Activation and Characterization of Procarboxypeptidase B from Human Plasma

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ABSTRACT: Recently we reported the isolation and cloning of a novel plasma procarboxypeptidase B that binds plasminogen [Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., & Drayna, D. (1991) *J. Biol. Chem. 266,* 21833−21838]. This plasma procarboxypeptidase is structurally similar to tissue procarboxypeptidases, and initial substrate studies showed that this plasma protein behaves like a basic carboxypeptidase and is now known as human plasma procarboxypeptidase B (pro-pCPB). However, unlike the tissue procarboxypeptidases, pro-pCPB is extremely unstable to trypsin activation. Trypsin cleaves pro-pCPB at two sites: Arg-92 and Arg-330. Cleavage at Arg-92 releases the activation peptide and generates an active enzyme. However, cleavage at Arg-330 inactivates pCPB. This renders the characterization of pCPB difficult. We have found that 6-amino-*n*-hexanoic acid (ϵACA), a competitive inhibitor of basic carboxypeptidases, selectively limits trypsin cleavage of pro-pCPB. In the presence of ϵACA, trypsin cleavage at Arg-330 is significantly limited while the cleavage at Arg-92 is unaffected. Using this approach, active pCPB can now be obtained. Kinetic characterization shows that pCPB behaves like other known basic carboxypeptidases. pCPB is more specific for substrates with C-terminal arginine than those with C-terminal lysine for all the natural and synthetic peptides tested. It also hydrolyzes the synthetic ester substrate more efficiently than the synthetic peptide substrate, especially at high pH. The active site Zn^{2+} can be replaced with other metals with change in substrate specificity. Binding studies using either Lys-plasminogen or Glu-plasminogen with pro-pCPB or pCPB show that pro-pCPB has a 10-fold higher affinity to both forms of plasminogen than pCPB. This suggests that the glycosylated activation peptide mediates the high-affinity binding of pro-pCPB to plasminogen. Ligand blot binding studies show that the binding site for pro-pCPB to plasminogen is inhibited by α_2-antiplasmin, suggesting a similar site of interaction.

Recently, we reported the purification and cloning of a novel procarboxypeptidase B from human plasma that specifically binds plasminogen (Eaton et al., 1991). This human plasma procarboxypeptidase B (pro-pCPB)[1] is synthesized in the liver and released as a glycosylated zymogen into the circulation. pro-pCPB is structurally similar to tissue procarboxypeptidases in that it shares ~40% sequence identity and contains the amino-terminal activation peptide of ~95 residues followed by an ~300 amino acid catalytic domain. The catalytic domains of pCPB and tCPB share ~50% sequence identity (Eaton et al., 1991). Unlike tissue carboxypeptidase B (tCPB), whose function is to serve as a digestive enzyme, the physiological function of pCPB is unknown. However, its binding to plasminogen suggests a role in fibrinolysis.

Initial characterization of pCPB has been hampered by our inability to generate active pCPB by trypsin. Activation of pro-tCPB by trypsin is the result of single-site cleavage that releases the amino-terminal activation peptide (Neurath, 1984, 1990; Pascual et al., 1989; Cox et al., 1962; Reeck & Neurath, 1972; Burgos et al., 1991). The resultant active tCPB is resistant to further trypsin degradation. In contrast, activation of pro-pCPB by trypsin ultimately results in generation of an inactive fragment (Eaton et al., 1991). This inactive fragment is generated by tryptic cleavage at Arg-92 and Arg-330. By analogy to tissue procarboxypeptidases (Neurath, 1984, 1990; Pascual et al., 1989; Cox et al., 1962; Reeck & Neurath, 1972; Burgos et al., 1991), cleavage at Arg-92 releases the activation peptide and initially generates an active enzyme. However, cleavage at Arg-330 results in the loss of a C-terminal fragment of the catalytic domain that inactivates pCPB. Here we report a novel approach that selectively limits trypsin cleavage to allow generation of sufficient active pCPB for characterization. In addition, binding studies of pro-pCPB with plasminogen and other plasma proteins are also presented.

EXPERIMENTAL PROCEDURES

Materials

6-Amino-*n*-hexanoic acid (ϵACA), 5-aminopentanoic acid, 7-aminoheptanoic acid, hippuryl-L-arginine (Bz-Gly-Arg), hippuryl-L-lysine (Bz-Gly-Lys), D-arginine, L-arginine, D-lysine, L-lysine, *n*-hexanoic acid, benzamidine, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (tranexamic acid), *p*-aminobenzoic acid, and carboxypeptidase B type I from porcine pancreas (tCPB) were purchased from Sigma Chemical Co. N^{α}-*p*-tosyl-L-arginine methyl ester (TAME), *N*-[3-(2-furylacryloyl)]-L-alanyl-L-arginine (FA-Ala-Arg), *N*-[3-(2-furylacryloyl)]-L-alanyl-L-lysine (FA-Ala-Lys), hippuryl-L-argininic acid, and bradykinin were obtained from Bachem Bioscience Inc.; Lys^6-Met^5-enkephalin, Arg^6-Met^5-enkephalin, Lys^6-Leu^5-enkephalin, and Arg^6-Leu^5-enkephalin were

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[1] Abbreviations: ϵACA, 6-amino-*n*-hexanoic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; PBS, phosphate-buffered saline; pro-pCPB, plasma procarboxypeptidase B; pCPB, plasma carboxypeptidase B; tCPB, tissue carboxypeptidase B; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; tPA, tissue plasminogen activator.

