

Plasmin alters the activity and quaternary structure of human plasma carboxypeptidase N

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Human CPN (carboxypeptidase N) is a tetrameric plasma enzyme containing two glycosylated 83 kDa non-catalytic/regulatory subunits that carry and protect two active catalytic subunits. Because CPN can regulate the level of plasminogen binding to cell surface proteins, we investigated how plasmin cleaves CPN and the consequences. The products of hydrolysis were analysed by activity assays, Western blotting, gel filtration and sequencing. When incubated with intact CPN tetramer, plasmin rapidly cleaved the 83 kDa subunit at the Arg⁴⁵⁷–Ser⁴⁵⁸ bond near the C-terminus to produce fragments of 72 and 13 kDa, thereby releasing an active 142 kDa heterodimer, and also cleaved the active subunit, decreasing its size from 55 kDa to 48 kDa. Further evidence for the heterodimeric form of CPN was obtained by re-complexing the non-catalytic 72 kDa fragment with recombinant catalytic

subunit or by immunoprecipitation of the catalytic subunit after plasmin treatment of CPN using an antibody specific for the 83 kDa subunit. Upon longer incubation, plasmin cleaved the catalytic subunit at Arg²¹⁸–Arg²¹⁹ to generate fragments of 27 kDa and 21 kDa, held together by non-covalent bonds, that were more active than the native enzyme. These data show that plasmin can alter CPN structure and activity, and that the C-terminal 13 kDa fragment of the CPN 83 kDa subunit is a docking peptide that is necessary to maintain the stable active tetrameric form of human CPN in plasma.

Key words: carboxypeptidase, kininase I, leucine-rich repeat, plasmin, plasminogen activation, proteolysis.

INTRODUCTION

Zinc metallo-carboxypeptidases can be clearly divided into two different groups when categorized based on sequence identity – those that are similar to pancreatic CPA (carboxypeptidase A) and CPB (the CPA/B subfamily), and others having properties and sequences closer to those of CPN and CPE (the ‘regulatory’ or CPN/E subfamily) [1,2]. Alternatively, they can be classified by their substrate specificity; CPA-type enzymes preferentially cleave C-terminal hydrophobic residues, and CPB-type enzymes readily cleave only C-terminal Arg or Lys residues [1,3]. Interestingly, all active CPN/E subfamily members are of the CPB type, exhibiting a strict specificity for C-terminal Arg or Lys residues [1,2,4]. However, in spite of similar specificities, their functions differ, depending largely on their enzymatic properties and localization [5]. In the circulation, the two known CPs have CPB-type specificity, and are synthesized in and secreted by the liver into the blood; CPN (kininase I; anaphylatoxin inactivator; lysine CP; EC 3.4.17.3) was the first member of the CPN/E subfamily to be discovered, and CPU [plasma CPB; thrombin-activatable fibrinolysis inhibitor (TAFI) CPR; EC 3.4.17.20] is a member of the CPA/B subfamily [1,6–10]. CPU in plasma is an inactive proenzyme of 60 kDa bound to plasminogen [11]. It is proteolytically activated during coagulation to the active enzyme of 35 kDa by the thrombin–thrombomodulin complex [9,10].

CPN was discovered in the early 1960s as an enzyme that cleaves bradykinin, and thus has also been called kininase I [12]. It exists as a 280 kDa tetramer in the plasma at a concentration of approx. 100 nM [1,6,13–15]. It is made up of two catalytic sub-

units, ranging in size from 48 to 55 kDa, and two regulatory/non-catalytic subunits of 83 kDa each [13–17]. (The calculated molecular mass of the catalytic subunit based on its sequence is 50 kDa. However, in SDS/PAGE, the purified enzyme exhibits two major forms of 48 kDa and 55 kDa. The 55 kDa form represents the complete protein, whereas the 48 kDa form is generated by *in vivo* proteolytic cleavage at the C-terminus, which contains many basic residues. As the catalytic subunit is not glycosylated, the reason for the somewhat higher molecular mass in SDS/PAGE is unknown, but may relate to its high hydrophobicity.) The regulatory subunits are glycosylated, functioning to carry and stabilize the catalytic subunits in the circulation [1,16,17]. The 83 kDa subunit can affect the ability of CPN to interact with certain substrates (e.g. anaphylatoxin C3a) and inhibitors (protamine) [1,18]. CPN is constitutively active and plays an important role in inactivating potent peptide hormones such as kinins and anaphylatoxins that are released into the circulation [6,12,19]. In a patient, genetically determined low levels of plasma CPN were associated with recurrent bouts of angioedema, probably due to enhanced levels of intact kinins [20]. Recently, a frameshift mutation was identified in this patient’s DNA encoding exon 1 of the catalytic subunit, the possible cause of the low CPN level [21].

The plasminogen system is involved in thrombolysis/fibrinolysis, extracellular matrix degradation, tissue remodelling and repair. The binding of plasminogen to C-terminal Lys residues on cell surface proteins and fibrin clots enhances its rate of activation up to 1000-fold [7,22–24]. Thus CPU and CPN, because they cleave C-terminal Lys residues, can down-regulate plasminogen activation [7,24]. Whereas CPU reduces plasminogen binding

Abbreviations used: BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium; CP, carboxypeptidase; dansyl-Ala-Arg, 5-dimethylamino-naphthalene-1-sulphonyl-L-alanyl-L-arginine; DFP, di-isopropyl fluorophosphate; LRR, leucine-rich repeat; TAFI, thrombin-activatable fibrinolysis inhibitor; TBS, Tris-buffered saline; tPA, tissue plasminogen activator.

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both on cell surfaces and in fibrin clots, for unknown reasons the effect of CPN is restricted largely to removal of C-terminal Lys residues of plasma membrane proteins [7,24]. Because these types of proteins constitute the major cellular plasminogen receptors, CPN decreases plasminogen binding to cells [24] and may thereby decrease extracellular matrix degradation and cellular migration [7,25]. For example, freshly isolated monocytes, exposed to CPN in the plasma, bind approx. 30-fold lower levels of plasminogen than monocytes cultured for 18 h or more [26]. In addition, proteolytically cleaved CPN was reported to be more effective in reducing cellular plasminogen binding than native CPN [24].

Although it is known that CPN is sensitive to cleavage by plasmin and other serine proteases [16,27], the cleavage sites in CPN have never been identified and the effects of proteolysis on the activity and quaternary structure of CPN have not been carefully investigated. Because plasmin could indirectly alter the generation of more plasmin from plasminogen by hydrolysing CPN, we investigated in detail how plasmin cleaves CPN and how this affects CPN's structure and activity. In the present study, we have identified the cleavage sites in both the regulatory and catalytic subunits of CPN, and show that hydrolysis of the 83 kDa subunit results in dissociation of the tetramer into an active heterodimer and that the catalytic subunit is even more active after a covalent bond is split. The facts that plasmin is a critical component of the coagulation/fibrinolytic system and that CPN is considered a life-sustaining plasma enzyme [4,13] render the study of the interaction of the human enzymes particularly relevant.

EXPERIMENTAL

Materials

Aprotinin, BSA, gelatin, soybean trypsin inhibitor, *o*-phenanthroline, BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium) and D-Val-Leu-Lys-*p*-nitroanilide were from Sigma. Human plasminogen, plasmin and tPA (tissue plasminogen activator) were from Calbiochem. Hepes, Triton X-100, Tween-80 and chloroform were from Fisher Scientific. The CPN substrate dansyl-Ala-Arg (5-dimethylaminonaphthalene-1-sulphonyl-L-alanyl-L-arginine) was synthesized as described [28]. Polyclonal anti-plasminogen antibody was purchased from Biodesign International. Polyclonal antiserum to CPN and its isolated subunits was elicited in rabbits as described [16], and IgG fractions were separated by chromatography on Protein A-Sepharose.

