Unique cleavage specificity of 'prohormone thiol protease' related to proenkephalin processing

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Abstract

'Prohormone thiol protease' (PTP) represents the major enkephalin precursor processing activity in chromaffin granules. In this study, cleavage specificity of PTP for paired basic and monobasic residues was examined with a series of model peptide-MCA (-methylcoumarinamide) substrates. Monobasic peptides were cleaved at the COOH- and NH_2-terminal sides of the single basic residue. Dibasic peptides, however, were preferentially cleaved at the NH_2-terminal side of the pair, or between the two basic residues, with low cleavage at the COOH-terminal side of the pair. Inhibition by the peptide inhibitor (D-Tyr)-Glu-Phe-Lys-Arg-CH_2Cl provided further evidence for PTP's specificity for the dibasic Lys-Arg site. Inhibition by Z-Leu-Val-Gly-CHN_2 and Z-Arg-Leu-Val-Gly-CHN_2 suggests involvement of Val-Gly in substrate binding to PTP; these two cystatin C-related inhibitors also indicate PTP as a cysteine protease. These results demonstrate PTP's unique cleavage specificity that differs from other processing endopeptidases, including the subtilisin-related proprotein convertases, PC1/PC3, and PC2, as well as the pituitary proopiomelanocortin-converting enzyme, PCE. This study provides further evidence for PTP as a novel prohormone processing enzyme that belongs to the class of cysteine proteases.

Key words: Prohormone processing; Cysteine protease; Proenkephalin; Neuropeptide; Peptide-MCA

1. Introduction

Investigation of proenkephalin processing with recombinant enkephalin precursor has resulted in the identification and purification of a novel 'prohormone thiol protease' (PTP) that represents the major processing enzyme activity for production of (Met)enkephalin in adrenal medullary chromaffin granules [1–3]. PTP is a single-chain 33 kDa glycoprotein with a pI of 6.0, and a pH optimum of pH 5.5. PTP's requirement for dithiothreitol (DTT) and inhibition by iodoacetate, p-hydroxymercuribenzoate, mercuric chloride, and cystatin C, indicate that PTP is a cysteine protease [1]. In vitro processing of recombinant enkephalin precursor by PTP resembles proenkephalin processing in vivo, with production of multiple high molecular weight intermediates that possess the NH_2-terminal segment of the precursor [1]. Importantly, PTP cleaves the enkephalin-containing intermediates, BAM-22P, peptide E, and peptide F, at paired basic and monobasic residues to generate the final (Met)enkephalin product [1–3]. Of interest was the finding that PTP cleaved at the NH_2-terminal side of paired basic residues, as well as between the two basic residues of the pair. In contrast, other prohormone processing enzymes, including the proprotein convertases [4] PC1/3 [5–7] and PC2 [7,8], and the aspartyl protease known as 'proopiomelanocortin converting enzyme' (PCE) [9], cleave primarily at the COOH-terminal side or between the paired basic residues. To obtain further characterization of PTP cleavage site specificity, this study has examined PTP cleavage at dibasic and monobasic sites with model peptide-MCA (methylcoumarylamide) substrates, and has examined the effects of active site-directed peptide inhibitors.

2. Experimental

2.1. Materials

Peptide-MCA substrates and AMC (7-amino-4-methylcoumarin) standard were obtained from Peninsula Laboratories (Belmont, CA). Hog kidney aminopeptidase M was from Sigma (St. Louis, MO). (D-Tyr)-Glu-Phe-Lys-Arg-CH_2Cl was a gift from Dr. Nabil Seidah at the Clinical Research Institute of Montreal, Canada; Z-Leu-Val-Gly-CHN_2 and Z-Arg-Leu-Val-Gly-CHN_2 were gifts from Dr. A. Grubb at the University of Lund, Sweden.