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from Peninsula Laboratories Inc.; Glu-plasminogen, Lys-plasminogen, α_2-antiplasmin, prothrombin, tissue plasminogen activator, and the plasminogen fragments were obtained from American Diagnostica Inc.; 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was purchased from Boehringer Mannheim. All other chemicals and solvents were of reagent grade or better.

Methods

Purification of Plasma Procarboxypeptidase B (pro-pCPB). Human plasma, 20 units, was fractionated with 0.35 M ammonium sulfate and centrifuged at 5000 rpm for 30 min using a Sorvall RC-3B refrigerated centrifuge. The supernatant was then exhaustively dialyzed against PBS. The dialyzed supernatant was passed through an affinity column (2.5 × 20.0 cm) prepared by coupling pro-pCPB monoclonal antibody to Bio-Rad Affi-Gel 10 according to the manufacturer's specifications. The antibody column was first washed with PBS and then with 10 column volumes of PBS with 1.0 M NaCl. The enzyme was eluted from the column with 0.2 M glycine hydrochloride buffer, pH 2.20. The fractions were analyzed by SDS−PAGE. Fractions containing pro-pCPB were neutralized, combined, and dialyzed overnight against 25 mM Tris-HCl, pH 7.50. The enzyme solution was applied to a Pharmacia Mono Q HR 5/5 column and pro-pCPB eluted with a 0.0−1.0 M NaCl salt gradient in 25 mM Tris-HCl, pH 7.50. Fractions containing pro-pCPB were pooled and subjected to chromatography on a Pharmacia HiTrap protein A column to remove IgG. Following this step the pro-pCPB was homogeneous as judged by SDS−PAGE.

Activation of pro-pCPB and Isolation of the Active Enzyme. Purified pro-pCPB in 50 mM Tris−acetate, pH 7.50, containing 0.01% *n*-octyl β-D-glucopyranoside was incubated with trypsin at a 1:3 trypsin:pro-pCPB (w/w) ratio for 30 min at 37 °C in the presence and absence of 20 mM ϵACA or structural analogs of ϵACA. The reaction was terminated by the addition of AEBSF (1.0 mM, final concentration). Activation was assessed by SDS−PAGE. Active pCPB was isolated from the activation mixture using a Pharmacia Mono S HR 5/5 column eluted with a 0.0−1.0 M NaCl salt gradient in 25 mM Tris-HCl buffer, pH 7.50, containing 5% glycerol, 0.02% *n*-octyl β-D-glucopyranoside, and 1 mM AEBSF. The active fractions were combined and concentrated using a Centricon 10 microconcentrator. The N-terminal amino acid sequence of the pCPB was determined on Applied Biosystems 470A and 473A vapor-phase sequenators as described before (Hewick et al., 1982; Henzel et al., 1987).

The stability of active pCPB to trypsin was examined in the presence and absence of ϵACA. The concentration of trypsin and ϵACA was the same as in the activation study. Aliquots were taken periodically from the incubation mixture for assay of activity and for SDS−PAGE analysis.

