Rat Mast Cell Carboxypeptidase: Amino Acid Sequence and Evidence of Enzyme Activity within Mast Cell Granules†

Karen R. Cole, Santosh Kumar, Hai Le Trong, Richard G. Woodbury, Kenneth A. Walsh, and Hans Neurath*
Department of Biochemistry, University of Washington, Seattle, Washington 98195
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ABSTRACT: The amino acid sequence of rat mast cell carboxypeptidase has been determined. The major form has 308 residues; a minor form has an additional (glutamyl) residue at the amino terminus that may indicate an alternate cleavage site during zymogen activation. The enzyme is homologous to pancreatic carboxypeptidases A and B, with conservation of the functional amino acid residues of the active site. The putative substrate binding site resembles that of carboxypeptidase A, although other structural features bear more similarity to carboxypeptidase B. Mast cell carboxypeptidase retains enzymatic activity toward a peptide substrate (angiotensin I) while bound within the granular matrix of the rat connective tissue mast cells. Evidence is presented to suggest that a cluster of positively charged lysyl and arginyl residues binds the enzyme to the negatively charged heparin of the granular matrix but leaves the active site exposed to bind and cleave peptide substrates.

Carboxypeptidase is one of several proteolytic enzymes contained in rat connective tissue mast cells. This enzyme was first isolated and characterized by Everitt and Neurth (1980), and its properties have been reviewed by Woodbury et al. (1981) and Katunuma et al. (1986). Its molecular weight, composition, metal dependence, substrate specificity, and inhibition by potato carboxypeptidase inhibitor all suggest that it may resemble pancreatic carboxypeptidases A and B (Everitt and Neurath, 1980; Woodbury et al., 1989). The enzyme is segregated, together with chymase and tryptase, in the granules of rat connective tissue mast cells. Upon stimulation by IgE and other secretogogues, the cells release the proteases as a macromolecular complex with proteoglycans, i.e., high molecular weight heparin. Dissociation of the complex by treatment with 2 M KCl releases the enzymes in an active, soluble form. The most effective method of isolation of rat mast cell carboxypeptidase (RMC-CP)[1] involves affinity chromatography on a column of immobilized potato carboxypeptidase inhibitor (Everitt & Neurath, 1980). The association of the enzyme–inhibitor complex is so tight ($K_i \sim 5$–50 nM) that it requires elution at pH 11.5 to dissociate the enzyme from the adsorbent. This destabilizes the enzyme with a concomitant loss of activity. Hence, rigorous characterization of the enzymatic properties of the enzyme and of its stability is still lacking. In addition, it has been difficult to avoid partial degradation during purification and to obtain a purified enzyme that migrates in gel electrophoresis with a single boundary.

In an effort to establish the relationship of mast cell carboxypeptidase to its pancreatic counterpart and as a starting point of a more detailed investigation of its structure and biological functions, we have undertaken the determination of the amino acid sequence of rat mast cell carboxypeptidase. In addition, we have established that the enzyme is active and relatively stable when bound to the isolated granular matrix of rat connective tissue mast cells. While this work was in progress, Reynolds et al. (1989a,b) reported the cDNA sequences of mouse peritoneal and human lung and skin mast cell carboxypeptidases. The corresponding amino acid sequences are closely related to those reported herein and give credence to the belief that these granulocyte carboxypeptidases belong to the gene superfamily of mammalian zinc carboxypeptidases.

Materials and Methods

Sprague-Dawley rats were obtained from Tyler Labs (Bellevue, WA). Potato carboxypeptidase inhibitor was a generous gift of Dr. C. A. Ryan (Washington State University, Pullman, WA). It was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer specifications. Cyanogen bromide and citraconic anhydride were purchased from Eastman Kodak. Endoproteinase Asp-N was obtained from Boehringer Mannheim. *N*-(*p*-Tosyl)-L-phenylalanine chloromethyl ketone treated trypsin (TPCK–trypsin) and α-chymotrypsin were from Worthington Biochemicals. *Achromobacter* protease I was a generous gift of T. Masaki (Ibaraki University, Ibaraki, Japan). Poly(vinylidene difluoride) (PVDF) membranes (Immobilon) were obtained from Millipore. Narrow-bore butyl-300 and RP-300 columns were from Pierce. A Synchropak RP-18 column was from Synchrom, Inc.; TSK-G2000 SW columns were from LKB. Angiotensin I was purchased from Sigma.

Assay of Carboxypeptidase and Chymotryptic Activities. Carboxypeptidase A activity was assayed spectrophotometrically at 254 nm for 3–5 min in a Beckman DU-6 spectrophotometer employing 150 μmol of hippuryl-L-β-phenyllactate in 0.025 M Tris-HCl, pH 7.5, as the substrate (McClure et al., 1964). Mast cell extract, 10–25 μL, was added to sufficient substrate solution to achieve a final volume of 3.0 mL. The activity of rat mast cell protease I (RMCP I) was measured for 3–5 min at 256 nm with benzoyltryosine ethyl ester as described by Woodbury et al. (1981). Substrate, 1.5 mL, was added to 1.5 mL of 80 mM Tris-HCl, pH 7.8, and the solution was incubated with 10–25 μL of mast cell extract.

Preparation of Rat Mast Cell Carboxypeptidase. The enzyme was isolated from peritoneal mast cells by a modifi-

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[1] Abbreviations: RMC-CP, rat mast cell carboxypeptidase; MMC-CP, mouse mast cell carboxypeptidase; PVDF, poly(vinylidene difluoride); RMCP I, rat mast cell protease I; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; BOCPA, bovine carboxypeptidase A.

