THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 1, Issue of January 7, pp. 351–358, 2000
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Printed in U.S.A.

Formation of Enzymatically Active, Homotypic, and Heterotypic Tetramers of Mouse Mast Cell Tryptases

DEPENDENCE ON A CONSERVED Trp-RICH DOMAIN ON THE SURFACE*

(Received for publication, August 17, 1999, and in revised form, October 18, 1999)

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Mouse mast cell protease (mMCP) 6 and mMCP-7 are homologous tryptases stored in granules as macromolecular complexes with heparin and/or chondroitin sulfate E containing serglycin proteoglycans. When pro-mMCP-7 and pseudozymogen forms of this tryptase and mMCP-6 were separately expressed in insect cells, all three recombinant proteins were secreted into the conditioned medium as properly folded, enzymatically inactive 33-kDa monomers. However, when their propeptides were removed, mMCP-6 and mMCP-7 became enzymatically active and spontaneously assumed an ~150-kDa tetramer structure. Heparin was not required for this structural change. When incubated at 37 °C, recombinant mMCP-7 progressively lost its enzymatic activity in a time-dependent manner. Its *N*-linked glycans helped regulate the thermal stability of mMCP-7. However, the ability of this tryptase to form the enzymatically active tetramer was more dependent on a highly conserved Trp-rich domain on its surface. Although recombinant mMCP-6 and mMCP-7 preferred to form homotypic tetramers, these tryptases readily formed heterotypic tetramers *in vitro*. This latter finding indicates that the tetramer structural unit is a novel way the mast cell uses to assemble varied combinations of tryptases.

Mouse mast cell (MC)[1] protease (mMCP) 6 (1, 2) and mMCP-7 (3, 4) are homologous tryptases stored in abundance in their mature forms in the acidic secretory granules of numerous populations of MCs ionically bound to heparin and/or chondroitin sulfate E containing serglycin proteoglycans (1, 5, 6). Both tryptases are exocytosed when MCs are activated via their high affinity immunoglobulin (Ig) E receptors. mMCP-7 inhibits the formation of fibrin/platelet clots (7), whereas mMCP-6 induces the extravasation of neutrophils (8). Thus, the two tryptases often work in concert in the acute phases of MC-mediated inflammatory responses.

A small amount of exocytosed mMCP-7 is retained in tissues for >1 h (5). However, most exocytosed mMCP-7 dissociates from its exocytosed protease/proteoglycan macromolecular complex in the extracellular matrix to allow the rapid diffusion of this tryptase away from the MC (5, 6). Because its proteoglycan-binding domain has more Lys and Arg residues than the corresponding domain in mMCP-7, exocytosed mMCP-6 cannot dissociate easily from its serglycin proteoglycan at neutral pH. Thus, due to its >10 million-dalton size, very little of the exocytosed mMCP-6/proteoglycan complex is able to enter the circulation.

Pancreatic trypsin and most other serine proteases are enzymatically active in their ~30-kDa monomer states. Thus, it was a surprise when Schwartz and co-workers (9, 10) and then others (11–16) discovered that human MC tryptases purified from different tissues exist as tetramers. While it has been proposed that heparin is needed for human lung- and skin-derived tryptases to form stable, enzymatically active tetramers (10, 14–16), mMCP-7 is able to circulate in the blood of the V3 mastocytosis mouse for >1 h as an enzymatically active, homotypic tetramer free of proteoglycan (5). Thus, the domain on the surface of mMCP-7 that binds to proteoglycan (6) does not appear to be the most important structural determinant for either inducing or maintaining the tetramer state of this tryptase. Based on a multiple sequence alignment and comparative protein structure modeling approach, Johnson and Barton (4) first noted that most human (17–19), dog (20), and mouse (1–4) tryptases have a number of other conserved motifs on their surfaces, including Trp-, Pro-, and Tyr-rich domains and glycosylation sites. Analysis of the crystallographic structure of human tryptase II/β complexed to 4-amidinophenyl pyruvic acid (21) did not explain why the Trp-rich domain on the face opposite that of the substrate-binding cleft was conserved. Nevertheless, it indicated that the Tyr- and Pro-rich domains are conserved in order that they can physically interact with one another to form the basic ringed structural unit (21).

We have been able to induce insect cells to secrete tryptase pseudozymogens (*e.g.* pro-EK-mMCP-7 (Ref. 7) and pro-EK-mMCP-6 (Ref. 8)) into the conditioned medium which could be activated with enterokinase (EK) after their purification. In the present study, we reexamined the tetramer issue at the molecular level using these two recombinant tryptases, as well as recombinant pro-mMCP-7 (6) and newly derived Trp → Leu and glycosylation mutants of mMCP-7. We now report that both recombinant mMCP-6 and mMCP-7 must form a tetramer structure to become enzymatically active. However, in neither case is formation of the tetramer dependent on heparin. Based

* This work was supported in part by Grants AI-23483, GM-54762, HL-36110, and HL-63284 from the National Institutes of Health and Grant BIR-9601845 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MC, mast cell; CD, circular dichroism; EK, enterokinase; FPLC, fast protein liquid chromatography; Ig, immunoglobulin; mMCP, mouse MC protease; PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanilide; TBST, Tris-buffered saline with Tween 20.

on a multiple sequence alignment, we noted that recently cloned gerbil (22) and rat (23–25) MC tryptases also have the conserved residues that form the Trp-rich domain on the surface of mature mouse and human tryptases (4–7, 21). Using site-directed mutagenesis, we now show that mMCP-7 requires this domain to form the enzymatically active tetramer. Finally, we demonstrate that recombinant mMCP-6 and mMCP-7 can form heterotypic complexes *in vitro*. The latter discovery offers an explanation why a portion of exocytosed mMCP-7 is retained for an extended period of time in connective tissues.

EXPERIMENTAL PROCEDURES

Expression of Pro-EK-mMCP-6, Pro-mMCP-7, Pro-EK-mMCP-7, and Various Mutants of Pro-EK-mMCP-7 in Insect Cells—Recombinant pro-EK-mMCP-6 (8), pro-mMCP-7 (6), and pro-EK-mMCP-7 (7) were expressed in baculovirus-infected High Five insect cells (Invitrogen, San Diego, CA) in serum-free, X-press medium (BioWhittaker, Walkersville, MD), as described previously. Both pro-EK-mMCP-6 and pro-EK-mMCP-7 have the EK-susceptible peptide, Asp-Asp-Asp-Asp-Lys, between the natural propeptide and the mature portion of the enzyme. To facilitate their purification from the insect cell-conditioned medium by means of an immunoaffinity column (International Biotechnology Inc., New Haven, CT) (26), each recombinant pseudozymogen also contained the 8-residue FLAG peptide attached to its C terminus.