Steady-State Kinetics. Initial rates of hydrolysis were measured in 25 mM Tris, pH 7.80, containing 150 mM NaCl at 25 °C in a Kontron Uvikon 860 spectrophotometer equipped with a thermostated six-cell changer. With hippuryl-L-arginine (Bz-Gly-Arg) or hippuryl-L-lysine (Bz-Gly-Lys) or hippuryl-L-argininic acid, the reaction was followed spectrophotometrically at 254 nm and with *N*-[3-(2-furylacryloyl)]-L-alanyl-L-arginine (FA-Ala-Arg) or *N*-[3-(2-furylacryloyl)]-L-alanyl-L-lysine (FA-Ala-Lys) at 340 nm (Clarke et al., 1962; Plummer & Kimmel, 1980). Hydrolysis of bradykinin, Arg^6- or Lys^6-Leu^5-enkephalin, and Arg^6- or Lys^6-Met^5-enkephalin was measured by incubating the substrate (six different concentrations), ranging from 2 mM to 50 μM (depending on the substrate), with pCPB (500 ng) in a final volume of 500 mL at 25 °C for 0 to 120 min. At set time intervals, an aliquot (180 mL) was taken out, the reaction was quenched with 20 mL of 5% trifluoroacetic acid, and a 20-mL sample was analyzed with a Spectra Physics HPLC system. Peptide products were separated on a Synchropak C18 column (250 × 4.6 mm) with an increasing linear gradient of acetonitrile with 0.1% TFA (solvent B) in H_2O with 0.1% TFA (solvent A) at a flow rate of 1.0 mL/min. Peptides were detected at 220 nm. The gradient was 10−70% solvent B in 30 min (Skidgel et al., 1984, 1988). All values presented were averaged from at least three determinations, and the standard deviation was within 5%. The constants K_m and k_{cat} were determined by regression analysis from the data by the method of Lineweaver−Burk. Hydrolysis of hippuryl-L-arginine (peptide substrate) and hippury-L-argininic acid (ester substrate) as a function of pH was examined at 25 °C. The final concentration of the substrate in the assay cuvettes was 1 mM. To ensure constant ionic strength throughout the pH range, a three-buffer system made up of 100 mM ACES, 52 mM Tris, and 52 mM ethanolamine was used (Ellis & Morrison, 1982).

The effect of divalent cations on pCPB activity was also examined. pCPB was incubated without divalent cations or with either 1 mM $CoCl_2$ or 1 mM $CdCl_2$ for 30 min at 25 °C. The activity of the sample was then determined as described above. The concentration of the divalent cations was maintained in the assay cuvettes.

Binding Studies with Plasminogen (Glu or Lys). A Pharmacia BIAcore biosensor system was used to study the interaction between plasminogen (Glu or Lys) with pro-pCPB or pCPB and tissue carboxypeptidase B. Human plasminogen (Glu or Lys) was covalently linked to the hydrogel matrix of the BIAcore sensor chip. The hydrogel matrix was activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-[3-(diethylamino)propyl]carbodiimide (EDC). Plasminogen (Glu or Lys) at 100 μg/mL in 10 mM sodium acetate (pH 4.8) was injected for 15 min at 25 °C with a flow rate of 5 μL/min. The unreacted sites on the matrix were blocked with 1 M ethanolamine hydrochloride. Immobilization of plaminogen (Glu or Lys) on the BIAcore sensor chip resulted in an increase of the resonance signal by about 6000 resonance units (RU). This indicated that 6.0 ng/mm^2 of plasminogen (Glu or Lys) was cross-linked to the hydrogel surface. Binding studies were done at 25 °C, and the running buffer was Tris-buffered saline (10 mM Tris, pH 7.4, 150 mM NaCl) with 0.02% Tween 20 at a flow rate of 2 μL/min. The association and dissociation rate constants were calculated using software provided with the instrument (Karlsson et al., 1991).

For ligand blot binding, pro-pCPB was iodinated using Enzymobeads (Bio-Rad) as described in the manufacturer's instructions. Approximately 5 μg of plasminogen, kringle 1−3, kringle 4, kringle 5, tPA, and prothrombin was resolved on a 4−20% SDS−polyacrylamide gel and transferred to nitrocellulose. After the nitrocellulose was blocked, it was incubated with [^{125}I]pro-pCPB. For the plasminogen blot the incubation was carried out in the presence or absence of

