Human Carboxypeptidase M

PURIFICATION AND CHARACTERIZATION OF A MEMBRANE-BOUND CARBOXYPEPTIDASE THAT CLEAVES PEPTIDE HORMONES

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A membrane-bound neutral carboxypeptidase B-like enzyme was solubilized from human placental microvilli with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and purified to homogeneity by ion-exchange chromatography and affinity chromatography on arginine-Sepharose. It gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent Mr of 62,000 with or without reduction. The enzyme is a glycoprotein as shown by its high affinity for concanavalin A-Sepharose and reduction in mass to 47,600 daltons after treatment with 2-mercaptoethanol. The enzyme shares some properties with other carboxypeptidases, including its localization on the plasma membrane and optimal activity at neutral pH, carboxypeptidase B (EC 3.4.17.2) was extensively characterized and is a prototype for this class of enzymes (1). Carboxypeptidase N (arginine carboxypeptidase, kininase I, anaphylatoxin inactivator, creatine kinase conversion factor, EC 3.4.17.3) discovered in human plasma over 26 years ago, is an important plasma enzyme as it cleaves many endogenous peptides and proteins (1) (e.g. kinins (2,3), anaphylatoxins (4), fibrinopeptides (5), Arg'- or Lys'-enkephalins (6), creatine kinase (7), albumin propeptide (8), complement factor B (10), etc.). A metallo-carboxypeptidase with an acid pH optimum, originally described in pancreatic islets (11), was purified from adrenal medulla, brain, pituitary (12), and pancreatic islets (13).

We discovered an arginine/lysine carboxypeptidase from human plasma and named it carboxypeptidase H. The enzyme arginine- and lysine carboxypeptidase from human plasma and named it carboxypeptidase H. Because of its localization on the plasma membrane and optimal activity at neutral pH, carboxypeptidase M could inactivate or modulate the activity of peptide hormones either before or after their interaction with plasma membrane receptors.

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glucoside. The enzyme was finally eluted with buffer containing 0.25 mM NaCl + 0.5 mM α-methyl-d-glucoside (not shown).

Chemical deglycosylation of carboxypeptidase M with trifluoromethanesulfonic acid decreased its apparent size by an average of 14,400 daltons on sodium dodecyl sulfate-PAGE to 47,600 ± 1,000 (±S.E., n = 5), indicating a carbohydrate content of 23% by weight (Fig. 2).

Effect of pH—The peptidase activity of carboxypeptidase M was tested with Bz-Ala-Lys substrate at pH values ranging from 5.0 to 9.0. The maximal activity was obtained at pH 7.0, but the enzyme still retained about 90% of its activity at pH 8.0 and 60% at pH 9.0. The activity dropped sharply at acid pH, with only 47% at pH 6.0, 25% at pH 5.5, and no activity at pH 5.0.

To test its stability at acid pH, carboxypeptidase M was incubated for 1 h at room temperature in buffers with pH values ranging from 4.0 to 5.0 and then tested for activity at pH 8.5 with Bz-Gly-argininic acid. Carboxypeptidase M was stable between pH 4.5 and 5.0 but lost 17% of its activity after an hour at pH 4.25 and 33% at pH 4.0.

Inhibition—The hydrolysis of Bz-Gly-Lys by carboxypeptidase M was completely inhibited by 1 mM α-phenanethrone or 10 mM DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid. Aprotinin (100 units/ml) and 1 mM phenylmethylsulfonfluoride (serine protease inhibitors) and 0.1 mM p-chloromercuriphenylsulfonate (which inhibits sulfhydryl processes and carboxypeptidase H) had no appreciable effect. As measured by HPLC with bradykinin (0.1 mM) as substrate, 1 μM DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid completely inhibited bradykinin hydrolysis but 1 μM captorpril (an angiotensin 1 converting enzyme inhibitor) or 1 μM phosphoramidon (an inhibitor of neutral endopeptidase 24.11) had no effect.

Effect of Metal Ions—As with other mammalian basic carboxypeptidases, preincubation for 2 h with 1 mM CoCl₂ increased the peptidase activity of carboxypeptidase M (5-fold with Bz-Gly-Lys, 1.5-fold with Bz-Gly-Arg (Table II) and 1.4-fold with dansyl-Ala-Ala-Arg) and 0.1 mM cadmium acetate inhibited it (49% with Bz-Gly-Lys and 51% with dansyl-Ala-Ala-Arg). However, preincubation for 2 h with 1 mM cadmium acetate or 1 mM CoCl₂ had no effect on the esterase activity of carboxypeptidase M with Bz-Gly-argininic acid.