To evaluate the functional role of certain Trp residues and *N*-linked glycans in mMCP-7, the cDNA that encodes the pseudozymogen form (7) of mMCP-7 (3) was subcloned into the pALTER®-1 vector (Promega, Madison, WI). Site-directed mutagenesis was then performed with the Altered Sites® II *in vitro* mutagenesis system (Promega), according to the manufacturer's instructions. The mutagenic oligonucleotide 5′-AACAAGTGGCCCTTGCAGGTGAGCCTG-3′ (corresponding to nucleotides 112–138 of our mMCP-7 cDNA) was used to convert Trp^{14} to Leu, whereas the mutagenic oligonucleotide 5′-ACCTTGCTGCAGGCAGGCGTGGTC-3′ (corresponding to nucleotides 696–720 of our mMCP-7 cDNA) was used to convert Trp^{206} to Leu. These Trp residues were mutated to Leu residues rather than to Asp, Glu, Arg, or Lys residues in order to maintain the general hydrophobic nature of the Trp-rich domain on the surface of folded mMCP-7. A two-step polymerase chain reaction approach was then used to obtain a carbohydrate-deficient form of mMCP-7. The mutagenic oligonucleotides 5′-AGCCTGCGTGCCCAAGACACCTACTGG-3′ and 5′-ACAAACCCTGTGCAAATTTCTGACTAT-3′ (corresponding to nucleotides 132–159 and 375–402 of our mMCP-7 cDNA, respectively) were used to convert Asn^{21} and Asn^{102} to Gln. After each mutated cDNA was isolated and sequenced, it was subcloned into pVL1393 and expressed in insect cells, as described for recombinant pro-EK-mMCP-7 (7).

Recombinant pro-EK-mMCP-6, pro-EK-mMCP-7, and mutants of pro-EK-mMCP-7 were purified from the resulting conditioned medium with an anti-FLAG Ig affinity column (7, 8). Mature mMCP-6, mature mMCP-7, and the mMCP-7 mutants were obtained after EK treatment to proteolytically remove the bioengineered propeptide. The enzymatic activities of the resulting recombinant tryptases were monitored with the chromogenic substrate tosyl-Gly-Pro-Lys-*p*-nitroanilide (pNA) (Sigma), as described previously for wild-type tryptases (7, 8, 27).

Generally, a 1-μl sample of each activation mixture was placed in 1 ml of assay buffer containing 50 μg of substrate, 1 mM EDTA, and 25 mM sodium phosphate, pH 7.4. The change in optical density at 405 nm was determined after a 2–5-min incubation at room temperature.

Fast Protein Liquid Chromatography (FPLC) and Circular Dichroism (CD)—Gel filtration was carried out with a FPLC system (5). Various amounts of pro-mMCP-7 (20 μg), mMCP-6, mMCP-7, and the mMCP-7 mutants (20 μg) were suspended separately in 1 ml of 150 mM NaCl, 10 mM EDTA, and 10 mM sodium phosphate, pH 7.4, and applied to a Superose 12 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) that had been equilibrated at 4 °C in the same buffer. The column was eluted at 4 °C at a flow rate of 0.25 ml/min, and 30 0.5-ml fractions were collected. Portions (5 μl) of each fraction were analyzed for enzymatic activity with tosyl-Gly-Pro-Lys-pNA.

The CD spectra of enzymatically inactive pro-mMCP-7 and enzymatically active mMCP-7 over the range of 225–250 nm were determined with an Aviv 62DS CD spectrophotometer, according to standard procedures (15). Briefly, 200 μl of a 0.4 mg/ml solution of pro-mMCP-7 or mature mMCP-7 in 50 mM sodium acetate, 5 mM calcium chloride, pH 5.2, was placed in a cuvette with a 1-cm path length. The change in absorbance (A) of the sample was measured at room temperature for 1 s at each wavelength. Five consecutive scans were averaged and a base line of buffer solution was subtracted from the CD spectra of each protease sample. Trypsin (Sigma) and chymotrypsin (Sigma) were used to determine the optimal protein concentration needed to generate the CD spectra of a serine protease. The A was converted to molar ellipticity ($[\Theta]$ in degrees cm^2/dmol) by the following equation: $[\Theta] = (3300)\ [\Delta A/(c)(l)](MRW)(0.001)$, where ΔA = millidegree, c = mg/ml concentration of the protein solution, MRW = mean residue weight of 112, and l = 1-cm length of path of cuvette.

Immunoblot and N-terminal Amino Acid Analysis—Protein blots of the varied FPLC fractions were analyzed with either anti-mMCP-6 Ig (5), anti-mMCP-7 Ig (28), or anti-FLAG Ig (International Biotechnology) (26, 29). A 50-μl sample of each FPLC fraction was vacuum-transferred to an Immobilon-P membrane (Millipore, Bedford, MA) with a slot-blot apparatus. The membrane was washed three times with 200 μl of Tris-HCl-buffered saline and then incubated for 1 h at room temperature in Tris-HCl-buffered saline containing 5% nonfat milk and 0.02% Tween 20 (TBST buffer). After three washes with TBST buffer, the protein blot was incubated with a 1:500 dilution of affinity-purified rabbit anti-mMCP-6 Ig or anti-mMCP-7 Ig (~1.5 μg/ml final concentration) or with mouse anti-FLAG Ig (~5 μg/ml final concentration) in TBST buffer for 1 h at room temperature. After three washes with TBST buffer, the protein blot was incubated for 1 h in a 1:1000 dilution of an alkaline phosphatase conjugate of goat anti-rabbit Ig or goat anti-mouse Ig (1 ng/ml final concentration) in TBST buffer. Immunoreactive proteins were visualized with nitroblue tetrazolium (0.2 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.1 mg/ml) as substrates.

In some instances, samples of the FPLC fractions were diluted in SDS-polyacrylamide gel electrophoresis (PAGE) buffer (1% SDS, 1% β-mercaptoethanol, 0.1% bromphenol blue, and 500 mM Tris-HCl, pH 6.8) and boiled for 5 min before being loaded onto a 12% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Blue or was placed in a Bio-Rad immunoblotting apparatus. The resolved proteins were transferred for 2–4 h at 200 mA to an Immobilon-P membrane in a solution consisting of 20% methanol, 16 mM Tris-HCl, and 120 mM glycine, pH 8.3. The resulting protein blot was probed with the relevant antibody. For N-terminal amino acid analysis, certain SDS-PAGE protein blots were briefly stained with Ponceau S Red (Sigma). The visualized protein bands were isolated and subjected to automated Edman degradation by the Harvard Microchemistry Facility (Harvard Biological Laboratories, Cambridge, MA).

