33, and 20 kDa bands in reducing SDS−PAGE gels (Figure 4c), after electrophoresis under nonreducing conditions they were visualized as bands of 88, 81, and 36

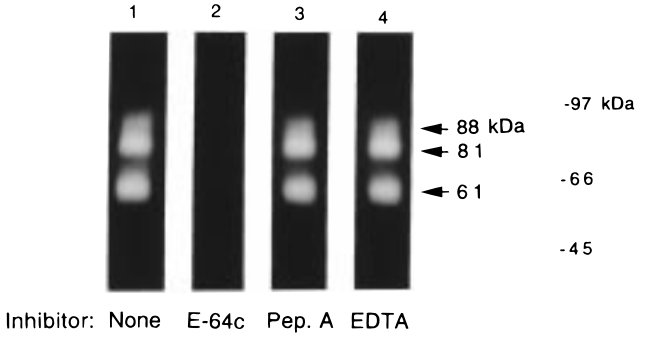

FIGURE 6: Inhibition of the PTP protease complex by a cysteine protease inhibitor. PTP from the Mono S step (1 μL aliquot corresponding to 1 μg of protein) was assayed in ^{35}S-enkephalin precursor radiozymogram gels containing 0.1% SDS without protease inhibitor (lane 1) or in the presence of E-64c (lane 2), pepstatin A (lane 3), or EDTA (lane 4) that inhibit cysteine protease, aspartyl protease, or metalloprotease, respectively. Inhibitors were present at final concentrations of 10 mM.

kDa (Figure 5b). The 88 and 81 kDa protein bands corresponded to two of the PTP activity bands (Figure 5a). At the position of the gel where the 61 kDa PTP activity was detected, no protein band was visible, indicating low levels of protein corresponding to this activity.

It was noted that slight differences in relative intensities of the 88, 81, and 61 kDa bands of PTP activity (detected in radiozymogram gels) obtained from purified PTP compared to PTP obtained from the Mono S step may involve differences in enzyme stabilities. Furthermore, PTP purified to apparent homogeneity by preparative native gel electrophoresis was active for only approximately 24 h, indicating the instability of purified native PTP. This observation suggests the possibility that purification of native PTP resulted in removal of a stabilizing factor.

Cysteine Proteases Represent Multicatalytic Activities of the PTP Complex: Activation by Secretory Vesicle Glutathione as an Endogenous Reducing Agent. Protease inhibitors were used to determine the protease classes(s) of the three proteolytic activities associated with the PTP complex (Figure 6). All PTP-associated activities detected in radiozymogram gels were inhibited by E-64c, a cysteine protease inhibitor. Inhibitors of aspartyl protease and metalloprotease, pepstatin A and EDTA, respectively, had no effect. These results provide evidence that the native PTP complex consists of multiple (three) cysteine proteases.

The requirement of a reducing agent such as DTT (dithiothreitol) to detect PTP activity is consistent with the cysteine protease nature of this protease. PTP activity was optimum at 1−5 mM DTT, with much activation occurring above 0.1 mM DTT (Figure 7a). ^{35}S-Enkephalin precursor radiozymograms demonstrated that DTT (1 mM) activated the three associated proteolytic activities (Figure 7b); no activity was detected in the absence of DTT. These results indicate that reducing conditions are required for PTP activity.

DTT, however, is not a natural, biological reducing agent that could activate PTP in vivo. Therefore, reducing agents that are known to be present in biological tissues (*34*) were tested for their effectiveness in activating PTP (Table 1). Reduced glutathione (GSH) at 10 mM, but not oxidized glutathione (GSSG, 10 mM final concentration), activated PTP. Homocysteine (10 mM) was also an effective activator

