Carboxypeptidase U, a Plasma Carboxypeptidase with High Affinity for Plasminogen*

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A novel basic carboxypeptidase clearly different from carboxypeptidase N has been isolated from human plasma. It circulates as an enzymatically inactive precursor enzyme bound to plasminogen. During fibrinolysis, it can be converted to its active form, carboxypeptidase U, through the action of plasmin. The active enzyme has an apparent molecular weight of 53,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It hydrolyzes the synthetic peptides hippuryl-L-arginine and hippuryl-L-lysine but, in contrast to other human basic carboxypeptidases, has only a limited esterase activity. After its activation, carboxypeptidase U tends to be very unstable.

The role of basic carboxypeptidases, i.e. enzymes that cleave COOH-terminal basic amino acids lysine and arginine, has gained increasing interest in medical science since it became evident that this type of enzyme can be involved in peptide hormone maturation. Most peptide hormones are initially synthesized as precursor proteins that are frequently cleaved at the COOH-terminal basic amino acids lysine and arginine; Hip-Lys, hippuryl-L-lysine; pNA, p-nitroanilide. We suggested the presence of an arginine carboxypeptidase activity which stands for "unstable" carboxypeptidase activity. CPU was separated from carboxypeptidase N and partially purified. Some of its characteristics were determined (14). In this paper we describe the purification and characterization of the enzymatically active subunit of carboxypeptidase U and compare it with the purified active subunit of carboxypeptidase N. Furthermore, we present more evidence that indeed a pro-carboxypeptidase U (pro-CPU) is present in human plasma and that it is physically associated to plasminogen. pro-CPU needs to undergo maturation through the action of plasmin (during fibrinolysis) to generate the enzymatically active CPU.

EXPERIMENTAL PROCEDURES

Chemicals

Hippuryl-L-arginine (Hip-Arg), hippuryl-L-lysine (Hip-Lys), and Val-Leu-Lys-pNA were from Bachem Feinchemischen (Bubendorf, Switzerland). dl-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGÉTA), guanidinoethylmercaptosuccinic acid (GEMSÁ), and HEPES were purchased from Calbiochem. Human serum albumin and lyophilized human plasmin were obtained from Sigma, and dithiothreitol and guanidine HCl were obtained from Janssen Pharmaceutica (Beerse, Belgium). Disopropyl fluorophosphate (DFP) and CoCl₂·6H₂O were from Aldrich. DEAE-Sepharose, arginine-Sepharose 4B, lysine-Sepharose 4B, Sephacryl S-100, Sephacryl S-200, Sephacryl S-300, Mono Q-Sepharose, molecular mass standard proteins, and the Excel Gel® for SDS-PAGE were obtained from Pharmacia (Uppsala, Sweden). Hippuryl-L-argininic acid was kindly provided by Dr. Yehuda Levin of the Weizmann Institute of Science, Rehovot, Israel. o-Methylhippuric acid was synthesized from glycine and o-methylbenzoyl chloride (Union Chimique Belge, Drogenbos, Belgium) by a procedure analogous to that used for the synthesis of hippuric acid (15). o-Aminocaproic acid, 1,10-phenanthroline, and all other reagents used were of high purity grade and were from Merck (Darmstadt, Germany).

Instruments

The pipetting of sample serum and reagents was performed with a Durrétted dispensier (Boehringer, Mannheim, Germany). For colorimetric determinations, a Hewlett-Packard 8540 ultraviolet-visible diode array spectrophotometer was used with a quartz flow cell (10 mm optical pathway). The high pressure liquid chromatography system consisted of a 303 solvent delivery system, an 802 C manometric module, a high performance liquid chromatography system, and a 100 × 8-mm (inner diameter) C18 reversed-phase µ-Bondapak column fitted used (12). By comparing the arginine carboxypeptidase activity in fresh human serum with serum that was first placed for 2 h at 37 °C, we suggested the presence of an arginine carboxypeptidase in fresh human serum, which differed from carboxypeptidase N in terms of substrate specificity with peptide substrates, esterase activity, pH optimum, and influence of various inhibitors and activators (13). Furthermore, the novel enzyme activity proved to be very labile. We named this activity CPU activity, which stands for "unstable" carboxypeptidase activity. CPU was separated from carboxypeptidase N and partially purified. Some of its characteristics were determined (14). In this paper we describe the purification and characterization of the enzymatically active subunit of carboxypeptidase U and compare it with the purified active subunit of carboxypeptidase N. Furthermore, we present more evidence that indeed a pro-carboxypeptidase U (pro-CPU) is present in human plasma and that it is physically associated to plasminogen. pro-CPU needs to undergo maturation through the action of plasmin (during fibrinolysis) to generate the enzymatically active CPU.