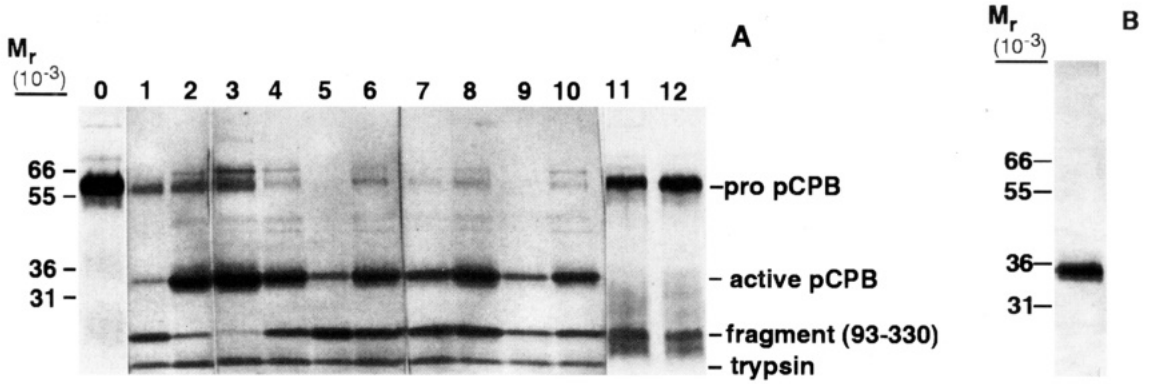

FIGURE 1: (A) Activation of plasma procarboxypeptidase B (pro-pCPB) in the absence and presence of ϵACA or its structural analogs. The conditions for the activation studies are described in Methods. Lanes: (0) pro-pCPB alone, (1) control (pro-pCPB and trypsin), (2) with 6-amino-*n*-hexanoic acid (ϵACA), (3) with 5-aminopentanoic acid, (4) with 7-aminoheptanoic acid, (5) with hippuryl-L-lysine, (6) with hippuryl-L-arginine, (7) with D-arginine, (8) with L-arginine, (9) with D-lysine, (10) with L-lysine, (11) with *trans*-4-aminomethyl)-cyclohexanecarboxylic acid, and (12) with *p*-aminobenzoic acid. (B) Purification of plasma carboxypeptidase B (pCPB). Active plasma carboxypeptidase B (pCPB) was eluted from the Mono S column with a 0.0−1.0 M NaCl salt gradient in 25 mM Tris-HCl buffer, pH 7.50, containing 5% glycerol, 0.02% *n*-octyl β-D-glucopyranoside, and 1 mM AEBSF. The homogeneity of the purification product was examined by SDS−PAGE.

either α_2-antiplasmin (0.5 mg/mL) or ϵACA (200 mM) in blocking buffer for 1 h. Filters were washed three times with blocking buffer and subjected to autoradiography.

For dot blot binding, approximately 10 μg of plasminogen, kringle 1−3, kringle 4, kringle 5, tPA, and prothrombin was blotted on nitrocellulose. After the nitrocellulose was blocked, the blot was incubated with [^{125}I]pro-pCPB in blocking buffer for 3 h. The blot was washed three times with blocking buffer and then subjected to autoradiography.

RESULTS

Activation of pro-pCPB. To circumvent the problem of unwanted proteolysis, a novel approach was employed to activate pro-pCPB. On the basis of the knowledge that pro-pCPB is eluted from the plasminogen affinity column with a high concentration of ϵACA and that ϵACA is a reversible inhibitor for tCPB (Folk, 1956; Folk & Gladner, 1958; Wintersberger et al., 1962), we predicted that ϵACA may alter the cleavage of pro-pCPB by trypsin. Analogs of ϵACA, 5-aminopentanoic acid and 7-aminoheptanoic acid, a series of active site directed compounds, and antifibrinolytic amino acids such as *trans*-4-(aminomethyl)cyclohexanecarboxylic acid and *p*-aminobenzoic acid were also tested.

Figure 1A shows the results of activation of pro-pCPB in the absence and presence of ϵACA or its structural analogs. After 30 min of incubation with trypsin, only the inactive fragment (M_r = 25 000) was found in the control experiment. Activation of pro-pCPB in the presence of ϵACA or its analogs resulted in diminished cleavage at Arg-330 but not at Arg-92. Among all the compounds tested, ϵACA and 5-aminopentanoic acid were the most effective in reducing cleavage at Arg-330. 7-Aminoheptanoic acid, hippuryl-L-arginine, L-arginine, and L-lysine limited cleavage at Arg-330 to a similar extent. The results with ϵACA and the active site directed compounds suggest that a free carboxyl group and a basic side chain are required to limit the unwanted trypsinolysis at Arg-330. Also, because 7-aminoheptanoic acid was less effective than either ϵACA or 5-aminopentanoic acid, the length of the methylene chain separating the free carboxyl group and the basic group may influence the effectiveness of these compounds. In general, the arginine substrates and the arginine isomers are more effective than their lysine counterparts (Figure 1A). This latter observation suggests that pCPB is more specific toward substrates with a C-terminal arginine. The results with the Arg and Lys stereoisomers indicate that the site where all these compounds interact is very specific and likely in the active site region.

Table 1: Activity of Active Plasma Carboxypeptidase B (pCPB) after Trypsin Activation in the Presence or Absence of Active Site Directed Compounds

active site directed compound	activity (μmol/min)[a]	active site directed compound	activity (μmol/min)[a]
none	0.77 ± 0.02	D-arginine	3.4 ± 0.1
6-amino-*n*-hexanoic acid	7.6 ± 0.1	L-arginine	4.6 ± 0.1
5-aminopentanoic acid	6.3 ± 0.2	D-lysine	2.1 ± 0.1
7-aminoheptanoic acid	4.8 ± 0.1	L-lysine	3.3 ± 0.1
hippuryl-L-lysine	2.6 ± 0.1	transexamic acid	1.1 ± 0.03
hippuryl-L-arginine	3.6 ± 0.1	*p*-aminobenzoic acid	0.93 ± 0.04

[a] The substrate used in the assay was hippuryl-L-arginine (1.2 mM, final concentration). The conditions for the activation and for the measurements of enzymatic activity were described in Methods. The activity reflected the amount of active pCPB in each of the activation studies.