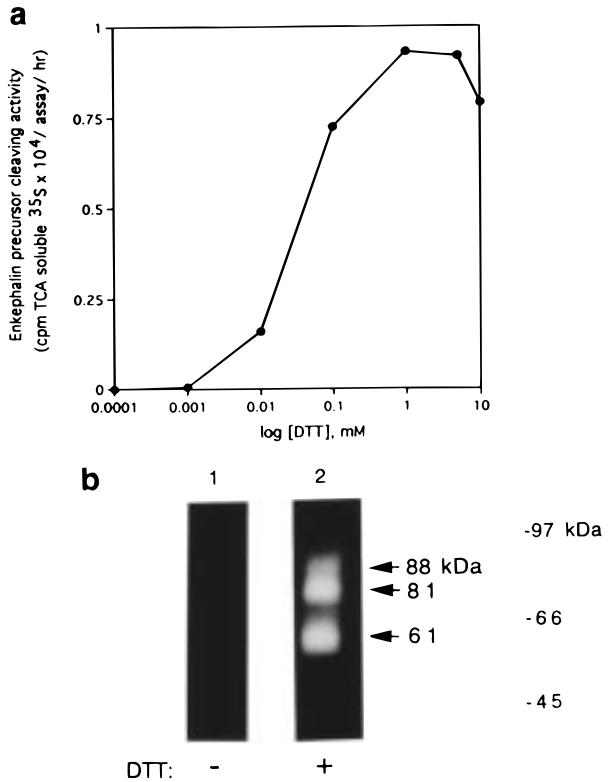

FIGURE 7: DTT activation of PTP. Panel a: DTT concentration dependence for PTP activation. The effect of DTT (0−10 mM) on PTP activity (obtained from the Mono S step, 80 ng of protein) was assessed by in vitro assays that monitored the conversion of the ^{35}S-enkephalin precursor into TCA-soluble radioactivity. Panel b: DTT activates the multicatalytic PTP protease complex. The ^{35}S-enkephalin precursor radiozymogram gel (with 0.1% SDS) of PTP was assayed in the absence (lane 1) or presence of 1 mM DTT (lane 2). PTP was obtained from the Mono S column (2 μL corresponding to 8 μg).

Table 1: Effects of Reducing Agents on the Prohormone Thiol Protease (PTP) Processing Enzyme[a]

reducing agents (10 mM)	% PTP activity compared to DTT (100%)
none	0
DTT	100
GSH	132
GSSG	2
NADPH	0
thiourea	4
homocysteine	108
ascorbic acid	0
ascorbic acid + MgATP	0

[a] PTP (80 ng of PTP protein per assay) obtained from the Mono S column was assayed in the absence or presence of reducing agents (10 mM final concentrations) by monitoring the conversion of the ^{35}S-enkephalin precursor to TCA-soluble radioactive peptide products, as described previously (*10*).

of PTP. Both GSH and homocysteine stimulated PTP to the same extent as DTT. However, PTP was not stimulated by the biological reducing agents NADPH, thiourea, or ascorbic acid (10 mM) that are capable of providing reducing conditions for other enzymes (*35*, *36*). These results indicated that PTP possesses preferences for certain reducing agents.

Further characterization indicated that reduced GSH was effective at millimolar concentrations, with much activation of PTP occurring at 5−10 mM GSH (Figure 8a). ^{35}S-Enkephalin precursor radiozymogram gel assays indicated

