Mechanism for Activation of Mouse Mast Cell Tryptase: Dependence on Heparin and Acidic pH for Formation of Active Tetramers of Mouse Mast Cell Protease 6[†]

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ABSTRACT: Tryptase, a serine protease with trypsin-like substrate cleavage properties, is one of the key effector molecules during allergic inflammation. It is stored in large quantities in the mast cell secretory granules in complex with heparin proteoglycan, and these complexes are released during mast cell degranulation. In the present paper, we have studied the mechanism for tryptase activation. Recombinant mouse tryptase, mouse mast cell protease 6 (mMCP-6), was produced in a mammalian expression system. The mMCP-6 fusion protein contained an N-terminal 6 × His tag followed by an enterokinase (EK) site replacing the native activation peptide (6×His−EK−mMCP-6). In the absence of heparin, barely detectable enzyme activity was obtained after enterokinase cleavage of 6×His−EK−mMCP-6 over a pH range of 5.5−7.5. However, when heparin was present, 6×His−EK−mMCP-6 yielded active enzyme when enterokinase cleavage was performed at pH 5.5−6.0 but not at neutral pH. Affinity chromatography analysis showed that mMCP-6 bound strongly to heparin-Sepharose at pH 6.0 but not at neutral pH. After enterokinase cleavage of the sample at pH 6.0, mMCP-6 occurred in inactive monomeric form as shown by FPLC analysis on a Superdex 200 column. When heparin was added at pH 6.0, enzymatically active higher molecular weight complexes were formed, e.g., a dominant ∼200 kDa complex that may correspond to tryptase tetramers. No formation of active tetramers was observed at neutral pH. When injected intraperitoneally, mMCP-6 together with heparin caused neutrophil influx, but no signs of inflammation were seen in the absence of heparin. The present paper thus indicates a crucial role for heparin in the formation of active mast cell tryptase.

Mast cells are of critical importance in various inflammatory conditions, in particular, allergic inflammation (*1*, *2*). When they are activated by various stimuli they respond by releasing the contents of their secretory granules to the extracellular space. The mast cell granules contain a wide spectrum of inflammatory mediators, e.g., histamine, cytokines, heparin proteoglycan and various proteolytic enzymes. The proteolytic enzymes include tryptases and serine proteases with trypsin-like substrate specificities, as well as chymases (with chymotrypsin-like properties) and carboxypeptidase A (*3*). Interestingly, mast cell proteases in which the same enzyme displays both trypsin- and chymotrypsin-like substrate cleavage properties have also been identified (*4*). The specific expression patterns for the mast cell proteases vary between different animal species and show, in addition, variability between different mast cell populations within the same animal. For example, mouse mast cells of the connective tissue subtype express two homologous tryptases, mouse mast cell protease 6 (mMCP-6)[1] and mMCP-7, the chymases mMCP-4 and -5, and carboxypeptidase A (*5*−*8*). In contrast, mast cells of the mucosal subtype show preferential expression of the chymases mMCP-1 and -2 (*9*, *10*). Of the two mouse tryptases, mMCP-7 appears to be expressed preferentially in ear tissue (*11*), whereas mMCP-6 is expressed at high levels both in ear tissue and in peritoneal mast cells (*12*−*14*). Previous studies have shown that mast cell tryptase has a unique structure, in which the active enzyme is present as a tetramer (*15*). The crystal structure of human β-tryptase has revealed that in these tetramers, the active sites are faced toward a central pore (*16*).

Tryptases are stored in their active forms, i.e., with their original activation peptides removed, as tight complexes with heparin proteoglycan and are released as such macromolecular complexes during mast cell degranulation. Heparin proteoglycan consists of a protein core to which negatively charged, highly sulfated heparin glycosaminoglycan chains

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[1] Abbreviations: mouse mast cell protease, mMCP; PBS, phosphate buffered saline; EK, enterokinase site; mMCP, mouse mast cell protease; DMEM, Dulbecco's modification of Eagle's medium; TBS, Tris-buffered saline; EBNA-1, Epstein Barr nuclear antigen-1; FCS, fetal calf serum.

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are attached (*17*, *18*). Heparin proteoglycan has recently been shown to be essential for proper storage of tryptase and other mast cell granule components (*14*, *19*). Further, the interaction of heparin proteoglycan with human tryptase has previously been shown to be essential for preserving enzyme activity. In the absence of heparin, tryptase quickly dissociates into monomers and thereby loses activity (*20*). The latter process has been shown to be accompanied by conformational changes in the tryptase that may result from the dissociation from heparin (*21*−*23*).

Although the role of heparin in the maintenance of tryptase activity has been extensively investigated previously, the possible role of heparin during tryptase activation has not been studied in detail. The first step in the tryptase activation process is the release of the activation peptide. Previous investigations have shown that autocatalytic processing of the activation peptide in human β-tryptase only occurred in the presence of added heparin, indicating a role for heparin in this step (*24*). The next step involves assembly of the inactive monomers into the active tetrameric form of the enzyme. This crucial step is poorly characterized, and the possible role of heparin is not fully understood. In this paper, we have addressed this issue. Mouse tryptase mMCP-6, the main tryptase expressed by mouse mast cells (*14*), was expressed with an N-terminal 6 × His tag followed by an enterokinase site, substituting for the native activation peptide. Enterokinase cleavage of this fusion protein thus yields the native monomeric form of mMCP-6. Here we show that heparin is of crucial importance for the assembly of active tryptase tetramers and that the heparin-dependent formation of enzymatically active tryptase only occurs at acidic pH.

MATERIALS AND METHODS

pCEP-Pu2 Vector. The pCEP-Pu2 vector originates from the pCEP4 vector (Invitrogen). The pCEP4 vector was used to produce a modified vector, the pCEP-Sh (*25*). The phleomycin (*Shble*) resistance gene in pCEP-Sh was then replaced with the puromycin (*pac*) resistance gene obtained from the pSV_2pac vector (*26*). In addition, a 105-bp region consisting of some of the 5′-UTR and the signal peptide sequence region of human BM40 [obtained from pRc/Ac (*27*)] has been inserted into the *Hin*dIII/*Nhe* I restriction sites downstream of the CMV promotor. The obtained vector was named pCEP-Pu/BM40s (*28*). We have made some additional modifications to this vector. An *Eco*RI site located upstream of the ampicillin resistance gene has been removed by cleavage with *Eco*RI followed by fill-in treatment and religation. We have also modified the multiple cloning site by inserting an *Eco*RI and a *Not*I site between the *Nhe*I and *Xho*I sites.