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in a radial compression module (Millipore, Brussels, Belgium). The fast protein liquid chromatography system, Biopilot system, and Smart® system were obtained from Pharmacia. Proteins were concentrated with Centriprep-10 concentrators (Amicon, Inc., Beverly, MA). Dialysis sacks (30 × 2.1 cm) were from Sigma.

For centrifugation, a Sorvall RC-5 DuPont NEN) was used. Electrophoresis was performed on a Mini-Precoat II 2-D Cell from Bio-Rad, and isocromafocussing was performed on a protein fractionator model RF3 (Rainin Instrument Co., Woburn, MA).

**Methods**

**Assay for Carboxypeptidase Activity**

The carboxypeptidase activities using the substrates hippuryl-L-arginine and hippuryl-L-lysine were determined by a high pressure liquid chromatography-assisted assay as described elsewhere (18) with the following modifications: the buffered substrate solutions were both prepared at pH 8.0 (pH measurements were made at room temperature, 18–21°C), and the incubation time was 30 min. The esterase activity, determined with the substrate hippuryl-L-argininic acid, was measured as follows: to 40 μl of substrate solution (10 μM hippuryl-L-argininic acid, 50 μmol/liter HEPES pH 8.0), 10 μl of the sample was added; this mixture was incubated for 10 min, and the reaction was stopped with 50 μl of 1 M HCl. After addition of 10 μl of the internal standard o-methylhippuric acid, the hippuric acid was extracted and determined by liquid chromatography as described below (16). Triplet determinations were performed for all enzyme assays. 1 unit hydrolyzes 1 μmol of substrate/min.

**Protein Concentration Determinations**

Protein concentrations were measured with the Pierce BCA protein assay (Pierce Chemical Co.). Human serum albumin was used as the standard.

**Purification of Carboxypeptidase N and Carboxypeptidase N Subunits**

Intact CPN was purified from human serum by liquid chromatography-assisted assay as described before (4). The CPN subunits were purified at 4°C by gel filtration chromatography on Sephacryl S-300 (1.6 × 60 cm) at a flow rate of 0.5 ml/min and eluted with 20 μM Tris, pH 7.4, containing 100 mM NaCl. The column was developed with this buffer, and 2-ml fractions were collected and assayed for arginine carboxypeptidase activity. Active fractions were concentrated to a final volume of 2 ml.

**Purification of Carboxypeptidase U Subunits**

**Step 1: Dissociation of CPU Subunits**—To 5 ml of the concentrated intact CPU (fraction after the Sephacryl S-300 chromatography) guanidine HCl was added to a final concentration of 3 M, and this solution was placed in ice for 1 h.

**Step 2: Gel Filtration Chromatography on Sephacryl S-100**—This solution was loaded at a flow rate of 0.5 ml/min onto a Sephacryl S-100 column (1.6 × 60 cm) previously equilibrated with 20 μM Tris buffer, pH 7.4, containing 100 mM NaCl. The column was developed with this buffer, and 2-ml fractions were collected and assayed for arginine carboxypeptidase activity.

**Step 3: Mono Q-Sepharose Chromatography**—1 ml of the pooled enzymatically active fractions from step 2 was mixed with 1 ml of buffer A (20 μM Tris, pH 7.8) and loaded onto a Mono Q PC column (0.16 × 5 cm, previously equilibrated with buffer A) at a flow rate of 0.1 ml/min (using the SMART® system). The column was washed with buffer A at the same flow rate until the A280 returned to base line. Proteins were eluted using a linear gradient from 0 to 40% buffer B (20 μM Tris, 1 mM NaCl, pH 7.8) in 25 min. Fractions of 100 μl were collected and assayed for carboxypeptidase activity. Enzymatically active fractions were evaluated with SDS-PAGE using ExcelGel®.

**Determination of Molecular Mass**

The apparent molecular mass of the CPU active subunit was determined by SDS-PAGE using standard procedures from the manufacturer. The low molecular mass proteins of the electrophoresis calibration kit (Pharmacia) were used as marker proteins.

**Determination of Isoelectric Point**

The isoelectric point of intact CPU was determined using isoelectric focusing at 4°C on a protein fractionator RF3 using standard procedures from the manufacturer. Ampholytes, pH 3–10, were used to generate the pH gradient. After 1 h of prefocusing, 5 μl of purified CPU solution was loaded. The focusing was continued for 2.5 h. 30 fractions of 3 ml each were collected. The pH of each fraction was measured at room temperature, and 600 μl of a 1 M Tris buffer (pH 8.0) was immediately added to each fraction to neutralize the pH. All fractions were subsequently assayed for arginine carboxypeptidase activity.