Degradation of Fibrinogen by mMCP-7 and Its Mutants—Mouse fibrinogen (Sigma) (20 μl containing ~100 μg of substrate), 40 μl of digestion buffer (25 mM sodium phosphate, 1 mM EDTA, pH 7.4), and 10 μl of mature wild-type recombinant mMCP-7, its carbohydrate mutant, or its W206L mutant were mixed and incubated at room temperature for 3 h. The resulting digests were subjected to SDS-PAGE analysis, as described previously (7).

Heparin Chromatography of Homotypic and Heterotypic Complexes of mMCP-6 and mMCP-7—Recombinant pro-EK-mMCP-6 and pro-EK-mMCP-7 were mixed in various molar ratios and treated with EK. The resulting complexes were then applied to replicate 3-ml columns of heparin-Sepharose that had been equilibrated in 100 mM NaCl and 50 mM NaAc, pH 5.0. After each column was washed with 10 ml of equilibration buffer, it was subjected to a 40-ml gradient in which the NaCl concentration was increased linearly from 0.1 M to 4.0 M. Samples of the 1-ml eluate fractions were assessed for their enzymatic activities with tosyl-Gly-Pro-Lys-pNA. In some experiments, samples of the eluate fractions were subjected to immunoblot analysis with anti-mMCP-6, anti-mMCP-7, or anti-FLAG Ig.

RESULTS

Conversion of Inactive Tryptase Monomers to Active Tetramers—Recombinant pro-mMCP-7 (data not shown), pro-EK-mMCP-6 (data not shown), and pro-EK-mMCP-7 (Fig. 1*A*) were all secreted into the insect cell-conditioned medium as inactive monomers with apparent molecular masses of ~33 kDa. When their propeptides were proteolytically removed, mature mMCP-6 and mMCP-7 spontaneously formed tetramers with apparent molecular masses of ~150 kDa (Fig. 1*B*). As assessed by CD (Fig. 2), mMCP-7 underwent a conformational change in its secondary structure during this activation process. The most striking difference in the CD spectra of the inactive and active forms of mMCP-7 occurred at 230 nm.

Recombinant mMCP-6 and mMCP-7 became enzymatically active after their bioengineered propeptides were removed; op-

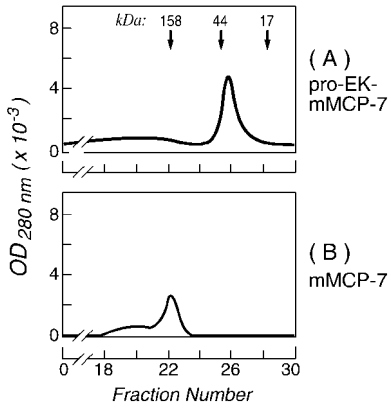

FIG. 1. **FPLC of recombinant pro-EK-mMCP-7 and mature mMCP-7.** Purified pro-EK-mMCP-7 (*A*) and EK-activated mature mMCP-7 (*B*) were applied to replicate FPLC columns. Fractions (0.5 ml) were collected, and the OD_{280} nm of each fraction was determined. Arrows in *A* indicate the elution positions of the three standard proteins having molecular masses of 158, 44, and 17 kDa. In the depicted experiment in *B*, mMCP-7 was activated with EK in the absence of heparin or glycerol. Immunoblot analyses of various column fractions revealed that the peaks indicated in *A* and *B* contained recombinant mMCP-7 (data not shown).

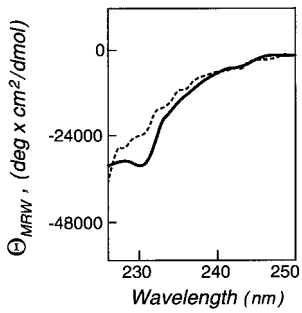

FIG. 2. **CD spectra of pro-EK-mMCP-7 and mature mMCP-7.** The CD spectra of enzymatically inactive pro-EK-mMCP-7 monomers (- - -) and enzymatically active mMCP-7 (——) tetramers were determined.

timal enzymatic activity was obtained when these tryptases were activated at pH 5.5 (Fig. 3). Recombinant mMCP-7 formed a tetramer whether it was activated with EK in the absence or presence of heparin or glycerol (Fig. 4*A*). The enzymatic activity of mature recombinant mMCP-7 generated in the absence of heparin or glycerol was maintained for 12 h at 37 °C in pH 5.2 buffer, but it slowly decreased during the next 15 h of incubation (Fig. 4*B*). Although nearly 3-fold more enzymatically active mMCP-7 was generated if heparin or glycerol was present during the initial EK-activation step, the subsequent rate of decay of enzymatically active mouse tryptase was not substantially different from that of recombinant mMCP-7 activated in the absence of either agent.

Although the amount of enzymatically active mMCP-7 in the sample decreased nearly 75% after the 27-h incubation at 37 °C, SDS-PAGE/immunoblot analysis revealed that the mMCP-7 monomers in the preparation were still ~33 kDa in size (Fig. 4*C*). N-terminal amino acid analysis revealed that the recombinant form of mMCP-7 that had lost most of its enzymatic activity after the 27-h incubation had an intact N terminus of Ile-Val-Gly-Gly-Gln-Glu-Ala-His-Gly-Asn-Lys. SDS-PAGE/immunoblot analysis also revealed that it contained the FLAG peptide at its C terminus (Fig. 4*C*).

Identification of Two Trp Residues in mMCP-7 Required for Its Ability to Form Tetramers—The CD spectra (Fig. 2) suggested that one or more Trp residues in mMCP-7 underwent a conformational change when the zymogen was converted to

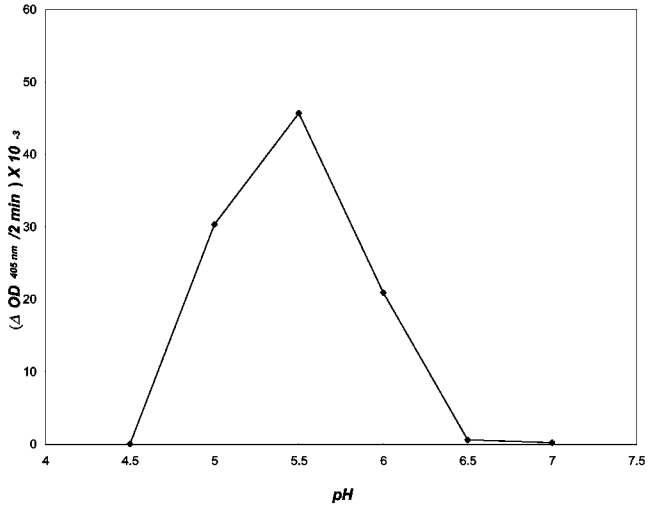

FIG. 3. **pH-dependent activation of recombinant mMCP-7.** In these experiments, equivalent amounts of recombinant pro-EK-mMCP-7 (~6 μg) were suspended in buffers (♦; 30 μl) whose pH values ranged from 4.5 to 7.0. EK (~0.4 unit corresponding to ~3 ng) was added and each sample was incubated at 37 °C for 3 h. At the end of this activation step, 1 μl of each reaction mixture was evaluated for its tryptic activity using tosyl-Gly-Pro-Lys-pNA as the substrate. Because EK exhibits a broad pH optimum and is more active at neutral pH than pH 5.5, the optimal tryptase activity of the mMCP-7 sample seen at pH 5.5 is not the result of increased efficiency in removal of the bioengineered propeptide.

