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Amino Acid Sequence of a Mouse Mucosal Mast Cell Protease†

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ABSTRACT: The amino acid sequence has been determined of a mouse mucosal mast cell protease isolated from the small intestines of mice infected with *Trichinella spiralis*. The active protease contains 226 residues. Those corresponding to the catalytic triad of the active site of mammalian serine proteases (His-57, Asp-102, and Ser-195 in chymotrypsin) occur in identical positions. A computer search for homology indicates 74.3% and 74.1% sequence identity of the mouse mast cell protease compared to those of rat mast cell proteases I and II (RMCP I and II), respectively. The six half-cystine residues in the mouse mast cell protease are located in the same positions as in the rat mast cell proteases, cathepsin G, and the lymphocyte proteases, suggesting that they all have identical disulfide bond arrangements. At physiological pH, the mouse and rat mucosal mast cell proteases have net charges of +3 and +4, respectively, as compared to +18 for the protease (RMCP I) from rat connective tissue mast cells. This observation is consistent with the difference in solubility between the mucosal and connective tissue mast cell proteases when the enzymes are extracted from their granules under physiological conditions.

The presence of high levels of proteolytic enzymes in basophilic secretion granules is a characteristic feature of mast cells. Benditt and Arase (1959) first detected in rat mast cells a protease with chymotrypsin-like specificity. Later, a second similar, yet distinct, protease was found in an unusual subtype of rat mast cells known as "atypical" or mucosal mast cells. They differed in histochemical properties from those of the peritoneal type and were exclusively localized in mucosal tissues of normal rats (Woodbury et al., 1978a). The protease from peritoneal mast cells is referred to as rat mast cell protease I (RMCP I)[1] and that from mucosal mast cells as RMCP II (Woodbury & Neurath, 1978). More recently, proteases resembling in substrate specificities and other properties those of rat peritoneal mast cells have been isolated from dog and human mast cells (Schechter et al., 1983; Powers et al., 1985). Additionally, proteases with trypsin-like or carboxypeptidase A like activity have been reported to be present in peritoneal rat mast cells (Everitt & Neurath, 1980; Kido et al., 1985; Serafin et al., 1987).

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[1] Abbreviations: MMMCP, mouse mucosal mast cell protease; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; CM, *S*-(carboxymethyl); HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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More recently, Newlands et al. (1987) and co-workers isolated a chymotrypsin-like protease from the small intestines of mice infected with *Trichinella spiralis* that resembles RMCP II. Antibodies against this protease were used to demonstrate the localization of this enzyme to mucosal mast cells of the gut. The antibodies also recognized an antigenically similar protein in the mast cells of skin and tongue (Newlands et al., 1987).

On the basis of the amino acid sequences of RMCP I (Le Trong et al., 1987) and RMCP II (Woodbury et al., 1978b) and the cDNA structures of human neutrophil cathepsin G (Salvesen et al., 1987) and of two serine proteases of cytotoxic T-cells (Lobe et al., 1986; Schmid & Weissmann, 1987), it has been proposed that the chymotrypsin-like proteases of mast cells, granulocytes, and lymphocytes are more closely related to each other than to the pancreatic or plasma serine proteases (Woodbury & Neurath, 1981; Le Trong et al., 1987).

The present study reports the determination of the amino acid sequence of a serine protease from mouse mucosal mast cells. The structure is compared to those of RMCP I and RMCP II in an attempt to further document and clarify the structure/function relationships of these enzymes. This information should also prove useful to investigate the distribution, heterogeneity, and differentiation of mouse mast cells and to compare these properties to those in the rat.

Materials and Methods

Mouse mucosal mast cell protease (MMMCP) was purified by the procedures described by Newlands et al. (1987). Iodoacetic acid (Sigma) was twice recrystallized from chloroform. Cyanogen bromide and citraconic anhydride were purchased from Eastman Kodak. Trifluoroacetic acid was obtained from Pierce Chemical Co. Acetonitrile was a product of Burdick and Jackson. *N*-(*p*-Tosyl)-L-phenylalanine chloromethyl ketone–trypsin (TPCK–trypsin) and *Staphylococcus aureus* V8 protease were purchased from Millipore.