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cation of the method of Everitt and Neurath (1980). Cells were harvested by centrifugation of peritoneal lavage fluid from 50 rats at 200g for 5 min at 4 °C. The enzyme was extracted from associated heparin by stirring the cell pellet for 30 min at 4 °C in 3–4 mL of 0.1 M Na_2CO_3, pH 8.0, 2 M KCl, 1% protamine sulfate, 1 mM phenylmethanesulfonyl fluoride, and 1 mg/mL lima bean trypsin inhibitor. The latter two components were included to inhibit RMCP I and tryptase. The extraction mixture was centrifuged at 17500g for 20 min at 4 °C and the supernatant analyzed for carboxypeptidase A and chymotryptic activity as described above. Four to five successive extractions were performed until the carboxypeptidase activity in the supernatant was below 5% of the initial level. The combined extracts were applied to a potato carboxypeptidase inhibitor–Sepharose 4B column (1.0 mL) equilibrated in 0.1 M Na_2CO_3, pH 8.0, containing 2 M KCl. Unbound material and excess salt were washed through the column with 0.1 M Na_2CO_3, pH 8.0. Bound RMC-CP was eluted with 50 mM triethylamine, pH 11.5, and the fractions were immediately neutralized by addition of 1 M Tris-HCl, pH 7.5. The purified RMC-CP was stored in lyophilized form.

Preparation of Mast Cell Granules. Mast cell granules were isolated from highly purified rat peritoneal mast cells as described by Le Trong et al. (1987). Isolated mast cells were suspended in Milli-Q water and subjected to six freeze–thaw cycles by incubating in a dry ice/ethanol bath for 10 min and then thawing at 37 °C. The suspension of lysed cells was centrifuged at 150g for 10 min at 4 °C to remove coarse cellular debris. The supernatant was centrifuged at 3000g for 30 min at 4 °C, and the granule-containing pellet was suspended in 3 mM potassium phosphate buffer, pH 8.0. This procedure yields granules devoid of their perigranular membranes (Uvnäs, 1974). To eliminate any proteases that had dissociated from the granular matrix, the granule suspension was washed extensively prior to each assay.

Electroblotting to PVDF Membranes. Protein samples (50–100 pmol) were electrophoresed on 12% $NaDodSO_4$–polyacrylamide minigels (Laemmli, 1970) at 150 V for 45–60 min. Low molecular weight standards ranging from 14 to 97 kDa were from Bio-Rad. Gels were electroblotted to PVDF membranes in a Bio-Rad minitransfer apparatus at 70 V for 90 min (Matsudaira, 1987), stained with Coomassie blue, and stored at −20 °C until subjected to sequence analysis.

Separation of Peptides. Fragments generated by cyanogen bromide digestion were initially separated by size on tandem TSK-G2000 SW (7.5 × 600 mm) gel permeation HPLC columns in 6 M guanidine hydrochloride and 10 mM phosphate, pH 6, at a flow rate of 1.0 mL/min. Pooled fractions were purified on reversed-phase HPLC narrow-bore columns, Aquapore RP-300-C8 (2.1 × 100 mm, 7 μm), RP-18 (2.1 × 30 mm, 5 μm), or Aquapore RP-butyl-300 (2.1 × 100 mm, 7 μm), as appropriate, on a Hewlett-Packard Model 1090 HPLC system equipped with a diode array detector. Fragments were eluted at a flow rate of 0.3 mL/min with linear gradients of 0–60% acetonitrile generated in 0.09% trifluoroacetic acid (Mahoney & Hermodson, 1980). Peaks were detected at 206 and 275 nm and the corresponding pools analyzed by a Hewlett-Packard Model 300 series computer. Isolation and desalting of peptides from the other digests were achieved on the appropriate reversed-phase HPLC narrow-bore column as described above.

Amino Acid Analyses and Sequencing. Reduction and pyridylethylation were performed before or after digestion according to Friedman et al. (1970). Excess reagents were removed by either dialysis against 9% formic acid or desalting over reversed-phase HPLC. Compositions were determined on a Waters 712 WISP HPLC system according to the method of Bidlingmeyer et al. (1984). Automated Edman degradations were performed on an Applied Biosystems 470A gas-phase sequencer with an on-line Model 120A HPLC (Hunkapiller et al., 1983).

Cleavage at Methionyl Residues. Cyanogen bromide digestion of 12 nmol of RMC-CP was carried out as described by Titani et al. (1984). Following digestion, the protein was reduced and pyridylethylated and the resulting fragments were separated as described above.

Cyanogen bromide digestion of RMC-CP was conducted after electrophoresis on a 12% $NaDodSO_4$–polyacrylamide gel following a modification of the method of Nikodem and Fresco (1979). A Coomassie-stained 38-kDa band, containing 10–20 μg of RMC-CP, was excised and placed in 1 mL of 88% formic acid. Cyanogen bromide, 30 mg, dissolved in 50 μL of acetonitrile, was added and digestion was allowed to proceed for 18 h in the dark at 37 °C. The supernatant of the digest was removed, diluted 5-fold with Milli-Q water, and lyophilized twice. This sample was redissolved in gel sample buffer, electrophoresed on a 15% $NaDodSO_4$–polyacrylamide gel, and electroblotted to a PVDF membrane as previously described.

Subdigestion of Cyanogen Bromide Fragments with Chymotrypsin. One of the cyanogen bromide fragments was subdigested with chymotrypsin (1% M/M) overnight in 0.1 M NH_4HCO_3, pH 8.0, at 37 °C. The resulting fragments were separated on an RP-300-C8 column.

Subdigestion of Cyanogen Bromide Fragments at Lysyl Residues. A second cyanogen bromide digest was performed on 10 nmol of RMC-CP. The material was subsequently dried and redissolved in 6 M guanidine hydrochloride containing 1.5 M Tris-HCl and 5 mg/mL EDTA, pH 8.6. The protein solution was diluted with 50 mM Tris-HCl to a final guanidine hydrochloride concentration of 1.0–1.5 M and treated with *Achromobacter* protease I, 1% (w/w), for 16 h at 37 °C. The digest was then dried, redissolved in 6 M guanidine hydrochloride, reduced, and pyridylethylated as previously described. The resulting fragments were separated and desalted on an RP-300-C8 column. Further purification of the fragments was achieved on either RP-butyl-300 or RP-18 columns.