**Activators and Inhibitors**

The effect of the enzyme inhibitors and activators on intact CPN, CPN subunits, intact CPU, and CPU subunits was evaluated with the hippuryl-L-arginine substrate using the high pressure liquid chromatography-assisted assay (16). To compare the enzymes under identical conditions, the purified preparations were diluted in 20 μM Tris buffer, pH 7.4, containing 150 mM NaCl to yield similar arginine carboxypeptidase activities of about 40 units/liter (final enzyme activities during assay). The activators and inhibitors tested were preincubated with the purified enzyme solutions as described in the following paragraphs.

**Phenanthroline**—20 μl of the enzyme dilutions were preincubated with 10 μl of a 17.5 mM solution of CoCl2 • 6H2O in buffer A (20 μM Tris, pH 7.4, containing 250 mM NaCl) and 5 μl of a solution of human serum albumin (60 mg/ml human serum albumin in 50 mM HEPES pH 8.0) for 1 h in ice (the final Co2+ concentration during assay was 1 mM).

**Phenanthroline**—20 μl of the enzyme dilutions were preincubated with 10 μl of a 17.5 mM solution of 1,10-phenanthroline in buffer A and 5 μl of the human serum albumin solution for 1 h in ice (the final phenanthroline concentration during assay was 1 mM).

**Methionine**—10 μl of a freshly prepared stock solution of 1,200 μM MERGETPA in distilled water, which was first devoid of oxygen by passing nitrogen for 30 min, was added to 40 μl of the substrate solution (hippuryl-L-arginine) before assaying the enzyme samples for activity (the final concentration of MERGETPA during assay was 200 μM). The stock solution of MERGETPA should be used within the hour to avoid loss of inhibitory capacity.

**Dithiothreitol**—20 μl of the enzyme solutions were preincubated for 1 h on ice with 10 μl of a 3.5 mM solution of dithiothreitol in buffer A and 5 μl of the human serum albumin solution (the final concentration of dithiothreitol during assay was 0.2 mM).

**Purification of Plasminogen**

Plasminogen was purified as described by Deutsch and Mertz (17).
600 ml of human plasma was diluted to 1,200 ml with 50 mM phosphate buffer (pH 7.5). This solution was loaded at a flow rate of 60 ml/h onto a column of lysine-Sepharose (2.6 × 11 cm) previously equilibrated with 50 mM phosphate buffer (pH 7.5). The gel was washed with the same buffer until the A_280 nm returned to base line and subsequently washed with 50 mM phosphate buffer containing 0.5 M NaCl (pH 7.5) to remove unspecifically bound proteins. The plasminogen was eluted with 100 ml of 0.2 M e-aminoacproic acid in H_2O at a flow rate of 100 ml/h and concentrated to 10 ml using the Centriprep-10 concentrators before storage at −80 °C. A similar purification of plasminogen was performed, this time using 1 mM DFP in the diluted plasma sample and in the equilibration buffer.

Characterization of Carboxypeptidase Activity Present in Purified Plasminogen Sample

Aliquots of the purified plasminogen sample were preincubated at 4 °C and at 37 °C in the presence or absence of 1 mM DFP. Subsequently, the carboxypeptidase and esterase activities were determined and compared with the activities of the plasminogen sample not previously preincubated.

Gel Filtration Chromatography of Purified Plasminogen Sample

5 ml of the purified plasminogen sample (from the plasminogen purification with addition of DFP) was loaded onto a Sephacryl S-200 column (1.6 × 60 cm) at a flow rate of 0.35 ml/min and eluted at the same flow rate with 0.5 mM Tris buffer, pH 7.4, containing 100 mM NaCl (all operations were performed at 4 °C). Fractions of 2.1 ml were collected. Aliquots of these fractions were used for enzyme assays.

Assay for Plasmin Activity

The chromogenic substrate p-Val-Leu-Lys-pNA (2HCl) was used to assay the amidolytic activity of plasmin by measurement of the release of p-nitroaniline (18). In the standard assay, 50 μl (0.005 casein unit) of a solution containing plasmin was incubated in 0.9 ml of phosphate-buffered saline containing 1 mM substrate and 0.1 ml of polyethylene glycol (5 g/liter, average molecular mass 6,000 Da). The increase in absorbance was monitored at 404 nm; 1 unit of lyophilized human plasma carboxypeptidase was defined as an amount sufficient to hydrolyze 20 nmol of the substrate per 1 h at 37 °C.