Purification of CPN and its subunits

CPN was purified from outdated human plasma by ion exchange chromatography followed by affinity chromatography on an arginine-Sepharose column as described previously [17]. The individual subunits were isolated from native CPN by treatment of the enzyme with 3 M guanidine/HCl followed by gel filtration chromatography using a modification [17] of the original method [16].

In some experiments, the recombinant catalytic subunit of CPN was used. The cDNA of the catalytic subunit of CPN (a gift from Dr Wolfgang Gebhard, University of Munich, Klinikum Grosshadern, Munich, Germany) was cloned into the open reading frame of yeast secretion expression vector pPIC9 (Invitrogen) after deletion of its own signal peptide coding sequence. This resulted in a construct, confirmed by sequencing, with the *Saccharomyces cerevisiae* α -factor secretion signal coding sequence upstream of that coding for the mature N-terminal sequence of the catalytic subunit of CPN: ... *LEKREAEAYVEFDKLAFRHHRY* ... (yeast α -factor sequence is shown in italics, and CPN catalytic subunit sequence is in bold). Normal yeast

processing of the α -factor sequence would predict cleavage of the Arg-Glu bond by Kex2 and then sequential removal of the two Glu-Ala dipeptides by Ste13, resulting in a catalytic subunit with extra N-terminal residues with a sequence of YVEFDKLAFR ... instead of the native N-terminal sequence of VTFR ... This modification resulted from the use of available restriction sites to generate the construct. CPN50-pPIC9 DNA was linearized with SalI and transfected into GS115 (*Pichia pastoris*). The recombinant strains were selected by growing transformed cells on histidine-deficient plates. Each purified yeast strain was then tested for expression on a small scale. Strains that secreted high amounts of CP activity at pH 7.4 with dansyl-Ala-Arg as substrate were then used for large-scale expression.

Single colonies of CPN50-GS115 yeast were gradually amplified in buffered glycerol/complex medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % yeast nitrogen base, 1 % glycerol) to 1000 ml in 3 days by changing the medium and increasing the volume every 24 h. Yeast cells were then collected by centrifugation and resuspended in 400 ml of buffered methanol/complex medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % yeast nitrogen base, 3 % methanol). Expression was carried out in 2000 ml shaking flasks at 30 °C and > 225 rev/min. After 48–60 h, the expression medium was collected and tested for CP activity.

To purify the expressed catalytic subunit, ammonium sulphate (40 % saturation) was used to precipitate > 90 % of the activity from the medium. The precipitate was collected by centrifugation and then dissolved in PBS. The activity in the supernatant was precipitated again at 30 % ammonium sulphate, and the resulting pellet was dissolved in 20 % ammonium sulphate in PBS. After centrifugation at 16 000 rev./min for 30 min, the supernatant (containing 90 % of the activity) was loaded on to a butyl-Sepharose column (Pharmacia) equilibrated with 20 % ammonium sulphate in 20 mM Mes, pH 7, and eluted with a reverse linear gradient of ammonium sulphate from 20 % to 0 % in 20 column volumes. The active fractions were pooled and loaded on to a *p*-aminobenzoylarginine-Sepharose affinity column [29] equilibrated with 20 mM Mes, pH 7.0. The column was washed extensively first with the equilibrating buffer, and then with 0.5 M NaCl in the same buffer. The recombinant 50 kDa protein was eluted from the column with 1 mM guanidinoethylmercaptosuccinic acid in the washing buffer, concentrated and then purified further by gel filtration on a Hiloal 16/60 Superdex 200 FPLC column (Pharmacia). The purified protein was highly active, with a specific activity of 50 μ mol/min per mg with 200 μ M dansyl-Ala-Arg, and N-terminal sequencing gave the sequence predicted above, i.e. YVEFDKLAFR.

SDS/PAGE and Western blot analysis

Samples were boiled for 5 min in sample buffer (50 mM Tris/HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0.1 % Bromophenol Blue) without dithiothreitol, and the proteins were separated using a 9 % or 12 % (w/v) polyacrylamide gel in the presence of SDS. The gel was then electroblotted on to a PVDF membrane and blocked with 3 % (w/v) BSA in TBS (Tris-buffered saline; 0.02 M Tris, pH 7.4, containing 0.5 M NaCl). The membrane was incubated with either polyclonal anti-CPN IgG at a concentration of 4 μ g/ml or anti-plasminogen antiserum at a 1:4000 dilution (in 1 % BSA/TBS) at 25 °C overnight and then washed four times with TBS containing 0.1 % Tween-20. The blot was then incubated for 4 h at room temperature with a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase at a 1:30 000 dilution in 1 % BSA/TBS. The blot was washed and the bands developed with the alkaline phosphatase substrate BCIP/NBT. The reaction was stopped by

rinsing with distilled water, and the developed blots were quantified by densitometric scanning.

Proteolysis of CPN

CPN and plasmin (44 nM each) were mixed in 90 μ l of 0.1 M Hepes, pH 7.4, containing 0.01 % Tween-80 and incubated at 37°C for various times (0–5 h). Aprotinin (2 μ g in 10 μ l) was added to terminate plasmin activity. In controls, aprotinin was added at the beginning of the 37°C incubation and 10 μ l of water was added at the end. CPN (33 nM) was incubated with trypsin (0.65 nM; a 1:50 trypsin/CPN ratio) for various times and the reaction was stopped with 1 μ M soybean trypsin inhibitor. In control experiments, soybean trypsin inhibitor was added at the beginning. Aliquots were assayed for CPN activity and analysed by SDS/PAGE and Western blotting.

For N-terminal sequencing of proteolytic fragments, CPN (100 pmol) was digested with plasmin (33 pmol) in a final volume of 45 μ l of 0.1 M Hepes, pH 7.4, containing 0.01 % Tween-80 for 15 h at 37°C. The proteins were separated by SDS/12%–PAGE and blotted on to a PVDF membrane. The membrane was then stained briefly (30 s) with Coomassie Blue to identify the protein bands, which were cut out and sequenced by the Protein Sciences Facility at the University of Illinois at Urbana Champaign. For determination of the trypsin cleavage site in the active subunit, the catalytic subunit was first isolated from intact CPN as described [16,17]. The catalytic subunit (1 nmol) was incubated with 20 pmol of trypsin in 500 μ l of 0.1 M Tris/HCl, pH 8.0, for 1 h at 37°C, and the reaction was stopped with 2.5 μ g of aprotinin in 2.5 μ l. The protein fragments were separated on a Waters HPLC system using a μ Bondapak C18 column and eluted with a 30 min linear gradient of 30–80 % (v/v) acetonitrile in water containing 0.05 % trifluoroacetic acid. The 21 kDa fragment, which eluted at 25.5 min (as confirmed by SDS/PAGE), was collected, lyophilized and sequenced by the Protein Research Laboratory of the University of Illinois at Chicago.

The CPN fragments generated by plasmin were also analysed by gel filtration. CPN (7 pmol) was incubated at a 1:1 molar ratio with plasmin for 30 min or 3 h at 37°C in 0.1 M Hepes, pH 7.4, containing 0.01 % Tween-80 (final volume 45 μ l) and then 1 mM DFP (di-isopropyl fluorophosphate) to inhibit plasmin. Duplicate reactions were pooled, centrifuged briefly at 14000 *g* and then applied to a Superdex 200HR column (Pharmacia), equilibrated with 50 mM Hepes (pH 7.4) containing 0.2 M NaCl with or without 0.5 % CHAPS. Addition of 0.5 % CHAPS reduced the non-specific binding of protein to the column and enhanced recovery, but otherwise did not alter the profile of protein or the enzyme activity eluted (results not shown). CPN and plasmin activities in the fractions were assayed and the protein peaks were pooled, concentrated by ultrafiltration and analysed by SDS/PAGE and Western blotting.