Preparation of CM-Mouse Mucosal Mast Cell Protease. S-Carboxymethylated MMMCP was prepared by incubation of 16 nmol of protein in 0.5 mL of 6 M guanidine hydrochloride, 0.5 M Tris-HCl, 10 mM EDTA, and 45 mM dithiothreitol, pH 8.6, under argon for 2.3 h at room temperature. A solution of 50 mM iodoacetic acid in 100 μL of 1 N NaOH was added, and the mixture was incubated in the dark for 30 min. This mixture was then acidified, and the CM-MMMCP was desalted by reversed-phase HPLC on an Ultrapore RPSC-C3 column (Altex).

Specific Cleavage of CM-MMMCP. CM-MMMCP (6 nmol) was cleaved at methionyl residues (Gross, 1967) with cyanogen bromide (300-fold molar excess over methionyl residues) in 70% (v/v) formic acid.

For cleavage at arginyl residues, CM-MMMCP was N-citraconylated (Atassi & Habeeb, 1972) and digested with TPCK–trypsin. Citraconylation was performed by adding 12 μL of citraconic anhydride in small aliquots to CM-MMMCP (10 nmol) dissolved in 6 M guanidine hydrochloride at room temperature over a period of 1 h while maintaining the pH at 8.8 with a pH stat. The citraconylated protein was dialyzed against 50 mM NH_4HCO_3 at pH 8.7, and TPCK–trypsin was added in two aliquots over a 5-h period at 37 °C, with the final protease:substrate ratio at 2.2% (w/w). The digestion mixture was lyophilized, and citraconyl groups were removed by incubating the digest in 8.8% formic acid (v/v) for 4 h at 37 °C.

Tryptic subdigestion of peptide M7 was performed with TPCK–trypsin in 100 mM NH_4HCO_3, pH 8.0, for 6 h at 37 °C and with a protease:substrate ratio weight of 2%. The large peptides M2 and R7 were subdigested by cleavage at glutamyl residues using *S. aureus* V8 protease in 50 mM NH_4HCO_3, pH 8.0, with a protease:substrate weight ratio of 1:50 at 37 °C for 17–24 h. Peptide R7 was cleaved at aspartyl residues by incubating in 2% formic acid at 110 °C for 4.5 h.

Peptides were initially separated by gel permeation HPLC on TSK-2000 SW columns (LKB) connected in tandem, using buffer containing 6 M guanidine hydrochloride and 10 mM phosphate at pH 6.0. Peptides were further purified by reversed-phase HPLC with a Hewlett-Packard 1090 liquid chromatograph equipped with an analytical chemistry station for HPLC, on a C8 microbore column RP-300 (Pierce) using an acetonitrile gradient in aqueous trifluoroacetic acid (Mahoney & Hermodson, 1980).

Amino acid compositions were determined with a Waters Picotag system (Bidlingmeyer et al., 1984). Amino acid sequences were analyzed with an Applied Biosystems 470A sequencer equipped with a Model 120A PTH on-line analyzer (Hunkapiller et al., 1983).

The sequence of MMMCP was compared with those of RMCP I and RMCP II and other related proteases with a VAX/VMS computer using the ALIGN program of Dayhoff et al. (1983).

Results

General Strategy of Sequence Analysis. Six nonoverlapping fragments generated by cleavage with cyanogen bromide, five additional subdigestion fragments, and the intact CM-MMMCP were sequenced. The sequence analysis of these fragments provided 90% of the amino acid sequence of MMMCP. The overlaps and the remaining sequence were obtained by cleavage of MMMCP at arginyl residues and by cleavage at aspartyl or lysyl residues in the arginyl peptide R7. Details of the proof of the sequence are summarized in Figure 1.

Cleavage at Methionyl Residues. CM-MMMCP (6 nmol) was specifically cleaved at methionyl residues with cyanogen bromide. The mixture of peptides was first separated into pooled fractions by gel filtration on two TSK-2000 SW columns in tandem. Figure 2A illustrates the primary separation of the products of cyanogen bromide cleavage. Each pool was applied to a C8 microbore column and eluted by reversed-phase HPLC for further fractionation and desalting. Sequence analysis of the largest peptide M2 (residues 16–91) (M_r 8562) provided 25 residues. Subdigest of M2 by cleavage at glutamyl residues was performed with 300 pmol of peptide. The mixture of peptides was separated by reversed-phase HPLC on a C8 microbore column to obtain fragments M2-E3, M2-E4, and M2-E5 (chromatogram not shown); their sequences provided much of the sequence of peptide M2. Peptide M7 (300 pmol) was subdigested with trypsin to identify its C-terminal residues. Two small tryptic fragments (M7-T3 and M7-T4) were purified on a C8 microbore column and sequenced.