cDNA Subcloning. The cDNA encoding mMCP-6 was cloned as previously described (*29*). A PCR approach was used to produce a construct for expression of recombinant mMCP-6 in a mammalian system. The 5′ primer used in the PCR contained a 6 × histidine tag, which was added to simplify purification. In addition, an enterokinase susceptible peptide (Asp−Asp−Asp−Asp−Lys; EK), replacing the natural activation peptide (*13*), was inserted between the histidine tag and the mature mMCP-6 tryptase. The PCR product was ligated into the pCEP-Pu2 vector using the *Nhe*I and *Eco*RI sites in frame with the BM40 signal sequence. Competent *E. coli* (DK-1) were transformed with the ligated vector, plated on ampicillin plates, and grown overnight. Single colonies were picked and transferred to a nitrocellulose filter and grown overnight. Positive clones were detected with a probe obtained after random priming labeling (α-^{32}P-dCTP) of the PCR fragment. Two positive colonies were sequenced using Thermo Sequenace radiolabeled terminator cycle sequencing kit (Amersham Int., Amersham, England), with the use of vector specific primers.

Transfection of 293-EBNA Cells. The human kidney cell line, 293-EBNA, expresses the gene for the Epstein Barr nuclear antigen-1 (EBNA-1) protein. This protein is a DNA binding protein that interacts with the OriP, which is the Epstein Barr Virus (EBV) origin of replication. The OriP is located in the pCEP-Pu2 vector and is required for a stable episomal maintenance of the vector in the 293-EBNA cell line. The pCEP-Pu2 vector (13 μg) containing mMCP-6 cDNA insert was dissolved in sterile water and mixed with 1.6 mL of serum-free DMEM (Dulbecco's modified Eagle's medium, Gibco, Life Technologies). The DMEM medium was supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin (both from Gibco, Life Technologies), and 0.15 μg/mL lipofectamine (Gibco, Life Technologies). The mixture was vortexed and incubated at room temperature for 45 min. The DNA/DMEM/lipofectamine mixture and a control without DNA was carefully added to two 9-cm^2 wells (in a 6-well culture dish) containing approximately 100% confluent 293-EBNA cells ($\sim 5 \times 10^6$ cells/well). The wells had been prewashed with 8 mL of PBS each and contained 4.4 mL of serum-free DMEM (total volume 6 mL/well). The plates were incubated for 16 h at 37 °C (5% CO_2 at a relative humidity of 80%). After 16 h, 6 mL of DMEM supplied with 20% FCS (fetal calf serum, Gibco, Life Technologies) was added to each well, and the cells were left to grow overnight. Twenty-four hours later, the cells were split 1:4 and left to recover overnight. Selection of transfectants was performed in DMEM supplied with 10% FCS, 2 mM L-glutamine, 50 μg/mL gentamicin, and 0.5 mg/mL puromycin dihydrochloride (Sigma-Aldrich). The selection pressure was increased to a maximum level of 5 mg/mL puromycin, to favor cells with high copy numbers of the vector.

Purification of Recombinant Tryptase. Recombinant 6×His−EK−mMCP-6 was purified from conditioned media collected from 293 EBNA cells transfected with the mMCP-6-containing pCEP-Pu2 vector. Conditioned medium was centrifuged (418*g*, 10 min), and the supernatant was stored at −20 °C. One milliliter of Ni-NTA agarose (QIAGEN GmbH, Hilden, Germany) was added to 1000 mL of thawed conditioned medium. The mixture was rotated at +6 °C for 6 h and centrifuged (290*g*, 5 min). The Ni-NTA-agarose beads were loaded on a 10-mL Poly-Prep Chromatography column (Bio-Rad, Hercules, USA) and washed with PBS buffer (pH 7.0, 1 M NaCl, and 0.1% Tween 20) until no impurities were detected in the washing fractions as analyzed by SDS−PAGE analysis in a Laemmli system. Imidazole (100 mM in PBS, pH 7.0) was used to elute the bound tryptase. Fractions containing mMCP-6 fusion protein were pooled and stored at 4 °C. The concentration of mMCP-6 was determined by measuring A_{280}, using a calculated molar extinction coefficient of 68 810 M^{-1} cm^{-1}.

Enterokinase Cleavage. To remove the 6×His−EK peptide, 6×His−EK−mMCP-6 (2−15 μg) was incubated with enterokinase (Boehringer Mannheim GmbH, Mannheim, Germany), generally at a 6×His−EK−mMCP-6/enterokinase ratio (w/w) of 200−700:1 in PBS (pH 6.0) at 37 °C overnight. In some experiments, enterokinase cleavage was performed in PBS buffers with various pH ranging from 5.5 to 7.5.

Enzymatic Assays. Tryptase activity was measured in 96-well microtiter plates. In standard conditions, enterokinase-cleaved mMCP-6 (100−500 ng) was diluted with PBS (10 mM phosphate, 0.14 M NaCl, and 2.7 mM KCl, pH 6.0) to a final volume of between 130 and 250 μL. In some experiments, the enterokinase-treated 6×His−EK−mMCP-6 was diluted with PBS buffers with varying pH (5.5−7.5). The activity of mMCP-6 was measured either in the absence of heparin or after preincubation (30−60 min at room temperature) with pig mucosal heparin ("commercial heparin"; gift from Ulf Lindahl, Department of Medical Biochemistry and Microbiology, Uppsala University) at final concentrations between 47 ng/mL and 465 μg/mL. mMCP-6 activity was recorded after addition of 20 μL (2−4 mM) of the chromogenic peptide substrate S-2288 (H-D-Ile-Pro-Arg-pNA) (Chromogenix, Mölndal, Sweden). The absorbance at 405 nm was monitored with a Titertek Multiscan spectrophotometer (Flow Laboratories). Activities were determined as the average from three measurements, and the results are given ± standard deviation.

Heparin-Sepharose Chromatography. Affinity chromatography was performed on a 10-mL Poly-Prep Chromatography column containing ∼1 mL heparin-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with binding buffer (PBS, pH 6.0 or pH 7.4). A total of 10 μg of mMCP-6 in 100 μL was loaded on the column followed by washing of the column with binding buffer (4 × 500 μL). The column was eluted stepwise (4 × 500 μL fractions) with 10 mM sodium phosphate buffers (pH 6.0 or 7.4) containing 0.5−4.0 M NaCl. A total of 10 μL from each fraction was mixed with 260 μL of PBS (pH 6.0) and 10 μg of heparin (in 10 μL). After 30 min at room-temperature, mMCP-6 activity was determined after addition of S-2288 as above.

