Isolation and characterization of the subunits of human plasma carboxypeptidase N (kininase I)

(kinins/anaphylatoxins/kallikrein/proteases/carboxypeptidase B)

YEHUDA LEVIN^{*†}, RANDAL A. SKIDGEL^{*}, AND ERVIN G. ERDÖS^{*‡§}

Departments of ^{*}Pharmacology and [†]Internal Medicine, University of Texas Health Science Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235

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ABSTRACT Carboxypeptidase N (kininase I, arginine carboxypeptidase; EC 3.4.17.3) cleaves COOH-terminal basic amino acids of kinins, anaphylatoxins, and other peptides. The tetrameric enzyme of M_r 280,000 was purified from human plasma by ion-exchange and arginine-Sepharose affinity chromatography. Treatment with 3 M guanidine dissociated the enzyme into subunits of 83,000 and 48,000 molecular weight, which were separated and purified by gel filtration or affinity chromatography. When tested with hippurylarginine, hippurylargininic acid, benzoylalaninyllysine, or bradykinin, the M_r 48,000 subunit was as active as the intact enzyme and was easily distinguished from human pancreatic carboxypeptidase B (EC 3.4.17.2). However, the M_r 48,000 subunit was less stable at acid pH or at 37°C than the intact enzyme was. The carbohydrate-containing M_r 83,000 subunit was enzymatically inactive but stabilized the M_r 48,000 subunit at 37°C. Trypsin, plasmin, and plasma or urinary kallikrein cleaved carboxypeptidase N into lower molecular weight active fragments, which were unstable at 37°C. Cleavage of the M_r 48,000 subunit with the same enzymes increased activity and yielded fragments of M_r 29,000 or less. Antibodies to the M_r 83,000 or M_r 48,000 subunits crossreacted with the intact enzyme, and antibody to carboxypeptidase N also recognized both subunits. However, antibody to the M_r 83,000 subunit did not recognize the M_r 48,000 subunit and antibody to the M_r 48,000 subunit did not crossreact with the M_r 83,000 subunit. Thus, this study indicates that carboxypeptidase N is composed of two immunologically distinct subunits, a M_r 48,000 subunit that is responsible for the enzymatic activity and a M_r 83,000 subunit that may stabilize the enzyme in blood.

Carboxypeptidase N or arginine carboxypeptidase (EC 3.4.17.3) is an important inactivator of potent peptides such as kinins (1), anaphylatoxins (2, 3), and fibrinopeptides (1). It also cleaves COOH-terminal basic amino acids of a variety of other peptide substrates (1, 4). The enzyme exists in plasma as a M_r 280,000 tetrameric complex, even when plasma is stored for several months (5, 6). Purification of the enzyme from plasma on ion-exchange and affinity columns leads to an unstable preparation (5–7); this can be stabilized with protease inhibitors (7). Thus, dilution of naturally occurring inhibitors or simultaneous adsorption of the enzyme and contaminating proteases (e.g., plasmin) during purification leads to the appearance of lower molecular weight derivatives (5–8).

The aims of the present study were (i) to dissociate purified carboxypeptidase N and isolate its subunits; (ii) to determine the enzymatic activity, stability, and function of the isolated subunits; (iii) to determine the effect of various proteolytic enzymes on the activity and stability of both the intact enzyme and its subunits.

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MATERIALS AND METHODS

Outdated human plasma was obtained from the Parkland Memorial Hospital blood bank (Dallas, TX). Hippurylargininic acid (Vega Biochemicals, Tucson, AZ), bradykinin (Bachem Fine Chemicals, Torrance, CA), guanidine-HCl (Chemalog, South Plainfield, NJ), trypsin (Worthington, Freehold, NJ), and human plasmin [Committee on Thrombolytic Agents (CTA) Standard, 10 units/ml, the American National Red Cross] were used as received. Human urinary kallikrein was donated by H. Fritz (Munich, Federal Republic of Germany) and human plasma kallikrein by A. Kaplan (Stony Brook, NY). Benzoylalaninyllysine (Bz-Ala-Lys) and guanidinoethylmercaptosuccinic acid (GEMSA) were synthesized by modifications of published procedures (9–11). L-Arginine-Sepharose was prepared from epichlorohydrin-activated Sepharose 6B (12). Human pancreatic carboxypeptidase B (EC 3.4.17.2) was purified from autopsy samples as reported (13).