**RESULTS**

**Purification of Carboxypeptidase N—**The carboxypeptidase N activity in human serum was found to be stable at room temperature for several days. Consequently, the purification was performed at ambient temperature. The source used for the purification was human serum, obtained from the healthy blood donors, that had been standing at room temperature for 8–12 h. DFP, which does not inhibit carboxypeptidase N, was added to a final concentration of 1 mM to inhibit traces of proteolytic activity. The affinity chromatography on arginine-Sepharose was based on the work of Levin et al. (4) and Plummer and Hurwitz (3) and adjusted to the conditions described before (14). We used a two-step affinity chromatography with a first elution with 600 mM NaCl and a second, more specific elution with 150 mM NaCl combined with 1 mM GEMSA, a competitive inhibitor of carboxypeptidase N. The final result was an 800–1000-fold purification in specific activity with a 20–30% recovery. To obtain CPN purified to homogeneity, a subsequent Mono Q-Sepharose chromatography needs to be performed (14).

**Purification of Carboxypeptidase N Subunits—**When purified carboxypeptidase N (after the second arginine-Sepharose purification step) is treated with 3 mM guanidine HCl, it is transformed to its separate subunits, and no intact CPN (280 kDa) can be detected on gel filtration chromatography (Fig. 1). The enzymatically active subunit of CPN elutes in a single peak on Sephacryl media but at a much higher elution volume than can be expected from its molecular mass (55 kDa). This suggests a hydrophobic interaction between the CPN subunits and the Sephacryl media, as was also observed by Erdős et al. (19), and is compatible with the idea that hydrophobic regions of the CPN subunits may facilitate aggregation and stabilize the protein as a large molecular mass aggregate. The purified active subunits of CPN showed a sharp band on SDS-PAGE. Amino-terminal sequencing of the first 25 amino acids of this band (VFFHRHRHYDLVRXXKQVQEXPG) confirmed the identity of the CPN active subunit. Results of the purification scheme are summarized in Table 1.

**Purification of Carboxypeptidase U—**As was reported previously, carboxypeptidase U activity is only present in fresh human serum and is very unstable; 50% of the activity is lost by keeping the enzyme at 30 °C for 1 h (13). Decreasing the temperature to 4 °C has an important stabilizing ability. As a source for the enzyme, we therefore clotted the blood at 4 °C and performed further purification also at 4 °C. We noticed that working even at this temperature, a lot of CPU activity is lost during every purification step. Therefore, we made it our objective to first separate the CPU activity from the CPN activity and then perform some other purification steps to obtain a partially purified CPU devoid of CPN. DEAE-Sepharose chromatography using elution with a linear salt gradient provided us with an excellent tool to separate CPU from CPN (Fig. 2). CPU was further purified by using gel filtration chromatography on Sephacryl S-300. Results of the purification scheme are summarized in Table II.

**Purification of Carboxypeptidase U Subunits—**We failed to purify CPU to homogeneity mainly due to the marked instability of the enzyme activity. CPU on gel filtration chromatography exhibits a very high apparent molecular mass of 435 kDa (14). This is most likely due to aggregation with itself or with other plasma proteins. We therefore used a similar approach as was found to be successful for the purification of CPN subunits. Indeed, after treatment with 3 mM guanidine HCl, the active CPU subunit eluted even later as compared with the CPN active subunit using gel filtration chromatography on Sephacryl media. 40% of the active CPU is in its aggregated form and elutes in the void volume (Fig. 3). A further purification on Mono Q-Sepharose yields a broad peak of CPU activity (Fig. 4). The molecular mass of the active subunit of CPU was estimated to be 53 kDa by SDS-PAGE of the fractions that contain a high CPU activity and a single protein band on the electrophoretic pattern. Since only the first two fractions containing CPU activity show a single band on SDS-PAGE, the specific activity of CPU can merely be estimated, and not enough pure CPU is available to perform amino-terminal amino acid sequencing.