Size-Exclusion Gel Chromatography. Size exclusion gel chromatography was performed in an FPLC system (Amersham Pharmacia Biotech) using a Superdex 200 column (10 × 300 mm). The column was equilibrated with PBS (pH 6.0 or pH 7.4) and was run at a flow rate of 0.5 mL/min. The column was calibrated with the following gel filtration standards (Sigma): carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), α-amylase (200 kDa), and blue dextran (2000 kDa). Recombinant mMCP-6 (10 μg) was analyzed either alone or after preincubation (30 min at room temperature) with 5−150 μg of heparin. A sample volume of 200 μL was injected. Fractions (0.5 mL) were collected and were analyzed for tryptase activity as described above.

Benzamidine-Sepharose Chromatography. Samples (5 μg in 65 μL PBS, pH 6.0) of enterokinase-digested 6×His−EK−mMCP-6 were loaded on a ∼0.2 mL column of benzamidine-Sepharose (Amersham Pharmacia Biotech) that was equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl. Samples of mMCP-6 were loaded either in the absence of heparin or after preincubation (30 min at room temperature) with 75 μg of heparin (in 7.5 μL of H_2O). The column was washed with 1 mL of equilibration buffer. Fractions (0.2 mL) were collected and analyzed for protein content using Quantigold (Diversified Biotech, Newton Centre, MA) according to the procedure described by the manufacturer. The Quantigold method is sensitive to ∼10 ng of protein/sample.

mMCP-6/Heparin-Induced Inflammation. Samples (10 μg in 100 μL PBS) of either enterokinase-digested 6×His−EK−mMCP-6 or a combination of enterokinase-digested 6×His−EK−mMCP-6 and 50 μg of heparin were injected into the peritoneal cavity of BALB/c mice (females; 6−10 weeks). As controls, PBS alone or 50 μg of heparin in PBS containing enterokinase (same amount as used for 6×His−EK−mMCP-6 digestion) were injected. After 24 h, the mice were killed followed by peritoneal washing with 10 mL of PBS (4 °C) containing 5 mM EDTA. Cells were pelleted by centrifugation (300*g*, 10 min), washed once with PBS/5 mM EDTA, counted, and used for preparation of cytospin slides. The cytospin slides were subsequently either stained with May-Grünwald/Giemsa or stained immunohistochemically for mast cell carboxypeptidase A, a mast cell granule marker (see below).

Immunohistochemical Staining. The cells were fixated in 1% glutaraldehyde in TBS (50 mM Tris, pH 7.4, and 150 mM NaCl) for 1 h. The sections were permeabilized by incubation in methanol for 2 min, followed by quenching (incubation in TBS/0.1% $NaBH_4$, pH 8, for 15 min). To avoid unspecific antibody binding, the cell sections were blocked by preincubation with rabbit serum. Next, the sections were incubated with a polyclonal rat anti-mouse carboxypeptidase A antiserum [diluted 1:500 in TBS (*14*)]. After slides were washed three times with TBS, they were incubated with a rabbit anti-rat Ig antiserum (Dako Z455; diluted 1:100 in TBS). Slides were washed three times with TBS followed by addition of alkaline phosphatase/anti-rabbit Ig complex (Dako D488L; diluted 1:50 in TBS). The Dako Fast Red substrate system (Dako K595) was used for development.

RESULTS

Expression of mMCP-6. Recombinant mMCP-6 was produced in a mammalian expression system, the human 293 cells. The fusion protein contained an N-terminal 6 × His region followed by an enterokinase site (Asp−Asp−Asp−Asp−Lys) substituting for the natural activation peptide. Native mMCP-6 monomer is thus formed after enterokinase cleavage of the fusion protein. The 293 cells produced high levels (∼2 mg/L of culture medium) of fusion protein after transfection with the vector containing the mMCP-6 construct. After purification on Ni−NTA-agarose, a pure ∼35 kDa band was observed (Figure 1). The somewhat fuzzy appearance of the band indicates that the protein is glycosylated, which is expected considering that mMCP-6 has one potential glycosylation site (*13*). Cleavage of the fusion protein with enterokinase gave a reduction of the molecular mass to approximately ∼32 kDa, closely resembling the known molecular weight of mMCP-6 (Figure 1). The present paper thus describes, to our knowledge for the first time, the expression of mast cell tryptase at high levels in a mammalian system. Previous studies have mainly utilized

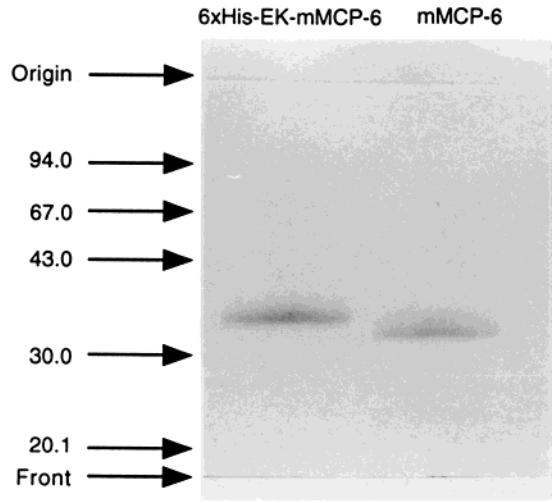

FIGURE 1: Purity of recombinant mMCP-6. The left lane shows SDS−PAGE analysis at reducing conditions of purified 6×His−EK−mMCP-6 fusion protein. The right lane shows the same amount of fusion protein after cleavage with enterokinase.

insect cells or yeast for expression of various tryptases (*24*, *30*−*33*).

Formation of Active mMCP-6. The 6×His−EK−mMCP-6 fusion protein was cleaved with enterokinase at various pH values, either in the absence or presence of heparin (Figure 2). Enterokinase cleavage of the mMCP-6 fusion protein over a pH range from 5.5 to 7.5 resulted in only very low or undetectable amounts of active mMCP-6 in the absence of heparin. In contrast, when enterokinase cleavage was performed in the presence of heparin, enzymatic activity toward S-2288 (H-D-Ile-Pro-Arg-pNA) was obtained. Markedly higher activities were obtained when heparin was added after the enterokinase cleavage step. This indicates that the removal of the activation peptide and the activation of the tryptase may not necessarily be associated in time. Possibly, cleavage of the activation peptide may take place in an early compartment in the secretory pathway (e.g., the ER), whereas heparin binding and tryptase activation may occur later. However, active tryptase was only obtained when enterokinase digestion was performed at pH 5.5−6.5. The amount of active tryptase formed was reduced drastically at pH values of 6.5 and higher, and at neutral pH (pH 7.5) tryptase activity was undetectable (Figure 2). As a control, SDS−PAGE analysis revealed complete enterokinase cleavage over the entire pH range tested (Figure 2B).