Enzyme Activity. The activity of carboxypeptidase N was measured in a spectrophotometer with the ester, hippurylargininic acid, or the peptide, Bz-Ala-Lys, substrate at 254 nm in 0.1 M Hepes or Tris (pH 8.0) at 37°C (14, 15).

The hydrolysis of bradykinin was measured either by bioassay or with an amino acid analyzer (6). The incubation mixture for bioassay contained 2–5 μ g of enzyme protein, 5 μ g of bradykinin (10 μ M), and 0.1 M Hepes buffer, pH 8.0, in 0.5 ml. The inactivation of bradykinin was measured on the isolated, atropinized guinea pig ileum.

The release of Arg-9 from bradykinin was determined in a Beckman 121 amino acid analyzer. Bradykinin (1 mM), 1–5 μ g of enzyme, and 0.1 M Hepes, pH 8.0, in 0.375 ml were incubated at 37°C for 5–60 min. The reaction was stopped with 0.375 ml of 3% sulfosalicylic acid (6).

Purification of Carboxypeptidase N. The enzyme was purified as published (5–7, 11) with the following modifications. Two liters of outdated human plasma, pretreated with 1 mM phenylmethylsulfonyl fluoride and 1 mM diisopropyl fluorophosphate, were diluted to 4 liters with 0.05 M Tris-HCl, pH 7.2, added to 1 liter of settled DEAE-cellulose (DE-52, Whatman) slurry, and adjusted to pH 7.2 with 1 M HCl. After stirring for 1–2 hr, the cellulose, recovered by filtration, was washed with 1 liter of 0.05 M NaCl in 0.05 M Tris-HCl, pH 7.2, then resuspended in the same buffer and poured into a 5 $\times$ 60 cm column. The enzyme was eluted with a linear gradient of 0.05–0.25 M NaCl (4 liters each) in the same buffer. To the active pool (1,600 ml) were added proteolytic inhibitors and arginine-Sepharose (100 ml of settled gel). The gel-bound en-

Abbreviations: Bz-Ala-Lys, benzoylalaninyllysine; GEMSA, guanidinoethylmercaptosuccinic acid; CTA, Committee on Thrombolytic Agents.
[†] Present address: Dept. of Biophysics, The Weizmann Institute of Science, Rehovot, Israel 76100.

[§] To whom reprint requests should be addressed.

zyme was recovered by filtration, poured into a column (5 × 5 cm), washed with 0.2 M NaCl followed by 0.25 M NaCl in 0.01 M sodium phosphate (pH 7.0) with 1% 1-butanol, and eluted with 1 mM GEMSA/0.25 M NaCl in the same buffer. The enzyme was purified an average of 2,665-fold with a 20–30% yield, similar to the results of Plummer and Hurwitz (7).

Treatment of Carboxypeptidase N with Guanidine. To 5–7 mg of purified carboxypeptidase N (1–2 mg/ml in 0.1 M NH_4HCO_3) was added guanidine-HCl to 3 M at 4°C. The mixture was applied to a column (2.5 × 100 cm) of Sephadex G-75 superfine preequilibrated with 0.01 M Tris-HCl, pH 7.2/0.05 M NaCl and eluted with the same buffer at 25 ml/hr. The two protein peaks eluted were pooled separately and concentrated by ultrafiltration on Amicon YM-10 membranes. The second peak was concentrated in 2 M NaCl followed by elution from the membrane with 2 ml of water/glycerol, 1:1 (vol/vol).

Gel Electrophoresis. Polyacrylamide gel electrophoresis was conducted in 7.5% or 10% gels containing 0.1% NaDodSO₄ (16). Polyacrylamide slab gel electrophoresis was done according to Laemmli (17). Proteins were stained with Coomassie blue R250 or G250 and carbohydrates were stained with Schiff reagent (18).

Proteolysis of Carboxypeptidase N. Aliquots (25–50 µg) of intact carboxypeptidase N or M_r 48,000 subunit were incubated at room temperature for 1–24 hr in 0.1 M Tris-HCl, pH 8, with: trypsin (10 µg), chymotrypsin (10 µg), plasmin (0.1 CTA unit), urinary kallikrein (1.5 µg), hog pancreatic kallikrein (10 µg), or plasma kallikrein (3 µg). Proteolysis was stopped with aprotinin (130 units).