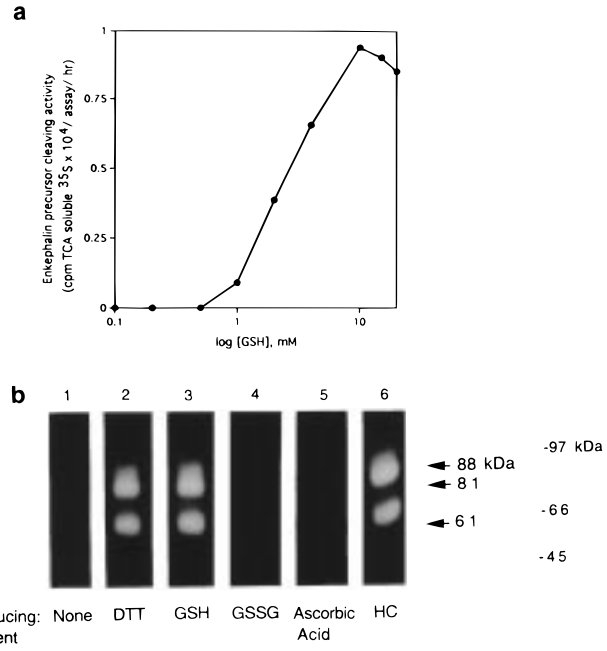

FIGURE 8: Activation of PTP by reduced glutathione. Panel a: Concentration dependence of PTP activation by reduced glutathione (GSH). The effect of GSH (0−10 mM) on PTP activity was assessed by in vitro assays that monitored the conversion of the ^{35}S-enkephalin precursor into TCA-soluble radioactivity. PTP was obtained from the Mono S step (80 ng of protein). Panel b: Glutathione activates the multicatalytic PTP protease complex. PTP was assayed in situ in ^{35}S-enkephalin precursor radiozymogram gels (with electrophoresis in 0.1% SDS) without reducing agent (lane 1) or with DTT (1 mM, lane 2), GSH (10 mM, lane 3), GSSG (10 mM, lane 4), ascorbic acid (10 mM, lane 5), or homocysteine (10 mM HC, lane 6).

that GSH (10 mM), but not oxidized GSSG (10 mM), was capable of activating all three PTP-associated proteases of 88, 81, and 61 kDa (Figure 8b). Homocysteine was also an effective activator of PTP. Radiozymogram assays also indicated that ascorbic acid (10 mM), a natural component of chromaffin granules (*37*), did not activate PTP.

To activate PTP in vivo, GSH must be colocalized with PTP and enkephalin peptides within chromaffin granules, secretory vesicles of the adrenal medulla. However, glutathione has not previously been reported to be localized in chromaffin granules or other secretory vesicles. Therefore, total glutathione (GSH and GSSG, Figure 9a) and oxidized GSSG (Figure 9b) levels were determined in isolated chromaffin granules. Levels of reduced GSH were calculated as the difference between total glutathione and oxidized GSSG (Figure 9). The standard curve for glutathione illustrated the linear rate of TNB (5-thio-2-nitrobenzoic acid) formation at 0.1−1 nmol of glutathione (Figure 9a). The rate of TNB formation by chromaffin granule extracts occurred in the linear range of the glutathione assay (Figure 9a). Similarly, oxidized GSSG in chromaffin granules was measured (Figure 9b). Results indicated that GSH and GSSG represent approximately 53% and 47%, respectively, of the total chromaffin granule glutathione (Figure 9c). The estimated concentration of reduced glutathione, 0.254 nmol of GSH/mg of granule protein, predicts GSH at millimolar concentrations within chromaffin granules in vivo which would allow effective activation of PTP for processing proenkephalin into active enkephalin neuropeptides.