2.2. Purification of PTP

PTP was purified from chromaffin granules of bovine adrenal medulla as described previously [1]. Briefly, PTP from isolated chromaffin granules was purified by chromatography on concanavalin A-Sepharose, Sephacryl S200, Polybuffer exchanger 94, and thiolpropyl-Sepharose [1]. The assay of PTP utilized, as substrate, recombinant enkephalin precursor in the form of [35S](Met)-preproenkephalin ([35S](Met)-

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Abbreviations: PTP, prohormone thiol protease; MCA, methylcoumarinamide; AMC, 7-amino-4-methylcoumarin; APM, aminopeptidase M; -CHN_2, diazomethane; -CH_2Cl, chloromethylketone; Z-, carboxybenzoyl; Boc-, tert-butoxycarbonyl; Bz-, benzoyl; DTT, dithiothreitol; PC, proprotein convertase.

PPE), synthesized from the corresponding cDNA by in vitro transcription and translation as previously described [1,10]. PTP activity in column fractions was detected by measuring the conversion of [^{35}S](Met)-PPE to TCA- (trichloroacetic acid) soluble radioactivity as described previously [1,10].

2.3. PTP cleavage of peptide-MCA substrates

Enzymatic hydrolysis of peptide-MCA substrates was carried out according to the method of Barrett and Kirshke [11]. Peptide-MCA substrates (25 μM final concentration) were each incubated with purified PTP (5 ng/assay) at 37°C for 30 min in a total volume of 160 μl PTP assay buffer (0.1 M sodium citrate, pH 5.0, containing 1 mM DTT, 1 mM EDTA, and 10 mM CHAPS). The reaction was stopped by the addition of 10 μl of 1% TFA (trifluoroacetic acid) and 2 ml H$_2$O; the fluorescent AMC liberated from the peptide-MCA substrate was then measured with a Perkin-Elmer 650-40 fluorimeter with excitation and emission wavelengths of 385 and 465 nm, respectively. Standard AMC was used for quantitation of AMC.

In some experiments, after incubation of peptide-MCA substrates with PTP, the reaction was neutralized with 8 μl of 1.5 M Tris-HCl buffer, pH 8.8, aminopeptidase M (2 μg) was added, and incubation continued for 1 h at 37°C. Reactions were terminated as described above and cleavage of peptide-MCA substrates was fluorimetrically quantitated by measuring production of AMC.

2.4. Determination of PTP kinetic constants (K_m and V_{max}) for Z-Phe-Arg-MCA

Affinity (K_m) and maximal velocity (V_{max}) of PTP was obtained by measuring PTP activity at 1.5-40 μM Z-Phe-Arg-MCA, and by determining kinetic constants by a reciprocal Lineweaver-Burk plot of 1/[S] and 1/v [12], where [S] represents substrate concentration and v is enzyme velocity.

2.5. Rate of PTP inactivation by peptide inhibitors

Rates of PTP inactivation by irreversible chloromethyl ketone and diazomethane peptide inhibitors (D-Tyr)-Glu-Phe-Lys-Arg-CH$_2$Cl, Z-Leu-Val-Gly-CHN$_2$, and Z-Arg-Leu-Val-Gly-CHN$_2$, were determined as described by Barrett et al. [13]. PTP (5 ng/assay) was incubated with inhibitors at pH 5.0 at room temperature; at 5, 10, 15, 20 and 30 min time intervals, 6 μl aliquots were removed, diluted 1:25 in PTP assay buffer, and residual PTP activity was determined by incubation with 100 μM Z-Phe-Arg-MCA for 30 min at 37°C. Second-order rate constants (k_2) were computed by determining the observed rate of inactivation, k, as $0.693/t_{0.5}$. The k_2 was calculated as $k/[I]$, where [I] represents inhibitor concentration [14,15].

3. Results

3.1. PTP cleavage specificity with monobasic and dibasic peptide-MCA substrates

PTP cleavage of several monobasic and dibasic peptide-MCA substrates was highest with the monobasic substrates Z-Phe-Arg-MCA and Bz-Val-Leu-Lys-MCA (Table 1, column 1). PTP activity was 1.7-fold higher with Z-Phe-Arg-MCA than with Bz-Val-Leu-Lys-MCA. PTP also cleaved Bz-Arg-MCA and Boc-Gln-Gly-Arg-MCA, while Ac-Lys-MCA was not cleaved.