CPN proteolysis during plasminogen activation by tPA was investigated after preincubation of human Glu-plasminogen and CPN (30 nM each) for 15 min at 25°C in 100 μ l of 0.1 M Hepes buffer, pH 7.4, containing 0.01 % Tween-80 and 0.05 % gelatin. Then tPA (3 nM) was added to start the reaction at 37°C. Aliquots were removed at 30 and 60 min for Western blotting with antibody to CPN.

Immunoprecipitation experiments

CPN (32 nM) was treated with plasmin (32 nM) for 30 min at 37°C in 45 μ l of 0.1 M Hepes buffer, pH 7.4, containing 0.01 % Tween-80 and 1 mM DFP was added to stop the reaction. Polyclonal IgG antibodies to the 83 kDa subunit (30 μ g), catalytic subunit (60 μ g) or control rabbit IgG (50 μ g) were then added to

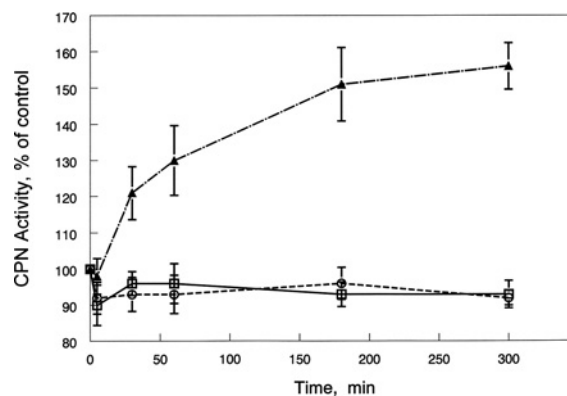


Figure 1 Effect of plasmin on CPN activity

CPN (44 nM) was incubated with plasmin (44 nM) at 37°C. Aliquots were taken at various time points, then aprotinin was added to inhibit plasmin and samples were assayed for CPN activity (\blacktriangle). In control experiments, aprotinin (200 μ g/ml) was added before incubation (\circ), or CPN was incubated alone (\square). Results are the means \pm S.E.M. of four experiments.

the mixtures and incubated at 4°C for 2 h with mixing. Protein A–Sepharose (20 μ l) was then added to a total volume of 100 μ l and incubated for 2 h at 4°C with mixing. The Protein A–Sepharose complexes were pelleted by centrifugation (8000 *g* for 10 min) and CPN activity in the supernatant was assayed.

CP activity

CP activity was measured with dansyl-Ala-Arg as substrate in 0.1 M Hepes, pH 7.5, containing 0.1 % Triton X-100 [28], and fluorescence was measured in a Perkin Elmer spectrofluorimeter at excitation and emission wavelengths of 340 and 495 nm respectively.

RESULTS

Effect of plasmin treatment on CPN activity

To determine whether plasmin alters the activity of intact CPN, equimolar amounts of plasmin and CPN were incubated at 37°C, and aliquots were taken at various times and assayed with dansyl-Ala-Arg as substrate (Figure 1). Plasmin enhanced CPN activity in a time-dependent manner, increasing it to approx. 130 % of control levels within 1 h and reaching a maximum of approx. 150 % after 3 h (Figure 1). In controls, the activity of CPN alone or CPN plus plasmin in the presence of the added inhibitor aprotinin remained constant (Figure 1), indicating that plasmin enhanced CPN activity by proteolysis, not by simple binding. However, because plasmin and CPN were incubated at a 1:1 ratio, it is possible that a binding interaction, mediated through the plasmin active site, would be inhibited by a plasmin inhibitor. To address this question, plasmin was added to CPN at a 1:1 molar ratio in two sets of reactions, which were allowed to incubate for 3 h (at which time the activation of CPN is essentially complete). Then, aprotinin was added to one set of reactions, to inhibit further plasmin activity (control), and then a second aliquot of CPN was added (to yield a plasmin:CPN ratio of 1:2) to both series, and the incubation was continued for an additional 3 h, at which time CPN activity was measured. CPN activity in the sample without aprotinin increased further to 144 % of that in the control (plasmin + aprotinin) sample (average value from two separate experiments), similar to the increase in CPN activity seen in the first 3 h incubation. If the activation had been due to a stoichiometric 1:1 binding interaction, the second aliquot of

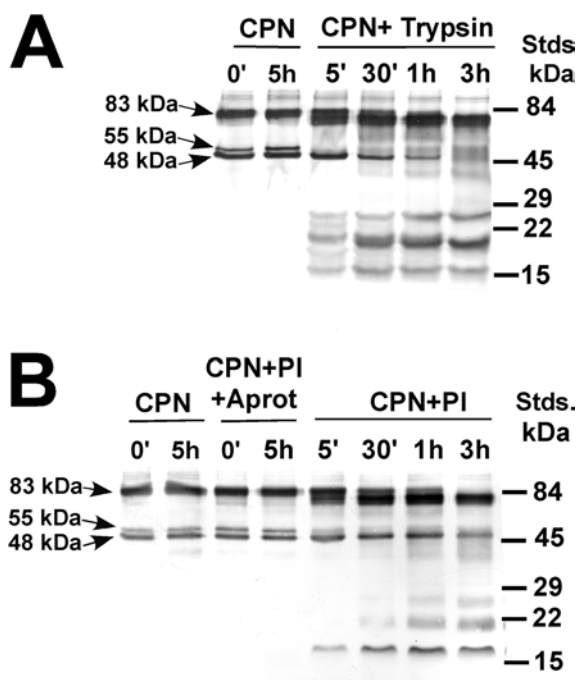


Figure 2 Western blot of CPN cleaved by trypsin or plasmin

Samples of CPN treated with trypsin or plasmin were analysed by Western blotting using anti-CPN IgG as the primary antibody. **(A)** CPN (33 nM) was incubated with trypsin (0.7 nM) at 37°C for various times and the reaction was stopped with 1 μ M soybean trypsin inhibitor. CPN incubated alone (5 h) or without incubation (0') was used as controls. **(B)** CPN (44 nM) was incubated with 44 nM plasmin (PI) at 37°C and the reaction was stopped with aprotinin (200 μ g/ml) at various times. In control experiments, CPN was analysed without incubation (0') or after 5 h of incubation alone, or aprotinin was added prior to incubation (CPN + PI + Aprot).

CPN should not have been activated further, as the plasmin would already be bound. These data indicate that plasmin increases CPN activity by catalytically cleaving it as a substrate.

Further evidence for the activation of CPN by proteolysis was obtained using trypsin, which cleaves CPN much faster and, although it is less specific than plasmin, yielded a similar pattern of cleavage (Figure 2). Trypsin at a 1:50 ratio (trypsin/CPN) hydrolysed as much CPN in 1 h as did plasmin at a 1:1 ratio in 3 h, as determined by SDS/PAGE (Figure 2). Trypsin also enhanced CPN activity similarly to plasmin, increasing it to $174 \pm 11\%$ of control ($n = 5$). Trypsin inactivated by soybean trypsin inhibitor (1 μ M) did not affect CPN activity ($101 \pm 3\%$; $n = 4$).