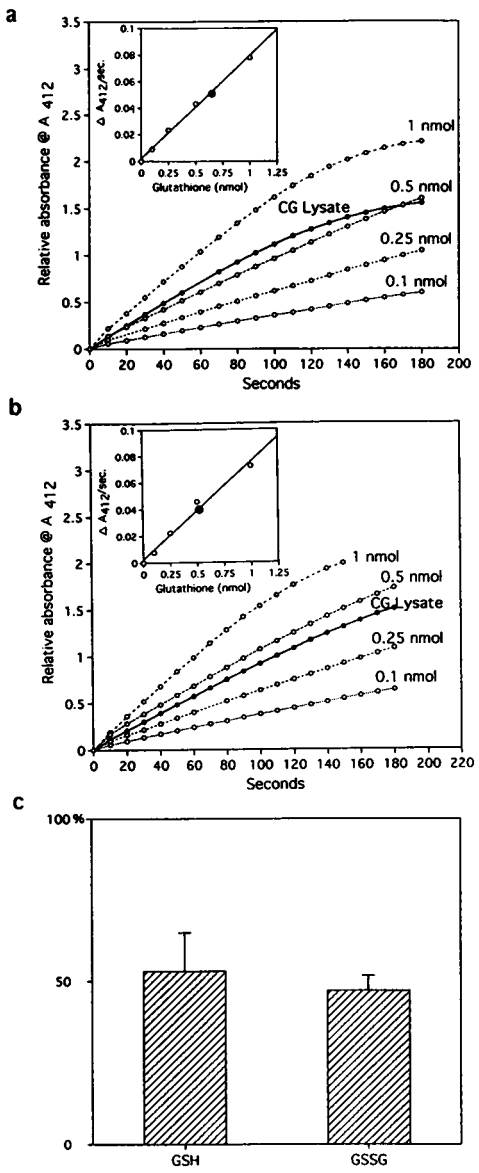

FIGURE 9: Glutathione content in chromaffin granules. Panel a: Total glutathione (GSH + GSSG) in chromaffin granules. Total glutathione (GSH + GSSG) in chromaffin granules was determined by comparing the initial rates of TNB formation, detected spectrophotometrically at 412 nm, from chromaffin granule extracts (25 μL corresponding to 375 μg of protein) and from standard amounts of glutathione (0.1, 0.25, 0.5, and 1.0 nmol of glutathione). The inset shows the standard curve of glutathione measured as ΔA_{412} per second, as well as the initial rate of TNB formation generated by the chromaffin granule sample. Control assays without glutathione or granules had low blank values that were less than 10% of those measured for glutathione (data not shown). Panel b: Oxidized glutathione (GSSG) in chromaffin granules. The content of oxidized glutathione (GSSG) in chromaffin granules was determined by comparing the initial rates of TNB formation, detected at 412 nm, by granule extracts and from standard amounts of glutathione (0.1, 0.25, 0.5, and 1.0 nmol). The inset shows the standard curve of glutathione measured as the rate of TNB formation, ΔA_{412} per second, and shows the initial rate of TNB formation generated by the granule sample. Panel c: Comparison of reduced GSH and oxidized GSSG content in chromaffin granules. The percentages of total chromaffin granule glutathione as reduced GSH and oxidized GSSG are indicated (expressed as the mean ± sem, $n = 3$). GSH and GSSG contents in chromaffin granules were 254 and 243 nmol/mg of granule protein, respectively. When NEM (10 mM) was included in buffers during isolation of chromaffin granules to prevent oxidation of GSH, the same levels of chromaffin granule GSH were measured (data not shown), thus indicating accurate determination of reduced glutathione content in chromaffin granules.

DISCUSSION

The prohormone thiol protease (PTP) represents the major proenkephalin processing enzyme activity in secretory vesicles of adrenal medulla (also known as chromaffin granules) (*10*−*13*, *22*). This study provides evidence for the novel finding that PTP exists as a multicatalytic cysteine protease complex that can be activated by endogenous glutathione localized to chromaffin granules. The high molecular mass nature of native PTP of ∼185 kDa was demonstrated by gel filtration chromatography and by a single band of ^{35}S-enkephalin precursor cleaving activity in in situ radiozymogram gel assays conducted under native buffer conditions. In contrast to native radiozymogram gels that indicated a single PTP activity, the presence of SDS (0.1%) during electrophoresis resolved PTP into three cysteine protease activities with apparent molecular masses of 88, 81, and 61 kDa. These three cysteine protease activities cleave at basic residues, demonstrated by cleavage of the substrates Z-Phe-Arg-MCA and Boc-Val-Leu-Lys-MCA by in situ protease assays in SDS−PAGE gels; these results are consistent with PTP processing of proenkephalin at basic residues (*10*−*12*). Preparative native gel electrophoresis indicated that purified PTP consisted of four protein subunits of 66, 60, 33, and 29 kDa; moreover, the four protein subunits of purified native PTP accounted for the three proteolytic activities of the PTP protease complex. These cysteine protease activities require activation by reducing agents; importantly, endogenous secretory vesicle glutathione may serve as the in vivo reducing agent for PTP activity. These results provide evidence that PTP exists as a novel cysteine protease complex, activated by endogenous secretory vesicle glutathione. PTP thus represents a unique secretory vesicle protease complex that participates in the biosynthesis of enkephalins and possibly other neuropeptides.