PTP cleaved the dibasic peptide-MCA substrates Z-Arg-Arg-MCA, Boc-Gln-Arg-Arg-MCA, Boc-Gly-Arg-Arg-MCA, Z-Arg-Val-Arg-Arg-MCA, Boc-Gly-Lys-Arg-MCA, and Boc-Glu-Lys-Lys-MCA (Table 1, column 1), but with lower activity than with the monobasic peptides Z-Phe-Arg-MCA and Bz-Val-Leu-Lys-MCA. PTP cleaved substrates containing Arg-Arg, Lys-Arg, and Lys-Lys pairs with similar levels of activity (same order of magnitude). Arg in the P$_4$ position [16] of Z-Arg-

Val-Arg-Arg-MCA, a substrate for the processing enzyme furin [17], did not significantly enhance PTP activity. PTP cleavage at dibasic and monobasic sites of these model peptide-MCA substrates is consistent with PTP processing of the enkephalin precursor and enkephalin-containing intermediates BAM-22P, peptide E, and peptide F (Fig. 1).

It must be noted that this fluorimetric assay does not detect peptide-MCA products, since only AMC, and not peptide-MCA products, is fluorimetrically detected. Thus, while PTP activity with peptide-MCA substrates indicates cleavage at the COOH-terminal side of the basic residue adjacent to -MCA, cleavages at the NH$_2$-terminal side of the basic residue(s) would not be detected. Therefore, following PTP hydrolysis, aminopeptidase M (APM) was used to convert peptide-MCA products to free AMC as a means to detect cleavage at the NH$_2$-terminal side of basic residue(s). Assays with PTP plus APM showed several fold higher activity compared to PTP alone (Table 1, column 2), indicating that PTP cleaves at the NH$_2$-terminal side of basic residue(s).

Cleavage at the NH$_2$-terminal side of basic residue(s) was calculated as activity detected in the presence of APM, minus the activity in the absence APM. Comparison of cleavage at COOH- and NH$_2$-terminal sides showed that with monobasic substrates, PTP cleaves both sides of the basic residue (Fig. 2a). Preference for cleaving at either NH$_2$- or COOH-terminal sides appears to depend on the particular monobasic peptide substrate. However, with all dibasic peptide-MCA substrates

Table 1

Hydrolysis of peptide-MCA substrates by PTP, without and with aminopeptidase M

Substrate	Proteolytic activity (μmol AMC/h/mg)	
	−APM	+APM
Monobasic:		
1. Z-Phe-Arg-MCA	1,176	1,915
2. Bz-Arg-MCA	7	24
3. Boc-Gln-Gly-Arg-MCA	6	84
4. Bz-Val-Leu-Lys-MCA	695	885
5. Ac-Lys-MCA	0	3
Dibasic:		
1. Z-Arg-Arg-MCA	16	49
2. Boc-Gln-Arg-Arg-MCA	15	118
3. Boc-Gly-Arg-Arg-MCA	7	42
4. Z-Arg-Val-Arg-Arg-MCA	19	50
5. Boc-Gly-Lys-Arg-MCA	21	73
6. Boc-Glu-Lys-Lys-MCA	7	26

PTP (5 ng/assay) activity was measured with peptide-MCA substrates (25 μM) in the absence and presence of aminopeptidase M (APM), as described in section 2. Control assays showed that these NH$_2$-terminally blocked peptide-MCA substrates were not cleaved by APM alone, and the amount of APM (2 μg/assay) was sufficient to completely hydrolyze Arg-MCA (25 μM).
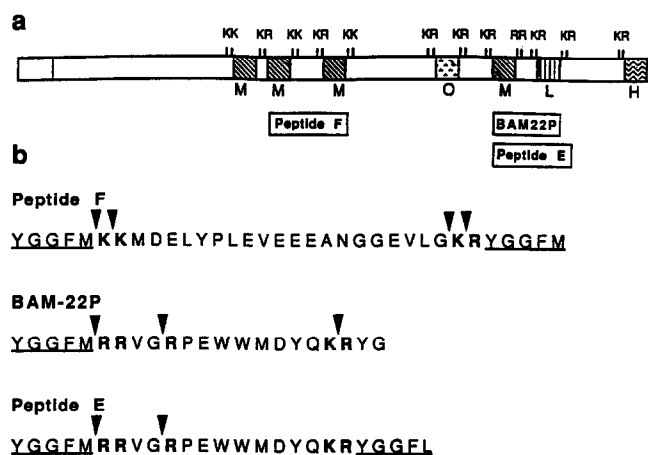
Fig. 1. PTP cleavage sites within enkephalin-containing peptides. (a) Proenkephalin-derived intermediates BAM-22P, peptide F, and peptide E are indicated. The bioactive peptides are represented by: M, (Met)enkephalin (Tyr-Gly-Gly-Phe-Met); L, (Leu)enkephalin (Tyr-Gly-Gly-Phe-Leu); O, M-Arg^6-Gly^7-Leu^8; H, M-Arg^6-Phe^7; K, Lys; R, Arg. (b) Major PTP cleavage sites [1–3] within peptide F, BAM-22P, and peptide E are indicated by arrows. The sequence of (Met)enkephalin, the final product of proenkephalin processing, is underlined. Paired basic residues (KR, KK, RR) and monobasic (R) cleavage sites are indicated by bold letters.