Dose-response experiments showed that maximal tryptase activity was obtained, at pH 6.0, at ∼50 μg/mL of heparin (Figure 3). At this optimal heparin concentration, the specific activity of mMCP-6 is ∼0.02 pmol of S-2288 hydrolyzed s^{-1} (ng of mMCP-6)$^{-1}$. All subsequent experiments were performed at approximately this heparin concentration. At neutral pH, tryptase activity was undetectable at heparin concentrations up to 50 μg/mL. However, at higher heparin concentrations some S-2288-hydrolyzing activity was seen also at pH 7.4 (Figure 3).

Benzamidine is an active site-directed, general inhibitor of trypsin-like serine proteases. After enterokinase cleavage of 6×His−EK−mMCP-6 at pH 6.0 and subsequent addition of heparin, all of the obtained protein was bound to a column of benzamidine-Sepharose (not shown). This indicates that most, if not all, of the enterokinase-cleaved 6×His−EK−mMCP-6 molecules were correctly folded and contained intact active sites.

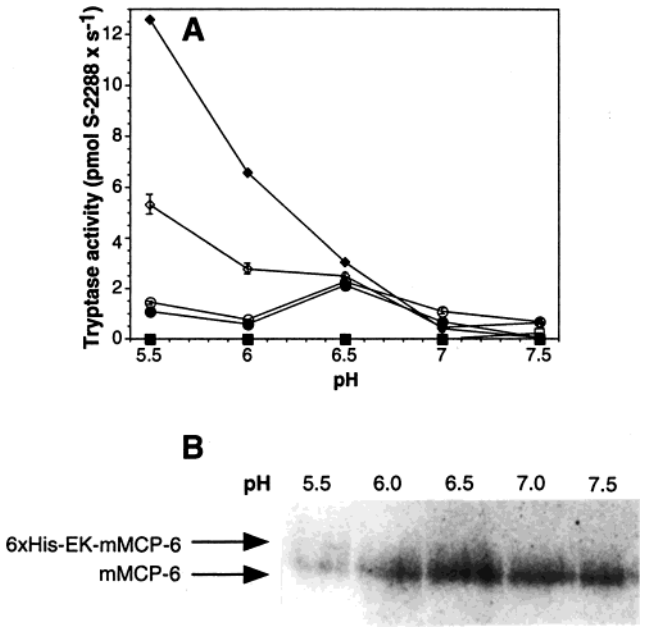

FIGURE 2: pH-dependence for activation of mMCP-6. (A) 6×His−EK−mMCP-6 (2.4 μg) was incubated overnight with enterokinase in PBS buffer (pH 5.5−7.5) either in the presence (○, ●; total volume 222 μL) or absence (◇, ◆, ■; total volume 210 μL) of 12 μg of heparin. After overnight incubation, heparin (12 μg in 12 μL) was added to samples that had been enterokinase-digested in the absence of heparin (◇, ◆). Subsequently, 10-μL samples from each incubation mixture were mixed with either 200 μL of PBS with the same pH as that used for enterokinase digestion (○, ◇) or PBS, pH 7.4 (●, ◆), followed by determination of enzymatic activity with S-2288. Samples that had been treated with enterokinase in the absence of heparin were also assayed without addition of heparin (■; no detectable activity when assayed at either at pH 7.4 or the same pH as that used during enterokinase cleavage). The results shown represent the mean of triplicate determinations ± standard deviation. Error bars frequently fall within the symbols. (B) As a control, SDS−PAGE analysis showed complete enterokinase digestion of 6×His−EK−mMCP-6 at each pH tested.

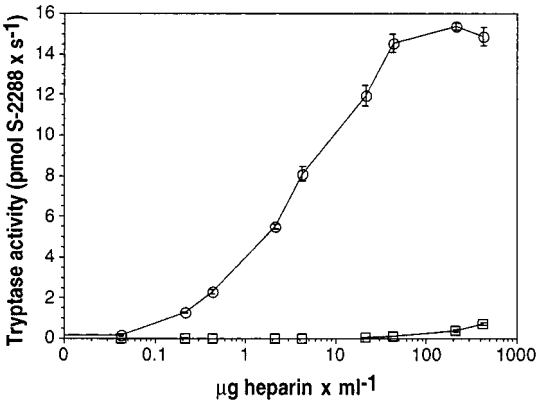

FIGURE 3: Dose dependence for heparin-induced activation of mMCP-6. 6×His−EK−mMCP-6 was digested with enterokinase at pH 6.0. A total of 0.55 μg of enterokinase-digested 6×His−EK−mMCP-6 (in 5 μL of PBS, pH 6.0) was diluted to 200 μL, either with PBS, pH 6.0 (○) or PBS, pH 7.4 (□). Heparin (10 ng−100 μg; in 10 μL H_2O) was added and after 45 min, tryptase activity was measured after addition of 20 μL (2 mM) of S-2288.

Time Course for mMCP-6 Activation. Activation of mMCP-6 after addition of heparin was rapid (Figure 4). Nearly maximal activity was obtained already 1 min after the addition of heparin. Interestingly, the highest activity was

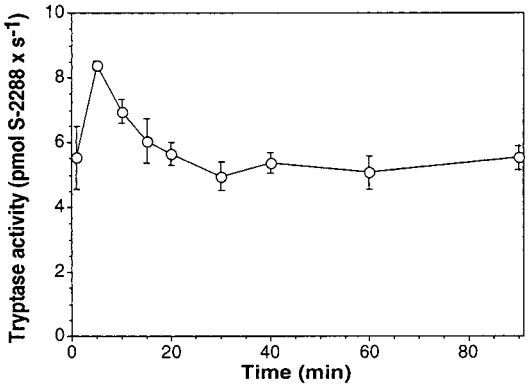

FIGURE 4: Time course for stimulation of mMCP-6 activity by heparin. 6×His−EK−mMCP-6 was digested with enterokinase in the absence of heparin. Heparin (2 μg) was added to enterokinase-digested 6×His−EK−mMCP-6 (0.4 μg) in 100 μL of PBS, pH 6.0. After various periods of time tryptase activity was measured after adding 20 μL of S-2288 (2 mM).