Our suggestion that the binding site for ϵACA is at or near the active site pocket of pCPB was further tested using the two antifibrinolytic amino acids: *trans*-4-(aminomethyl)-cyclohexanecarboxylic acid and *p*-aminobenzoic acid which are structurally very similar to ϵACA. Each compound has a free carboxyl group and a basic side chain separated by the same distance (7 Å). However, unlike ϵACA, the antifibrinolytic amino acids are not inhibitors of tissue carboxypeptidases. They also do not limit the cleavage at Arg-330 (Figure 1A), thereby supporting the notion that the binding site for ϵACA is most likely the active site of pCPB.

Table 1 shows the activity of pCPB following each of the activation studies described in Figure 1A. The results compare favorably with those of Figure 1A. Activation of pro-pCPB in the presence of ϵACA or 5-aminopentanoic acid yielded the highest enzymatic activity while the enzymatic activities geneated in the presence of 7-aminoheptanoic acid, hippuryl-L-arginine, L-arginine, D-arginine, and L-lysine were least. Activation with the two antifibrinolytic amino acids, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid and *p*-aminobenzoic acid, yielded the lowest enzymatic activity, showing the inability of these two compounds to reduce

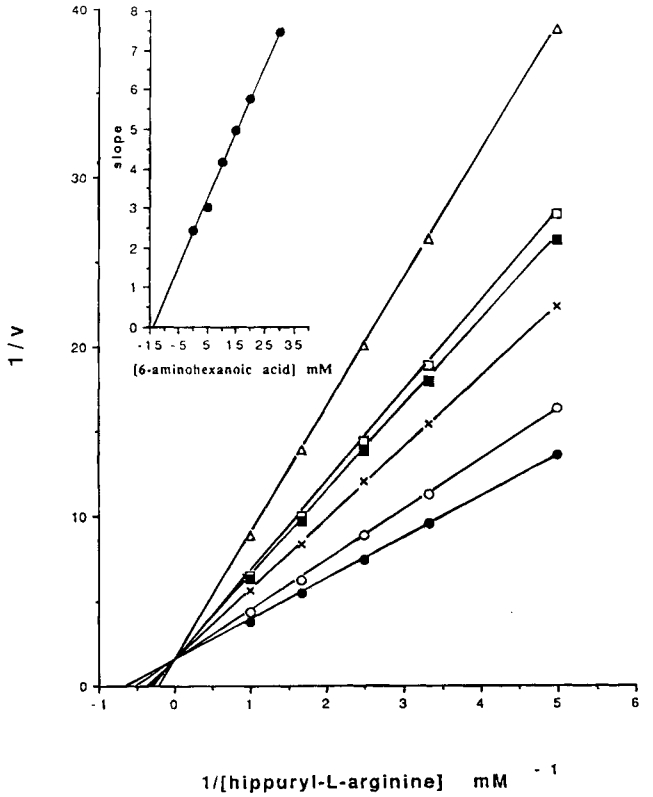

FIGURE 2: Double-reciprocal plots for the inhibition of active pCPB with ϵACA. The inhibitor concentrations were 0 (●), 5.0 (○), 10.0 (×), 15.0 (■), 20.0 (□), or 30 mM (△). The secondary plot (insert) shows that the K_i is 13 mM.

cleavage at Arg-330. Although these two compounds do have a free carboxyl group and a basic side chain, they also have bulky ring systems that apparently hinder binding sterically.

The active pCPB was purified to homogeneity with ion-exchange chromatography (Figure 1B), and the identity of the purified protein was verified by N-terminal amino acid analysis. The specific activities of pCPB with hippuryl-L-arginine and hippuryl-L-lysine as substrate were 64 and 20 units/mg, respectively. These are lower than the values of 70 and 200 units/mg, respectively, obtained with pancreatic tissue basic carboxypeptidase (Folk, 1963). Purified active pCPB was stable to trypsin treatment (2 h at 37 °C) as shown by SDS−PAGE analysis (data not shown).

Figure 2 shows that ϵACA is a weak competitive inhibitor of pCPB with a K_i value of 13 mM. This compares well with our finding that 20 mM ϵACA was the most effective concentration for limiting trypsin cleavage at Arg-330 (data not shown). The data support the notion that binding of ϵACA to the active site of pCPB may result in a conformational change limiting trypsin cleavage at Arg-330. In a control experiment, ϵACA was shown to be a poor inhibitor of trypsin. At the concentration used for activation, ϵACA inhibited about 10% of trypsin activity.