tested, PTP clearly prefers to cleave at the NH$_2$-terminal side of the basic residues (Fig. 2b). PTP cleavage of Z-Arg-Arg-MCA at the NH$_2$-terminal side or between Arg-Arg was 2-fold greater than at the COOH-terminal side of dibasic site (Fig. 2b). Boc-Gln-Arg-Arg-MCA showed 7-fold greater cleavage at the NH$_2$-terminal side of basic residues compared to the COOH-terminal side (Fig. 2b).

3.2. Kinetics with Z-Phe-Arg-MCA as substrate

PTP showed the highest level of activity with Z-Phe-Arg-MCA compared to all other monobasic and dibasic peptide substrates tested (Table 1). Therefore, to assess PTP's affinity (K_m) and maximal velocity (V_{max}) for Z-Phe-Arg-MCA, PTP activity was measured at different concentrations of Z-Phe-Arg-MCA (Fig. 3). PTP demonstrated a K_m of 8 μM and a V_{max} of 5.3 mmol AMC/h/mg with Z-Phe-Arg-MCA.

3.3. Effect of active site-directed peptide inhibitors

Active site-directed peptide inhibitors can provide information concerning the specificity of the protease for specific recognition of amino acid residues at and near the cleavage site of peptide substrates. For studies of the effect of peptide inhibitors, PTP activity was measured with Z-Phe-Arg-MCA as substrate (100 μM). PTP was potently inactivated by the irreversible chloromethane and diazomethane peptide inhibitors (D-Tyr)-Glu-Phe-Lys-Arg-CH$_2$Cl, Z-Leu-Val-Gly-CHN$_2$, and Z-Arg-Leu-Val-Gly-CHN$_2$, as shown by plots of the rates of PTP

inactivation (Fig. 4). Calculation of the second-order rate constant k_2, indicated rapid rates of PTP inactivation by these peptide inhibitors (Table 2).