Cleavage at Arginyl Residues. Native RMC-CP (15 nmol) was digested with trypsin after citraconylation of the lysyl residues (Habeeb & Atassi, 1972). The protein was dissolved in 6 M guanidine hydrochloride and, 0.1 M $Na_2PO_4 \cdot 7H_2O$, pH 7.0, and the pH was maintained at 8.7 with a pH stat while 40 μL of citraconic anhydride was added in 5-μL aliquots at room temperature over 90 min. The solution was stirred for an additional 30 min at room temperature and incubated overnight at −20 °C. Subsequently, the protein was reduced, pyridylethylated, and extensively dialyzed against 0.1 M NH_4HCO_3, pH 8.8. Trypsin was added at an enzyme:substrate ratio of 1:100 (w/w) in two aliquots for 4 h each at 37 °C. After digestion, the protein solution was lyophilized, and citraconyl groups were removed by incubation in 9% formic acid for 3 h at 37 °C. As previously described, the fragments were separated over an RP-300-C8 column and where necessary rechromatographed on an RP-18 column.

Subdigestion of Arginyl Fragments with Trypsin. At least five arginyl peptides (500 pmol) eluted together from the RP-300-C8 column in 47% acetonitrile. This mixture was digested with trypsin (1% w/w) for 3 h at 37 °C in 0.1 M NH_4HCO_3, pH 8.0. The resulting fragments were purified over an RP-300-C8 column as previously described.

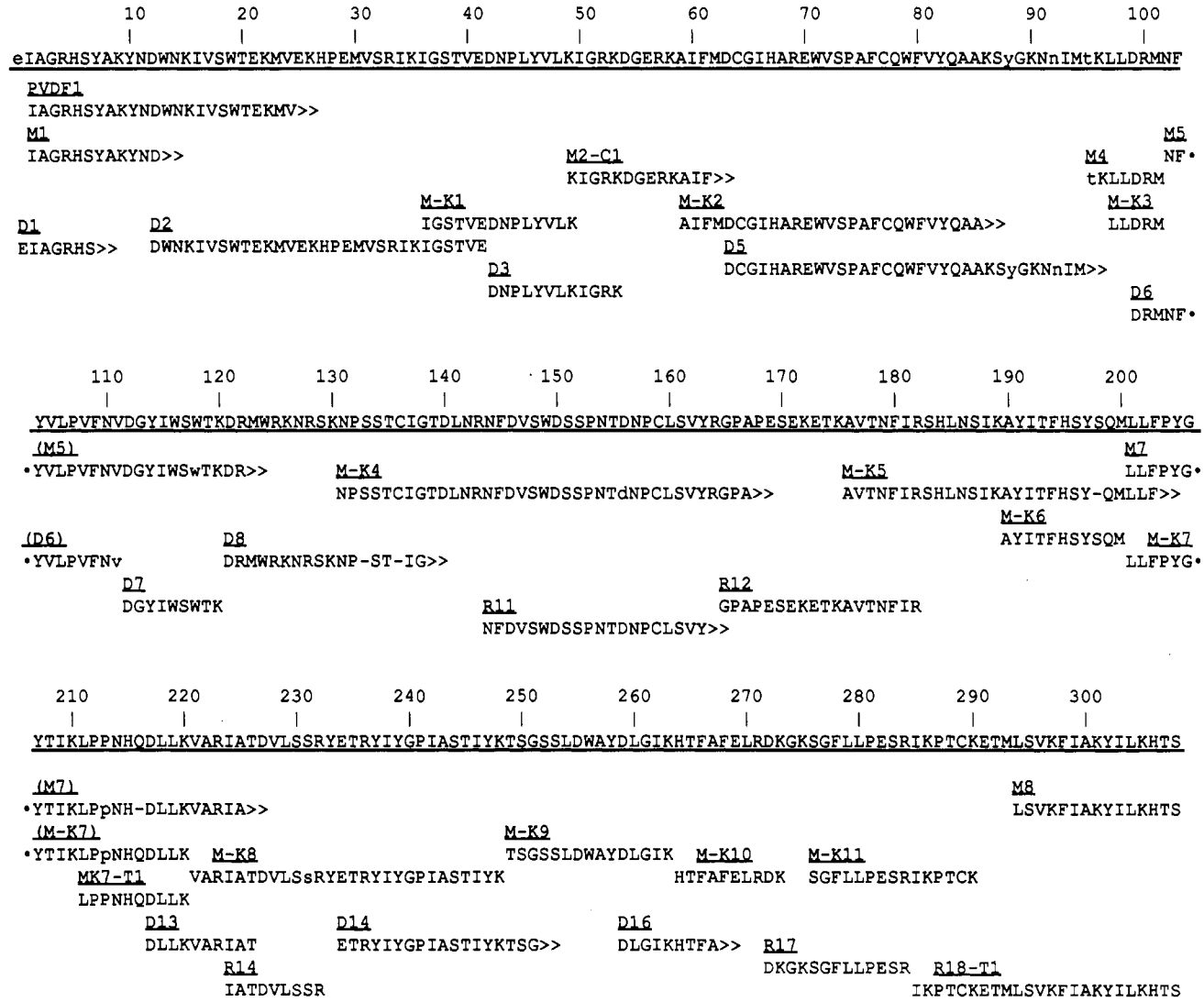

FIGURE 1: Summary of the proof of sequence of RMC-CP. The amino-terminal sequence of an electroblotted preparation is designated PVDF1. The sequences of specific peptides are given in single-letter codes below the sequence summary (underlined). Peptide names include prefixes M, D, and R, which refer to fragments obtained from digests at methionyl, aspartyl, and arginyl residues, respectively. M-C and M-K denote methionyl peptides subdigested with chymotrypsin and at lysine residues, respectively. Trypsin subdigests of methionyllysyl and arginyl peptides are designated as MK-T and R-T, respectively. Lower-case letters indicate tentative identifications. Dashes indicate residues that were not identified within the fragments. The arrowheads (>>) designate peptide sequences that did not reach their C-termini. Sequences continued on a second line are followed and preceded by the symbol (•).

Cleavage at Aspartyl Residues. Reduced, pyridylethylated RMC-CP (6 nmol) was incubated in 8 M urea for 15 min at 37 °C to promote unfolding. The solution was then diluted 4-fold with 0.1 M NH_4HCO_3, pH 7.7, and digested at aspartic acid residues overnight with endoproteinase Asp-N at an enzyme:substrate ratio of 1:275 (w/w) in 0.1 M NH_4HCO_3, pH 7.7, and 2 M urea at 37 °C. Following digestion, the fragments were purified and desalted on a RP-300-C8 column and then rechromatographed on an RP-18 column as required.