Steady-State Kinetics. Michaelis−Menten kinetics described adequately the kinetics of plasma carboxypeptidase B (pCPB). Kinetic constants for pCPB with synthetic peptide and synthetic ester substrates and the biologically active peptides are listed in Table 2. For the synthetic substrates, the K_m values are very similar for all the peptide substrates, but the k_{cat} values are higher for the arginine substrates. In general, pCPB catalyzes the hydrolysis of the

Table 2: Kinetic Constants for Substrate Hydrolysis by pCPB

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
hippuryl-L-Arg	180 ± 4	26 ± 1	0.144
hippuryl-L-Lys	270 ± 12	15 ± 1	0.0556
FA-Ala-Arg	210 ± 4	36 ± 2	0.171
FA-Ala-Lys	290 ± 14	29 ± 1	0.1
hippurylargininic acid	240 ± 10	169 ± 8	0.704
bradykinin	10 ± 0.2	121 ± 4	12.1
Arg^6-Leu^5-enkephalin	63 ± 1	748 ± 11	11.9
Lys^6-Leu^5-enkephalin	110 ± 1	282 ± 8	2.56
Arg^6-Met^5-enkephalin	140 ± 2	647 ± 10	4.62
Lys^6-Met^5-enkephalin	220 ± 4	290 ± 5	1.32

Table 3: Activity of Plasma Carboxypeptidase (pCPB) in the Presence of Divalent Cations

	hippuryl-L-arginine [μmol min^{-1} (mg of protein)$^{-1}$]	hippuryl-L-argininic acid [μmol min^{-1} (mg of protein)$^{-1}$]
none	64 ± 3	217 ± 7
Co^{2+}	22 ± 1	660 ± 12
Cd^{2+}	201 ± 6	73 ± 3

naturally found peptides more efficiently than the synthetic peptides. The k_{cat}/K_m values show that pCPB has a higher specificity for substrates with a C-terminal arginine.

Similar to other known tissue carboxypeptidases B, pCPB also catalyzes the hydrolysis of hippuryl-L-argininic acid, the synthetic ester analog of hippuryl-L-arginine (Folk, 1963; Zisapel & Sokolovsdy, 1975; Auld & Holmquist, 1974; Auld, 1987). Although the K_m values are very similar, the k_{cat} value for the synthetic ester substrate is much higher than those obtained with the synthetic peptides. Except for bradykin, pCPB catalyzes the hydrolysis of other natural peptides more efficiently than the synthetic ester substrate. The k_{cat}/K_m value for hippuryl-L-argininic acid is 0.704 mM^{-1} s^{-1}, which is 4-fold higher than the best synthetic peptide substrate (FA-Ala-Arg) and 12.5-fold higher than the worst synthetic peptide substrate (hippuryl-L-lysine). However, compared to natural peptides, the k_{cat}/K_m value for the synthetic ester substrate is about $^1/_{20}$th of the two best natural peptides.

The effect of pH on the rate of hydrolysis of hippuryl-L-arginine and hippuryl-L-argininic acid was examined. The observed turnover number for the synthetic peptide substrates was very low, which may be due to the activity not being measured at the pH optimum. With the peptide substrate, pCPB shows a pH optimum in the range of 7.5−8.0. However, with the ester substrate, there is a rather broad range (pH 7.5−9.0) of maximum enzyme activity with an optimum at pH 8.5, indicating that pCPB functions better as an esterase at higher pH. On the basis of the results, it appears that the conditions used to assay the activity were appropriate.

When Zn^{2+} was replaced with either cobalt or cadmium, pCPB displayed activities toward peptide and ester substrate that are different from those of the native zinc enzyme. In accord with published results (Auld & Holmquist, 1974; Auld, 1987; Hendriks et al., 1989; Auld & Vallee, 1987), with Co^{2+} at the active site the hydrolysis of the ester substrate was stimulated while the activity toward the peptide substrate was inhibited. The opposite trend was observed with Cd^{2+} at the active site. The peptidase activity was stimulated while the esterase activity was inhibited (Table 3).

Table 4: Binding Constants for Plasminogens with pro-pCPB, pCPB, and tCPB[a]

	Glu-plasminogen			Lys-plasminogen		
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)
pro-pCPB	$4.0 \times 10^4 \pm 1300$	0.012 ± 0.001	0.3	$1.6 \times 10^5 \pm 8000$	0.0057 ± 0.0007	0.035
pCPB	$2.4 \times 10^4 \pm 1300$	0.048 ± 0.006	2.0	$8.7 \times 10^4 \pm 6000$	0.033 ± 0.004	0.38
tCPB	24 ± 1	0.007 ± 0.008	298	522 ± 26	0.022 ± 0.005	40

[a] Depending on the affinity of the various carboxypeptidases for plasminogen, five serial dilutions (each 5-fold) were made. The starting concentration for pro-pCPB was 500 nM, for pCPB was 5000 nM, and for tCPB was 1 mM. All sensorgrams were recorded at a flow rate of 2 μL/min at 25 °C in Tris-buffered saline (10 mM Tris, pH 7.4, 150 mM NaCl) with 0.02% Tween 20. Regeneration after each binding experiment was performed by washing the flow cell with 5.0 M NaCl, and control experiments were done to show that this was sufficient to remove all the bound protein.