Inhibition by (D-Tyr)-Glu-Phe-Lys-Arg-CH$_2$Cl provided further evidence for the specificity of PTP for the paired basic residues Lys-Arg. Very potent inhibition of PTP by Z-Arg-Leu-Val-Gly-CHN$_2$ and Z-Leu-Val-Gly-CHN$_2$ (k_2 values of 308,000 and 220,000 M$^{-1}\cdot$s^{-1}, respectively) is consistent with the observation that these peptides resemble PTP's cleavage site, Val-Gly$\downarrow$Arg, within the enkephalin-containing peptides BAM-22P and peptide E [2,3]. These results suggest PTP recognition of the Val-Gly-Arg sequence. In addition, the sequences of Z-Arg-Leu-Val-Gly-CHN$_2$ and Z-Leu-Val-Gly-CHN$_2$ correspond to the Arg$_8$-Leu$_9$-Val$_{10}$-Gly$_{11}$ seg-
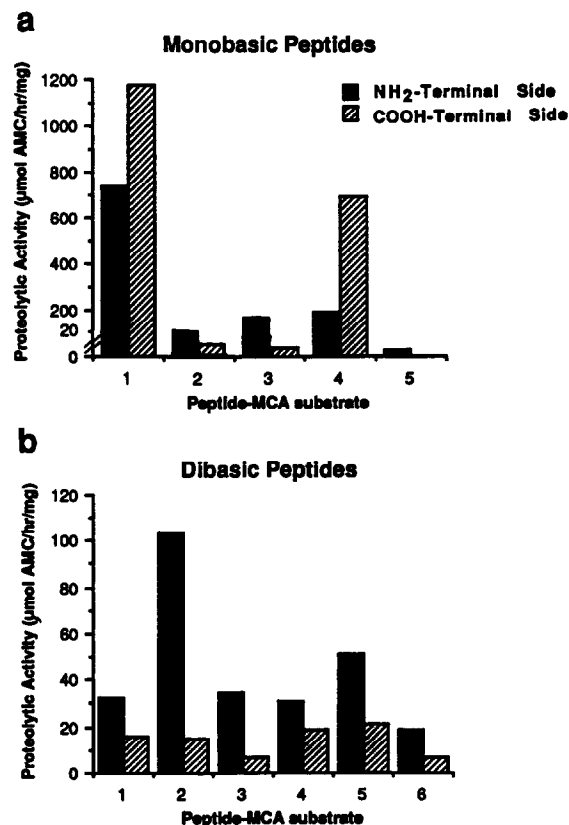

Fig. 2. PTP cleavage at NH$_2$- and COOH-terminal sides of basic residues. (a) Monobasic peptides. Cleavage at NH$_2$-terminal (filled bars) and COOH-terminal (hatched bars) sides of basic residues are represented by PTP activity with APM minus PTP activity without APM, and PTP activity without APM, respectively. Monobasic peptides numbered 1–5 are Z-Phe-Arg-MCA, Bz-Arg-MCA, Boc-Gln-Gly-Arg-MCA, Bz-Val-Leu-Lys-MCA, and Ac-Lys-MCA, respectively (as indicated in Table 1). (b) Dibasic peptides. Cleavage at NH$_2$-terminal (filled bars) and COOH-terminal (hatched bars) sides of dibasic peptides are represented by PTP activity with APM minus PTP activity without APM, and PTP activity without APM, respectively. Dibasic peptides numbered 1–6 are Z-Arg-Arg-MCA, Boc-Gln-Arg-Arg-MCA, Boc-Gly-Arg-Arg-MCA, Z-Arg-Val-Arg-Arg-MCA, Boc-Gly-Lys-Arg-MCA, and Boc-Glu-Lys-Lys-MCA, respectively (as indicated in Table 1). All experiments were performed three times (in duplicate) and the average of PTP activity is indicated; values from triplicate experiments varied by less than 10%.
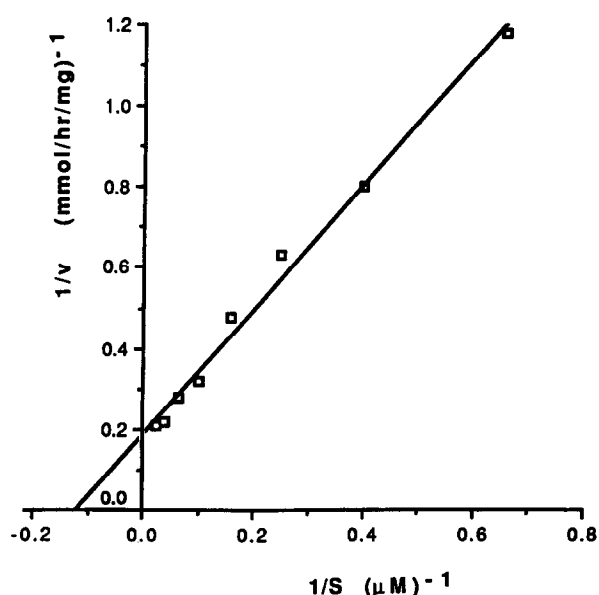
Fig. 3. Lineweaver–Burk plot of PTP with Z-Phe-Arg-MCA. PTP (0.1 ng/assay) was assayed with different concentrations of substrate Z-Phe-Arg-MCA [S]. Kinetic constants K_m and V_{max} were determined by $1/v$ vs. $1/S$.

ment of cystatin C that is essential for selective inhibition of cysteine proteases [18–20], thus, confirming PTP as a cysteine protease.