active enzyme. Nine Trp residues (Trp^{12}, Trp^{14}, Trp^{25}, Trp^{38}, Trp^{126}, Trp^{130}, Trp^{206}, Trp^{214}, and Trp^{236}) in mMCP-6 are conserved in mMCP-7 and various rat, gerbil, dog, and human MC tryptases. Only five of the conserved Trp resides are uniquely found in those mMCPs that form tetramers. Because comparative protein structure modeling suggested that Trp^{12}, Trp^{14}, Trp^{126}, and Trp^{206} are spatially close on the surface of the folded tryptase, a site-directed mutagenesis approach was used to create mMCP-7 mutants in which Trp^{14} and Trp^{206} were separately converted into Leu residues. The W14L and W206L mutants of mMCP-7 were readily expressed in insect cells and were secreted into the culture medium. In each instance, >80% of the expressed mutant bound to a heparin-Sepharose column (data not shown). Thus, the conversion of Trp^{14} or Trp^{206} to Leu did not cause an extensive alteration in the overall three-dimensional structure of mMCP-7.

The propeptides of both Trp mutants could be removed easily by EK treatment (Fig. 5*A*). However, the W14L mutant did not possess any enzymatic activity when its propeptide was removed (Fig. 5*B*). As assessed by FPLC, it also was unable to form a tetramer even if heparin glycosaminoglycan was present (data not shown). In contrast, the W206L mutant readily cleaved tosyl-Gly-Pro-Lys-pNA when analyzed immediately after a room temperature treatment with EK (Fig. 5*B*). Moreover, at least some of the EK-treated material was able to form a tetramer. The W206L mutant also exhibited a pH optimum comparable to that of the wild-type tryptase (data not shown). Because fibrinogen is a physiologic substrate of native mMCP-7 (7), the substrate specificity of the W206L mutant was examined. Freshly activated wild-type mMCP-7 and its W206L mutant readily degraded the α chain of mouse fibrinogen (Fig. 6).

Whereas the above findings indicated that Trp^{14} was important in some way for the enzymatic activity of mMCP-7, the initial data suggested that Trp^{206} was not. To examine this in more detail, the thermal-dependent stability of the W206L mutant was evaluated. Unlike wild-type recombinant mMCP-7, which only lost ~5% of its enzymatic activity after a 5-h incubation at 37 °C (Fig. 4*B*), the W206L mutant lost 85%