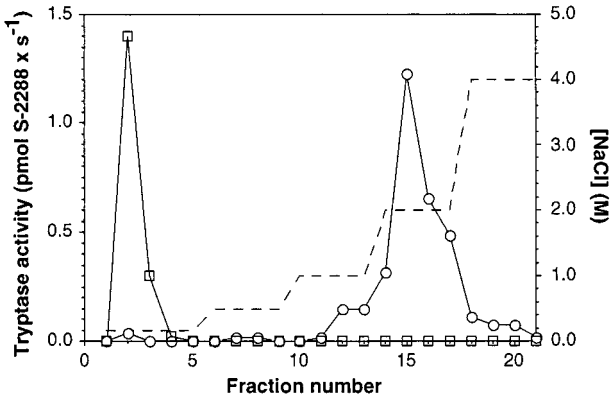

FIGURE 5: Affinity chromatography of mMCP-6 on heparin-Sepharose. A total of 10 μg of 6×His−EK−mMCP-6 (in 100 μL) was enterokinase-digested at pH 6.0 in the absence of heparin. The enterokinase-digested 6×His−mMCP-6 was applied to a column of heparin-Sepharose (0.3 mL) equilibrated with PBS, pH 6.0 (○) or PBS, pH 7.4 (□). The column was washed (4 × 500 μL) with equilibrating buffer followed by stepwise elution with PBS buffers of the same pH as the equilibrating buffer, containing 0.5, 1.0, 2.0, and 4.0 M NaCl (4 × 500 μL of each buffer). Samples (10 μL) from each fraction were mixed with 10 μg of heparin in 260 μL of PBS buffer (pH 6.0) and were assayed for tryptase activity after addition of 20 μL of S-2288 (2 mM). NaCl concentration (- - -). Note that samples obtained after affinity chromatography at pH 7.4 were brought to pH 6.0 before heparin addition and activity measurement. No detectable mMCP-6 activity was obtained when heparin was added and activity was measured at pH 7.4.

seen approximately 5 min after heparin addition, after which the activity decreased ~30% and leveled off.

Binding to Heparin. Affinity chromatography on heparin-Sepharose was used to study the heparin-binding properties of mMCP-6. After enterokinase cleavage of 6×His−EK−mMCP-6 at pH 6.0, all of the generated mMCP-6 bound to the heparin-Sepharose column in PBS (0.15 M NaCl), pH 6.0 (Figure 5). Stepwise elution of the column with increasing NaCl concentrations showed that mMCP-6 required NaCl concentrations above 1 M for elution, indicating high affinity for heparin. In contrast, when the heparin-Sepharose column was equilibrated with PBS, pH 7.4, no binding of mMCP-6 was detected (Figure 5).

Reversibility of pH Effects. Experiments were performed to determine whether neutral pH induced irreversible effects on mMCP-6 that would prevent possible subsequent activation. 6×His−EK−mMCP-6 was cleaved with enterokinase at either pH 6.0 or 7.4. A portion of the pH 6.0 incubation mixture was brought to neutral pH and, conversely, a portion of the pH 7.4 incubation mixture was brought to pH 6.0. Subsequently, heparin was added to all mixtures, and enzymatic activity was measured either at pH 6.0 or neutral pH. The results summarized in Table 1 show that the highest activity was obtained when mMCP-6 had been enterokinase-digested at pH 6.0 followed by heparin binding at pH 6.0 and activity assay at neutral pH. In contrast, when mMCP-6 monomer was brought to pH 7.4 before addition of heparin, tryptase activity was very low. Enterokinase cleavage at pH 7.4 did not prevent subsequent activation, if the pH was lowered to 6.0 before addition of heparin. However, the activity obtained under these conditions was lower than if the mMCP-6 monomer had been enterokinase-digested at pH 6.0. Moreover, mMCP-6 that had been first treated with pH 7.4 followed by lowering of the pH to 6.0 bound with similar affinity to heparin-Sepharose as the mMCP-6 monomer that had been incubated at pH 6.0 only (not shown). These results thus clearly indicate that the generation of active tryptase requires that heparin is bound to the mMCP-6 monomer at acidic pH. Further, it appears that exposure of the mMCP-6 monomer to neutral pH does not produce irreversible effects on the conformation of the protein that completely prevents subsequent activation if the pH is lowered before addition of heparin.

Table 1: Reversibility of pH Effects on Tryptase Activity[a]

	step 1 enterokinase cleavage	step 2 heparin binding	step 3 activity assay	activity (pmol of S-2288 s^{-1}; ± SD, n=3)
I	6.0	6.0	6.0	1.88 ± 0.40
II	6.0	7.3	7.5	0.088 ± 0.008
III	6.0	6.0	7.3	2.88 ± 0.31
IV	7.5	7.5	7.5	0.061 ± 0.008
V	7.5	6.0	7.5	1.08 ± 0.001
VI	7.5	7.5	6.0	0.067 ± 0.016

[a] Step 1: 6×His−EK−mMCP-6 (4.7 μg) was digested with enterokinase in PBS (86 μL), pH 6.0 (I−III), or pH 7.5 (IV−VI) in the absence of heparin. Step 2: 25-μL samples from the incubation mixtures were diluted with 215 μL of PBS, pH 6.0 (I, III, V) or pH 7.5 (II, IV, VI), followed by the addition of 10 μg of heparin (in 10 μL of H_2O). Step 3: after 30 min, 20 μL of the incubation mixtures were transferred to 96-well microtiter plates followed by addition of 180 μL of PBS, pH 6.0 (I, VI) or pH 7.5 (II−V). Twenty microliters of S-2288 (2 mM) was added followed by monitoring of enzymatic activity. Note that the neutral pH varies from 7.3 to 7.5, reflecting the resulting pH obtained after diluting samples as described above.