The time course of plasmin-mediated cleavage of CPN was analysed by Western blot and quantified by densitometry (Figures 2B and 3). The 83 kDa subunit and the 55 kDa form of the active subunit are most sensitive to proteolysis; significant hydrolysis became apparent within 5 min, and there was almost complete conversion into smaller products within 1 h (Figures 2B and 3). The 83 kDa subunit was converted into a 72 kDa fragment and a smaller band of approx. 13 kDa; the densities of the two products increased in parallel with time (Figures 2B and 3A). The 13 kDa fragment was derived from the 83 kDa subunit, as it stained positively for carbohydrate (results not shown), whereas the catalytic subunit does not have carbohydrate [16,27]. The quantity of the 13 kDa and 72 kDa fragments increased more rapidly than the corresponding decline in the intact 83 kDa subunit, reaching over 80% of the maximum by 30 min (at which time about half of the 83 kDa subunit was degraded) and remaining relatively constant thereafter (Figure 3A). This was probably due to generation of the fragments being balanced by their further

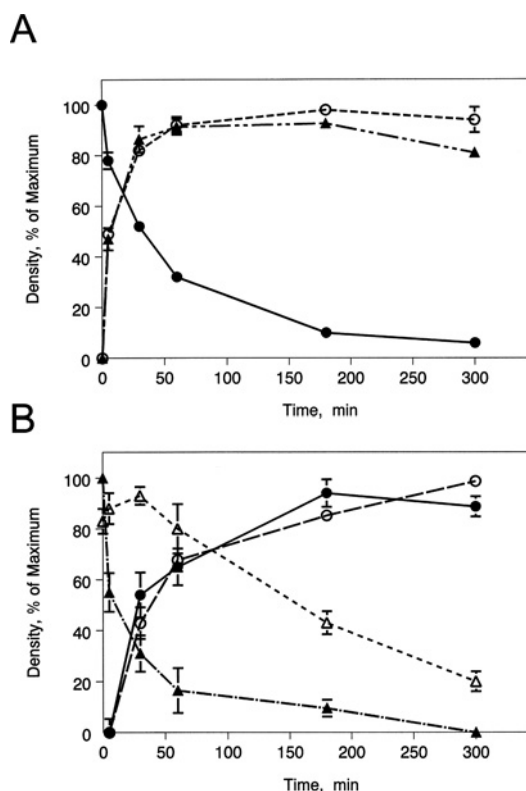


Figure 3 Densitometric analysis of CPN fragments

(A) Analysis of the CPN 83 kDa subunit and its proteolytic fragments: ●, 83 kDa subunit of CPN; ○, 72 kDa fragment; ▲, 13 kDa fragment. **(B)** Analysis of the catalytic subunit of CPN and its proteolytic fragments: ▲, 55 kDa subunit; △, 48 kDa subunit; ●, 27 kDa fragment; ○, 21 kDa fragment. The results are plotted as a percentage of the maximum density obtained against time, and are means \pm S.E.M. of four separate experiments. Note that the appearance of split products increased in parallel with the decrease in intact CPN constituents.

degradation by plasmin at later time points. This is supported by a decline in the quantity of the 13 kDa band at the last time point and the appearance of a diffuse 50 kDa band derived from the 72 kDa band at later times (see below).

The 55 kDa subunit was converted first into the 48 kDa form, and then the 48 kDa subunit was cleaved into peptides of 27 and 21 kDa (Figures 2 and 3B). Although the relative intensity of the 27 kDa band increased in parallel with that of the 21 kDa band (Figure 3B), the absolute intensity of staining in Western blotting was lower because it binds less to the antibody used for detection (Figure 2). The presence of a diffuse band at approx. 48 kDa at 3 and 5 h, times when the generation of the 27 and 21 kDa bands is maximal (Figure 2B), is due to the appearance of small amounts of 40–50 kDa degradation fragments derived from the 72 kDa band, as determined by Western blotting with antiserum specific for the regulatory subunit in separate experiments (results not shown). This was corrected for in the densitometric analysis of the 48 kDa band (Figure 3B). The increase in CPN activity was correlated with the rate of hydrolysis of the 48 kDa subunit to the 27 and 21 kDa fragments (Figure 3B); the first notable increase was seen at 30 min, and a maximum was reached between 3 and 5 h.

Identification of the cleavage sites in CPN

The 55 kDa form of the catalytic subunit of CPN is converted into the 48 kDa form by cleavage in the C-terminal region, as the N-terminal sequence of the 48 kDa protein matches that of the mature full-length catalytic subunit [30,31]. The cleavage site

in the 48 kDa subunit that generates the 27 and 21 kDa fragments following cleavage by trypsin or plasmin was determined by separation on HPLC or by SDS/PAGE and blotting, followed by N-terminal sequence analysis as described in the Experimental section. The N-terminal sequence of the 21 kDa fragment released by plasmin hydrolysis was RTASTPT.... This is identical with an internal sequence in the active subunit (Arg²¹⁹–Thr²²⁵) [31], and consequently shows that plasmin cleaves at the Arg²¹⁸–Arg²¹⁹ bond and that the 27 kDa derivative is the N-terminal fragment. When the 21 kDa fragment released by trypsin was analysed, two sequences were obtained: RTASTPTDCKLFQK and TAST-PTDCKLFQKL. Thus trypsin cleaves at both the Arg²¹⁸–Arg²¹⁹ and the Arg²¹⁹–Thr²²⁰ bonds. Trypsin was more active than plasmin, probably because it prefers to cleave after Arg residues, whereas plasmin cleaves preferentially after Lys.

The site of plasmin-mediated cleavage of the 83 kDa subunit of CPN was also determined by protein sequencing. The N-terminal sequence of the 72 kDa fragment is consistent with that of the 83 kDa subunit ([32]; see also [32a]), i.e. CPMGCD... (the actual sequence was XPMGXD, because cysteine was not detected). The N-terminal sequence of the 13 kDa fragment is SQXTYSN, corresponding to the sequence Ser⁴⁵⁸–Asn⁴⁶⁴ in the C-terminal region of the 83 kDa subunit of CPN. Thus plasmin cleaves the Arg⁴⁵⁷–Ser⁴⁵⁸ bond and releases a 13 kDa fragment that is not covalently attached by S–S bonds, as dithiothreitol is not necessary to dissociate it. In addition, gel filtration separates the 13 kDa fragment (see below), further evidence that it is not attached to the 72 kDa fragment after cleavage by plasmin.

Effect of plasmin on the structure of CPN

CPN exists in plasma as a tetramer of approx. 280 kDa, with estimates ranging from 270 to 330 kDa, of which 216.6 kDa would be contributed by the protein, as calculated from the subunit protein sequences, with the balance being carbohydrate [13,16,17,27]. To investigate whether cleavage of CPN by plasmin alters the quaternary structure of the enzyme, the products were separated by gel filtration on a Superdex 200HR column and the collected fractions were assayed for CPN activity (Figure 4) and analysed by Western blotting (Figure 5). Native, untreated CPN eluted from the column in a single high-molecular-mass peak of protein that exactly matched the CP activity (Figure 4A). After treatment with plasmin for 30 min (Figure 4B) or 3 h (Figure 4C), four main peaks with absorbance at 280 nm were observed, none of which coincided with the control CPN peak (Figure 4A). The first and major protein peak eluted with an apparent molecular mass of 147 kDa, had CPN activity and contained the 72 kDa fragment derived from the 83 kDa subunit and intact catalytic subunit (the 48 kDa form), as revealed by Western blotting (Figure 5). These data indicate that initial cleavage by plasmin alters the quaternary structure of CPN.

Because we have shown that plasmin binds to CPN (M. O. Quagraine, V. LeRay, F. Tan and R. A. Skidgel, unpublished work), we had to rule out the possibility that this interaction would survive gel filtration and alter the elution of CPN apart from its proteolytic effect. To investigate this, plasmin was pretreated with 2 mM DFP for 1 h at 4 °C to inhibit it, then preincubated with CPN as above for 30 min at 37 °C, followed by gel filtration. Under these conditions, CPN eluted at exactly the same position as untreated CPN (results not shown), indicating that the association of plasmin and CPN is not tight enough to survive in gel filtration. Thus initial cleavage of CPN by plasmin dissociates the tetramer into two heterodimers.