**Enzymatic Activity of CPN, CPU Subunits, CPU, and CPN Subunits—**The peptidase and esterase activity of both the CPN, CPU subunits, CPU, and CPN subunits was determined...
**TABLE I**
Purification of carboxypeptidase N active subunit from human serum

| Fraction                  | Total protein | Total activity | Specific activity | Yield |
|---------------------------|---------------|----------------|------------------|-------|
|                           | mg            | units          | units/g           | %     |
| Human serum               | 7,050         | 6.50           | 0.92             | 100   |
| Arginine-Sepharose (I)    | 18.2          | 2.86           | 157              | 44    |
| Arginine-Sepharose (II)   | 2.0           | 1.76           | 880              | 27    |
| Sephacryl S-100           | 0.07          | 1.10           | 15,700           | 17    |

**FIG. 3.** Purification of carboxypeptidase U active subunits.

Partially purified carboxypeptidase U (after the gel filtration step on Sephacryl S-300) was dissociated with 3 M guanidine HCl and subsequently passed through a column of Sephacryl S-100. Carboxypeptidase U complexed to other plasma proteins elutes in the void volume, whereas the active carboxypeptidase U active subunit elutes much later.

**TABLE II**
Purification of carboxypeptidase U active subunit from fresh human serum

| Fraction                  | Total protein | Total activity | Specific activity | Yield |
|---------------------------|---------------|----------------|------------------|-------|
|                           | mg            | units          | units/g           | %     |
| Recalcified human plasma  | 2,300         | 2.16           | 0.94             | 100   |
| DEAE-Sepharose            | 182           | 1.13           | 6.2              | 52    |
| Sephacryl S-300           | 25            | 0.94           | 37               | 43    |
| Sephacryl S-100           | 0.067         | 0.15           | 2,240            | 7     |
| Mono Q-Sepharose*         | <0.001        | 0.02           | >20,000          | <1    |

*Since only the fractions containing CPU activity without contaminating proteins are taken into consideration (see Fig. 4), the total protein and specific activity can only be estimated.

using the synthetic peptide substrates hippuryl-L-arginine and hippuryl-L-lysine (Table III). CPN and CPN active subunit cleave off lysine much faster than arginine. However, both intact CPU and CPU subunits have an arginine carboxypeptidase activity with a similar \( k_{cat} \) as their lysine carboxypeptidase activity.

Besides its peptidase activity, CPN, as other human basic carboxypeptidases, exhibits an esterase activity (19). CPN has a high esterase activity, measured with hippuryl-L-argininic acid, as compared with its peptidase activity. We also found this to be the case for the purified CPN as well as for the CPN subunits. However, purified CPU and CPU subunits exhibit a very low esterase activity using this substrate.

**Stability of CPN, CPN Subunits, CPU, and CPU Subunits**—To have a sufficient stability during assay of the purified enzymes and to ensure optimal conditions for comparing the enzymes, the purified CPN, CPU, CPN subunits, and CPU subunits were diluted to a similar protein concentration and activity as described under "Experimental Procedures." The arginine carboxypeptidase activity of all four diluted enzyme solutions was measured using incubation times of 10, 20, and 45 min. The activities were linear in this range, suggesting a stabilizing effect of the substrates used (correlation coefficients are all ranging from 0.991 to 0.999 using all enzyme preparations and both Hip-Lys and Hip-Arg). Consequently, for further...
studies, incubation periods of 30 min were chosen.

The stability of the enzyme preparations was evaluated by incubating the enzymes under similar conditions at 37 °C for different time intervals (Fig. 5). Carboxypeptidase N is stable during the 4-h incubation period. The CPN active subunits have a decreased stability as reported before (4). The partially purified intact CPN is very unstable under these conditions (t ½ is 15 min), whereas the purified CPU subunits exhibit a somewhat better stability (t ½ is 55 min).

Molecular Mass and Isoelectric Point of CPU—Using gel permeation chromatography on Sephacryl S-300, the apparent molecular mass of carboxypeptidase U was estimated at 435 kDa (14). The molecular mass of the active subunit of CPU was estimated to be 53 kDa by SDS-PAGE of the first fractions of the Mono Q-Sepharose purification step. The isoelectric point of intact CPU as determined by isocratic focusing was determined at pl 5.2.

Michaelis-Menten Constants (K m) —K m constants were determined by a direct linear plot (20) using the synthetic peptide substrates Hip-Arg and Hip-Lys at substrate concentrations ranging from 0.2 to 10 mM. CPU exhibits a K m of 1.12 mM (Hip-Arg) and 1.45 mM (Hip-Lys) (Fig. 6).

Inhibition Constants (K i)—The CPU activity was measured at substrate concentrations (Hip-Arg) of 5, 10, and 20 mM at five different concentrations of the inhibitor in the range of 1–100 μM for GEMSA and 0.1–3 μM for MERGETPA. The K i as determined by the Dixon plot (21), is 58 μM for GEMSA and 0.75 μM for MERGETPA (Fig. 7). Competitive kinetics are confirmed by Cornish-Bowden plots (22), which give parallel lines. Purified CPN has a K i of 11 nM using MERGETPA (results not shown).