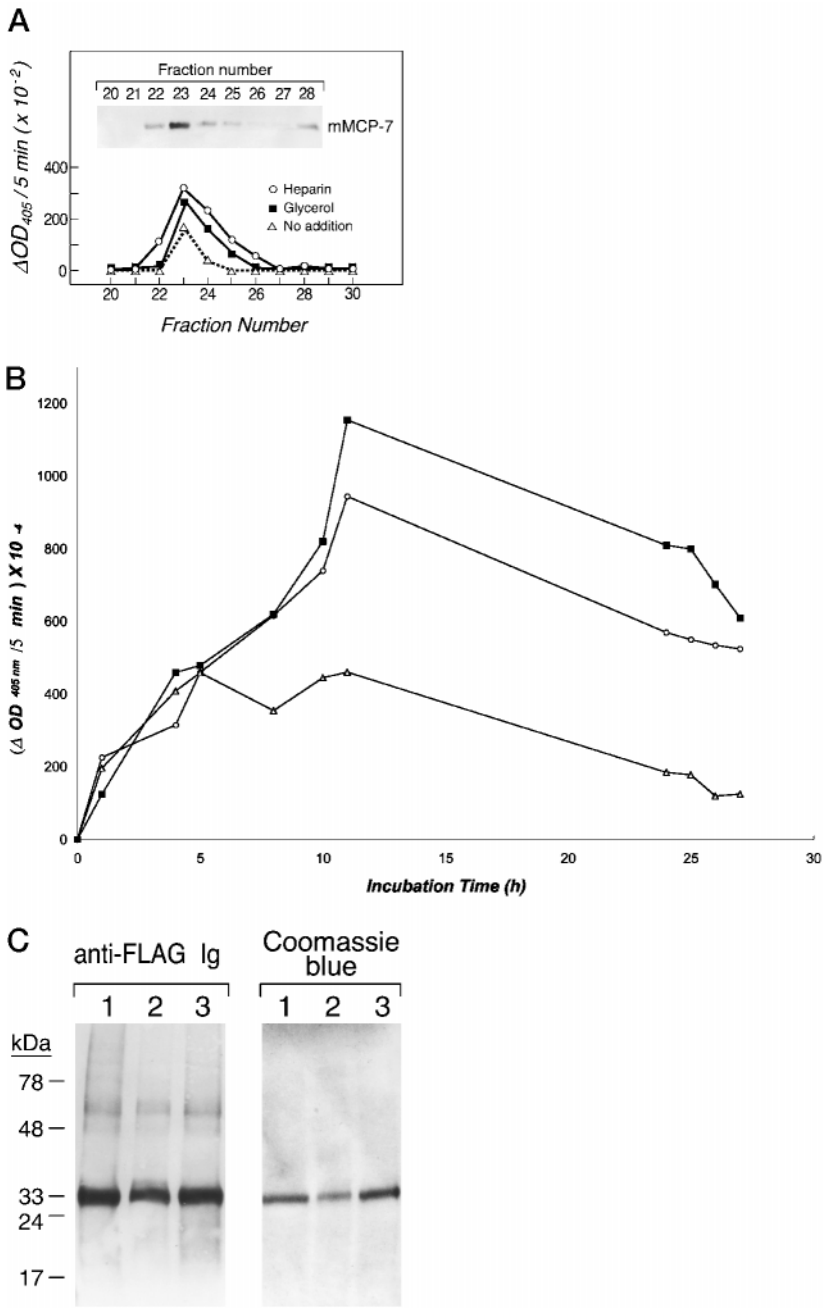

FIG. 4. **Activation of recombinant mMCP-7 in the presence or absence of heparin or glycerol.** In *panel A*, equivalent amounts of recombinant pro-EK-mMCP-7 (~12 μg) were suspended in pH 5.2 buffer (60 μl) lacking (△) or containing glycerol (■; 10% final concentration) or heparin (○; 6:1, tryptase to glycosaminoglycan wild-type ratio). EK (~0.8 unit) was added. Each sample was incubated at 37 °C for 3 h and then applied to replicate FPLC columns. Five-μl samples of the resulting 1-ml column fractions were evaluated for their ability to cleave tosyl-Gly-Pro-Lys-pNA during a 5-min incubation to generate optical density at 405 nm. Each column fraction (50 μl) was also analyzed for the presence of immunoreactive mMCP-7 with anti-mMCP-7 Ig. The *inset* depicts the immunoblot analysis of fractions 20–28 of mMCP-7 that had been activated in the presence of heparin. Similar immunoblot results were obtained with recombinant mMCP-7 that had been activated with EK in the absence of heparin and glycerol or in the presence of glycerol alone (data not shown). In *panel B*, the time curves of activation and denaturation of recombinant mMCP-7 at 37 °C and pH 5.2 are depicted in the absence of heparin and glycerol (△), in the presence of glycerol (■), or in the presence of heparin (○). Analogous to the experiment depicted in *A*, pro-EK-mMCP-7 (12 μg) was placed in pH 5.2 buffer (60 μl) containing EK (0.8 unit) alone or with heparin (2 μg) or glycerol (10% final concentration). At the indicated times, a 1-μl sample of each mixture was removed and assayed for its ability to cleave tosyl-Gly-Pro-Lys-pNA during a 5-min incubation at pH 7.0. In *panel C*, 10-μl samples of the three reaction mixtures obtained after the 27-h incubation in *B* were subjected to SDS-PAGE/immunoblot analysis. Two replicate gels were prepared. Protein samples were exposed to β-mercaptoethanol before electrophoresis to disrupt disulfide bonds. The gel on the *right* was stained with Coomassie Blue to evaluate gross proteolysis of the recombinant tryptase. The proteins in the gel on the *left* were transferred to a membrane, and the resulting blot was stained with anti-FLAG Ig to evaluate proteolysis at the C terminus. Depicted in each gel are recombinant material incubated 27 h in the absence of heparin and glycerol (*lane 1*), in the presence of heparin (*lane 2*), or in the presence of glycerol (*lane 3*).

of its activity during the same time period (Fig. 7*A*). SDS-PAGE/immunoblot analysis revealed that neither the W14L mutant nor the W206L mutant underwent proteolysis. Even after a 24-h incubation at 37 °C, both mutants were ~33 kDa in size (Fig. 7*B*). Moreover, amino acid sequence analysis and immunoblot analysis with anti-FLAG Ig revealed that both mutants possessed intact N and C termini.

Role of the N-linked Glycans Bound to mMCP-7—Although

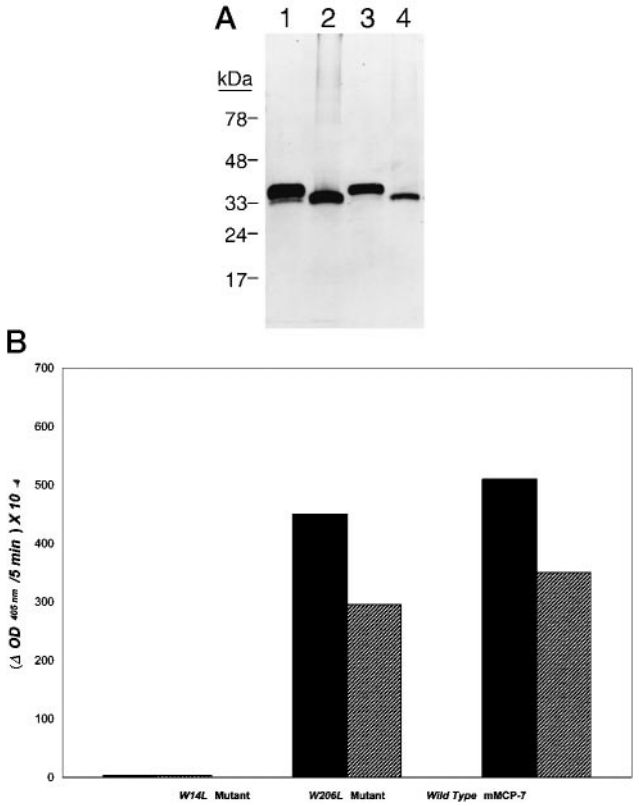

FIG. 5. **Biochemical and functional analysis of Trp mutants of mMCP-7.** *A*, SDS-PAGE/immunoblot analysis of the W14L (*lanes 1* and *2*) and W206L (*lanes 3* and *4*) mutants of mMCP-7 before (*lanes 1* and *3*) and after (*lanes 2* and *4*) an overnight incubation at room temperature with EK. Molecular size standards are shown on the *left*. *B*, the enzymatic activities of wild-type mMCP-7 and its W14L and W206L mutants activated in the presence (■) or absence (▨) of heparin were determined as described above.

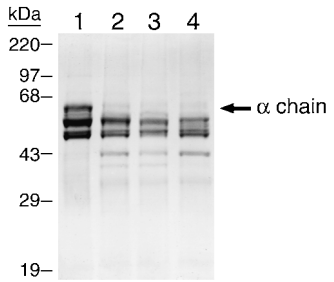

FIG. 6. **Digestion of fibrinogen by recombinant mMCP-7, its carbohydrate mutant, and its W206L mutant.** Purified mouse fibrinogen was incubated in buffer alone (*lane 1*) or in buffer containing wild-type mMCP-7 (*lane 4*), its carbohydrate mutant (*lane 3*), or its W206L mutant (*lane 2*). Molecular size standards are shown on the *left*. The *arrow* on the *right* refers to the α chain of fibrinogen that is selectively cleaved by wild-type mMCP-7, its carbohydrate mutant, and its W206L mutant.

the carbohydrate mutant of mMCP-7 was secreted into the conditioned media, in many instances it was difficult to proteolytically remove its bioengineered propeptide (data not shown). Nevertheless, FPLC analysis indicated that the portion of the expressed pseudozymogen whose propeptide could be proteolytically removed spontaneously assumed the tetramer structure (data not shown). Moreover, the carbohydrate mutant was enzymatically active. Not only could it cleave tosyl-Gly-Pro-Lys-pNA (Fig. 7*A*), it also was able to selectively cleave the α chain of fibrinogen (Fig. 6). The most notable feature of the carbohydrate mutant was its instability at 37 °C (Fig. 7*A*). The carbohydrate mutant was less stable at 37 °C than the W206L