4. Discussion

Investigation of the cleavage specificity of the 'prohormone thiol protease' (PTP) with peptide-MCA substrates revealed that PTP cleaves at monobasic (Arg and Lys) and paired basic (Arg-Arg, Lys-Arg, Lys-Lys) residues. At monobasic sites, PTP cleaved at both the COOH- and NH$_2$-terminal sides of the single Arg and Lys residues, with preference for either side of the basic residue dependent on the particular peptide substrate. Among the monobasic peptides tested, PTP showed highest activity with Z-Phe-Arg-MCA. Also, PTP seems to prefer mono-arginyl compared to mono-lysyl substrates. At dibasic sites, PTP clearly prefers to cleave at the NH$_2$-terminal side or between the pair of basic residues, with a much lower degree of cleavage at the COOH-terminal side of the paired basic residues. These results show that PTP's cleavage specificity is consistent with its role in processing the enkephalin precursor [1,3] and possibly other prohormones at paired basic and monobasic residues flanking the bioactive peptide within its precursor.

Importantly, these results indicate that PTP's cleavage specificity differs from that of other prohormone processing proteases, including the subtilisin-related proprotein convertases PC1/3 [5–7] and PC2 [7,8], as well as the

'proopiomelanocortin converting enzyme' (PCE) [9]. In contrast to PTP, PC1/3 and PC2 in co-transfection experiments cleave prohormones at the COOH-terminal side of paired basic residues [21–23]. Also, PC1/3 cleaves on the COOH-terminal side of a single Arg [24]. In addition, furin, another subtilisin-related processing protease, cleaves at the COOH-terminal side of the Arg-X-Lys/Arg-Arg motif [17]. The PCE aspartyl protease from bovine pituitary intermediate lobe [9] cleaves dibasic sites of proopiomelanocortin (POMC) between and at the COOH-terminal side of paired basic residues. Recently, a yeast aspartyl proteinase encoded by the YAP3 gene was shown to be involved in processing pro-α-mating factor in KEX2-deficient mutants [25]. Yeast aspartyl protease, YAP3, also cleaves paired basic residues of mammalian POMC [26] and anglerfish prosomatostatin-I [27] between and at the COOH-terminal side of dibasic residues, as well as at the COOH-terminal side of a monobasic site of prosomatostatin-II [27]. Clearly, the cleavage site specificity of PTP differs from other processing enzymes.

Comparison of PTP with several other putative monobasic-cleaving processing enzymes indicates that PTP is the only cysteine protease that cleaves at both monobasic and paired basic residues at an acidic pH of 5.0–5.5, which is consistent with the intragranular environment of pH 5.5–5.8 [1–3]. A putative dynorphin-converting activity represented by a thiol protease cleaves dynorphin B29 at either side of Arg[14], but does not cleave the dibasic Arg-Arg site within dynorphin B29 [28], and, therefore, differs from PTP. Monobasic-cleaving serine proteases involved in processing prosomatostatin [29] and procholecystokinin [30] also differ from PTP since they preferentially cleave at the COOH-terminal side of monobasic residues. These comparisons illustrate that PTP differs from previously reported monobasic-cleaving proteases that may be involved in prohormone processing.

Inhibition of proteases by active site-directed chloromethyl ketone and diazomethane peptide inhibitors provides knowledge of protease specificity for the P_1 position and can indicate residues at the P_2, P_3, or P_4 positions that may be involved in substrate binding. The P_1 residue refers to the amino acid at the NH$_2$-terminal side of the cleaved peptide bond, and P_2 to P_4 are adjacent to P_1 [16]. Inhibition of PTP by (D-Tyr)-Glu-Phe-

Table 2
Effect of peptide inhibitors on PTP activity

Inhibitors (concentration)	k_2 (M$^{-1} \cdot$ s^{-1})
(D-Tyr)-Glu-Phe-Lys-Arg-CH$_2$Cl (10^{-7} M)	10,312
Z-Leu-Val-Gly-CHN$_2$ (3×10^{-9} M)	220,000
Z-Arg-Leu-Val-Gly-CHN$_2$ (3×10^{-9} M)	308,000