Antibody Production. Antisera to purified carboxypeptidase N and to the high and low molecular weight subunits (obtained after treatment with guanidine) were raised in rabbits.

"Rocket" Immunoelectrophoresis. Rocket immunoelectrophoresis was performed according to Laurell (19). Antisera (0.42 ml) mixed with 15.6 ml of 1% agarose (Sigma, low electro end osmosis) in sodium barbital buffer (pH 8.6) at 55–65°C were poured onto a sheet of Gel Bond film (Marine Colloids Division, FMC, Rockland, ME). Antigen was applied to 2.5-mm wells and electrophoresed at 10 V/cm for 3 hr at 14°C, and the gel was then washed, pressed, and stained (20).

Acid Stability of Carboxypeptidase N. Aliquots of carboxypeptidase N (4 µg) or M_r 48,000 subunit (2 µg) were incubated

at room temperature for 1 hr in 0.05 M sodium acetate (pH 4.0–5.0) and then tested for activity with hippurylargininic acid. Controls were diluted in 0.1 M Tris-HCl buffer, pH 8.0.

Heat Stability of Carboxypeptidase N. Carboxypeptidase N was treated with proteolytic enzymes as stated above. The purified M_r 48,000 subunit (18 µg) was preincubated for 30 min at 4°C in buffer alone or together with either the M_r 83,000 subunit (154 µg) or bovine serum albumin (160 µg). All samples were diluted to 1.2 ml with 0.1 M Tris-HCl buffer, pH 8.0, and incubated at 37°C.

RESULTS

Separation of Subunits. Carboxypeptidase N was purified from outdated human plasma by a two-step procedure simplified from published methods (5–7, 11). The enzyme appeared as a single band in 7.5% polyacrylamide gel electrophoresis but in 10% gels with NaDodSO₄ dissociated to a M_r 83,000 subunit that stained positively for carbohydrate and two low molecular weight subunits of M_r 48,000 and 56,000 that did not stain for carbohydrate (7, 8).

Purified human carboxypeptidase N retained 85–95% activity after treatment with 3 M guanidine even though the enzyme completely dissociated. After guanidine treatment, two peaks were obtained after gel filtration on a Sephadex G-75 column (Fig. 1). The first protein peak eluted in the void volume and was inactive. The second peak had all of the enzymatic activity but contained less protein. The material from the first peak in 10% polyacrylamide gel electrophoresis with NaDodSO₄ gave a single carbohydrate-positive band of M_r approximately 83,000. Protein from the second peak gave a single band of M_r 48,000 without carbohydrate. The M_r 48,000 subunit was retained on the Sephadex G-75 column and co-eluted with the salt at pH 7.2 (Fig. 1). However, at pH 8.7 the M_r 48,000 subunit eluted in fraction 42, as expected for its molecular weight. This suggests a hydrophobic interaction between the subunit and gel support because increasing pH decreases the strength of hydrophobic interactions (21).

The subunits after guanidine treatment were also separated by affinity chromatography. After dissociation by guanidine, the enzyme was applied to a column of arginine-Sepharose. The M_r 83,000 subunit eluted with a buffer of low ionic strength (0.05 M NaCl), but the M_r 48,000 subunit was retained by the affinity

FIG. 1. Chromatography of the active and inactive subunits of carboxypeptidase N on a Sephadex G-75 column after dissociation with 3 M guanidine. Fractions (6.4 ml) were tested for protein (○, A₂₈₀) and activity (▲, substrate: hippurylargininic acid). First peak, M_r 83,000 inactive subunit; second peak, M_r 48,000 active subunit.

Table 1. Comparison of carboxypeptidase N with the isolated M_r 48,000 subunit

Enzyme	Substrate (1 mM)	Hydrolysis, mmol·min ⁻¹ · μmol ⁻¹ *	K_m , mM	GEMSA I_{50} , [†] μM
Carboxypeptidase N (M_r 280,000)	Bradykinin	0.10 [‡]	—	—
	Hippurylargininic acid	5.4	0.14	25
	Bz-Ala-Lys	9.0	0.25	9
Active subunit (M_r 48,000)	Bradykinin	0.11 [‡]	—	—
	Hippurylargininic acid	5.6	0.08	34
	Bz-Ala-Lys	7.5	0.28	11

* μmol refers to active site, assuming the M_r 280,000 enzyme has two active sites and the M_r 48,000 subunit has one.