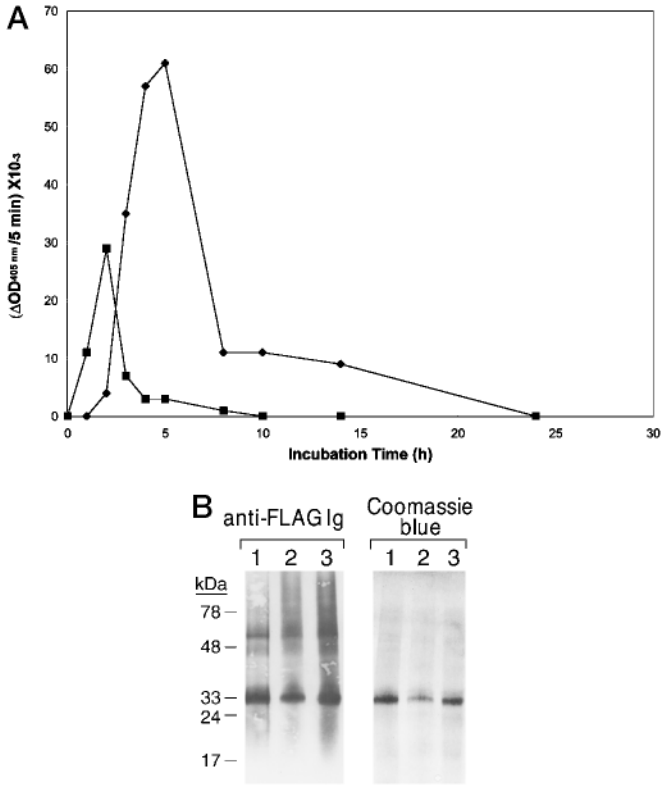

FIG. 7. **Activation and denaturation of mMCP-7 mutants.** *A*, the W206L (♦) and carbohydrate (■) mutants of pro-EK-mMCP-7 were suspended in pH 5.2 buffer containing EK and heparin, and the resulting solutions were incubated at 37 °C for 1–24 h as described above for wild-type pro-EK-mMCP-7. At the indicated times, samples were removed and assayed for their ability to cleave tosyl-Gly-Pro-Lys-pNA. In the depicted experiment, the maximal activity of the carbohydrate mutant (■) was substantially less than the W206L mutant (♦) because it was not possible to remove the bioengineered propeptide in more than 50% of the former zymogen. *B*, SDS-PAGE/immunoblot analyses were carried out at the end of the 24-h incubation on the W206L mutant incubated in the absence of glycerol or heparin (*lane 3*), in the presence of glycerol (*lane 2*), or in the presence of heparin (*lane 1*). Protein samples were exposed to β-mercaptoethanol before electrophoresis to disrupt disulfide bonds. The gel on the *right* was stained with Coomassie Blue. The protein blot on the *left* from the replicate gel was stained with anti-FLAG Ig.

mutant.

Formation of Enzymatically Active, Heterotypic Complexes of mMCP-6 and mMCP-7—Recombinant mMCP-6 and mMCP-7 homotypic tetramers could be separated from one another by heparin-Sepharose chromatography (Fig. 8, *A* and *C*). If recombinant pro-EK-mMCP-6 and pro-EK-mMCP-7 were incubated in varied molar ratios before EK activation and the resulting mature tryptase products were subjected to heparin-Sepharose chromatography, a separate peak was obtained that eluted from the column at an ionic strength intermediate of that of mMCP-6 and mMCP-7 (Fig. 8*B*).

DISCUSSION

We show in this study that recombinant mMCP-6 and mMCP-7 spontaneously assume tetramer structures when they are converted from inactive zymogen monomers to active enzymes and that a conserved Trp domain on the surface is required for this structural change. Although mMCP-6 and mMCP-7 prefer to exist as homotypic tetramers, we also show that the two tryptases can form heterotypic complexes. This latter discovery has important functional implications considering that the tissue retention and substrate specificities of the two mouse tryptases are different when they are in their homotypic forms.

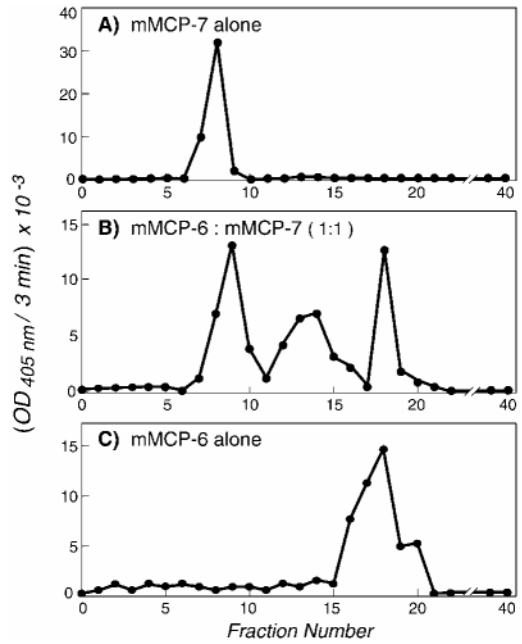

FIG. 8. **Heparin-Sepharose chromatography of homotypic and heterotypic tetramer complexes of recombinant mMCP-6 and mMCP-7.** Pro-EK-mMCP-6 and pro-EK-mMCP-7 were incubated alone (*A* and *C*) or in an ~1:1 molar ratio (*B*) and were activated with EK. The resulting tetramers were subjected to heparin-Sepharose chromatography. A new column was used in each analysis. The resulting eluate fractions were assessed for their ability to cleave tosyl-Gly-Pro-Lys-pNA. The intermediate peak in *B*, corresponding to heterotypic tetramers, was obtained whether pro-EK-mMCP-6 and pro-EK-mMCP-7 were premixed in 3:1, 2:1, 1:2, or 1:3 molar ratios before exposure to EK (data not shown). Although we suspect that it is possible to obtain every possible combination of mMCP-6 and mMCP-7 monomers in a heterotypic tetramer, this remains to be determined. Peaks corresponding to homotypic mMCP-7 and homotypic mMCP-6 tetramers were detected in all experiments.