Second order rate constant, k_2, was computed from plots of inactivation of PTP by these peptide inhibitors in Fig. 4, as described in section 2.
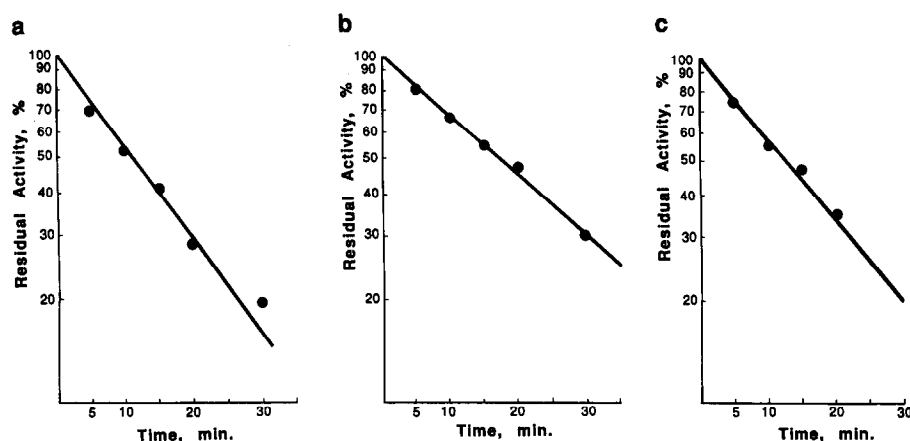
Fig. 4. Inactivation of PTP by peptide inhibitors. Inactivation of PTP by (D-Tyr)-Glu-Phe-Lys-Arg-CH_2Cl (10^{-7} M), Z-Leu-Val-Gly-CHN_2 (3×10^{-9} M), and Z-Arg-Leu-Val-Gly-CHN_2 (3×10^{-9} M) as a function of time of preincubation of PTP with each inhibitor is shown in panels a, b, and c, respectively.

Lys-Arg-CH_2Cl provides additional support for PTP's specificity for the dibasic Lys-Arg site. Potent inhibition of PTP by Z-Leu-Val-Gly-CHN_2 and Z-Arg-Leu-Val-Gly-CHN_2 suggests the involvement of Val-Gly in PTP binding to peptide substrates. These results are consistent with PTP cleavage of BAM-22P and peptide E at the Val-Gly↓Arg sequence (Fig. 1) [2,3]. It is also important to note that Z-Leu-Val-Gly-CHN_2 and Z-Arg-Leu-Val-Gly-CHN_2 are selective and potent inhibitors of cysteine proteases, since their peptide sequences are identical to the protease binding domain of the natural cysteine protease inhibitor, cystatin C [18–20]. Thus, in addition to results of previous studies [1], inhibition of PTP by these cystatin C-related peptide inhibitors provides further support for PTP as a cysteine protease.

Subsequent to endoproteolytic processing of prohormones, resultant peptide intermediates require removal of COOH- and NH_2-terminal residues by carboxypeptidase H (CPH, also known as carboxypeptidase E or enkephalin convertase) [31,32] and an aminopeptidase [33], respectively. However, prohormone intermediates generated by PTP cleavage at the NH_2-terminal side of dibasic or monobasic residues would require only an aminopeptidase, and not CPH, for further processing. Our finding of PTP as the major enkephalin precursor processing enzyme in chromaffin granules suggests that CPH may not be necessary for processing all proenkephalin-derived intermediates. Results of this study also indicate that PTP could generate peptides possessing X-Gly at their COOH-terminus that may serve as substrates for peptidylglycine α-amidating monooxygenases (PAM) [34]. Many neuropeptides require COOH-terminal α-amidation for biological activity. Future studies of the co-regulation of PTP, aminopeptidase, CPH, and PAM will be important in understanding how PTP participates in the post-translational processing of prohormones.

In summary, use of peptide-MCA substrates and active site-directed peptide inhibitors demonstrates that PTP possesses unique cleavage site specificity compared to other prohormone processing enzymes. These results provide further evidence for PTP as a novel prohormone processing enzyme belonging to the class of cysteine proteases.

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