The next two proteins peaks that eluted (labelled PK2 and PK3 in Figure 4) were not well resolved on the column and activity

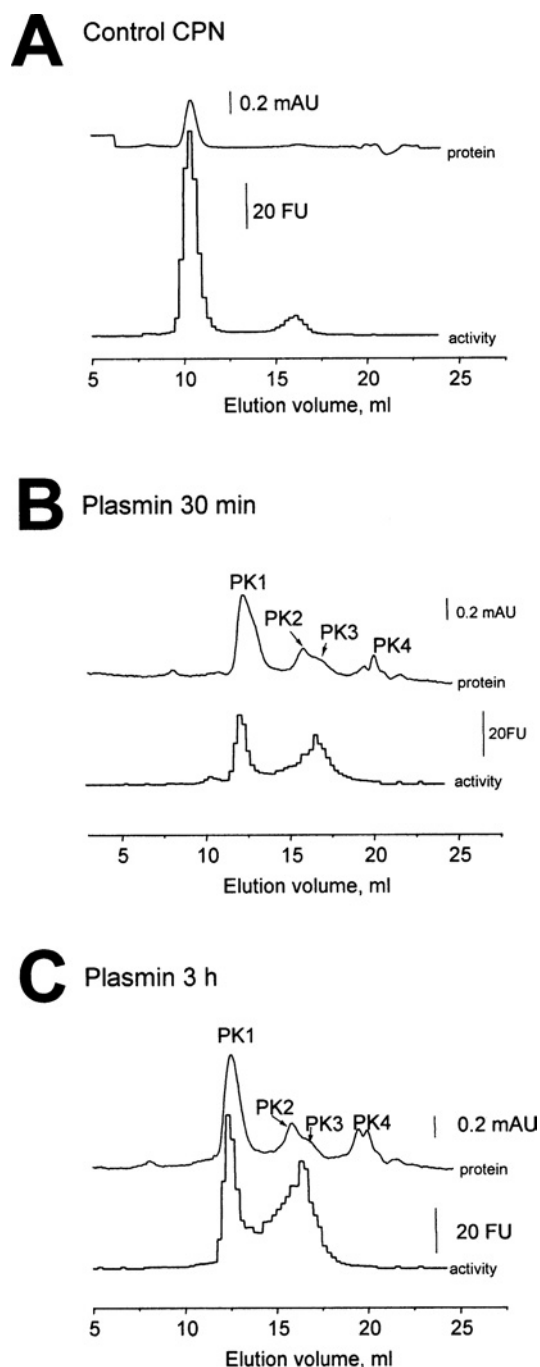


Figure 4 Gel filtration of CPN hydrolysis products

As a control (A), CPN was incubated alone at 37 °C for 30 min. To determine the effect of plasmin on the quaternary structure, CPN (7 pmol) was incubated at a 1:1 molar ratio with plasmin at 37 °C for 30 min (B) or for 3 h (C). The reaction mixture was analysed by FPLC on a Superdex HR200 gel filtration column and CPN activity in the fractions was determined. Upper tracings, UV absorbance at 280 nm; lower tracings, CPN activity, assayed with dansyl-Ala-Arg, in relative fluorescence units (FU). Peaks (PK) 1, 2, 3 and 4 indicate fractions collected and pooled for Western blot analysis (see Figure 5). The figure is representative of the results of six separate experiments.

measurements gave a broad peak, but the highest activity coincided with peak 3. In Western blots, this peak contained primarily the 48 kDa catalytic subunit, whereas peak 2 contained mainly the 13 kDa fragment derived from the 83 kDa subunit, although there

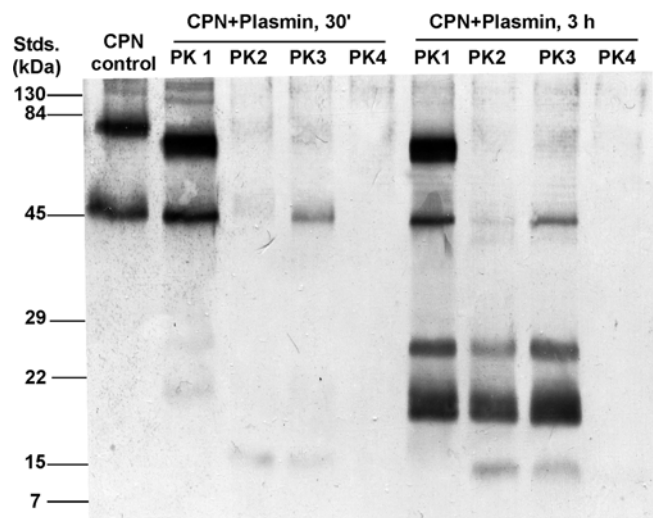


Figure 5 Western blot of CPN cleavage products separated by gel filtration

CPN was incubated at a 1:1 molar ratio with plasmin for 30 min or 3 h, as indicated in Figure 4, and the peaks obtained after Superdex HR200 gel filtration chromatography were analysed by Western blotting. Peaks (PK) 1, 2, 3 and 4 indicate fractions that were collected and pooled as shown in Figure 4.

was an overlap due to the incomplete separation (Figure 5). Peak 4 did not contain CPN fragments or activity.

The protein and activity profile obtained after 3 h of plasmin treatment (Figure 4C) was remarkably similar to that seen after 30 min of treatment (Figure 4B). However, Western blot analysis revealed differences in the form of the catalytic subunit, as it was cleaved predominantly into 27 and 21 kDa fragments in 3 h, apparent in all three protein peaks eluted (Figure 5). The cleaved catalytic subunit was eluted from the gel filtration column in the same position as the uncleaved subunit. This was independent of being bound to the 72 kDa fragment of the regulatory subunit or when eluted alone. This suggests that the 27 and 21 kDa fragments of the catalytic subunit remained associated after proteolysis. The two fragments are held together by non-covalent bonds, as the two proteins are separated during SDS/PAGE in the absence of reducing agent (Figure 5).

Additional evidence for the heterodimeric form of CPN

To obtain more evidence that plasmin generates a heterodimer from the tetramer, the recombinant catalytic subunit was incubated for 1 h at 4 °C with approximately equimolar amounts of the isolated, purified 72 kDa fragment of the 83 kDa subunit and then separated on a Superdex 200HR column (results not shown). Whereas the recombinant catalytic subunit alone eluted in the same position as peak 3 above, when incubated with the 72 kDa fragment, approx. 30% of the activity eluted at a higher molecular mass, in the same position as the heterodimer (peak 1 above).

To further support the results of the gel filtration studies, we used immunoprecipitation with antibodies specific to the regulatory subunit to determine whether the 72 kDa fragment remained associated with the catalytic subunit after plasmin treatment (Table 1). After 30 min of plasmin treatment, antibodies specific to the 83 kDa subunit immunoprecipitated 63% of the CP activity from the solution compared with the rabbit IgG control, suggesting that a substantial portion of the catalytic subunit was still associated with the 72 kDa fragment after the 13 kDa fragment was released by plasmin (Figure 4B and Table 1). In control experiments with the catalytic subunit and intact CPN, 12%

Table 1 Immunoprecipitation of CPN with subunit-specific antibodies after plasmin treatment

CPN was treated with plasmin at a 1:1 molar ratio at 37 °C for 30 min (CPN + plasmin). Polyclonal antibodies to the 83 kDa or catalytic subunits were used to immunoprecipitate the samples and CPN activity remaining in the supernatant was assayed. To confirm the specificity of the antibodies, immunoprecipitations were carried out with intact CPN or the recombinant catalytic subunit (r50K) alone. Activity is expressed as a percentage of control activity obtained in immunoprecipitations with non-immune rabbit IgG. Results shown are the means \pm S.E.M. of three to four experiments.