To further investigate the nature of the essential metal ion, carboxypeptidase M was preincubated with 1 mM α-phenanethrone for 2 h on ice and then diluted to a final concentration of either 0.01 or 0.6 mM α-phenanethrone in the assay with dansyl-Ala-Arg as substrate. Under these conditions, the enzyme retained only 7% of its activity when assayed in the presence of 0.6 mM α-phenanethrone while dilution to 0.01 mM resulted in complete recovery of its activity. This indicates that the enzyme has a high affinity for the metal ion which reassOCIates after dilution of the chelator. To determine whether carboxypeptidase M could be reactivated after removal of its active site metal ion, it was dialyzed overnight against 0.05 mM Tris-HCl pH 7.5, with 0.5% CHAPS and 1 mM o-phenanethrone, then preincubated for 2 h at 4°C with 0.1 mM HEPEs, pH 7.7, containing either no addition, 1 × 10⁻⁵ M zinc acetate or 1 mM cobalt chloride. After dialysis, the enzyme had only 16% of the initial activity with dansyl-Ala-Arg while zinc acetate increased the activity to 39% and cobalt chloride to 127%. Higher concentrations of zinc inhibited (e.g. 1.6 × 10⁻⁴ M zinc acetate inhibited the native enzyme by 73%). These data indicate that carboxypeptidase M is a metalloenzyme, probably containing zinc in the active center as do other mammalian carboxypeptidases (1, 2, 32, 33).

Hydrolysis of Synthetic Substrates—Carboxypeptidase M hydrolyzed peptides containing COOH-terminal Arg or Lys including Bz-Ala-Lys, Bz-Gly-Lys, and Bz-Gly-Arg (Table II). However, the ester substrate (1 mM Bz-Gly-argininic acid) was cleaved the fastest, about 46 times as fast as the corresponding peptide substrate, Bz-Gly-Arg (5 mM). Carboxypeptidase M cleaved Bz-Gly-Arg about 1.7-fold faster than Bz-Gly-Lys, showing a preference for COOH-terminal Arg over Lys. The penultimate amino acid also had a significant effect on the hydrolysis rate as 1 mM Bz-Ala-Lys was cleaved about 17 times faster than 5 mM Bz-Gly-Lys. Carboxypeptidase M (8 μg) did not hydrolyze the peptide dipeptidase substrate furylacryloyl-Phe-Gly-Gly, or the carboxypeptidase A substrate Bz-Gly-phenylactic acid and furylacryloyl-Phe-Phe. Under the same conditions, the latter two substrates were rapidly cleaved by 0.1 μg of bovine carboxypeptidase A.

Hydrolysis of Naturally Occurring Peptides—Carboxypeptidase M readily hydrolyzed COOH-terminal arginine or lysine of several active peptides (Table III). Of the peptides tested, Met⁶-Arg⁶-enkephalin was cleaved most favorably with the highest kₐ (934 min⁻¹) and a relatively low Kₐ of 46 μM to give the highest specificity constant (kₐ/Kₐ = 20.3 μM⁻¹ min⁻¹). Carboxypeptidase M again showed a preference for arginine because changing the COOH-terminal amino acid to lysine (Met⁶-Lys⁶-enkephalin) resulted in a large increase in the Kₐ (to 375 μM) and a decrease in the kₐ (to 663 min⁻¹), and a specificity constant less than one-tenth of that of Met⁶-Arg⁶-enkephalin. The penultimate amino acid also significantly affected the specificity constant as shown with Met⁶-Arg⁶-enkephalin and Leu⁶-Arg⁶-enkephalin (Table III). In this case, the Kₐ of Leu⁶-Arg⁶-enkephalin was only slightly higher (63 μM) but the kₐ was almost an order lower than that of Met⁶-Arg⁶-enkephalin, resulting in a 12 fold lower specificity constant. Of the peptides tested, bradykinin had the lowest activity.