Cleavage of Arginyl Residues. CM-MMMCP (10 nmol) was cleaved by trypsin at arginyl residues after N-citraconylation. The peptides were separated on TSK-2000 SW columns as shown in Figure 2B. Two large peptides R7 (residues 70–142) and R9 (residues 148–211) were recovered in a yield of 50–60% after desalting on a C8 column by reversed-phase HPLC. Peptide R7 was isolated as a quartet peak by reversed-phase HPLC. It is probable that oxidation of its single methionyl residue, *S*-(carboxymethyl)cysteine residue, or two tryptophanyl residues account for the anomalous chromatographic behavior. Peptide R10 was also recovered in two forms, probably due to partial oxidation of its tryptophanyl residue.

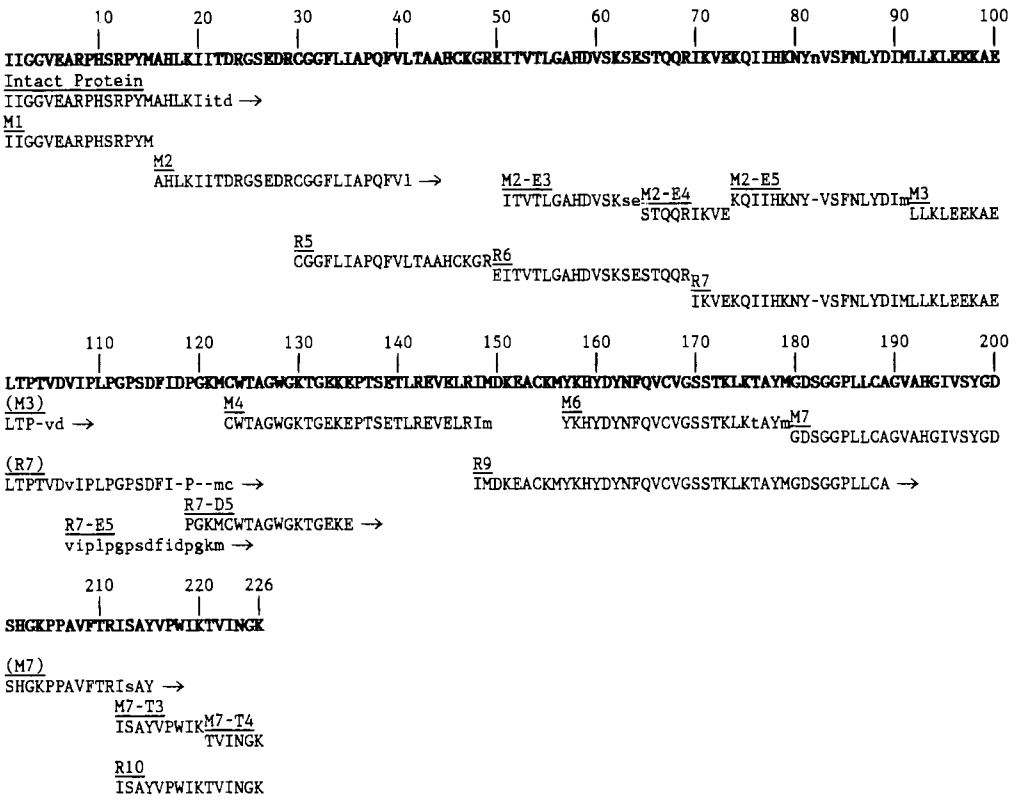

FIGURE 1: Detailed summary of the proof of the sequence of MMMCP. The proven sequences of specific peptides (names are underlined) are given in single-letter code below the summary sequence (bold type). Prefixes M and R denote peptides generated by cleavage of the CM protein at methionyl and arginyl bonds, respectively. Subpeptides are identified in hyphenated suffixes, with E, T, and D designating enzymatic cleavage by *S. aureus* V8 protease or by trypsin or chemical cleavage with dilute acid, respectively. Peptide sequences written in upper case letters were proven by Edman degradation; those in lower case letters indicate tentative identification. A dash designates a residue within a peptide that could not be identified. An arrow indicates that the C-terminal region of the peptide was not identified. The location of peptide R6 was assigned by its sequence similarity to this region of RMPC II (Woodbury et al., 1978b). The sequence from residues 42 to 60 is identical in MMMCP and RMCP II with the exception of residue 54. Asn-82 (n) was assigned by homology to other serine proteases. The putative sequence Asn(82)-Val-Ser and the lack of an amino acid at the corresponding cycles of peptides M2-E5 and R7 suggest that Asn-82 is glycosylated.