Antibody	CPN activity remaining in supernatant (%)		
	CPN	r50K	CPN + plasmin
Anti-(83 kDa subunit)	9 \pm 1	93 \pm 3	37 \pm 8
Anti-(50 kDa subunit)	2 \pm 1	2 \pm 1	2 \pm 1

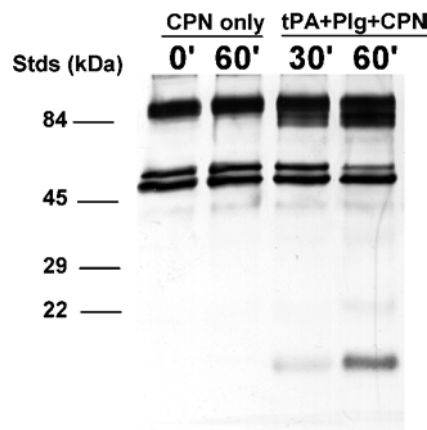


Figure 6 Proteolysis of CPN activity during plasminogen activation by tPA

Plasminogen (Plg) was activated by incubation with tPA at a 10:1 ratio (plasminogen/tPA) at 37 °C in the presence of CPN (the CPN/plasminogen ratio was 1:1). Aliquots of the activation reaction mixture were taken at 30 or 60 min and analysed by Western blotting with antibody to CPN. The blot shown is representative of three separate experiments. Note the appearance of the 13 kDa docking peptide concomitant with the presence of the 72 kDa fragment of the regulatory subunit.

and 91% of the CP activity was immunoprecipitated respectively (Table 1), showing that the antibody to the regulatory subunit does not precipitate the catalytic subunit, but is capable of efficient immunoprecipitation of CPN. As a positive control, antibody to the catalytic subunit immunoprecipitated essentially all of the CPN activity in all of the samples (Table 1). Subtraction of the control value indicates that approx. 50% of the catalytic subunit remained associated with the 72 kDa fragment of the regulatory subunit. This is consistent with the measurement of activity in the gel filtration fractions, where about half of the activity was associated with the heterodimer in peak 1 and about half with the free catalytic subunit (Figure 4).

CPN cleavage during plasminogen activation

To determine whether CPN is cleaved during plasminogen activation, tPA was used to activate plasminogen in the presence of CPN and aliquots of the activation mixture were analysed by Western blotting (Figure 6). Under the conditions used for this reaction (1:1 molar ratio of CPN/plasminogen and a 10:1 ratio of plasminogen/tPA), the rate of plasminogen activation was still linear at 60 min (results not shown). Hydrolysis of the 83 kDa subunit was already apparent after 30 min (Figure 6), and became

more significant at 60 min, as indicated by conversion into the 72 kDa fragment and generation of the 13 kDa band. Under these conditions, conversion of the catalytic subunit from the 55 kDa to the 48 kDa form was apparent at 60 min, and barely detectable amounts of the 27 and 21 kDa bands also began to appear at 60 min. In control experiments, CPN was not cleaved by tPA alone under our conditions (results not shown).

DISCUSSION

CPN is stable in normal plasma, even when stored at 4 °C for prolonged periods. However, it is readily cleaved during purification unless high concentrations of protease inhibitors are added [16,17,27], which has been attributed to the separation of plasma protease inhibitors from their cognate proteases, resulting in activation and proteolysis. We have identified the two primary sensitive sites in CPN that yield a characteristic pattern of fragments after serine protease-mediated hydrolysis: Arg²¹⁸–Arg²¹⁹ in the catalytic subunit and Arg⁴⁵⁷–Ser⁴⁵⁸ in the 83 kDa subunit (Figure 7). We found that the consequences and the rates of hydrolysis at these two proteolytically sensitive sites are different. Plasmin cleaves the 83 kDa subunit faster, resulting in dissociation of the 280 kDa tetramer into two heterodimers consisting of the 72 kDa fragment of the regulatory subunit and the catalytic subunit. This also is likely to lead to a less stable association between the catalytic and non-catalytic subunits; about half of the CP activity was recovered free after separation by gel filtration chromatography, whereas essentially no free catalytic subunit was found during gel filtration of the intact 280 kDa tetramer. Initial hydrolysis of the 83 kDa subunit was probably the cause of dissociation of the tetramer into the heterodimer. The lower-molecular-mass product was formed early, when mainly the 83 kDa subunit was cleaved, and the heterodimer form (peak 1) lacked the 13 kDa fragment released from the 83 kDa subunit by plasmin (Figure 7). Another possibility is that the early conversion of the 55 kDa form of the catalytic subunit into the 48 kDa form might have induced a dissociation of the tetramer into the heterodimer. However, this is unlikely, as the native enzyme, which usually contains roughly equal amounts of the 48 kDa and 55 kDa forms of the active subunit (e.g. see Figures 2 and 6), always elutes as a single high-molecular-mass tetrameric peak in gel filtration.

Hydrophobic analysis of the sequence and predictions of chain flexibility indicate that the C-terminal domain of the 83 kDa subunit is more flexible, hydrophilic and exposed to the exterior, and the residues around the cleavage site are relatively non-bulky. Thus the Arg⁴⁵⁷–Ser⁴⁵⁸ cleavage site in the C-terminal domain of the 83 kDa subunit is probably in an exposed loop on the exterior of the molecule, accessible to proteases. The lack of cleavage at other sites may be due to the presence of a more defined secondary or tertiary structure in those regions. For example, tryptic digestion of the 83 kDa subunit for sequencing releases peptides from either the N- or the C-terminal domain, but none from the LRR (leucine-rich repeat) sequence [32,32a]. The trypsin cleavage site on the 83 kDa subunit was not determined directly in these studies, but because trypsin cleaves after Arg and gave a pattern of fragments identical to that obtained after plasmin-mediated hydrolysis, the Arg⁴⁵⁷–Ser⁴⁵⁸ bond is also likely to be cleaved by trypsin.

The 13 kDa fragment released after plasmin treatment contains 81 amino acids, yielding a lower predicted molecular mass of 7304 Da. However, this sequence contains a potential glycosylation site at Asn⁴⁹⁷, the only such site outside the LRR region (Figure 7B), which probably explains the difference in molecular mass, as the 13 kDa protein stained positively for carbohydrate. The 83 kDa subunits contain 15 Cys residues, eight of which are

C-terminal to the LRR domain, with four before the plasmin cleavage site and four C-terminal to it (Figure 7B). The 13 kDa fragment is released from the heterodimer, established in gel filtration or SDS/PAGE without reduction, indicating a lack of disulphide bonds between the four C-terminal Cys residues and any other Cys residues in the sequence. Thus any disulphide bonds in the 13 kDa fragment would be intrachain. Consequently, non-covalent interactions, including electrostatic interactions and hydrogen bonds, are important to maintain the integrity of the tetrameric structure. This agrees with previous findings that the subunits can be dissociated with SDS or 3 M guanidine in the absence of reducing agent, but not by neutral detergents [16].

The cleavage site in the catalytic subunit that results in conversion of the 55 kDa form into the 48 kDa form is somewhere in the C-terminal region, because the N-terminal protein sequence of the isolated 48 kDa subunit matches that of the mature catalytic subunit, as deduced from the cDNA sequence [30,31]. This is consistent with the presence of numerous basic residues in the C-terminal region of the catalytic subunit, which are potential cleavage sites for serine proteases with trypsin or plasmin-like specificity [31]. For example, 13 of the last 50 residues are basic amino acids, and in four cases they are clustered in di- or tri-basic sites: Lys³⁹⁶–Arg³⁹⁷, Arg⁴⁰⁶–Arg⁴⁰⁷, Arg⁴¹¹–Arg⁴¹² and Arg⁴²⁴–Lys⁴²⁵–Lys⁴²⁶. The major 55 kDa and 48 kDa forms of the catalytic subunit probably represent the uncleaved and completely C-terminally cleaved forms of the catalytic subunit, but cleavage at intermediate sites may occur, as minor intermediate forms that migrate in between the 48 and 55 kDa bands can be resolved on high percentage polyacrylamide gels (R. A. Skidgel, M. O. Quagrain and F. Tan, unpublished work).