Previous studies showed that PTP consisted of a 33 kDa protein (*10*) that cleaves proenkephalin and enkephalin-containing peptides at paired basic residues (*10*−*12*) to generate appropriate intermediate and final enkephalin peptide products (Figure 10). However, the observation that native PTP exists as a high molecular mass peak of activity suggested that PTP may be composed of a complex of proteins which includes the 33 kDa polypeptide. In this study, purification of a larger amount of PTP (15 times greater) by a modified series of purification steps consisting of concanavalin A−Sepharose, chromatofocusing, Mono S FPLC ion exchange, and preparative native gel electrophoresis (as described in Experimental Procedures) resulted in purified native PTP (apparently homogeneous) that was composed of four polypeptides of 66, 60, 33, and 29 kDa. Moreover, these four polypeptides displayed the three enkephalin precursor cleaving activities on radiozymogram gels. Therefore, the four candidate protein subunits of purified native PTP can account for the three associated activities of the PTP protease complex.

The electrophoretic mobilities of the four PTP protein subunits and ^{35}S-enkephalin precursor cleaving activities were compared on reducing and nonreducing SDS−PAGE gels. During electrophoresis under reducing conditions, the 66, 60, 33, and 29 kDa subunit proteins were visualized. However, radiozymogram gels showed that no PTP activity was detected after electrophoresis under reducing conditions

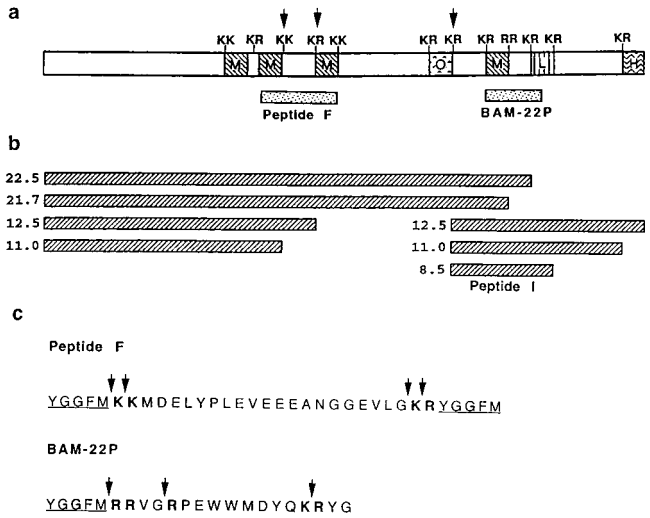

FIGURE 10: PTP processing of proenkephalin at paired basic and monobasic residue cleavage sites. (a) Proenkephalin structure. The structure of proenkephalin is schematically illustrated. Proenkephalin contains four copies of (Met)-enkephalin pentapeptide with the primary sequence YGGFM (M) and one copy of (Leu)-enkephalin corresponding to YGGFL (L), as well as the octapeptide that represents (Met)-enkephalin-Arg^6-Gly^7-Leu^8 (O) and the hexapeptide that represents (Met)-enkephalin-Arg^6-Phe^7 (H). Peptide F and BAM-22P correspond to two of several proenkephalin-derived products that are present within chromaffin granules. (b) PTP processing of proenkephalin. Recombinant proenkephalin was processed by PTP at paired basic residue cleavage sites, resulting in the illustrated high molecular mass intermediate products that reflect those in adrenal medulla in vivo (*12*). The arrows shown above proenkephalin in panel a represent PTP processing at paired basic residue sites within proenkephalin (*10*−*12*). (c) PTP cleavage of enkephalin-containing peptides. PTP processing sites (indicated by arrows) of the enkephalin-containing peptides peptide F and BAM-22P were determined by peptide microsequencing of proteolytic products (*10*, *11*). (Met)-enkephalin sequences are underlined within peptide F and BAM-22P amino acid sequences.