Influence of Activators and Inhibitors—The influence of some inhibitors and activators on CPN, CPU, and the active subunits of both enzymes is summarized in Table IV. The results are in agreement with our previous findings in fresh human serum (13). Co 2+ (1 mM), a well known activator of carboxypeptidase N, has little effect on CPU and CPU subunits. o-Phenanthroline (1 mM) inhibits CPN and, to a lesser degree, also CPU. Both findings could suggest that the active site Zn 2+ is more tightly bound in CPU as compared with CPN. The CPN inhibitor MERGETPA at the concentration used (0.2 mM) inhibits both CPN and CPU. Dithiothreitol (0.2 mM) strongly enhances the peptidase activity of intact CPN (340%) but inhibits the activity of the CPU subunit.

Interaction between pro-CPU and Plasminogen and Conversion of pro-CPU to CPU—Plasminogen was purified from human plasma using standard procedures as described under “Experimental Procedures.” However, we noticed that some carboxypeptidase activity (144 units/liter) was present in the purified plasminogen sample. This carboxypeptidase activity clearly proved to be CPU activity, as demonstrated by the ratio of activity on Hip-Arg and Hip-Lys (0.94) and the low esterase activity in comparison with the peptidase activity (0.18 ratio) (Table V). The presence of CPU activity in a purified preparation obtained from human plasma indicates that some of the pro-CPU is transformed to active CPU during purification since native plasma does not contain CPU activity (14). When the purified plasminogen sample is placed for 2 h at 37 °C, the carboxypeptidase activity increases substantially (from 100 to 173%). Since CPU is unstable at 37 °C, this can only mean that the rate of formation of active CPU from its precursor exceeds its inactivation rate. This hypothesis is strengthened by the results of a similar experiment in the presence of DFP. Incubating the purified plasminogen sample for 2 h at 37 °C in the presence of 1 mM DFP results in a strong decrease of CPU activity (from 100 to 2%). DFP, which does not inhibit CPU, clearly inhibits the rate of formation of active CPU from its precursor (Table V). The most logical explanation is that the purified plasminogen sample contains some plasmin and that this plasmin activates pro-CPU. Indeed, by assaying the plasmin activity in the purified plasminogen sample, we could demonstrate that 0.9% of the plasminogen has been transformed to active plasmin, which explains the transformation of pro-CPU to active CPU. Addition of MERGETPA, an inhibitor of CPU activity (14), does not influence the hydrolysis of the plasmin substrate, thereby excluding the possibility that CPU could act on D-Val-Leu-L-Lys-pNA 2HCl. The plasmin activity in the purified plasminogen sample can be completely abolished by the addition of DFP. In conclusion, the data in Table V indicate that the purified plasminogen sample contains pro-CPU as well as active CPU and that the factor that is able to transform pro-CPU to active CPU is still present.

To limit the presence of CPU activity in the purified plasminogen sample (in other words, to prevent the transformation of pro-CPU to active CPU during purification), we performed the purification of plasminogen in the presence of DFP. This resulted in a purified plasminogen sample that contained only trace amounts of active CPU (1.8 units/liter). DFP was not included in the buffer to elute plasminogen, which means that this purified plasminogen sample does not contain DFP. After adding a solution of plasmin (0.1 unit/ml) to the purified plasminogen sample (1:1) and subsequent incubation for 2 h at 37 °C, the CPU activity increased from 1.8 to 105 units/liter (×58 increase). This clearly indicates that plasmin can activate pro-CPU to active CPU and that pro-CPU indeed is present in the purified plasminogen sample. In a control experiment, the same concentration of plasmin was added to partially purified CPU from human serum and incubated for 2 h at 37 °C. The CPU activity was almost completely abolished (3% remaining activity), which indicates that CPU itself is not activated by plasmin and confirms the instability of CPU already demonstrated before.

The purified plasminogen sample, which contained low CPU activity, was applied on a Sephacryl S-200 gel filtration column, and the CPU activity was measured in the eluted fractions (Fig. 8). The recovery of CPU activity after this gel filtration was 237%, which indicates again that the sample undergoes some CPU activation during purification as a consequence of small amounts of plasmin (0.02 units/ml) present in the plasminogen sample. DFP (5 mM) was added to aliquots of the fractions, and these aliquots were subsequently incubated for 2 h at 37 °C. The CPU activity, being unstable, was no longer present. How-
**Human Plasma Carboxypeptidase U**

**FIG. 6.** Carboxypeptidase U activity as a function of substrate concentration of Hip-Arg (right) and Hip-Lys (left). Insets, direct linear plot of V against S. Each line represents one observation and is drawn with intercepts S on the abscissa and V on the ordinate. The points of intersection give the coordinates of the best fit values of $K_m$ and $V_{max}$. Each point represents the mean of triplicates.