Two subdigestions of arginyl peptide R7 (1.2 nmol each) were performed by cleavage at aspartyl residues and by cleavage at glutamyl residues. Each mixture of fragments was separated on a C8 microbore column, and peptides R7-D5 and R7-E5 (chromatogram not shown) were recovered. Digestion of R7 at glutamyl residues was incomplete. In addition, cleavage was observed at aspartyl residue 106. A poorly separated mixture of cleavage products was found in a pooled fraction on reversed-phase HPLC. The sequence of R7-E5 was deduced by analysis of this mixture and subtraction of the known sequence of peptides with N-termini starting at residues 74, 98, and 101, respectively.

Edman degradation of intact CM-MMMCP overlapped peptides M1 and M2. The sequence of R5 overlapped the amino-terminal region of M2 by 11 residues and extended the sequence to Arg-49. The alignment of R5 and R6 was not proven by the sequence of an overlapping fragment, but supported by the virtual identity of portions of their sequences with that of RMCP II (Woodbury et al., 1978b).

The sequences of peptides R6 and the largest arginyl peptide R7 are linked by M2-E4, providing a continuous sequence from residues Glu-50 to Glu-135. M4 overlaps R7 and extends the sequence to Ile-148. R9 overlaps M6 and M7 and contains a marginal overlap of M4. Since M7 overlaps R10, a continuous sequence of 226 residues is provided.

Confirmation of the C-terminal region of MMMCP was established as follows. Peptide R10, generated by arginyl cleavage, was the only peptide lacking Arg and must correspond to the C-terminus of the protein. Subdigestion of peptide M7 with TPCK–trypsin yielded two pure peptides, M7-T3 and M7-T4, corresponding to the same sequence. The agreement between the sequence and composition of R10 (Table I) indicates that the C-terminus was reached, and this conclusion is supported by the sequence of M7-T4.

Discussion

The amino acid sequence of the MMMPC was determined primarily from a set of methionyl-cleavage fragments that were overlapped by sequence analyses of the intact protein and of peptides generated by cleavage of the protein at arginyl and glutamyl residues. Seventy-two percent of the residues were identified at least twice in different peptides.

The amino acid sequence clearly establishes the homologous relationship to MMMPC to other mammalian serine proteases. A comparison of the amino acid sequences of MMMCP and bovine chymotrypsin A (not shown) indicates that approximately 35% of the residues are identical at analogous loci when seven gaps are introduced in the sequence to give optimum alignment. The residues comprising the "catalytic triad" of the active site of mammalian serine proteases (His-57, Asp-102, and Ser-195 in bovine chymotrypsin) are found in corresponding positions in MMMCP.

Analysis for homology indicates 74.3% and 74.1% sequence identity to RMCP I and RMCP II, respectively, without introducing gaps in the sequences to achieve optimal alignments. Interestingly, this is similar to the level of sequence identity (73%) of RMCP I and RMCP II (Le Trong et al., 1987). As in the case of RMCP I and RMCP II, MMMCP is structurally closely related to human neutrophil cathepsin G (Salvesen et al., 1987), a human lymphocyte protease (Schmid & Weissman, 1987), and a mouse cytotoxic T-cell protease (Lobe et al., 1986) as indicated by 50%, 48%, and 47% se-

Table I: Amino Acid Compositions[a] of Peptides Isolated after Cleavage with CNBr and Tryptic Peptides from S-Carboxymethylated and N-Citraconylated MMMCP