(data not shown), indicating that the individual 66, 60, 33, and 29 kDa proteins (detected on reducing SDS−PAGE gels) alone, under reducing conditions, do not possess proteolytic activity. In contrast, under nonreducing conditions, the four PTP subunits displayed the three characteristic proteolytic activities of apparent molecular masses of 88, 81, and 61 kDa, detected on radiozymogram gels. Protein bands of 88 and 81 kDa corresponded to two of the proteolytic activities of the PTP complex. However, the third proteolytic activity of 61 kDa appears to be associated with a low amount of enzyme protein that was not detectable. These results indicate that the protein subunits of PTP possess different electrophoretic mobilities under reducing compared to nonreducing conditions.

Future studies that will develop specific antibodies to each subunit will be useful for characterizing each subunit. It will be of interest to define which subunits are responsible for proteolytic activity and whether the protease complex includes noncatalytic subunits. Protease complexes, such as the proteosome, may contain regulatory subunits without proteolytic activity (*38*, *39*).

The three activities of the PTP complex were inhibited by E64-c, indicating that all three PTP-associated activities were cysteine proteases. These activities were not inhibited by pepstatin A or EDTA, which inhibit aspartyl protease and metalloprotease, respectively. Like most cysteine proteases, PTP requires reducing conditions for activity, suggesting that

reduced sulfhydryl groups may be important for enzymatic activity. Thus, the PTP complex consists of three associated cysteine protease activities.

Dithiothreitol (DTT) is routinely used as a reducing agent to detect PTP activity, but DTT is not a cellular constituent. Therefore, this study tested known biological reducing agents that could serve as reducing agent(s) for PTP in vivo. Cells utilize several reducing agents for in vivo functions such as glutathione, ascorbic acid, NADPH, thiourea, and others (*34*). In addition, ascorbic acid is present within chromaffin granules where PTP and enkephalins are located (*35*−*37*). Reduced glutathione (GSH), but not oxidized glutathione (GSSG), was an effective activator of PTP-associated protease activities. However, ascorbic acid, NADPH, and thiourea were ineffective. Thus, PTP prefers specific reducing agents to maintain its proteolytic activity.

Glutathione activation of PTP in vivo would require localization of GSH within secretory vesicles that contain PTP and enkephalin peptides. Indeed, chromaffin granules contain high levels of GSH, estimated at 0.25 nmol of GSH/mg of granule protein, which correspond to millimolar concentrations of GSH within chromaffin granules in vivo (*40*). Moreover, PTP is effectively activated by millimolar levels of reduced glutathione. These results, therefore, indicate that GSH is present in chromaffin granules at levels that could activate PTP in vivo.

This first demonstration of glutathione in secretory vesicles also suggests that GSH may serve as a previously unknown reducing agent for secretory vesicle function(s). GSH has been detected in rat brain synaptosomes that consist of nerve endings, which includes synaptic vesicles (*41*). Brain GSH is present at millimolar concentrations in rat (*42*), monkey (*43*), and human (*43*), suggesting that GSH in the central nervous system would be available for PTP in the processing of proenkephalin or other neuropeptide precursors. Glutathione is also present within pancreatic zymogen granules that store and secrete digestive enzymes (*44*). Morevoer, the established role of endogenous glutathione as a protectant against oxidants that mediate cellular damage (*45*, *46*) suggests that the effects of GSH could, in part, be mediated through modified production of peptide neurotransmitters or hormones.

The novel finding that PTP exists as a cysteine protease complex is the first report of a protease complex involved in proneuropeptide processing. Moreover, the polypeptide subunits and the cysteine protease nature of the PTP protease complex differ from those associated with other biologically important protease complexes including the ubiquitin/proteosome complex (*38*, *39*, *48*, *49*), the tricorn protease (*50*), and the ICE proteases (*51*, *52*). Many of these previously characterized protease complexes are involved in proteolysis of multiple proteins. This knowledge suggests that the PTP protease complex could possibly be involved in the proteolysis of multiple polypeptide substrates localized to secretory vesicles. In fact, PTP is capable of processing proenkephalin (*10*−*13*), as well as proneuropeptide Y (*47*). Clearly, it will be of interest in future studies to define the role of the PTP protease complex in the production of biologically active neuropeptides that mediate intercellular communication in the nervous and endocrine systems.

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