[†] I_{50} , concentration that inhibited activity with 1 mM substrate by 50%.

[‡] Measured by amino acid analyzer with 1 mM bradykinin. In bioassay (10 μM bradykinin) the rates of hydrolysis were 5.8 and 4.8 μmol·min⁻¹·μmol⁻¹ of active site for carboxypeptidase N and the M_r 48,000 subunit, respectively.

gel and eluted only with 1 mM GEMSA, a further indication that the active site resides in the M_r 48,000 subunit.

Activities. The activities of the M_r 48,000 subunit and the intact enzyme were compared in detail. The M_r 48,000 subunit was fully active in cleaving hippurylargininic acid, Bz-Ala-Lys, and bradykinin. On a molar basis, it was as active as the intact enzyme (Table 1), assuming the intact enzyme is a tetramer of two active M_r 48,000 subunits and two inactive M_r 83,000 subunits (5, 7, 8). The K_m values were similar for the intact enzyme and the M_r 48,000 subunit when measured with both ester and peptide substrates (Table 1). Thus, the M_r 48,000 subunit appeared to retain all the enzymatic activity of the intact carboxypeptidase-containing enzyme with ester and small peptide substrates. In addition, the I_{50} for GEMSA with the subunit and intact enzyme was similar with both substrates (Table 1).

Comparison to Carboxypeptidase B. The activities of intact carboxypeptidase N and its active subunit were compared with those of purified human pancreatic carboxypeptidase B to determine whether the separated M_r 48,000 subunit resembled human carboxypeptidase B or the intact enzyme. Carboxypeptidase B has a molecular weight of 34,000 (13, 22). Carboxypeptidase B cleaved hippurylarginine faster relative to Bz-Ala-Lys than did carboxypeptidase N or the M_r 48,000 subunit (Table 2) at the substrate concentration used. This is in accord with our earlier studies on these carboxypeptidases (5, 6). When the esterase and peptidase activities of the two enzymes were compared, carboxypeptidase B was found to hydrolyze Bz-Ala-Lys and hippurylargininic acid at a ratio of 0.7, whereas the ratios for intact carboxypeptidase N and its low molecular weight subunit were 3.3 and 2.4, respectively (Table 2). Cadmium acetate (0.1 mM) inhibited both the M_r 48,000 subunit and the intact carboxypeptidase N (6, 15) but enhanced the esterase activity of carboxypeptidase B (Table 2; ref. 23).

Table 2. Comparison of the activities of human pancreatic carboxypeptidase B, carboxypeptidase N, and the low molecular weight subunit

Substrates (1 mM)	Activity ratio		
	Carboxy- peptidase B	Carboxy- peptidase N	M_r 48,000 subunit
Bz-Ala-Lys/ hippurylarginine	2.5	29.4	24.4
Bz-Ala-Lys/ hippurylargininic acid	0.7	3.3	2.4
Hippurylargininic acid with Cd/without Cd*	1.6	0.33	0.40

* Enzyme was preincubated for 20 min (4°C) with 0.1 mM cadmium acetate.

Stability. Because the dissociated enzyme was less stable than the intact one at 37°C (6), we incubated the intact enzyme, M_r 48,000 subunit, and proteolytically treated carboxypeptidase N at 37°C. Intact carboxypeptidase N was stable at 37°C for 2 hr (Fig. 2), but after treatment with plasmin, chymotrypsin, urinary kallikrein, or plasma kallikrein the activity was rapidly lost (Fig. 2). Similarly, the isolated M_r 48,000 subunit lost 75% of its activity in 2 hr (Fig. 3). To test the stabilizing effect of the high molecular weight subunit, a 5-fold molar excess of the M_r 83,000 subunit was incubated with the M_r 48,000 subunit. Under these conditions, the activity was more stable, decreasing only 37% (Fig. 3). In control experiments, the same amount of bovine serum albumin had no protective effect.

The relative stabilities of the intact enzyme and the subunit at room temperature were tested at pH 4–5. The M_r 48,000 subunit was less stable than the intact enzyme (Fig. 4), losing 94% of its activity in 1 hr at pH 4.0, while the intact enzyme lost only 9%.