	M1 1–15	M2 16–91	M3 92–122	M4 123–149	M5 150–156	M6 157–179	M7 180–226	R9 148–211	R10 212–226	whole protein: from hydrolysis[c]	whole protein: from sequence
Asx (D/N)		5.4 (6)	2.7 (3)		(1)	1.8 (2)	2.5 (3)	4.3 (5)	0.8 (1)	13.4	15
Glx (E/Q)	1.1 (1)	7.8 (8)	3.0 (3)	5.0 (5)	(1)	1.8 (1)	0.5	2.5 (2)		18.3	15
CMC (C)		1.8 (2)		0.8 (1)	(1)	0.6 (1)	1.5 (1)	2.0 (2)		6.1	6
Ser (S)	1.3 (1)	5.7 (5)	1.4 (1)	1.2 (1)	(2)	2.6 (2)	4.9 (4)	4.6 (5)	1.1 (1)	14.3	14
Gly (G)	2.4 (2)	5.9 (5)	2.5 (2)	3.3 (3)		1.7 (1)	8.5 (8)	8.7 (8)	1.3 (1)	20.8	21
His (H)	1.0 (1)	3.3 (4)				0.8 (1)	1.8 (2)	3.2 (3)		7.0	8
Arg (R)	1.9 (2)	3.8 (4)		2.2 (2)		0.4	1.4 (1)	1.4 (1)		9.3	9
Thr (T)		4.4 (5)	1.9 (2)	4.3 (4)		1.6 (2)	2.0 (2)	3.1 (3)	1.1 (1)	14.6	15
Ala (A)	1.1 (1)	5.9 (5)	1.2 (1)	1.3 (1)	(1)	1.3 (1)	4.3 (4)	5.3 (5)	1.2 (1)	14	14
Pro (P)	1.6 (2)	0.7 (1)	5.0 (5)	0.7 (1)			3.2 (4)	3.3 (3)	1.1 (1)	11.2	13
Tyr (Y)	1.0 (1)	2.7 (3)				3.2 (4)	2.2 (2)	4.7 (5)	1.0 (1)	10.2	10
Val (V)	1.1 (1)	5.3 (5)	2.1 (2)	1.2 (1)		1.6 (2)	5.1 (5)	4.9 (5)	2.1 (2)	14.6	16
Met[b] (M)	0.9 (1)	0.9 (1)	0.7 (1)	0.8 (1)	(1)	0.6 (1)		2.1 (3)		5.2	6
Ile (I)	1.9 (2)	8.8 (8)	2.1 (2)	1.5 (1)		0.3	4.4 (4)	2.4 (2)	3.2 (3)	15.6	17
Leu (L)		5.3 (5)	5.2 (5)	2.2 (2)	(1)	1.6 (1)	2.7 (2)	3.4 (3)		14.6	15
Phe (F)		3.1 (3)	1.1 (1)			1.0 (1)	1.3 (1)	2.0 (2)		6.0	6
Lys (K)		4.8 (6)	3.0 (3)	2.2 (2)	(2)	2.5 (3)	2.8 (3)	5.5 (6)	2.1 (2)	19.1	19
Trp (W)				ND (2)			ND (1)		ND (1)	ND	3
total residues	15	76	31	27	7	23	47	64	15		
yield (%)	38	20	18	25		17	27	50	30		

[a] Residues per peptide from amino acid analysis (6 N HCl, 110 °C, 24 h). Values of 0.3 or less are not reported. Numbers in parentheses are derived from the sequence (Figure 1). ND = not determined. [b] Determined as homoserine in M1, M2, M3, M4, M6, and M7. [c] From the hydrolysis of S-carboxymethylated MMMCP for 24, 48, and 72 h. Thr and Ser values are extrapolated to $t = 0$. Val and Ile values were from the 72-h analysis.

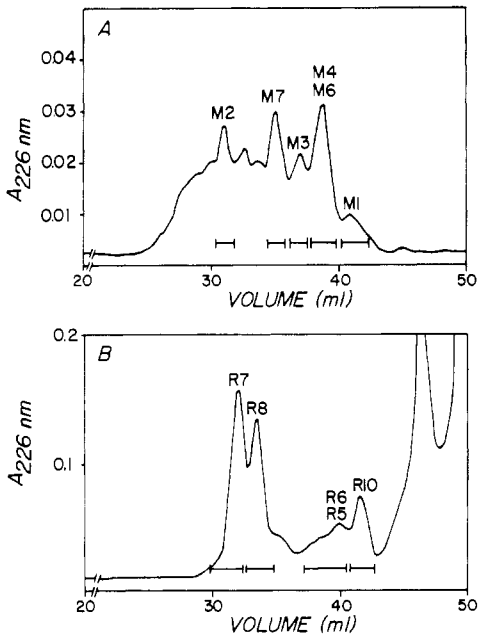

FIGURE 2: Primary separation of mixtures of peptides generated from the cleavage of CM-MMMCP (A) at Met (6 nmol of protein) with CNBr and (B) at Arg (10 nmol of citraconylated CM-MMMCP) with TPCK–trypsin. In each case, peptides were separated by size in 6 M guanidine hydrochloride and 10 mM sodium phosphate buffer, pH 6.0, at a flow rate of 0.5 mL/min on a tandem pair of TSK-2000 SW columns. Each pool of fractions was desalted and further fractionated by reversed-phase HPLC on a C8 microbore column R7-300 (100 × 2.1 mm) equilibrated in 0.09% trifluoroacetic acid with a linear gradient of acetonitrile.