Recombinant pro-mMCP-7, pro-EK-mMCP-6, and pro-EK-mMCP-7 (Fig. 1) were all secreted as inactive monomers. Recombinant pro-mMCP-7 does not have the EK-susceptibility site in its propeptide, nor does it have the FLAG peptide at its C terminus (6). That the natural tryptase zymogen is secreted as a monomer rather than a tetramer indicates that the inability of the inactive pseudozymogens to form tetramers is not a technical artifact due to their modified propeptides and C termini. When their propeptides were removed, mature mMCP-6 and mMCP-7 spontaneously formed tetramers (Fig. 1) that were enzymatically active (Fig. 3). The MC tryptases that have been purified from human lung exist as heparin-stabilized tetramers (10). Because certain human lung tryptases lose nearly all of their enzymatic activities *in vitro* within minutes after heparin is removed (16), it has been proposed that heparin is needed for the formation and stabilization of active human tryptase tetramers. Heparin-containing serglycin proteoglycans appear to play important roles in the packaging of the proteases in the MC secretory granules and physically retaining exocytosed mMCP-6 in extracellular matrices around activated MCs (5). It is possible that some tryptase tetramers are stabilized by serglycin proteoglycans in a species-dependent manner. However, in the V3 mastocytosis mouse (30) induced to undergo systemic anaphylaxis (5), mMCP-7 circulates in the blood free of glycosaminoglycans or proteoglycans. Thus, heparin is not needed for stabilization of this particular mouse tryptase *in vivo*. The observation that recombinant mMCP-6 is enzymatically active for hours in the absence of heparin (8) also indicates that serglycin proteoglycans are not needed for mMCP-6 to spontaneously form the enzymatically active tetramer. These findings are consistent with recent data obtained from a transgenic mouse, which is unable to express fully sulfated heparin due to targeted disruption of the *N*-deacetylase/*N*-sulfotransferase 2 gene (31). Although heparin does not influence the overall enzymatic activity of mMCP-6 for low molecular weight substrates, screening of a phage-display peptide library with recombinant mMCP-6 revealed that heparin can restrict the substrate specificity of this particular tryptase (8).

In many instances, the C terminus is critical for the proper folding of the initially translated protein and for its subsequent post-translational modification (32). Both recombinant tryptases have 8-residue FLAG peptides attached to their C termini. The observation that recombinant mMCP-6 and mMCP-7 are enzymatically active and spontaneously form tetramers after their propeptides are removed, coupled with the observation that the C termini of these and other MC tryptases are not conserved, suggests that the C terminus does not play a significant role in the activation process of either mouse tryptase, including their ability to form tetramers.

The activation process of mMCP-6 and mMCP-7 is exquisitely pH-dependent and recombinant mMCP-7 exhibited optimal enzymatic activity when activated at pH 5.5 (Fig. 3), the pH of the secretory granule (33). Thus, the mouse tryptases probably undergo their critical structural changes only after their exit from the *trans* region of the Golgi. The importance of low pH remains to be determined, but the conversion of inactive pepsinogen to active pepsin is also pH-dependent (34). During the activation process of this aspartic protease, a low pH environment is needed to disrupt critical salt bridges that hold its propeptide in place.

The enzymatic activity of a purified human lung tryptase is dramatically influenced *in vitro* by heparin and glycerol (16). Based on these observations, it has been assumed that glycerol and heparin help human tryptases maintain critical structural motifs that keep the monomers in their active state. Natural mMCP-7 circulates in the blood in the absence of heparin and glycerol. Nevertheless, we examined the role of these two agents in the *in vitro* generation and stability of mMCP-7. Because recombinant mMCP-7 was able to form an enzymatically active tetramer in the absence of either agent (Fig. 4), recombinant mMCP-7 appears to be more stable *in vitro* than those purified human lung tryptases that have been analyzed. Human MCs express at least five distinct tryptases (17–19, 35, 36). Thus, it remains to be determined whether or not the individual human tryptases differ in their heparin requirement to remain enzymatically active.

mMCP-7 is the most heavily glycosylated of those examined mouse MC granule proteases (28), and it possesses two *N*-linked glycan sites (3). The observation that all human MC tryptases that form tetramers have an *N*-linked glycosylation site which resides near the conserved Trp-rich domain (17–19) raised the possibility that high and/or complex mannose-type oligosaccharides might be needed for formation of this structural feature. Thus, both Asn^{21} and Asn^{102} were mutated to Gln to obtain a carbohydrate-deficient mutant of mMCP-7. The resulting data indicate that the *N*-linked glycans are not essential for enzymatic activity or tetramer formation, although they contribute substantially to the overall thermal stability of the tryptase.

Because MCs contain very low amounts of tryptase zymogens in their granules, it previously was not possible for investigators to determine whether or not a tryptase undergoes a structural change when its propeptide is removed. Nevertheless, a number of *in vitro* studies have been carried out in an attempt to understand the mechanism by which tryptase tet-

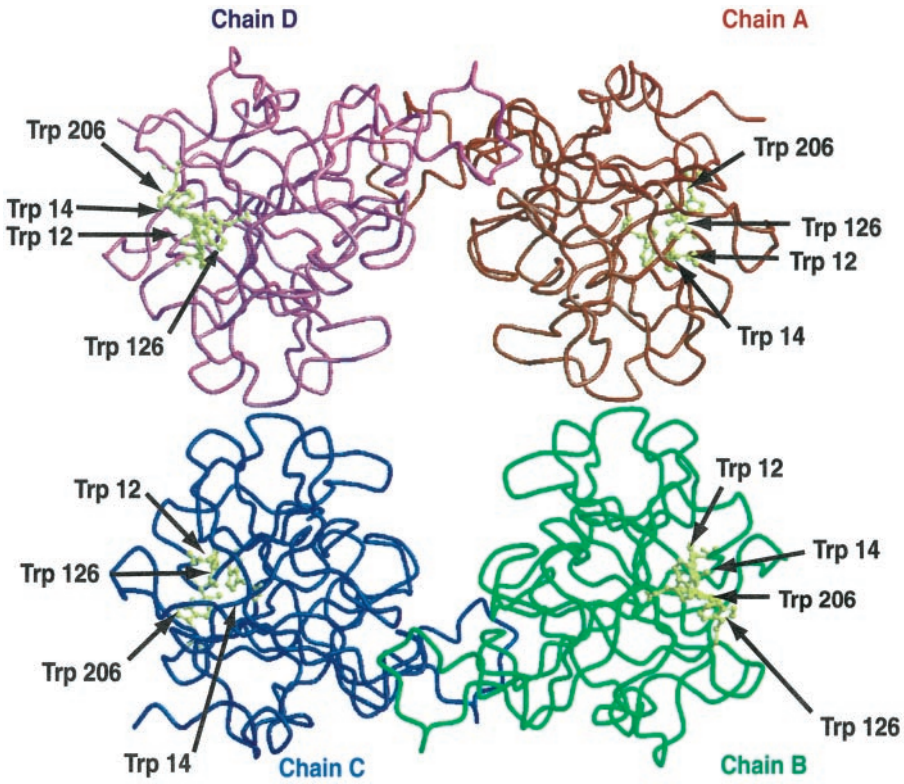

FIG. 9. **Three-dimensional model of mMCP-7 in its homotypic tetramer form.** The depicted three-dimensional model of the mMCP-7 homotypic tetramer was calculated with the program MODELLER (46, 49) based on the crystallographic structure of human tryptase II/β (21). Individual mMCP-7 monomers (chains A–D) in the tetramer are colored in *red*, *green*, *blue*, and *purple*. The Trp-rich domain on the surface of each monomer is indicated in *yellow*. Based on Pereira and co-workers' interpretation of their human tryptase II/β structure (21), the substrate-binding clefts of the mMCP-7 tetramer face the hole formed by the four monomers.

ramers slowly inactivate. The change in the CD spectra that occurred when pro-mMCP-7 was converted into mature mMCP-7 (Fig. 2) is consistent with the CD spectra obtained by others for a purified human tryptase, which rapidly lost its enzymatic activity when incubated in the absence of dextran sulfate (15, 37) and for a purified bovine tryptase induced to undergo thermal-dependent inactivation (38). Using a conformation-dependent monoclonal antibody, Schwartz and coworkers (37) also concluded that a human lung tryptase undergoes a change in its three-dimensional structure when it inactivates *ex vivo*. In neither of these instances did it appear that inactivation was a result of proteolysis. Although the amount of enzymatically active mMCP-7 in the sample decreased nearly 75% after a prolonged incubation at 37 °C (Fig. 4*B*), mMCP-7 also did not undergo autolysis (Fig. 4*C*).