A second digest was performed on 4 nmol of reduced, carboxymethylated RMC-CP in 1.5 M guanidine hydrochloride and 0.1 M NH_4HCO_3, pH 7.7, with endoproteinase Asp-N at an enzyme:substrate ratio of 1:45 (w/w). The digest proceeded for 48 h at 37 °C with two-thirds of the enzyme added for the first 24 h and the remaining one-third added for the second 24-h incubation. The resulting fragments were separated over an RP-butyl-300 column.

RESULTS

Electroblotting to PVDF. The affinity-purified preparation of RMC-CP displayed three components when electrophoresed on 12% $NaDodSO_4$–polyacrylamide gels. The material with the largest molecular mass (38 kDa) represented approximately 50% of the total material; the remaining material was evenly distributed between a 36-kDa band and a 34-kDa band. The 38-kDa component (100 pmol) was electroblotted and sequenced through 24 amino-terminal residues (PVDF 1) as shown in Figure 1. Sequencing of the 34-kDa band yielded the same N-terminal sequence, which suggests degradation at the C-terminus by tryptase or another PMSF-insensitive mast cell protease during the preparation procedure. Analysis of the 36-kDa component revealed a 50/50 mixture of two sequences from the N-terminus, beginning with IAGR and with EIAGR, respectively. The overlapping nature of these two sequences suggests that they are products of cleavage at alternate zymogen activation sites.

General Strategy of Sequence Analysis. Three primary digests (at methionine, aspartic acid, and arginine) and three subdigests were necessary to determine the sequence of RMC-CP. The analysis was difficult because the enzyme was poorly soluble even in strong denaturing agents such as 6 M guanidine hydrochloride and 8 M urea. Incomplete digestion and low yields did not always permit both compositional and

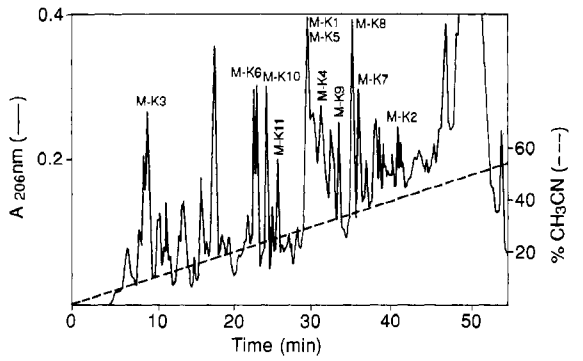

FIGURE 2: Primary separation of peptides after cleavage of RMC-CP at methionyl and lysyl residues. Peptides were eluted from the RP-300-C8 column (2.1 × 100 mm) with a gradient of 0–60% acetonitrile in 0.09% trifluoroacetic acid at a flow rate of 0.3 mL/min. Purified peptides that were sequenced bear the prefix M-K as in Figure 1.

sequence analysis. Amino acid analyses served primarily as an indication of peptide purity and location. Figure 1 illustrates the orientation of all the fragments and provides the proof of sequence.

Cyanogen bromide digestion yielded only five of the nine expected fragments; these included the amino- and carboxy-terminal peptides and three internal segments. A separate cyanogen bromide digest was subdigested at lysyl residues to provide 11 isolated fragments labeled with the prefix M-K in Figure 1. Two other digests provided overlaps and the remainder of the sequence. Enzymatic cleavage at aspartyl residues with endoproteinase Asp-N generated 10 of the 17 expected fragments, labeled D*x* in Figure 1. The sequences of four of the fragments isolated after cleavage at arginyl residues (R*x*) provided the remaining overlaps. Each digest is discussed in more detail below.

Cleavage at Methionyl Residues. Cleavage at eight methionine residues should have yielded nine fragments. However, a Met–Thr bond at residues 94–95 cleaved poorly, and the larger peptides did not separate from each other due to their similarity in size and hydrophobicity. Peptides M1, M4, M5, and M8 (Figure 1) were derived from reversed-phase HPLC fractionation of pooled fractions after gel filtration. The N-terminal sequence of M5 (NFYVL..., residues 102–123) was confirmed and extended by analysis of the corresponding fragment from an electroblot of a cyanogen bromide digest of the 38-kDa form of RMC-CP. A smaller fragment from the same electroblot had the N-terminal sequence LLFPYG... and is denoted as M7 in Figure 1 (residues 201–225). A mixture of cyanogen bromide fragments of intermediate size was subdigested with chymotrypsin to give fragment M2-C1 (residues 49–61).

Subdigestion of Methionyl Fragments at Lysyl Residues. Another cyanogen bromide digest of RMC-CP was immediately treated with *Achromobacter* protease I to generate the mixture fractionated on Aquapore RP-300-C8 (Figure 2). Eleven peptides were obtained and sequenced (Figure 1). Peptides M-K1 and M-K5 were first separated from each other on an RP-18 column (data not shown). With the exception of M-K2, M-K4, and M-K5, the fragments were sequenced to their C-terminal lysyl or homoseryl residues. Fragment M-K7 (residues 201–220) was the major component in a mixture with two minor sequences beginning at Ala-59 and Asp-63. Subdigestion of this mixture with trypsin gave MK7-T1, which confirmed a proline at position 213 previously indicated by weak data in M-K7 and M7.

Cleavage at Arginyl Residues. Four peptides isolated after a tryptic digest of citraconylated RMC-CP yielded the sequence and overlaps. R11 confirmed the identification of aspartyl residue 156, and R14 confirmed the identification of serine 231. A mixture of at least six other unresolved fragments was redigested with trypsin and separated on an RP-300-C8 column. The late-eluting fragment, R18-T1 (residues 285–308), in which incomplete digestion occurred, represented the major sequence in a mixture of several known sequences. The phenylthiohydantoin amino acid yield from R18-T1 exceeded that from the minor sequence (residues 70–86) by approximately 2-fold.

Cleavage at Aspartyl Residues. Ten fragments were isolated from the endoproteinase Asp-N digest of pyridylethylated RMC-CP (Figure 1). The sequence of fragment D1 indicated the presence of a glutamic acid residue at the amino terminus as was observed in the mixture of sequences derived from the 36-kDa component of the electroblotted native RMC-CP. We suspect that the glutamyl residue in RMC-CP may have resulted from alternate zymogen activation since in the mouse mast cell carboxypeptidase precursor the amino-terminal isoleucine is preceded by a glutamyl residue (Figure 3). Human mast cell carboxypeptidase has aspartic acid in this position.