quence identity, respectively. The homology among these "chymotrypsin-like" proteases of cellular origin suggests that they are more closely related to each other than to the pancreatic or plasma serine proteases. Interestingly, human neutrophil elastase is not more similar to MMMCP, or to any of the other proteases of cellular origin, than pancreatic chymotrypsin is to MMMCP. Analysis of percent sequence identities and alignment scores suggests that, with the exception of human neutrophil elastase, the mast cell/granulocyte/lymphocyte proteases have diverged less from each other during evolution than have the pancreatic serine proteases from each other (Le Trong et al., 1987).

MMMCP shares with the rat mast cell proteases certain structural features that may relate to function. For example, the six half-cystinyl residues in MMMCP align precisely with those of RMCP I and RMCP II. Additionally, MMMCP, RMCP I, and RMCP II all lack a disulfide bond near the active site seryl residue (Cys-191 to Cys-220 in chymotrypsin) that is invariantly present in pancreatic and plasma serine proteases (Woodbury et al., 1978b; Le Trong et al., 1987). The cDNA structures of human neutrophil cathepsin G (Salvesen et al., 1987), a human lymphocyte protease (Schmid & Weissmann, 1987), and two proteases from mouse cytotoxic T-cells and natural killer cells (Lobe et al., 1986) indicate that the disulfide bond arrangement of all these proteases is identical with that observed for the mast cell proteases. An analysis of the three-dimensional structure of RMCP II at 1.9-Å resolution, indeed, indicates that RMCP II has a hydrophobic pocket that can accommodate a phenylalanyl residue at the P_3 position of synthetic substrates (Remington et al., 1988). The formation of this pocket, which is absent in chymotrypsin, is possible only because RMCP II lacks that particular disulfide bond.

Since the amino acid sequence of MMMCP is equally similar to those of RMCP I (74.3%) and RMCP II (74.1%), it would not be possible to predict, solely on the basis of amino acid sequence similarity, from which type of mast cell (mucosal or connective) the mouse protease originated. Nor could such a distinction be made on the basis of the nature of the primary substrate binding site, which in RMCP I is a seryl residue at position 176 and an alanyl residue in RMCP II. In MMMCP a threonyl residue occupies position 176. At the corresponding site, an Asp/Ser substitution accounts for the difference in specificity of trypsin and chymotrypsin, respectively (Hartley, 1970). Reasonably, one would assume that MMMCP and

RMCP II have identical structures (at this site) and specificities.

The mouse and rat mucosal mast cell proteases, however, have similar net charges at physiological pH which differ from that of RMCP I. At pH 7.2, the net charge of mouse and rat mucosal mast cell proteases is +3 and +4, respectively, whereas that of RMCP I is +18. This is consistent with the experimental observations that MMMCP and RMCP II are readily extracted from mast cell granules in physiological solutions (Newlands et al., 1987; Woodbury & Neurath, 1978), in contrast to RMCP I which remains associated with the granule proteoglycan (heparin) when connective tissue mast cells are extracted with physiological solutions (Lagunoff & Pritzl, 1976). The extent of dissolution of the various mast cell granules following their secretion into an extracellular environment may thus depend largely on the relative charges of the proteases and the proteoglycans at the prevailing pH. The degree to which secreted mast cell granules dissolve in extracellular fluids, thereby releasing soluble enzymes and proteoglycans, may have important physiological consequences for the interaction of the proteases with substrates and inhibitors and for enzyme regulation and may determine the distance from the cells of origin over which the mast cell proteases exert their action (Woodbury et al., 1981; Le Trong et al., 1987).

The amino acid sequence of MMMCP provides further evidence that the chymotrypsin-like proteases of mast cell, granulocyte, and lymphocyte origin comprise a family of serine proteases that are distinct from those secreted by the pancreas and liver (Woodbury et al., 1978b; Woodbury & Neurath, 1981; Le Trong et al., 1987). With the primary structure of MMMCP now in hand, it should be possible to isolate and express cDNA and genomic clones of this protease and to relate them to those of rat mast cell origin (Benfey et al., 1987).

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Registry No. MMMCP, 117581-16-5; protease, 9001-92-7.

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