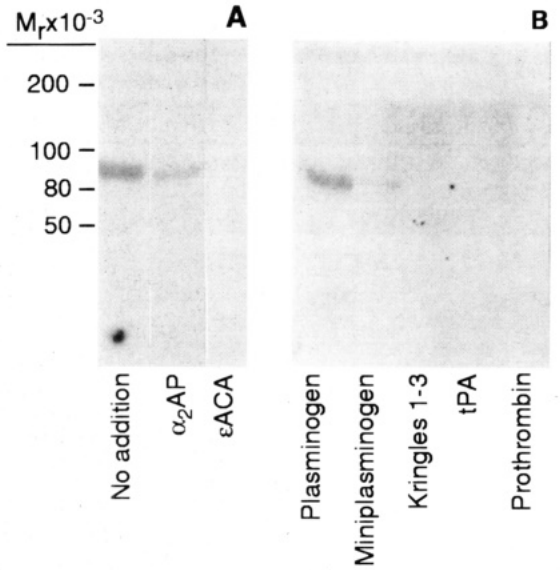

FIGURE 3: (A) Inhibition of [^{125}I]pro-pCPB binding to plasminogen. Binding was carried out in the absence (lane 1) or in the presence of α_2-antiplasmin (lane 2) or ϵACA (lane 3). (B) Lack of binding of [^{125}I]pro-pCPB to plasminogen fragments, tPA, and prothrombin. Lanes: (1) plasminogen, (2) miniplasminogen, (3) kringles 1−3, (4) tPA, and (5) prothrombin.

Plasminogen Binding for Basic Carboxypeptidases. Table 4 shows the binding constants for Glu-plasminogen and Lys-plasminogen with pro-pCPB, pCPB, and tCPB. pro-pCPB has a higher affinity for plasminogen than pCPB. With Glu-plasminogen, the K_D value for pro-pCPB is 7-fold higher than for pCPB, while it is 10-fold higher with Lys-plasminogen. Thus, removal of the glycosylated activation peptide of pro-pCPB reduces the affinity of pCPB for plasminogen. Lys-plasminogen interacts with higher affinity with both forms of pCPB than Glu-plasminogen does. In comparison, we found tCPB to bind weakly to both Lys- and Glu-plasminogen. The interaction between pro-pCPB and plasma proteins such as α_2-antiplasmin, prothrombin, tissue plasminogen activator, and the plasminogen fragments kringle 4, kringles 1−3, and kringle 5-protease was also examined. Both dot blot (data not shown) and ligand blot (Figure 3B) studies show that pro-pCPB only bind to plasminogen and that it only interacts with intact plasminogen and not a fragment of the molecule (Figure 3B). In a separate binding study, α_2-antiplasmin and ϵACA inhibit the binding of pCPB to plasminogen, suggesting that kringle 1 may mediate binding (Figure 3A).

DISCUSSION

In this paper, we present a novel way to selectively limit unwanted proteolysis during the activation of pro-pCPB by trypsin. Activation is carried out in the presence of a reversible inhibitor, 6-amino-*n*-hexanoic acid (ϵACA), which diminishes deleterious cleavage at Arg-330 while allowing the hydrolysis at Arg-92 required to produce active enzyme. It is also worth mentioning that activation of pro-pCPB requires the addition of relatively large quantities of trypsin (protease:pro-pCPB 1:3, by weight) to generate the maximum activity. This contrasts with tissue procarboxypeptidase B, which is quickly activated by a relatively low concentration of trypsin and with no further proteolysis of the active enzyme (Pascual et al., 1989; Cox et al., 1962; Reeck & Neurath, 1972; Burgos et al., 1991).

We further show that ϵACA is a competitive inhibitor for pCPB (K_i = 13 mM), that other structurally similar antifibrinolytic amino acids do not prevent cleavage at Arg-330, and that active pCPB resists further degradation by trypsin. This indicates that ϵACA does not limit trypsin cleavage at Arg-330 through direct interaction with the side chain of Arg-330 but that ϵACA binds noncovalently to the active site of pCPB to produce a conformational change in the protein which results in selectively shielding Arg-330 from the bulk solvent.

Kinetic characterization shows that pCPB behaves similarly to carboxypeptidase N, another known basic carboxypeptidase found in plasma. However, unlike carboxypeptidase N, which is more effective in hydrolyzing substrates with a C-terminal lysine residue (Skidgel, 1988; Levin et al., 1982), pCPB is more specific to substrates with a C-terminal arginine. This suggests the possibility that these two enzymes may work complementary to each other. pCPB hydrolyzes the synthetic ester substrate more efficiently than the synthetic peptide substrates. Kinetic constants (Table 2) show that the difference in synthetic ester and synthetic peptide hydrolysis is mainly due to the change in the rate-determining step. This is based on a higher k_{cat} value for the synthetic ester substrate versus synthetic peptide substrates, with the K_m values being similar for both substrates.