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc.</th>
<th>CoCl₂</th>
<th>Activity</th>
</tr>
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<tr>
<td>Bz-Gly-argininic acid</td>
<td>1</td>
<td>102 ± 14</td>
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<tr>
<td>Bz-Ala-Lys</td>
<td>1</td>
<td>22 ± 7</td>
<td></td>
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<tr>
<td>Bz-Gly-Lys</td>
<td>5</td>
<td>1.3 ± 0.2</td>
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<tr>
<td>Bz-Gly-Lys</td>
<td>5</td>
<td>6.6 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Bz-Gly-Arg</td>
<td>5</td>
<td>2.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Bz-Gly-Arg</td>
<td>5</td>
<td>3.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Bz-Gly-phenylactic acid</td>
<td>1</td>
<td>Not cleaved</td>
<td></td>
</tr>
<tr>
<td>FA-Phe-Phe</td>
<td>0.5</td>
<td>Not cleaved</td>
<td></td>
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</table>

* Enzyme was preincubated (+) for 2 h on ice in the presence of 1 mM CoCl₂.

* Results are the average values ± standard error of the mean obtained from three different preparations of carboxypeptidase M.
Carboxypeptidase M readily converted dynorphin A(1–13) (Tyr-Gly-Gly-Phε-Leur-Arg-Ile-Arg-Pro-Lys-Leu-Lys) to dynorphin A(1–12). At a substrate concentration of 100 μM, the rate was 2.0 μmol/min/mg, similar to the rates of hydrolysis of bradykinin (2.0 μmol/min/mg) or Met5-Lys6-enkephalin (2.2 μmol/min/mg) calculated at 100 μM substrate concentration. However, when assayed under the same conditions, carboxypeptidase M did not hydrolyze dynorphin A(1–13) amide, even after prolonged (23 h at 37°C) incubation. This indicates that the enzyme is indeed a carboxypeptidase requiring a free COOH at the COOH terminus of a substrate and that it does not act as an endopeptidase. The kinetic studies with the other peptides described above also show that carboxypeptidase M is specific for COOH-terminal basic amino acids and does not have endopeptidase activity because the HPLC analyses showed that the peptide substrates are not cleaved further after the removal of the COOH-terminal basic amino acid. In addition, Leu5-enkephalin (100 μM) was not hydrolyzed when incubated with carboxypeptidase M under the same conditions as the peptides with COOH-terminal arginine or lysine.

**Lack of Immunological Crossreactivity with Other Carboxypeptidases**—In a Western blot with polyclonal antiserum to carboxypeptidase N (1:2,000 dilution), darkly staining bands of Mr = 83,000 and Mr = 48,000 and a weaker band of Mr = 55,000, corresponding to the high and low molecular weight subunits, were seen in the lane containing 260 ng of carboxypeptidase N, while no bands were evident in the lane containing 460 ng of carboxypeptidase M (not shown). Conversely, polyclonal antiserum to carboxypeptidase M (1:1,000 dilution) gave a strong band with carboxypeptidase M (400 ng), but no bands were detected with carboxypeptidase N (400 ng) or either form (Mr = 54,000 or Mr = 25,000) of human pancreatic carboxypeptidase B (1 μg) (not shown). These results were confirmed in double immunodiffusion (1 μg of antigen and antiserum at 1:4 and 1:16 dilutions) where antiserum to carboxypeptidase N did not cross-react with carboxypeptidase M and antiserum to carboxypeptidase M did not cross-react with carboxypeptidase N or either form of human pancreatic carboxypeptidase B (not shown).

**DISCUSSION**

This report describes the purification and characterization of a human membrane-bound carboxypeptidase. We propose the name carboxypeptidase M to denote with the letter “M” that it is membrane-bound and to distinguish it from other known mammalian carboxypeptidases which are primarily soluble enzymes (1).

Carboxypeptidase M was purified seven times using somewhat different procedures (see “Materials and Methods”). We found the most reproducible procedure to be sequential chromatography on the following four columns in the order given: 1) DEAE-Trisacryl, 2) Q-Sepharose, 3) arginine-Sepharose, and 4) HPLC on Mono-Q HR.

Although carboxypeptidase M shares some properties with other carboxypeptidase B-like enzymes, it is a distinctly different enzyme. Thus, while carboxypeptidase M is a single chain protein with an Mr = 62,000, human plasma carboxypeptidase N is a tetramer (containing two high and two low molecular weight subunits) of Mr = 280,000 (1, 2, 17), human pancreatic carboxypeptidase B has an Mr of 34,250 (18), and carboxypeptidase H has an Mr of 50,000 to 52,000 (12–14). The NH2-terminal amino acid of carboxypeptidase M also differs from those of the other B-type carboxypeptidases.