FIG. 2. Stability at 37°C of the active fragments of carboxypeptidase N released by proteases. Carboxypeptidase N (25 μg) was incubated at room temperature for 24 hr with buffer alone (●), human urinary kallikrein (1.5 μg) (■), hog pancreatic kallikrein (10 μg) (Δ), or human plasma kallikrein (3 μg) (□); or for 6 hr with chymotrypsin (10 μg) (○) or plasmin (0.1 CTA unit) (○). Aprotinin (130 units) was added to stop proteolysis, and the samples were diluted 1:40 in assay buffer and incubated at 37°C. Substrate: hippurylargininic acid.

FIG. 3. Stabilizing effect of the M_r 83,000 subunit on the M_r 48,000 subunit at 37°C. The isolated M_r 48,000 subunit (18 μ g) was incubated at 37°C in the presence of buffer alone (○), bovine serum albumin (160 μ g) (●), or the M_r 83,000 subunit (154 μ g) (▲). Substrate: hippurylargininic acid.

Effect of Proteases. Plasmin and trypsin can cleave carboxypeptidase N to lower molecular weight active fragments. Plasmin or trypsin released from the intact enzyme two faster-moving fragments in 7.5% polyacrylamide gel electrophoresis that stained for carbohydrate, in agreement with the results of Plummer and Hurwitz (7). Cleavage of carboxypeptidase N by purified human urinary or plasma kallikrein produced three faster-moving fragments all of which contained carbohydrate.

The isolated M_r 48,000 subunit was also cleaved by proteolytic enzymes. Treatment of the subunit (50 μ g) with plasmin (0.05 CTA unit), trypsin (5 μ g), or urinary kallikrein (0.75 μ g)

FIG. 4. Activity of intact carboxypeptidase N (●) and the M_r 48,000 subunit (□) exposed to pH 4.0–5.0 for 1 hr at room temperature. Control activity at pH 8.0 = 100%.

for 5 hr at room temperature produced three similar patterns in NaDodSO₄/12% polyacrylamide gel electrophoresis. Plasmin and trypsin released from the M_r 48,000 subunit three protein bands of M_r 29,000, 28,000, and 23,000 and a diffuse band of M_r 26,000–27,000. The pattern after treatment with urinary kallikrein was somewhat different, and a major band of M_r 29,000, a minor diffuse band at M_r 28,000, and a minor band at M_r 23,000 were found. In all cases, proteolysis of the M_r 48,000 subunit yielded fragments more active than the native subunit. Treatment with plasmin, trypsin, or urinary kallikrein for 5 hr at room temperature increased esterase activity 40%, 54%, and 14% and peptidase activity 41%, 84%, and 44%, respectively, relative to nonincubated controls. A control sample, incubated in buffer alone under the same conditions, lost 30% of its esterase and 13% of its peptidase activity. This was not unexpected in light of the marked instability of the M_r 48,000 subunit at 37°C (Fig. 3).

Antibodies. Antibodies were raised in rabbits to native carboxypeptidase N and to the isolated M_r 83,000 and 48,000 subunits. The crossreactivity of the antibodies was studied by rocket immunoelectrophoresis. The M_r 83,000 subunit, the M_r 48,000 subunit, and native carboxypeptidase N produced distinct patterns with their respective antibodies (Fig. 5). In addition, the M_r 48,000 subunit crossreacted with antibody to the intact enzyme but not with antibody to the M_r 83,000 subunit. The M_r 83,000 subunit also crossreacted with antibody to intact carboxypeptidase N but not with antibody to the M_r 48,000 sub-

FIG. 5. Rocket immunoelectrophoresis of carboxypeptidase N and the isolated subunits in agarose containing antisera to the M_r 48,000 subunit (A), M_r 83,000 subunit (B), or intact carboxypeptidase N (C). In all cases the wells contained (from left to right) the M_r 48,000 subunit (5 μ g), the M_r 83,000 subunit (2 μ g), and intact carboxypeptidase N (4 μ g).

unit, and native carboxypeptidase N interacted with antibodies to either subunit.

DISCUSSION

Carboxypeptidase N of M_r 280,000 (1) was completely dissociated into its constituent subunits in 3 M guanidine, suggesting that ionic forces hold the protein together. When the subunits were isolated by either gel filtration or affinity chromatography and analyzed by polyacrylamide gel electrophoresis, the M_r 83,000 subunit contained carbohydrate but was inactive and the M_r 48,000 subunit contained no carbohydrate and all the activity. Rocket immunoelectrophoresis showed that the two subunits do not have common antigenic determinants and that antibodies to the intact enzyme recognized both isolated subunits.