The combined digests provided the sequence from residues 1 to Met-94 and from Lys-96 to Ser-308, although overlaps at residues 121–122 and 272–273 were only two residues each (Figure 1). The identifications of Asp-121 and Arg-122 were strong in M5 and D8. M-K10 and R17 provided equally convincing identifications of Asp-272 and Lys-273. Since R17 was derived from a tryptic digest of citraconylated protein, an arginine at position 271 may be inferred as an additional overlap.

To complete the sequence from Met-94 to Lys-96, it was necessary to rely on marginal data from the analysis of D5. This fragment appeared as a 50/50 mixture with a peptide beginning at residue 272. The data from residues 88–94 include two tentative assignments due to low yields of phenylthiodantoins in this portion of the peptide. Residues 95–101 were obtained from the sequence of the trace peptide M4 and its amino acid composition.

The lack of the lysyl peptides from residues 87–96 is a handicap. We were unable to purify peptides from this region, in yields acceptable for sequencing, from the Asp-N or tryptic digests. In each case, the fragment did not separate from other large peptides, and analysis of the mixtures led to ambiguous interpretations of the sequence. Attempts to subdigest, at tryptophan 79, a mixture of arginyl peptides containing residues 70–100 gave yields too low for definitive sequence information.

Activity of Granule-Bound RMCP I and RMC-CP. Angiotensin I (10–50 nmol) was incubated with active mast cell granules at an enzyme:substrate molar ratio of 1:70 in 150 μL of 3 mM potassium phosphate buffer, pH 8.0, at 37 °C. After incubation for 30 min, the reaction was stopped by acidification with trifluoroacetic acid and the solution centrifuged at 7000*g* for 10 min to remove the granules. In controls, angiotensin I was incubated with the supernatant of the granule preparation or with granules pretreated with 5 mM 1,10-phenanthroline to inhibit the carboxypeptidase. Degradation products were isolated by HPLC on a Synchropak RP-C18 column and subjected to amino acid analysis.

The results (Figure 4) indicate that proteolytic degradation of angiotensin I by mast cell granules involves the coordinated action of the granule-bound RMC-CP and RMCP I. In the presence of 1,10-phenanthroline, which allows only RMCP I action (Le Trong et al., 1987), a single bond is cleaved to