In addition to structural differences, the kinetic constants of some biologically active peptides obtained with carboxypeptidase M and N differed (6). For example, the kcat/Km of Met5-Lys6-enkephalin is much higher with carboxypeptidase N (28.7 μM−1 min−1) than with carboxypeptidase M (1.8 μM−1 min−1). The arginine derivative (Met5-Arg6-enkephalin) is the most favorable substrate for carboxypeptidase M (kcat/Km = 20.3 μM−1 min−1). The kcat/Km for the hydrolysis of bradykinin by carboxypeptidase M is about 3-fold higher than with carboxypeptidase N (6). In addition, the hydrolysis rates of smaller synthetic substrates by the two enzymes were different. These experiments show that carboxypeptidase M preferentially cleaves peptides containing COOH-terminal arginine over those containing COOH-terminal lysine, just the opposite of carboxypeptidase N which prefers COOH-terminal lysine (1, 2, 6).

Carboxypeptidase M differs from known B-type carboxypeptidases in many other ways. Carboxypeptidase M is less stable at acid pH than carboxypeptidase N (17). It does not cross-react with antiserum to carboxypeptidase N in Western blotting and antiserum to carboxypeptidase M does not cross-react with carboxypeptidase N or human pancreatic carboxypeptidase B. Cobalt and cadmium have no effect on the esterase activity of carboxypeptidase M. In contrast, cobalt inhibits and cadmium activates the esterase activity of carboxypeptidase B (17, 18, 33), while cadmium inhibits the esterase activity of carboxypeptidase N (2, 17). Carboxypeptidase M is a glycoprotein (as shown by its binding to concanavalin A-Sepharose and a decrease in molecular weight after chemical deglycosylation) while carboxypeptidase B and the active subunit of carboxypeptidase N are not glycosylated (1, 2, 17).

Carboxypeptidase M is also clearly distinct from carboxypeptidase H. Carboxypeptidase M has a neutral pH optimum, while pH 5.5 is optimal for carboxypeptidase H (11–14), and carboxypeptidase M is not inhibited by p-chloromercuriphenylsulfonate at a concentration which completely inhibits carboxypeptidase H (6, 12, 14). In addition, carboxypeptidase H is an intragranular enzyme (12–14) while carboxypeptidase M is on plasma membranes (6, 22).

We have recently isolated and sequenced the cDNA for human carboxypeptidase M.3 The deduced amino acid sequence is fully consistent with the data presented in this paper, including the presence of potential glycosylation sites, the molecular weight of the deglycosylated enzyme, and its uniqueness when compared with known carboxypeptidases.

Although carboxypeptidase M was purified from human placenta, the enzyme is present in other tissues as well. We previously detected significant carboxypeptidase activity in membrane fractions of various human and animal tissues as well as in several lines of cultured cells (6). Besides the placenta, these included human kidney, lung, pulmonary arterial endothelial cells, fibroblasts, and amniotic fluid, and bovine lung and pulmonary artery (6). More recently, others also reported the presence of a carboxypeptidase cleaving the COOH-terminal arginine of bradykinin or Leu5-Arg6-enkephalin in membrane fractions from porcine blood vessels and hog aortic endothelial cells (54).

The potential importance of cleaving COOH-terminal arginine or lysine in the activation or regulation of peptide hormone activity has generated a great deal of interest in this type of carboxypeptidase (1). For instance, it is now known that most peptide hormones are synthesized as part of a larger precursor protein which must be enzymatically processed in

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order to release the active hormone (35). In many cases, this involves initial enzymatic cleavage at paired basic residues in the precursor molecule to release a peptide containing additional COOH-terminal arginine or lysine residues which must be removed by a carboxypeptidase to generate a fully active peptide. These carboxypeptidases also inactivate peptide hormones (1). For example, anaphylatoxin C5a is inactivated by carboxypeptidase N (4, 36). In addition, removal of COOH-terminal basic residues could modulate the activity of peptide hormones (1). It could inactivate agonist for the receptor (37). Similarly, anaphylatoxin C5a has both spasmogenic and chemotactic properties (36). Removal of the COOH-terminal arginine by a carboxypeptidase abolishes the spasmogenic and histamine-releasing activity of C5a while the chemotactic effectiveness is retained (36).

The actual functional role that the various arginine/lysine carboxypeptidases play in vivo is probably related to their localization as well as their physical properties. Carboxypeptidase M, because of its presence on plasma membranes and optimal activity at neutral pH, is ideally situated to act on peptide hormones at local tissue sites (1). It could inactivate or modulate their activity either before or after their interaction with specific plasma membrane receptors. Placental microvilli are the sites at which the materno-fetal exchange takes place (38) and are rich in peptides such as angiotensin I converting enzyme, neutral endopeptidase 24.11, and amipopeptidase A (22). Carboxypeptidase M at this location could play a protective role, contributing to the inactivation of potentially deleterious peptides before they cross this important barrier.