**FIG. 7.** Dixon plots of the rate of hydrolysis of Hip-Arg by carboxypeptidase U as a function of the concentration of GEMSA (left) and MERGETPA (right). Activity measurements are made at substrate concentrations of 5, 10, and 20 mM. Each point represents the mean of triplicates.

**TABLE IV**

| Addition  | Concentration | CPN activity | CPN subunit activity | CPU activity | CPU subunit activity |
|-----------|---------------|--------------|---------------------|--------------|---------------------|
| None      |               | 100          | 100                 | 100          | 100                 |
| $Co^{2+}$ | 1             | 282          | 428                 | 63           | 103                 |
| o-Phenanthroline | 1   | 4            | 1                   | 57           | 53                  |
| MERGETPA  | 0.2          | 0            | 0                   | 6            | 1                   |
| Dithiothreitol | 0.2 | 340          | 98                  | 86           | 52                  |

However, addition of plasmin (0.1 unit/ml) to aliquots of the fractions after gel filtration and subsequent incubation for 2 h at 37 °C gave rise to active CPU (Fig. 8). This again demonstrates that pro-CPU is present in these samples and that it can be activated to CPU by plasmin.

**DISCUSSION**

Recently, we demonstrated the presence of a novel basic carboxypeptidase activity present in fresh human serum and absent in human heparinized plasma (12, 13). Because of the instability of this enzyme at room temperature and at 37 °C, we named it CPU (unstable carboxypeptidase). Carboxypeptidase U could only be partially purified, mainly because of its marked instability (14). We were able to demonstrate that partially purified CPU has characteristics (substrate specificity, influence of inhibitors and activators) that clearly distinguish it from plasma carboxypeptidase N and from other known basic carboxypeptidases (carboxypeptidase B, carboxypeptidase M, carboxypeptidase H, and urinary carboxypeptidase).

Different hypotheses for the presence of this unstable carboxypeptidase were investigated, including that the enzyme was not present in human blood cells and that it was not identical with the active subunit of CPN. Finally, it was concluded...
that a pro-CPU is present in human plasma and that it is transformed to an active basic CPU during the process of coagulation or fibrinolysis. An immediate physiological role for this enzyme in the coagulation system could not be established since inhibition of the enzyme did not significantly alter routine assays to evaluate the coagulation cascade (14).

Since then, our findings are confirmed by results of Campbell and Okada (23) and Campbell et al. (24), who noticed a "transient" basic carboxypeptidase activity in serum, which was derived from plasma components and not from blood cells or platelets and was unrelated to CPN or its subunits. The marked lability of the active enzyme is clearly shown in all of these publications and is probably also the reason why this enzyme activity was not noticed before.

Because of the high apparent molecular mass of active CPU (435 kDa), it seemed likely that either CPU is composed of subunits (as is the case with CPN) or that it is composed to some other plasma protein(s). We tried to reduce the interactions between CPU and other plasma proteins by treatment with 3 M guanidine HCl, an approach that has proved to be successful for the transformation of CPN into its subunits (4). By subsequently using gel filtration chromatography on Sephacryl media, followed by ion exchange chromatography on mono Q-Sephrose, we were able to purify the CPU active subunit. It shows an $M_r$ of 53,000 on SDS-PAGE, which is similar to that of the CPN active subunit. The CPU active subunit was characterized with regard to its peptidase and esterase activity and its inhibition pattern. This resulted in similar findings as previously reported for the intact enzyme (14). Most interestingly, CPU does not have a measurable esterase activity. We did not succeed in obtaining sufficient material for amino-terminal sequencing of the CPU subunit, mainly because only a limited amount of the active CPU subunit was pure after the mono Q-Sephrose step. Therefore, if one needs to obtain structural information, one has to try to purify the (stable) precursor of CPU (pro-CPU) from human plasma.

As is often the case in scientific progress, a breakthrough in this field came unexpectedly. In 1991, Eaton et al. (25) published the isolation and molecular cloning of a protein in human plasma that was bound to plasminogen-Sepharose. This protein turned out to be a procarboxypeptidase, which they named "human plasma procarboxypeptidase B." This protein could be cleaved in vitro by trypsin and plasmin. The resulting active carboxypeptidase was very unstable after its activation. The substrate specificity of this enzyme and its lability are identical to our active CPU.