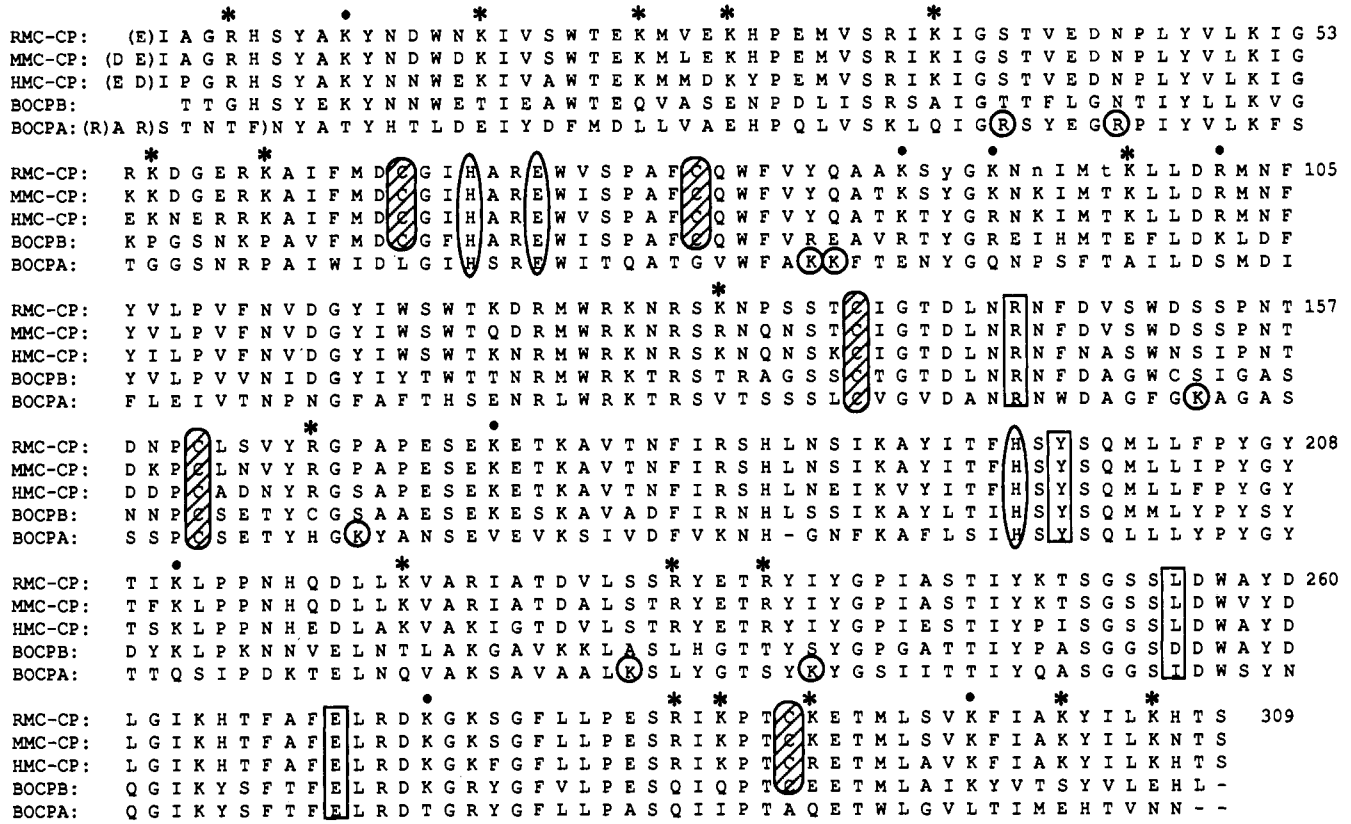

FIGURE 3: Comparison of the amino acid sequences of mast cell and selected pancreatic carboxypeptidases. Names of proteins are abbreviated as in Table I. Residue numbering is based on the sequence of bovine carboxypeptidase Aα. Catalytic residues are boxed, zinc-binding residues are circled, and cysteine residues are in shaded ovals. The amino-terminal Glu in RMC-CP was present in approximately 12% of the molecules. The 94-residue activation peptides of MMC-CP and HMC-CP predicted from cDNA and that of BOCPA determined from amino acid sequencing end in Asp-Glu, Glu-Asp (Reynolds et al., 1989a,b), and Arg (Wade et al., 1988) respectively. Residues within parentheses are removed during posttranslational activation processes. Three forms of BOCPA have been described, beginning with ARSTNTFNYA-, STNTFNYA-, and NYA-, respectively (Petra, 1970). Symbols: (*) basic residues exclusive to the three mast cell carboxypeptidases; (●) basic residues in all enzymes except BOCPA. Basic residues in BOCPA that are neutral in RMC-CP are circled.

Table I: Percentage Sequence Identities and Alignment Scores of Mast Cell and Pancreatic Carboxypeptidases[a]

protein	RMC-CP	MMC-CP	HMC-CP	RTCPB	BOCPB	RTCPA1	RTCPA2	BOCPA
RMC-CP		93.8%	85.7%	53.1%	56.2%	43.1%	43.9%	41.0%
MMC-CP	70.1		85.4%	53.4%	55.9%	42.8%	42.0%	41.6%
HMC-CP	59.7	57.8		54.1%	57.8%	43.1%	43.0%	40.0%
RTCPB	51.0	51.4	42.5		76.1%	46.4%	46.2%	45.7%
BOCPB	45.1	51.0	44.6	61.1		48.1%	45.7%	48.0%
RTCPA1	36.7	30.7	33.0	36.4	41.2		63.6%	79.2%
RTCPA2	36.6	30.8	36.7	41.4	36.6	46.5		63.0%
BOCPA	30.3	38.1	33.4	31.8	36.0	52.1	42.6	

[a] The upper right-hand side of the table shows the percentage of identical residues between the indicated proteins. The lower left-hand side displays the alignment scores (Dayhoff et al., 1983) expressed in standard deviation units from the mean of 100 randomly generated sequences. A score of >5 indicates homology. The abbreviations are as follows: rat mast cell carboxypeptidase (RMC-CP), mouse mast cell carboxypeptidase (MMC-CP), human mast cell carboxypeptidase (HMC-CP), rat pancreatic carboxypeptidase B (RTCPB), bovine pancreatic carboxypeptidase B (BOCPB), rat pancreatic carboxypeptidase A1 (RTCPA1), rat pancreatic carboxypeptidase A2 (RTCPA2), and bovine pancreatic carboxypeptidase A (BOCPA).

generate two peptides, P1 (DRVY) and P2 (IHPFHL) (Figure 4A,B). Under these conditions, the peptide CP3 (Figure 4A,C) did not arise from RMCP I action. In the absence of 1,10-phenanthroline, carboxypeptidase action removes two C-terminal residues to generate the octapeptide CP3 (DRVYIHPF) and removes the C-terminal residues of P1 and P2 to generate CP1 (DRV) and CP2 (IHPFH) (Figure 4A,C). Supernatant controls lacking granules generated no degradation products. Clearly both RMC-CP and RMCP I are active in the isolated granules.

Comparison with Other Carboxypeptidase Sequences. The sequence of RMC-CP was compared with those of mouse and human mast cell carboxypeptidases (Figure 3) and rat and bovine pancreatic carboxypeptidases A and B by using the ALIGN program (Dayhoff et al., 1983). Table I displays the percentage of sequence identity among the various proteins and the alignment scores.

Location of Basic Residues. A hypothetical, three-dimensional model of RMC-CP displaying the location of the lysyl and arginyl residues was constructed by using the QUANTA 2.1 program (Polygen Corp., Waltham, MA, 1989). The model of RMC-CP was generated from the tertiary structure of bovine pancreatic carboxypeptidase A (BOCPA) by substituting 29 neutral or acidic residues of BOCPA with lysine or arginine at positions corresponding to their location in RMC-CP and removing 7 basic residues in BOCPA that were neutral in all three mast cell carboxypeptidases (Figure 3). No alterations in the backbone conformation of BOCPA were executed to accommodate these sequence changes.

Figure 5 displays identical projections of the models of

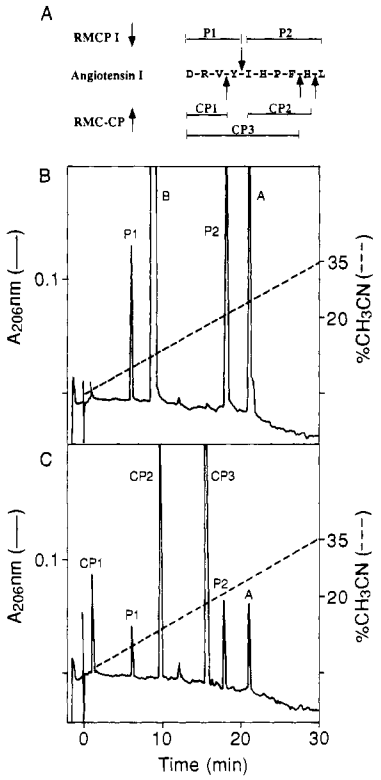

FIGURE 4: (A) Products of angiotensin I digestion by granule-bound RMCP I and RMC-CP. The downward and upward arrows indicate the RMCP I and RMC-CP cleavage sites, respectively. P1, P2, CP1, CP2, and CP3 represent the respective RMCP I and RMC-CP cleavage products. (B) Elution of cleavage products in the presence of 1,10-phenanthroline from an RP-18 column under conditions described under Materials and Methods. (A) angiotensin I; (B) 1,10-phenanthroline. (C) As in (B) in the absence of 1,10-phenanthroline.

BOCPA and RMC-CP. The active-site faces of the molecules (Figure 5, top) are compared with the projections obtained by rotating the molecules approximately 180° around the *y*-axis (Figure 5, bottom). There is clearly a clustering of positive charges on the side of RMC-CP opposite the active site.