Cleaved CPN is more active after the hydrolysis of the catalytic subunit at the Arg²¹⁸–Arg²¹⁹ bond. These results are consistent with our previous studies showing that when a serine protease cleaves the isolated, purified catalytic subunit, its activity increased by 41–84 % [16]. When the catalytic subunit bound to the 83 kDa subunit was cleaved in the present study, the distribution of free and heterodimer-associated catalytic subunits did not change when analysed by gel filtration. Thus proteolysis of the catalytic subunit does not result in rapid dissociation of the fragments, which remain associated by non-covalent forces in an active conformation. The cleaved catalytic subunit, if allowed to stand for prolonged periods, does dissociate into fragments that can be resolved on gel filtration, but no longer have any catalytic activity (R. A. Skidgel and E. G. Erdös, unpublished work). These data are also consistent with the location of the putative active-site residues in the sequence of the catalytic subunit [31,33]; the N-terminal 27 kDa fragment of the catalytic subunit contains the three zinc binding residues and substrate binding residues, whereas the C-terminal 21 kDa fragment contains the critical catalytic glutamic acid. Thus, one fragment alone would not comprise a functional CP. The reason for the increased catalytic activity after cleavage is not clear, but probably results from subtle conformational changes. Clarification of this point will require determination of the three-dimensional structures of the two forms to see how they differ.

The sequence of the catalytic subunit of CPN in the region of Arg²¹⁸–Arg²¹⁹, when compared with those of other CPN/E family members, contains an extra 4–12 residues [33]. In the three-dimensional structures of the two CPN/E family members that have been determined, i.e. domain 2 of duck CPD [34] and human CPM [35], the amino acids equivalent to those surrounding the Arg²¹⁸–Arg²¹⁹ cleavage site in the catalytic subunit are exposed in a surface loop on the exterior of the molecule. These data are taken as an indication that the unique sensitivity of the catalytic subunit to proteases arises from an extended surface-exposed loop that

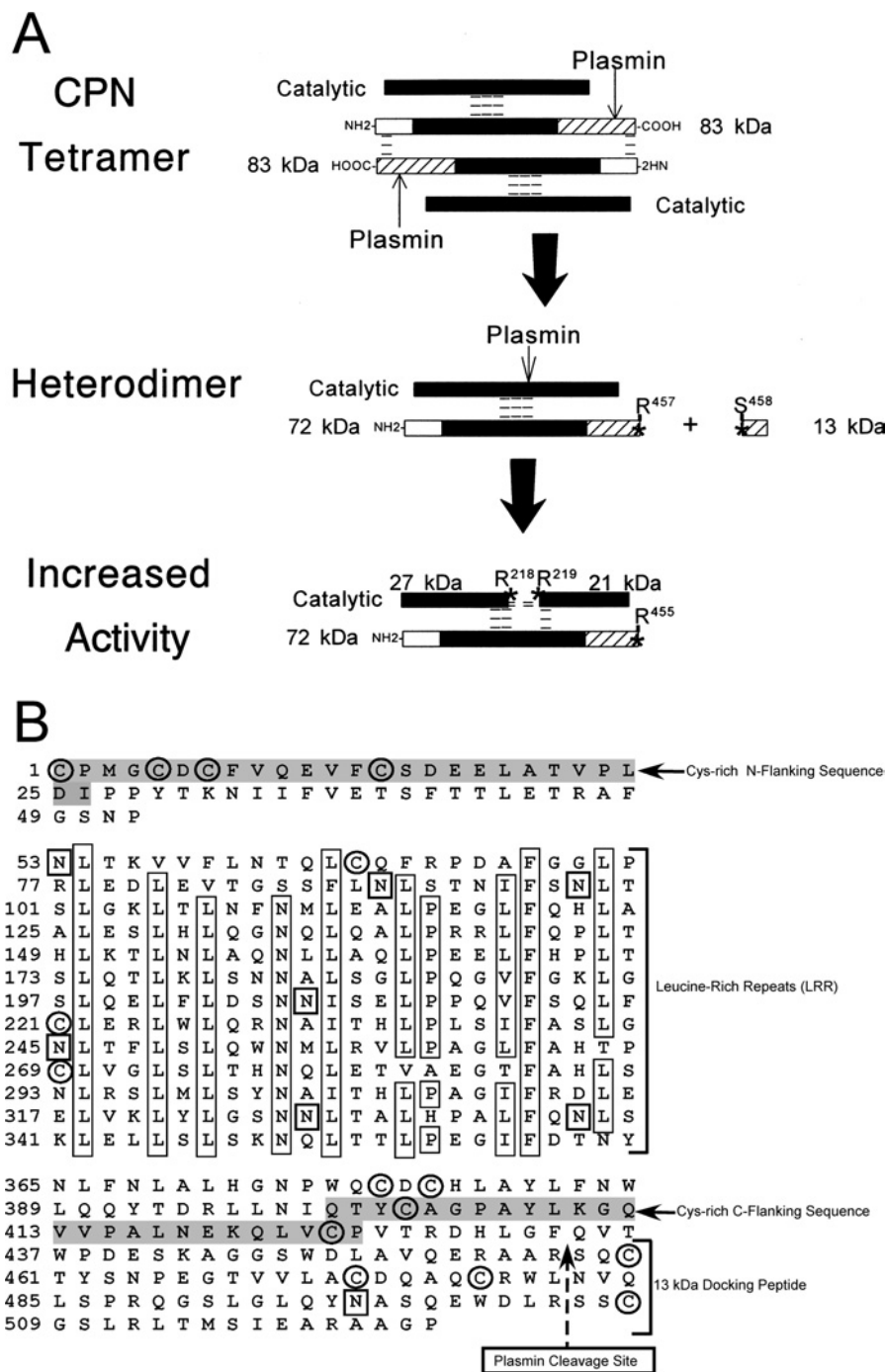


Figure 7 Cleavage of CPN by plasmin and domain structure of the 83 kDa subunit

(A) A hypothetical representation of the CPN tetramer is shown with the three major domains of the 83 kDa subunit designated as follows: empty box, N-terminal domain; solid box, LRR; hatched box, C-terminal domain. The eight potential Asn-linked glycosylation sites are not shown. In the absence of carbohydrate, the calculated molecular mass of the regulatory subunit is 58287 Da. Dashed lines represent inter- and intra-chain non-covalent interactions. The sites cleaved by plasmin are denoted by stars, and the amino acids at the cleavage sites are marked. The conversion of the catalytic subunit from its 55 kDa form to a 48 kDa form is not shown for the sake of clarity. (B) The amino acid sequence and domain structure of the 83 kDa subunit of CPN is shown, defining the location of the N-terminal domain, LRR and C-terminal domain containing the 13 kDa docking peptide. Highly conserved residues within the 24-amino-acid LRRs are boxed. The plasmin cleavage site at Arg⁴⁵⁷-Ser⁴⁵⁸ is marked by the broken arrow. Cys residues are circled and Asn residues that are potential glycosylation sites are marked with a box. The consensus Cys-rich N- and C-flanking sequences on either side of the LRR are shaded.

also contains a dibasic cleavage site not present in other members of the CPN/E family [33,36].

The sequence of the 83 kDa subunit of CPN identifies it as a member of the LRR family of proteins [32,32a], which belong to a larger superfamily called the 'solenoid protein' family [37].

Although we originally reported that the 83 kDa subunit contained 12 LRRs constituting one of four distinct domains [32,32a], we have re-evaluated the domain structure after correcting some early sequencing errors (accession no. J05158). In addition, we used the Pfam protein family database [38] to analyse the sequence, which

has an updated definition of the LRR motif, which by now has been found in over 2000 proteins from a variety of species [37,39]. The 83 kDa subunit can be divided into three major domains with the following features (Figure 7B): (1) a short 52-residue N-terminal domain that contains a cysteine-rich LRR N-flanking region (amino acids 1–27); (2) a 312-residue central domain that consists of 13 tandem LRRs of 24 residues each (amino acids 53–364); and (3) a C-terminal domain of 145 residues that contains a 26-residue cysteine-rich LRR C-flanking region (amino acids 400–425).