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REFERENCES


Continued on next page.
Material: Human carboxypeptidase M was purified to homogeneity from oesophageal juice and its subunits isolated after treatment of the sample with 3 M guanidine hydrochloride as described previously (1). Human carboxypeptidase M was poured into 3 M guanidine hydrochloride and a small amount of sample was taken for the determination of absorbance at 280 nm. The polypeptide was purified using electrophoresis on activated Sepharose 4 B as published previously (1). Recombinant polypeptides in 1/8 M Tris-Cl, pH 7.5, 1/8 M KCl, 1/2000 M di-thioerythritol were kindly provided by Dr. R. Ekstrom and the sequence of the carboxypeptidase was determined on a Beckman model 8900 sequencer.

Human carboxypeptidase M was purified to homogeneity from oesophageal juice using dialysis and desalting of the sample with dialysis tubing of 125,000 molecular weight cut-off. A 0.1 M Tris-Cl and 0.1 M KCl solution was prepared in the electrophoresis buffer (pH 7.5). The purified protein was then desalted and concentrated to a solution of 0.1 M Tris-Cl, pH 7.5 and subjected to anion exchange chromatography on a 1.0 cm x 25 cm column. Active fractions from the initial 0.1 M Tris-Cl column were pooled, concentrated as above, dialyzed against 0.05 M Tris-Cl, pH 8.0 with 0.5 M NaCl and applied to the C8 Sepharose column equilibrated with the dialysis buffer. The column was washed with 1.0 M NaCl at pH 8.0 and 0.5 M NaCl applied to the C8 Sepharose column equilibrated with the dialysis buffer. The column was washed with 1.0 M NaCl at pH 8.0 and 0.5 M NaCl applied to the C8 Sepharose column equilibrated with the dialysis buffer.

In some cases, ion exchange chromatography on a 2 cm x 40 cm column was used for further purification of human carboxypeptidase M. Active fractions were collected and dialyzed against 0.05 M Tris-Cl, pH 8.0 with 0.5 M NaCl and applied to the C8 Sepharose column equilibrated with the dialysis buffer. The column was washed with 1.0 M NaCl at pH 8.0 and 0.5 M NaCl applied to the C8 Sepharose column equilibrated with the dialysis buffer. The column was washed with 1.0 M NaCl at pH 8.0 and 0.5 M NaCl applied to the C8 Sepharose column equilibrated with the dialysis buffer.

The purified enzyme was assayed using a modification of the method of Sanger and is described in detail in the Methods section.
The first step utilized a DEAE-Trisacryl anion-exchange column. Under the conditions used (sample pH 7.3, elution buffer pH 9.9), 52% of the carboxypeptidase activity passed through the column and 38% of the activity was eluted with the salt gradient, while a majority of the contaminating proteins were tightly bound and eluted only with 0.1 M NaCl. Prior to chromatography on the second DEAE-Trisacryl column, the sample was dialyzed against the starting buffer (pH 8.3) and 5% of the activity bound to the column and was eluted with a 0-0.1 M NaCl gradient. In the final step we used an arginine-Sepharose affinity column (Fig. 1), eluting the carboxypeptidase with the inhibitor CGBA (1 mM). Sometimes, the activity in the column fractions was low or undetectable because of inhibition by CGBA, so all fractions collected after elution with the inhibitor were pooled and dialyzed to remove it. After dialysis, the activity of the pooled enzyme increased up to twenty-fold.

FIG. 1. Arginine-Sepharose affinity chromatography of human carboxypeptidase M. The enzyme was applied to the column in 0.25 M Tris-250 mM NaCl (pH 7.5) containing 0.5% CHAPS and eluted with the same buffer containing 1 mM CGBA. Protein bound noncovalently by charge were eluted with 0.5 M NaCl in the same buffer. Activity was measured with Bz-Gly-Arg-Arg-pNA. For further details, see Methods.

Human Carboxypeptidase M

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (Units)</th>
<th>Specific Activity (Units/mg)</th>
<th>Yield (%)</th>
<th>Purification (x-fold)</th>
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<td>Placental Microvilli</td>
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<td>231</td>
<td>3.32</td>
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<td>1.40</td>
<td>10.0</td>
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*One unit equals 1 nmol of Bz-Gly-Arg hydrolyzed per min at 37°C in the presence of 1 mM CaCl₂, assayed as described in Methods.