Therefore, pro-CPU is sequenced by this group. We intended to purify this pro-CPU to activate it and to prove that this activated enzyme is the same as active CPU purified from human serum. However, when we had purified plasminogen from human plasma according to a well known procedure that specifically elutes plasminogen from the affinity gel by using e-aminoacaproic acid, we noticed that a basic carboxypeptidase activity was already present in the concentrated purified plasminogen preparation. In further experiments, it became clear that this activity was indeed identical to the CPU activity and not to CPN. Moreover, the precursor of CPU (pro-CPU) was also present in this sample. Furthermore, we demonstrated that the concentrated plasminogen contained active plasmin, which is responsible for the conversion of active CPU from its inactive proenzyme (pro-CPU). Our results, together with the observations of Eaton et al. (25), strongly suggest that circulating pro-CPU is physically associated with plasminogen.

In conclusion, our final hypothesis is that a basic pro-CPU circulates in human plasma, bound to plasminogen, and that it is identical with the procarboxypeptidase cloned and sequenced by Eaton et al. (25). During fibrinolysis, when plasminogen is converted to plasmin by the tissue plasminogen activator (26), plasmin cleaves pro-CPU to form active CPU, which thus is released at the specific site of coagulation and fibrinolysis. It is generally accepted that an intricate interrelationship exists between the processes of coagulation, fibrinolysis, complement activation, and the kallikrein/kinin pathway. The basic carboxypeptidase CPU can be activated at the exact site where a lot of its potential substrates are being released during the activation of these different cascades. CPU could act on bradykinin (transforming it to des-Arg$^9$ bradykinin) and on released anaphylatoxins C$_{3a}$ and C$_{5a}$ and (maybe more important) it could play a role in the control of the fibrinolytic system.

Indeed, many interactions in the fibrinolytic system are promoted by the presence of lysine binding sites present in the kallikrein domains of plasminogen, prothrombin, coagulation factor XII, tissue plasminogen activator, urokinase, and apolipoprotein(a) (27). When tissue plasminogen activator is released from the endothelium and binds to fibrin, it selectively activates fibrin-bound plasminogen by the established mechanism of ternary complex formation (28). Plasmin degradation of fibrin exposes carboxyl-terminal lysine residues that are essential for the high affinity binding of plasminogen to fibrin (by means of their lysine binding sites). When native plasmin binds to these carboxyl-terminal lysines, it undergoes a unique conformational change that converts it into an optimum substrate for prourokinase (29). Thus, the activated CPU on site could control the rate of fibrinolysis by cleaving off part of the carboxyl-terminal lysine residues. Indeed, it has been demonstrated that the modification of the fibrinolytic effect by plasmin was nullified by a subsequent treatment of the clot with carboxypeptidase B, indicating that the plasmin effect was related to the exposure of carboxyl-terminal lysine residues on fibrin (30). Similarly lipoprotein(a) binding to plasminogen is lysine binding-dependent as it is inhibited by e-aminoacaproic acid. Lipoprotein(a) inhibits the binding of plasminogen to plasmin-modified immobilized fibrinogen, indicating that both molecules compete for similar lysine binding sites (31).

Another possible function of CPU could be situated in the interaction between the naturally occurring inhibitor $\alpha_2$-antiplasmin and plasmin. $\alpha_2$-Antiplasmin inhibits plasmin in a two-step reaction in which $\alpha_2$-antiplasmin reversibly binds to lysine binding sites of plasmin and then slowly complexes co-
valently with the enzyme's active site. The carboxyl-terminal lysine of α₂-antiplasmin has a key role in binding of the inhibitor to plasmin because by removal of the carboxyl-terminal lysine by treatment with a basic carboxypeptidase, α₂-antiplasmin lost its ability to inhibit plasmin rapidly (32). Thus, by removing this lysine from α₂-antiplasmin, CPU could protect the inactivation of plasmin, therefore enhancing the fibrinolysis.

Finally, the resemblance between the activation process of CPU and the digestive carboxypeptidases is striking. For instance, pancreatic procarboxypeptidase A can be stored and secreted as binary complexes with proproteinase or with chymotrypsinogen or even as ternary complexes that include both serine proteases (33). A similar complex formation between pro-CPU and plasminogen could also function as a control mechanism for the exact timing of potentiation of the activation process.

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