The change in CD spectra at 230 nm has been attributed to structural alterations in one or more Trp residues. Nine Trp residues are conserved in tryptases isolated from different species. Mouse MC chymases (39–45) are expressed as monomers and all have at least three Trp residues, two of which are conserved and correspond to Trp^{130} and Trp^{236} in mMCP-6 and mMCP-7. Trp^{126} is present in the chymases mMCP-1 (39, 43) and mMCP-2 (40), and Trp^{214} is also present in pancreatic trypsin. Thus, only 5 of the conserved Trp resides are uniquely found in mMCP-6 and mMCP-7. Because comparative protein structure modeling (Fig. 9) suggested that Trp^{12}, Trp^{14}, Trp^{126}, and Trp^{206} are spatially close on the surface of the folded tryptase, an expression/site-directed mutagenesis approach was used to create the W14L and W206L mutants of mMCP-7 (Fig. 5) to evaluate the importance of the Trp-rich domain in tetramer formation. Using a method that has been previously used to calculate surface areas of specific residues in other proteins (46), the relative solvent accessibilities of Trp^{12}, Trp^{14}, Trp^{126}, and Trp^{206} in the comparative model of mMCP-7 (Fig. 9) are 3, 4, 20, and 27%, respectively. These four Trp residues interact with each other and other residues in the same subunit but are unable to contact residues in adjacent subunits. It is conceivable that the mutation of the buried Trp^{14} into a smaller Leu residue triggers a small structural rearrangement that is transmitted across 15 Å to the nascent subunit interface, thereby destroying the precise shape complementarity needed for tetramer formation. This hypothesis is consistent with the fact that the W206L mutation does not impact the ability of mMCP-7 to form tetramers as much as the W14L mutation. Because Trp^{206} is significantly more exposed than Trp^{14}, mutation of residue 206 is less likely to disrupt the structure of the interface than mutation of residue 14. The hypothesis about the impact of the Trp^{14} mutation on subunit interactions is also consistent with the fact that native and mutant monomers and tetramers still bind to heparin-Sepharose even though the glycosaminoglycan binding site of each monomer is not further away from the site of mutation than the subunit interface. The reason is that the electrostatic interactions are generally more robust with respect to structural changes than are interactions based on shape complementarity, such as those involved in the tetramer formation.

Although the amino acid sequences of mMCP-6 and mMCP-7 are 71% identical, mMCP-6 has an overall positive charge at pH 5.0–6.0 that is greater than that of mMCP-7 (6). Thus, it was anticipated that recombinant mMCP-6 and mMCP-7 tetramers could be separated from one another by ion-exchange chromatography. As expected, a higher concentration of NaCl was needed to dissociate recombinant mMCP-6 tetramers from a heparin-Sepharose column than recombinant mMCP-7 tetramers (Fig. 8). If recombinant pro-EK-mMCP-6 and pro-EK-mMCP-7 were incubated in varied molar ratios before EK activation and the resulting mature tryptase products were subjected to heparin-Sepharose chromatography, a separate peak was obtained that eluted from the column at an ionic strength between that of mMCP-6 and mMCP-7. Although these and other studies document that recombinant mMCP-6 and mMCP-7 prefer to form homotypic complexes, the two tryptases can form enzymatically active heterotypic complexes *in vitro*.

In most experiments carried out in the V3 mastocytosis mouse, only immunoreactive mMCP-7 was detected in the blood 20 min after the animal was induced to undergo systemic anaphylaxis (5). Although this observation suggested that mMCP-6 and mMCP-7 are packaged in the secretory granules of MCs solely as homotypic tetramers, the possibility could not be ruled out that heterotypic tryptase complexes exist and are selectively retained in tissues. mMCP-6 and mMCP-7 are developmentally expressed at the same time when bone marrow-derived, MC-committed progenitors are cultured in the presence of interleukin 3 (3). Moreover, immunohistochemical studies carried out on serial sectioned tissue have revealed that heart, tongue, and jejunal submucosa MCs in the adult BALB/c mouse and MCs in the liver and spleen of the V3 mastocytosis mouse coexpress the two tryptases (5, 47). The ability of recombinant mMCP-6 and mMCP-7 to form heterotypic complexes *in vitro* now raises the possibility that the two tryptases form similar heterotypic complexes *in vivo*. If so, this would explain why a portion of exocytosed mMCP-7 is retained in the extracellular matrix for hours (5). Presumably any exocytosed mMCP-6/mMCP-7 heterotypic tetramers will be physically retained in a tissue if the serglycin proteoglycans in the macromolecular complexes are preferentially interacting with the mMCP-6 monomers.

Although recombinant mMCP-6 and mMCP-7 have restricted substrate specificities, they cleave different peptide sequences due to the substantial variation in the number of negatively charged amino acids in their substrate-binding pockets (4–8). Thus, the ability to form tetramers containing different combinations of mMCP-6 and mMCP-7 may be a novel mechanism the MC uses to control how extensively certain Lys/Arg-rich substrates are degraded. It also might be a mechanism for retaining a small amount of mMCP-7 in the inflammatory site so that it can inactivate any fibrinogen that diffuses into the site 1 h or more after the MC has degranulated.

Four cDNAs and their genes have been isolated (17–19, 35) that encode the highly homologous human MC tryptases I, II/β, III, and α. In addition, a fifth human tryptase (designated transmembrane tryptase) has been identified that is ~50% identical to the other members of its family (36). Preparations of tryptases purified from different tissues and different humans have been reported to degrade at least 17 substrates *in vitro*. Although the amino acid sequences of human tryptases α and II/β are 93% identical, the substrate specificities of these two tryptases are very different (48). If the varied human tryptases can form heterotypic complexes analogous to those formed by mMCP-6 and mMCP-7, some of the controversy as what are the physiologic substrates of the varied human tryptases could be resolved by identifying which tryptases are present in an analyzed preparation and how their combinations are structurally arranged in the enzymatically active tetramer.

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