Although the three-dimensional structure of the 83 kDa subunit of CPN has not been determined, in the 14 LRR family members that have been crystallized, the leucine-rich sequence results in an overall 'horseshoe' structure, with a curved β -sheet lining the inner, concave surface and other repeated secondary structures, such as α -helix or β -turn, flanking the outer circumference [37, 39]. Homology modelling of the 83 kDa subunit yields a structure within the LRR similar to those of other LRR proteins whose structures are known, but the N- and C-terminal domains cannot be modelled, as their sequences do not exhibit high enough similarity to proteins of known structure (R. A. Skidgel, unpublished work). Because LRR motifs are thought to be involved in protein–protein interactions [37], we hypothesized that the LRR domain of the 83 kDa subunit mediates its tight interaction with the catalytic subunit, which requires 3 M guanidine to dissociate [16,32, 32a]. Although only very few LRR proteins have been co-crystallized with their binding partners, a well studied example is the placental ribonuclease inhibitor, an LRR protein that forms a very tight interaction ($K_i < 1$ fM) with angiogenin. In this case, the interaction is mediated by contact of 24 angiogenin residues with 26 amino acids within the LRR domain of the ribonuclease inhibitor [40]. Based on these considerations, it is possible that the LRR domain of the 83 kDa subunit of CPN (Figure 7B) interacts with the C-terminal β -sheet domain of the catalytic subunit (a unique feature of the regulatory CPs [34,35]) to form the heterodimer, which would leave the catalytic CP domain free to interact with substrates.

With regard to tetramer formation, the C-terminal region must be important, as release of this 13 kDa fragment by plasmin results in dissociation of CPN into heterodimers. It is interesting to note that the placental ribonuclease inhibitor complexed with angiogenin also exists as a tetramer. In this case, two heterodimers of angiogenin and ribonuclease inhibitor (formed as described above) interact via the two N-terminal sequences of the ribonuclease inhibitor to form the tetramer [40]. Based on these considerations, it is possible that tetramer formation in CPN is mediated by interaction of the positively charged C-terminal domain of one 83 kDa subunit with the negatively charged N-terminal domain of the other 83 kDa subunit. The N-terminal 46 amino acids of the 83 kDa subunit contain eight acidic residues (Asp⁶, Glu¹¹, Asp¹⁶, Glu¹⁷, Glu¹⁸, Asp²⁵, Glu³⁷ and Glu⁴⁴) and only two basic residues (Lys³¹ and Arg⁴⁶) (Figure 7B), yielding a highly acidic isoelectric point for this region of 3.89. In contrast, the C-terminal 46 amino acids contain five basic residues (Arg⁴⁷⁹, Arg⁴⁸⁸, Arg⁵⁰⁵, Arg⁵¹² and Arg⁵²⁰) and three acidic residues (Glu⁵⁰¹, Asp⁵⁰³ and Glu⁵¹⁸) (Figure 7B), with a theoretical isoelectric point of 9.49 (not considering the possible effect of glycosylation). Proof of these interactions will require determination of the three-dimensional structure of the CPN tetramer.

The importance of heterodimer and tetramer forms of CPN goes beyond preventing the catalytic subunit from being removed by glomerular filtration, as indicated by the stabilization of the activity of the catalytic subunit at 37 °C by the 83 kDa subunit [16]. In addition, the presence of sialic acids on the glycosylated 83 kDa subunit [27] would protect CPN from uptake by liver lectins [41]. Overall, these properties result in the *in vivo* stability in blood

of CPN, which is also constitutively active. This is in marked contrast with the other blood-borne CP, CPU or TAFI, which is normally present as an inactive proenzyme and, once activated, is quite unstable in the circulation [9].

The cleavage of CPN by plasmin is relatively inefficient, requiring a high ratio (1:1) of plasmin to CPN, and this calls into question whether it is a physiologically meaningful reaction. Under normal circumstances, it is unlikely to be significant, even though the plasminogen (and potential plasmin) concentration in blood is approx. 2 μ M, 200 times higher than that of CPN [13,42]. This is because only a small fraction of the plasminogen is likely to be activated at any one time, and any plasmin generated would be inactivated rapidly by its plasma inhibitor, α_2 -antiplasmin, which has a blood concentration of approx. 1 μ M [42]. However, when a high percentage of plasminogen is activated, such as in decompensated disseminated intravascular coagulation, the natural inhibitor is overwhelmed, and unopposed plasmin activity results. One consequence is a lytic state resulting in serious bleeding problems due to plasmin-mediated cleavage of fibrinogen and coagulation factors [43–45]. Another situation that could result in high plasmin levels is thrombolytic therapy with plasminogen activators, administered intravenously at high concentrations to treat patients with myocardial infarction or occlusive strokes [46]. In addition, there is current interest in the development of active plasmin and microplasmin (lacking the kringle domains) for use as injectable drugs to treat peripheral arterial occlusion and deep vein thrombosis [47], which could also result in relatively high levels of active plasmin in blood. Thus there are several conditions where the plasmin concentration could reach the levels necessary for cleavage of CPN.

Based on our studies, the consequences of plasmin-mediated cleavage of CPN could be two-fold: net CPN activity would first increase in the plasma due to cleavage of the catalytic subunit, but, after a longer time, CPN levels could decrease due to the more ready dissociation of the catalytic subunit from heterodimers, leading to clearance via glomerular filtration. Thus, depending on the time frame, the ability of CPN to inactivate kinins and anaphylatoxins could be enhanced or diminished. These highly potent peptides are major physiological substrates for plasma CPN, which regulates their activity by cleaving the C-terminal Arg residues [4,6,12,19]. For example, inhibition of CPN activity in guinea pigs resulted in sudden death after complement activation due to a lack of degradation of the generated anaphylatoxins [48], and genetically determined low blood levels of CPN were associated with repeated attacks of angioedema in one patient, possibly due to increased kinin activity [20,49]. There are many conditions that could result in the concomitant generation of plasmin and release of kinins and anaphylatoxins, for example after the administration of streptokinase [50–52] or after activation of the plasma kallikrein/kinin system during sepsis [53] or cardiopulmonary bypass [54,55]. In these cases, increased CPN activity due to cleavage by plasmin could be a feedback mechanism to enhance the inactivation of kinins and anaphylatoxins and blunt the pathological consequences of their release. For example, protamine, an inhibitor of CPN, is given after cardiopulmonary bypass to reverse the effects of heparin, and in some patients causes a catastrophic reaction associated with kinin and anaphylatoxin release [56,57]. This syndrome might be due, in part, to the lack of inactivation of anaphylatoxins and kinins by CPN, because it can be reversed in some cases by re-administration of heparin [58], which reverses the inhibitory effect of protamine on CPN [18].

Plasmin could also enhance the ability of CPN to remove C-terminal Lys residues from cell surface proteins, thus acting as a feedback mechanism to limit cellular plasminogen activation [24].

Interestingly, CPN prefers cleaving C-terminal Lys over C-terminal Arg, and is thus better suited for this function compared with, for example, membrane-bound CPM, which prefers C-terminal Arg [1,5,13]. Although plasmin enhances CPN activity by cleavage of the catalytic subunit, it is also possible that conversion of the tetramer into heterodimers by hydrolysis of the 83 kDa subunit would enhance the ability of CPN to gain access to cell surface proteins. In addition, conversion into the heterodimer could double the effective concentration of enzyme in this process as, in the tetramer form, only one active site at a time may be functional in cleaving a fixed substrate such as a cell surface protein with limited diffusion capability. Finally, CPN is cleaved more rapidly by trypsin, indicating that other plasma serine proteases with similar specificity, such as members of the coagulation cascade or complement system, may also hydrolyse and enhance the activity of CPN in the circulation.

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