After many of the experiments reported here were completed, an abstract by Plummer and Kimmel was published (24). They showed in an elegant experiment that modification of carboxypeptidase N with dimethylmaleic anhydride released from the enzyme an active low molecular weight subunit and a high molecular weight subunit containing carbohydrate but no activity. They labeled the enzyme with *N*-bromoacetyl-D-[5- 14 C]arginine and found the label primarily in the small subunit (24).

When the M_r 48,000 subunit was tested for esterase activity with hippurylargininic acid or for peptidase activity with Bz-Ala-Lys or bradykinin, it was equally active on a molar basis to native carboxypeptidase N. When the activity of the M_r 48,000 subunit was compared to human pancreatic carboxypeptidase B with various substrates and inhibitors, it was found to be quite different from the pancreatic enzyme, just as the intact carboxypeptidase N was. This indicates that the M_r 48,000 subunit retains all the characteristics of the intact enzyme with respect to oligopeptides. Further experimentation should determine whether this holds true for higher molecular weight substrates.

While the enzymatic activities were similar, the stabilities of the M_r 48,000 subunit and the intact enzyme were strikingly different. The M_r 48,000 subunit was unstable at 37°C and at acid pH, whereas the intact enzyme was quite stable. The experiments at 37°C indicate that, although not required for enzymatic activity with oligopeptides, the M_r 83,000 subunit may be needed to stabilize the enzyme in plasma. The fact that addition of the M_r 83,000 subunit to the isolated M_r 48,000 subunit significantly enhanced stability at 37°C supports this hypothesis.

Native carboxypeptidase N was readily cleaved by plasmin or trypsin into two major lower molecular weight fragments containing carbohydrate that retained full activity (7). In addition, we found that human plasma or urinary kallikrein cleaved carboxypeptidase N into three major fragments that contained carbohydrate and moved faster than intact carboxypeptidase N on nondenaturing polyacrylamide gels. In all cases, proteolysis of purified carboxypeptidase N resulted in fragments that were active but much less stable at 37°C.

Oshima *et al.* (5, 6) reported that, in NaDodSO₄/polyacrylamide gel electrophoresis, carboxypeptidase N dissociated into high and low molecular weight subunits. Upon standing, carboxypeptidase N dissociated into two lower molecular weight "subunits," which were both active (6). It is likely that the subunits obtained during storage were fragments that resulted from trace proteolytic contamination of the pure enzyme, as suggested by Plummer and Hurwitz (7). In our studies, proteolysis of carboxypeptidase N yielded two or three lower molecular weight fragments, all of which contained carbohydrate. Presumably this resulted from cleavage of both subunits, which remained attached by ionic forces. Guanidine treatment, in contrast, dissociated carboxypeptidase N into an inactive carbohydrate-positive subunit (M_r 83,000) and an active carbohydrate-negative subunit (M_r 48,000).

drate-negative subunit (M_r 48,000).

Treatment of the isolated M_r 48,000 subunit with proteolytic enzymes yielded fragments with 14–54% higher esterase and 41–84% higher peptidase activity. NaDodSO₄/polyacrylamide gel electrophoresis of the enzyme-treated M_r 48,000 subunit revealed fragments of 29,000 and lower molecular weight. Thus, a fragment of carboxypeptidase N of M_r 29,000 or lower still retains enzymatic activity with small substrates.

Because carboxypeptidase N is the major blood-borne inactivator of potent peptides such as kinins (1) and anaphylatoxins (2, 3) the preservation of its activity in the blood is of great importance. The results of this study suggest conditions under which this important enzymatic activity could be compromised. For example, a decrease or loss of endogenous blood-borne proteolytic inhibitors may lead to cleavage of carboxypeptidase N. Because of the lower stability of the active proteolytic products at 37°C, the effective concentration of circulating enzyme would decrease, thereby increasing the half-life of blood-borne kinins and anaphylatoxins. Furthermore, if the M_r 48,000 subunit were released into the circulation without the M_r 83,000 subunit, it would rapidly lose activity and would be removed from the blood by glomerular filtration.

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