Discussion

The amino acid sequence of RMC-CP was determined primarily from peptides obtained after cleavage adjacent to methionyl, lysyl, or aspartyl residues, with overlapping segments provided by arginyl peptides. Although identifications of tyrosine 88, asparagine 92, and threonine 95 are only tentative, mouse and human mast cell carboxypeptidases have identical residues at positions 88 and 95 (Figure 3). The residue corresponding to rat asparagine 92 is lysine in the mouse and human sequences. No evidence of lysine at position 92 was observed in our sequence analysis, but we did note a weak increase in the asparagine signal above that of asparagine 91. The identification of Thr-95 relies on the sequence data and amino acid composition of the cyanogen bromide fragment M4, residues 95–101.

Figure 3 compares the complete amino acid sequence of RMC-CP with those of other mast cell and pancreatic carboxypeptidases. For simplicity, the sequences of only the bovine pancreatic enzymes are included. Table I indicates the percentage of sequence identity and the alignment scores among these carboxypeptidases. RMC-CP bears a striking resemblance (94% sequence identity) to the amino acid sequence of mouse mast cell carboxypeptidase deduced from the cDNA sequence of a clone isolated from a library constructed from a Kirsten sarcoma virus immortalized mouse mast cell line (Reynolds et al., 1989a). Likewise, the human mast cell carboxypeptidase sequence, determined from lung cDNA libraries (Reynolds et al., 1989b) and confirmed by N-terminal sequence analysis (Goldstein et al., 1989), is closely related to the rat mast cell enzyme (86% sequence identity).

When compared with the pancreatic carboxypeptidases (Table I), the mast cell carboxypeptidases displayed greater structural similarity with the carboxypeptidase B sequences than with those of carboxypeptidase A. The mast cell carboxypeptidases were 53–58% identical with bovine and rat pancreatic carboxypeptidase B sequences (Titani et al., 1975; Clauser et al., 1988), as compared with 40–44% identity shown toward the bovine and rat pancreatic carboxypeptidase A sequences (Clauser et al., 1988; Gardell et al., 1988; Bradshaw et al., 1969). Yet it is clear both from its specificity (Everitt & Neurath, 1980) and from the nature of residue 255 (Figure 3), the key determinant of specificity in the pancreatic carboxypeptidases (Schmid et al., 1974), that mast cell carboxypeptidase resembles pancreatic carboxypeptidase A in its function. Residue 255 is leucine in rat, mouse, and human mast cell carboxypeptidases (Figure 3), which clearly resembles the isoleucine of pancreatic carboxypeptidase A rather than the aspartyl residue that determines the specificity of carboxypeptidase B.

It is of interest to note the conservation of other functional and structural residues in the mast cell and pancreatic carboxypeptidases as shown in Figure 3. The three zinc-binding sites at His-69, Glu-72, and His-196 (Titani et al., 1975; Reynolds et al., 1989a,b) are conserved in all of the sequences. (The numbering of the residues is based on that of bovine pancreatic carboxypeptidase A.) The active-site residues of both bovine pancreatic carboxypeptidases, Arg-145, Tyr-198, and Glu-270, are also found in the mast cell enzymes.

There are five cysteine residues in the mast cell proteases, seven in the pancreatic B enzymes, and two in rat pancreatic carboxypeptidase A1 and in bovine carboxypeptidase A. Rat pancreatic carboxypeptidase A2 has four cysteines. In bovine pancreatic carboxypeptidase A, the cysteine residues at positions 138 and 161 are disulfide-bonded (Walsh et al., 1970). These residues are conserved in all the sequences shown in Figure 3. For bovine pancreatic carboxypeptidase B, additional disulfide-bonded pairs are Cys-66–Cys-79 and Cys-152–Cys-166, whereas Cys-290 has a free sulfhydryl (Titani et al., 1975). With the exception of Cys-152 and -166, these residues are present in all three mast cell carboxypeptidases. Homology with bovine pancreatic carboxypeptidase B suggests the placement of disulfide bonds in RMC-CP between Cys-66 and Cys-79 and between Cys-139 and Cys-161, leaving Cys-290 unpaired. The presence of an additional bond relative to pancreatic carboxypeptidase A and the absence of the third disulfide found in the pancreatic carboxypeptidase B enzyme(s) may reflect differences in enzyme stability or substrate specificities as in rat mast cell protease II, a chymotrypsin-like enzyme from mucosal mast cells (Remington et al., 1988).

The sequences of the activation peptides of the pro forms of bovine pancreatic carboxypeptidase A and of the mouse and human mast cell carboxypeptidases have been determined by direct amino acid sequencing (Wade et al., 1988) and from cDNA sequences (Reynolds et al., 1989a,b), respectively. Activation of the mouse and human mast cell enzymes occurs by cleavage after a glutamic acid or aspartic acid residue, respectively (Figure 3). Each of these enzymes also possesses an additional acidic residue in the −2-position of their pro forms, aspartic acid in the mouse protein and glutamic acid in the human protein. Similarly, the cDNA sequences of