Formation of mMCP-6 Tetramers. The results above indicate that heparin is necessary for tryptase activation. Active tryptase is present in a tetrameric form, and studies were therefore conducted to determine if heparin influences tetramer formation. In the absence of heparin, mMCP-6 (at pH 6.0) was present mainly in a monomeric form as shown by molecular sieve chromatography on a Superdex 200 column (Figure 6A). No enzymatic activity was present in the monomer fraction. Addition of heparin at increasing concentrations resulted in disappearance of the monomer peak and in the generation of higher molecular weight complexes (Figure 6, panels B−F). At low amounts of added heparin, a large portion of the generated complexes eluted in the void volume of the column. This material would thus correspond to macromolecular complexes with molecular

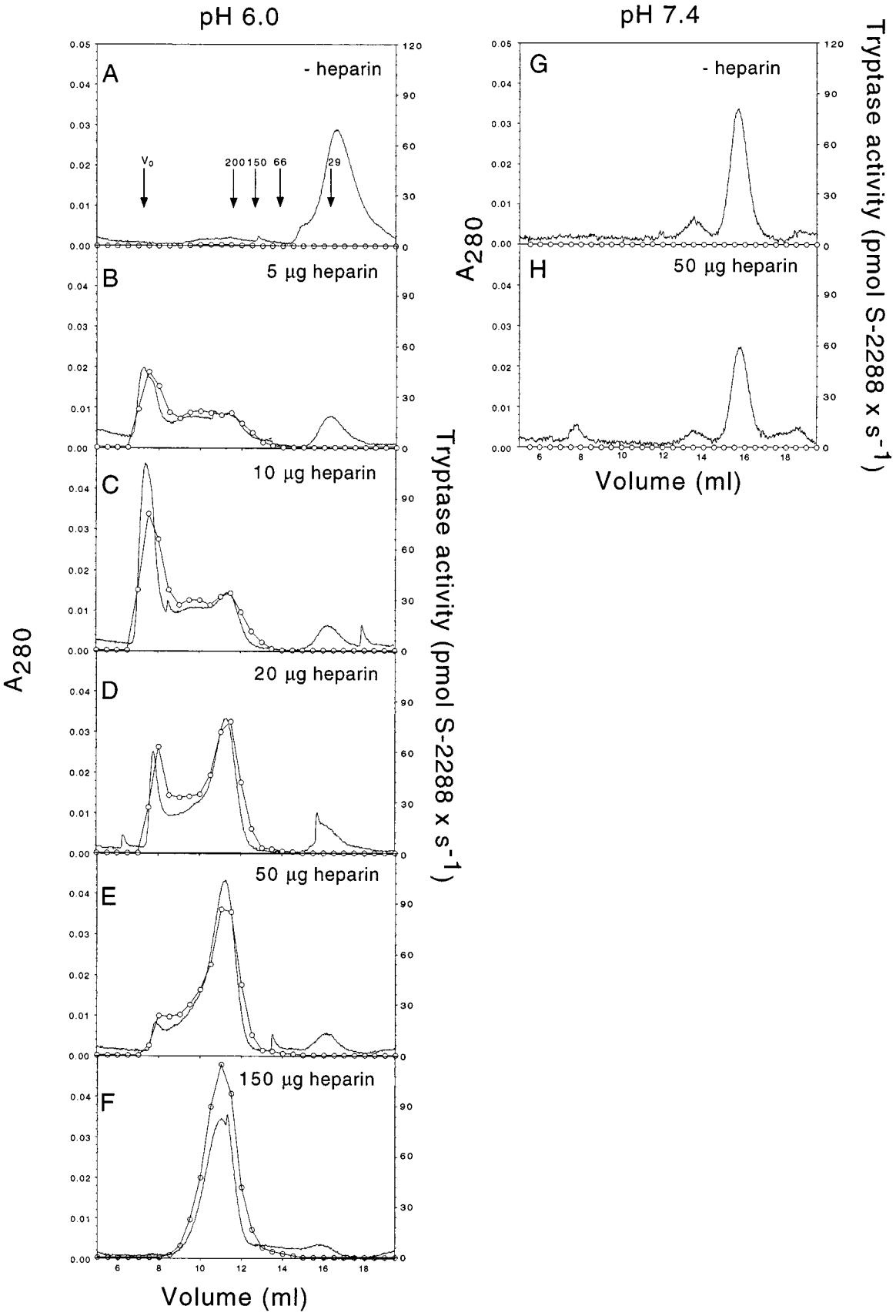

FIGURE 6: Effect of heparin and pH on mMCP-6 tetramer formation. 6×His−EK−mMCP-6 was digested with enterokinase in the absence of heparin. Enterokinase cleavage was performed either at pH 6.0 (A−F) or pH 7.4 (G and H). Samples of mMCP-6 (10 μg in 200 μL PBS, pH 6.0 or pH 7.4) were analyzed on a Superdex 200 column eluted with either PBS pH, 6.0 (A−F) or PBS, pH 7.4 (G and H). mMCP-6 samples were analyzed without addition of heparin (A and G) or after preincubation (45 min) with 5 μg (B), 10 μg (C), 20 μg (D), 50 μg (E, H) or 150 μg (F) of heparin. Absorbance at 280 nm (−−−) was recorded. Fractions (0.5 mL) were assayed for mMCP-6 activity with the chromogenic substrate S-2288 (O).

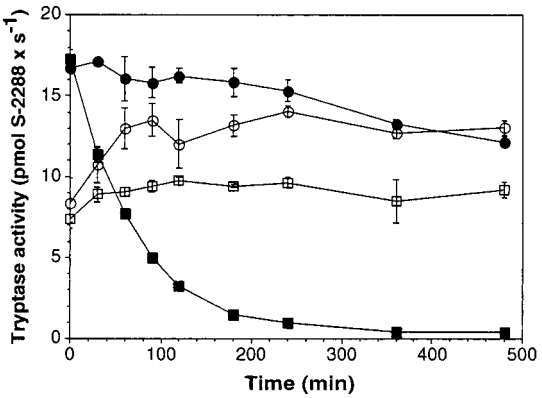

FIGURE 7: Stability of mMCP-6. A total of 14.4 μg of mMCP-6 was incubated with 145 μg of heparin for 30 min at room temperature to ensure initial optimal activity. Fifty-microliter samples of the mMCP-6/heparin mixtures were mixed with 450 μL of either PBS, pH 6.0 (○, □), or PBS, pH 7.4 (●, ■). These mixtures were subsequently incubated at either room temperature (○, ●) or 37 °C (□, ■). Samples (10 μL) were assayed with S-2288 for residual tryptase activities at the time points indicated. Residual tryptase activities were assayed at the same pH as was used during the incubation. The results shown represent the mean of triplicate determinations ± standard deviation. Error bars frequently fall within the symbols.

masses well above 700 kDa (thyroglobulin, M_r 669, is included in the Superdex 200 column; not shown). The void volume material displayed enzymatic activity toward S-2288. When increasing amounts of heparin were added to the mMCP-6 monomer, a gradual disappearance of the void volume peak was observed along with the formation of a lower molecular weight complex with an approximate size of 200 kDa. This size is in reasonable agreement with the expected size of a mMCP-6 tetramer bound to heparin. Again, enzymatic activity toward S-2288 was observed in the ~200-kDa peak. At pH 7.4, mMCP-6 eluted as a monomer, and heparin did not induce formation of active higher molecular weight compounds (Figure 6, panels G−H).