The order of magnitude tighter binding of plasminogen to pro than active pCPB reveals the importance of the activation peptide (Table 4). The glycosylated activation peptide may not only increase the half-life of pro-pCPB in circulation but may also serve as the domain that mediates binding to plasminogen. Significantly, the activation peptides of tissue procarboxypeptidases and pro-pCPB share less than 25% sequence identity, and tCPB is not glycosylated (Eaton et al., 1991). This may explain the low affinity of tCPB for plasminogen. Based on the plasma concentration of pro-pCPB (200−500 nM), it is tempting to speculate that pro-pCPB circulates complexes with plasminogen.

Activation of the proenzyme plasminogen to the active protease plasmin is the key step in fibrinolysis. Activation of plasminogen is largely dependent upon the recognition of the carboxyl-terminal lysines on fibrin or on plasminogen cell surface receptors by lysine-binding sites of the kringle domains of plasminogen (Miles et al., 1991; Pannell et al., 1988). It has recently been demonstrated that removal of these lysine residues with tCPB diminished the fibrinolytic effect of plasmin (Miles et al., 1991). pCPB could play a role in plasminogen activation. pCPB could also serve as a regulator of peptide hormone activity (Table 2). Even so, on the basis of the pCPB interaction with the biologically active peptides tested, we reiterate our previous suggestion that pCPB plays a role in fibrinolysis.

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REFERENCES

Auld, D. S. (1987) in *Enzyme Mechanisms* (Page, I. M., & Williams, A., Eds.) pp 240−258, The Royal Society of Chemistry, Burlington House, London.

Auld, D. S., & Holmquist, B. H. (1974) *Biochemistry 13*, 4355−4360.

Auld, D. S., & Vallee, B. L. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) pp 201−255, Elsevier, New York.

Burgos, F. J.; Salvà, M., Villegas, V., Soriano, F., Mendez, E., & Avilés, F. X. (1991) *Biochemistry 30*, 4082−4089.

Clarke, E., Schirmer, W. E. W., & Folk, J. E. (1962) *J. Biol. Chem. 237*, 3094−3099.

Cox, D. J., Wintersberger, E., & Neurath, H. (1962) *Biochemistry 1*, 1078−1082.

Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., & Drayna, D. (1991) *J. Biol. Chem. 266*, 21833−21838.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 62*, 405−426.

Folk, J. E. (1956) *J. Am. Chem. Soc. 78*, 3541−3543.

Folk, J. E. (1963) *Enzymes*, 57−59.

Folk, J. E., & Gladner, J. A. (1958) *J. Biol. Chem. 231*, 379−393.

Hendriks, D., Scharpé, S., van Sande, M., & Lommaert, M. P. (1989) *J. Clin. Chem. Clin. Biochem. 27*, 277−285.

Henzel, W. T., Rodriguez, H., & Watanabe, C. (1987) *J. Chromatogr. 404*, 41−52.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1982) *J. Biol. Chem. 256*, 7990−7997.

Karlsson, R., Michaelsson, A., & Mattson, A. (1991) *J. Immunol. Methods 145*, 229−240.

Levin, Y., Skidgel, R. A., & Erdös, E. G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4618−4622.

Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., & Plow, E. F. (1991) *Biochemistry 30*, 1682−1691.

Neurath, H. (1984) *Science 224*, 350−356.

Neurath, H. (1990) in *Proteolytic Enzymes: a practical approach* (Beynon, R. J., & Bond, J. S., Eds.) pp 1−13, IRL Press, New York.

Pannell, R., Black, J., & Gurewich, V. (1988) *J. Clin. Invest. 81*, 853−859.

Pascual, R., Burgos, F. J., Salvà, M., Soriano, F., Mèndez, E., & Avilés, F. X. (1989) *Eur. J. Biochem. 179*, 609−616.

Plummer, T. H., Jr., & Kimmel, M. T. (1980) *Anal. Biochem. 108*, 348−353.

Reeck, G. R., & Neurath, H. (1972) *Biochemistry 11*, 3947−3955.

Skidgel, R. A. (1988) *Trends Pharmacol. Sci. 9*, 299−304.

Skidgel, R. A., Johnson, A. R., & Erdös, E. G. (1984) *Biochem. Pharmacol. 33*, 3471−3478.

Skidgel, R. A., Deddish, P. A., & Davis, R. M. (1988) *Arch. Biochem. Biophys. 267*, 660−667.

Wintersberger, E., Cox, D. J., & Neurath, H. (1962) *Biochemistry 1*, 1069−1077.

Zisapel, N., & Sokolovsdy, M. (1975) *Eur. J. Biochem. 54*, 541−547.

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