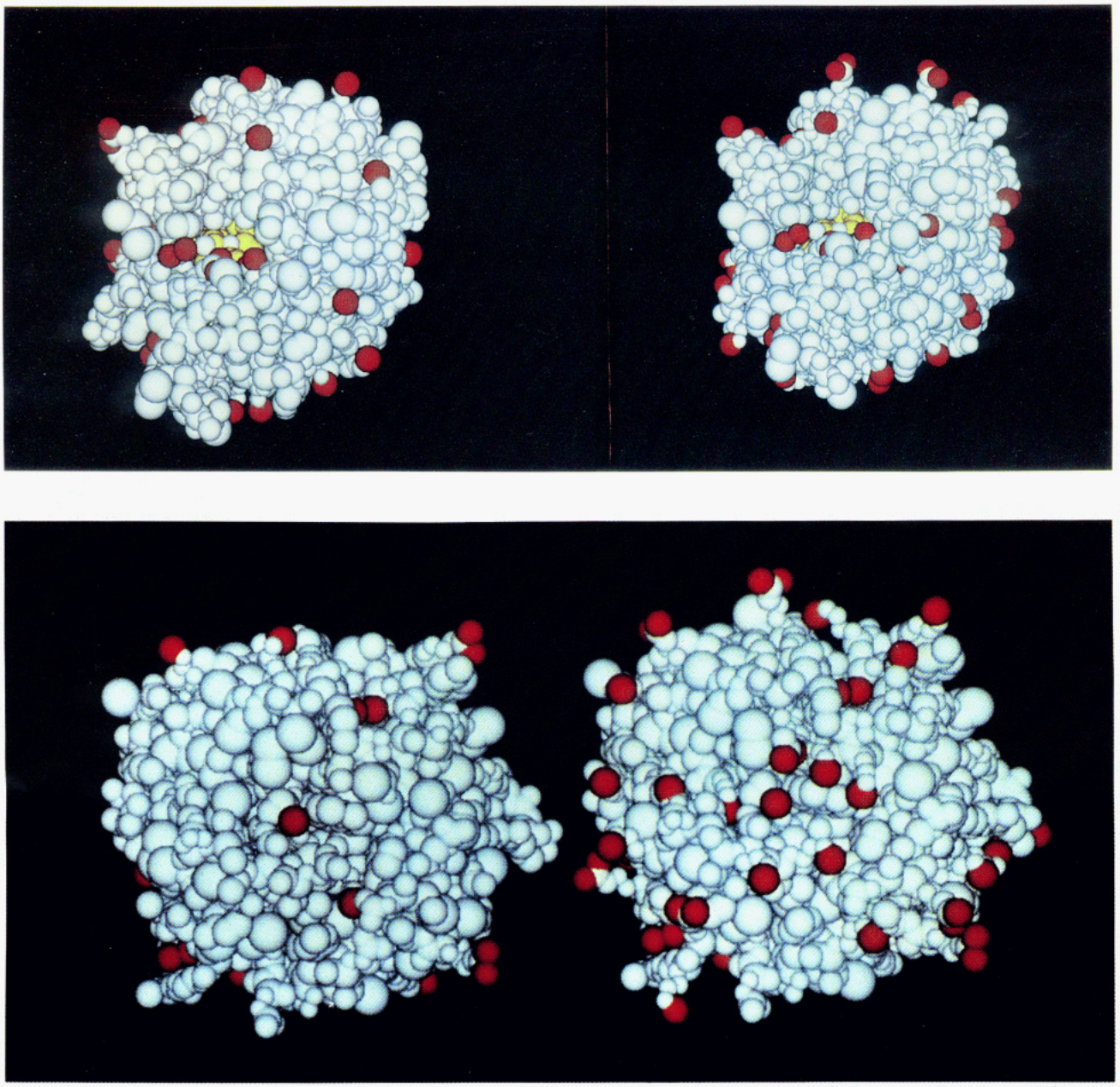

FIGURE 5: (Top) Space-filling model of the active-site face of native bovine carboxypeptidase A (left) and the same molecule (right) in which the positions of the lysine and arginine residues correspond to those in RMC-CP. The ϵ-amino nitrogens of lysine and the guanidinium nitrogens of arginine are colored in red. The zinc-binding residues, His-69, Glu-71, and His-196, are indicated in yellow. (Bottom) As in the upper model but with each molecule rotated approximately 180° around the y-axis to illustrate the difference in distribution and density of basic residues.

mouse mast cell chymase (Serafin et al., 1990) and rat mast cell protease II (Benfey et al., 1987) place two glutamyl residues prior to the amino-terminal isoleucine. Consequently, the specificity of the enzyme(s) responsible for activating all these proteases in mast cells may be directed at acidic residues rather than the basic residues common to many zymogens. The observation of a minor species of RMC-CP with an additional glutamic acid residue preceding the N-terminal isoleucine suggests occasional cleavage by the processing enzyme between, rather than after, the two acidic residues and the generation of a "ragged" amino terminus. Similarly, the precursor of bovine pancreatic carboxypeptidase A can be activated by bond cleavage at any of three loci (after an arginyl or a phenylalanyl residue), indicating that, while activation of the pancreatic zymogen requires the removal of the activation peptide, any of several cleavage sites are effective (Vendrell et al., 1990; Petra, 1970).

The data presented in Figure 4 provide direct experimental proof that granule-bound mast cell carboxypeptidase is enzymatically active and can act in tandem with RMCP I on peptide substrates such as angiotensin I. Binding to the granular matrix appears to stabilize the enzyme, as compared to free solution where it is relatively unstable (Everitt & Neurath, 1980).

Rat mast cell carboxypeptidase contains approximately the same number of histidine residues per molecule as the rat and bovine pancreatic enzymes, but 9–13 more lysine and 2–7 more arginine residues. The resulting net positive charge of 16 at pH 7 for RMC-CP accounts for its basic (calculated) isoelectric point of 9.8, well above 7.8 for bovine carboxypeptidase A. The ionic association between the mast cell enzymes and the acidic proteoglycan of the granular matrix, heparin, is presumed to be mediated through these numerous basic residues (Shick et al., 1984; Schwartz et al., 1982). As illustrated

by the molecular models of BOCPA and RMC-CP (Figure 5), the lysine and arginine residues are scattered over the surface of both molecules. Although the active-site faces of the two molecules (Figure 5, top) have similar distributions, the opposite face of the hypothetical structure of RMC-CP displays a much higher density of basic residues than that of BOCPA. Of the 29 basic residues unique to RMC-CP, 26 are also basic at corresponding loci in the mouse and human mast cell enzymes (Figure 3), but only 7 of these are basic in pancreatic carboxypeptidase B, indicating that a consistent, clustered, pattern of positive charges among the mast cell enzymes is not found on either pancreatic enzyme. Similarly, the other rat connective tissue mast cell granular protease, RMCP I, has a net positive charge of 18 at pH 7, a calculated p*I* of 10.2, and an asymmetric distribution of its lysyl and arginyl residues in patches at opposing ends of the molecule away from the active site (Remington et al., 1988). The observed clustering of the basic residues of both RMC-CP and RMCP I opposite their active sites indicates that both enzymes may bind the heparin matrix in such a way that their active sites remain accessible to substrate while embedded in that matrix.

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