Stability of mMCP-6. To examine the stability of active mMCP-6 under various conditions, mMCP-6 in complex with heparin was incubated at either pH 6.0 or 7.4 and at both room temperature and 37 °C. Samples of the incubation mixtures were assayed at various time points for residual tryptase activities. It is apparent from Figure 7 that mMCP-6 is largely stabile, at least for 8 h at pH 6, both in room temperature and 37 °C, and only slowly loses activity at pH 7.4 at room temperature. In contrast, mMCP-6 lost activity rapidly when incubated at pH 7.4 at 37 °C. Further, when molecular sieve chromatography of the mMCP-6 tetramer was performed with the Superdex 200 column equilibrated in PBS, pH 7.4, the majority of the mMCP-6 protein was recovered in the monomer fraction (not shown).

Pro-Inflammatory Properties of mMCP-6. Several previous studies have indicated that various mast cell tryptases have pro-inflammatory properties (*31*, *34*, *35*). We therefore investigated whether the recombinant mMCP-6 was an inflammatory stimulus in vivo. Injection of enterokinase-digested 6×His−EK−mMCP-6 into the peritoneum of BALB/c mice did not cause any signs of inflammation, i.e., no neutrophil influx or mast cell degranulation was observed (Figure 8A; Table 2). Likewise, injection of heparin together with enterokinase did not produce any signs of inflammation (Table 2). In contrast, injection of enterokinase-digested 6×His−EK−mMCP-6 together with heparin caused a marked influx of neutrophils into the peritoneum (Figure 8B; Table 2). It was also noted that some of the mast cells had degranulated. Further evidence for mast cell degranulation was obtained after immunostaining for carboxypeptidase A, an abundant component of the mast cell secretory granules. In controls obtained after injection of enterokinase-cleaved 6×His−EK−mMCP-6 without heparin, the carboxypeptidase A staining was exclusively intracellular (Figure 8C). In contrast, after injection of enterokinase-digested 6×His−EK−mMCP-6 together with heparin, intense carboxypeptidase A staining was seen both intracellularly in the mast cells and in exocytosed granules (Figure 8D).

DISCUSSION

Tryptase is one of the main inflammatory mediators secreted during mast cell degranulation. Considering the large amounts of tryptase that become available in the tissue during mast cell-mediated inflammation it is likely that tryptase fulfills an important role in the inflammatory response. Indeed, several recent studies have indicated that tryptase has pro-inflammatory properties in vivo (*31*, *34*−*37*). Further, it has been shown that inhibitors of mast cell tryptase may ameliorate inflammatory responses in vivo (*38*−*41*), and certain tryptase inhibitors are currently undergoing clinical trials for use in treatment of mast cell-mediated disease.

The biology of mast cell tryptase is tightly linked to its physiological ligand, heparin proteoglycan. Since mast cell tryptase is present in complex with heparin in the granules, it is relevant to regard the tryptase/heparin complex as a functional entity. Heparin is of crucial importance for the tryptases in several ways. First, heparin appears to be absolutely necessary in the packaging of tryptases in the secretory granules. In mice lacking sulfated heparin, mMCP-6 antigen and activity was virtually completely absent, although mMCP-6 expression was unaffected (*14*, *19*). This indicates that, in the absence of heparin, mMCP-6 is not properly targeted to the secretory granules and is either exocytosed or degraded intracellularly. Second, several studies have shown that purified human tryptase requires the presence of heparin for maintaining enzymatic activity over a prolonged time (see Introduction). Third, heparin has been implicated in the proteolytic removal of the N-terminal activation peptide in human β-tryptase (*24*). In the case of the other class of mast cell serine proteases, the chymases, it is known that heparin can alter their substrate specificity and can protect them from inhibition (*42*−*44*).

In the present study, we have examined the possible role of heparin during formation of active mouse tryptase from inactive monomers. As a model, we have utilized recombinant mMCP-6 expressed with a 6 × His tag, used for efficient purification, followed by an enterokinase site replacing the native activation peptide. Monomers, matching those present in vivo are thus produced by enterokinase cleavage of this fusion protein. The fusion protein was expressed in a mammalian cell system, ensuring that the glycosylation reactions and folding conditions closely resemble those present in the mast cells in vivo.

The data presented here indicate that formation of enzymatically active mMCP-6 from inactive monomers requires the presence of heparin. These results are thus in apparent

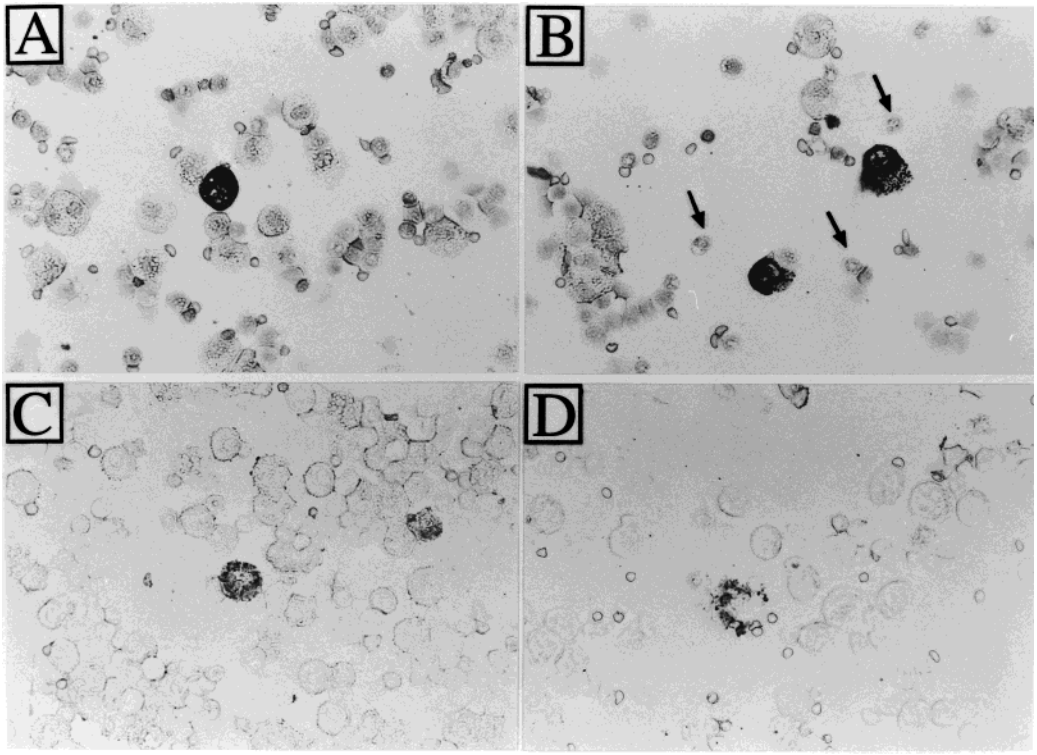

FIGURE 8: Pro-inflammatory effects of mMCP-6. Ten micrograms of enterokinase-digested 6×His−EK−mMCP-6 was injected into the peritoneal cavity of BALB/c mice, either alone (A and C) or after preincubation with 50 μg of heparin (B and D). After peritoneal lavage, cytospin slides were prepared and were stained either with May Grünwald/Giemsa (A and B) or with an antibody to the mast cell granule marker carboxypeptidase A (C and D). The arrows indicate neutrophils.

Table 2: Cells in Peritoneal Lavage Fluids[a]

injection	total cell no./ mouse	% neutrophils
PBS	$1.6 \pm 0.29 \times 10^6$	0.9 ± 0.16
enterokinase + heparin	$1.7 \pm 0.43 \times 10^6$	1.4 ± 0.42
enterokinase-digested 6xHis-EK-mMCP-6	$2.4 \pm 0.79 \times 10^6$	1.6 ± 0.42
enterokinase-digested 6xHis-EK-mMCP-6 + heparin	$3.6 \pm 0.53 \times 10^6$	14.0 ± 0.47

[a] Mice were injected as indicated. After 24 h, the mice were killed and cytospin slides were prepared. The percentage of neutrophils was calculated after May-Grünwald/Giemsa staining. A total of 300 cells were counted per slide. Results are shown as mean of triplicate determinations (±SD; $n = 3$).

contrast to the findings of Huang et al. (*31*, *45*), suggesting that mMCP-6 displays enzymatic activity in the absence of heparin. The reason for these discrepancies is not known. However, it should be noted that mMCP-6 in the previous study was expressed in insect cells, whereas we have expressed this tryptase in mammalian cells. Possibly, different folding conditions and/or glycosylation differences between these expression systems may influence the interaction with heparin. Further, the heavily charged C-terminal FLAG peptide (Asp−Tyr−Lys−Asp−Asp−Asp−Asp−Lys) that was introduced in the study by Huang et al. (*31*) may have affected the heparin binding properties of mMCP-6. We note that in some of our mMCP-6 preparations low but detectable enzymatic activity is observed without added heparin. However, the levels of activity are generally ≤1% of the activity obtained in the presence of optimal amounts of heparin.

Our results indicate that heparin is necessary for formation of the tetramer structure that is characteristic of all active tryptases studied. In the absence of heparin, mMCP-6 was mainly present in monomeric inactive form, as assessed by FPLC analysis on a Superdex 200 column. Addition of heparin resulted in formation of a ~200-kDa complex that displayed enzymatic activity toward S-2288. The size of this complex is thus in reasonable agreement with the expected size of a mMCP-6 tetramer bound to heparin, considering that each mMCP-6 monomer has a size of ~32 kDa and that heparin (pig mucosal heparin; "commercial heparin") has an average size of ~15 kDa. It is important to note that heparin glycosaminoglycan, due to its high anionic charge density, forms extended structures in solution and therefore displays an earlier elution position in gel chromatography when compared to a globular protein of similar molecular size. Interestingly, when low amounts of heparin were added to the mMCP-6 monomer (e.g., 5 μg of heparin to 10 μg of mMCP-6), larger mMCP-6 complexes were formed that eluted in the void volume of the Superdex 200 column. These macromolecular compounds displayed approximately equal enzymatic activity as the ~200 kDa complex. The exact nature of these high molecular weight complexes is not known. However, it seems reasonable to assume that they consist of several mMCP-6 tetramers that are bridged by heparin chains. Heparin may thus be capable of interacting with more than one tryptase tetramer unit simultaneously. The dependence on heparin for tryptase activity was also accentuated in in vivo experiments showing that mMCP-6 required the presence of heparin to display pro-inflammatory properties.

Activation of mMCP-6 showed a strong dependence on pH. When the 6×His−EK−mMCP-6 fusion protein was cleaved by enterokinase at neutral pH, very little or undetect-

able enzymatic activity was seen, even in the presence of heparin. However, when enterokinase cleavage and heparin binding was performed at pH 6.0, high levels of S-2288-hydrolyzing activity was obtained. Further studies revealed that the pH range compatible with tryptase activation showed a marked drop at ~pH 6.5, with only small amounts of active tryptase being formed after cleavage at higher pH. Moreover, affinity chromatography analysis demonstrated that mMCP-6 bound strongly to heparin-Sepharose at pH 6.0, whereas binding was undetectable at pH 7.4. It is interesting to note that also mMCP-7, in a recent study, showed similar dependence on pH for enzymatic activation (*45*). Taken together, this indicates that the strong dependence on acidic pH for mMCP-6 activation is because binding to heparin only occurs at acidic pH. The most probable mechanistic explanation for this is that histidine residues in mMCP-6 are involved in heparin binding. Because of their pK_a values (~6.5), histidine residues would be positively charged at acidic pH and capable of interacting with the negatively charged heparin chains. At higher pH, they lose their positive charge, which may abolish the interaction with heparin. This would be in agreement with an earlier study on mMCP-7 that showed that this tryptase interacted with heparin through a cluster of exposed histidine residues (*46*). However, mMCP-7 is believed not to require heparin for activation/tetramer formation (*45*). Our findings may also be related to a previous study suggesting that inactive monomers of human β-tryptase, purified from lung tissue, could undergo spontaneous reactivation at pH 6 but not at neutral pH (*47*).

Our results may have important implications for the in vivo mechanism for mMCP-6 activation. Considering the strong dependence of both heparin and on acidic pH, it is clear that mMCP-6 activation can only occur in a cellular location when these factors are present. Heparin biosynthesis is probably completed in the Golgi network, indicating that mMCP-6 activation does not occur during earlier stages in the secretory pathway. Further, it is known that the pH in the *trans*-Golgi network is ~6 (*48*) but is significantly higher in earlier compartments of the secretory pathway. It is thus conceivable that active tetramers complexed with heparin proteoglycan are formed in the *trans*-Golgi network and are subsequently exported to the secretory granules. However, it should be emphasized that the pH in the secretory granules (pH ~5.5) (*49*) is also compatible with heparin binding and tryptase activation. Thus, it cannot be ruled out that tryptase activation may occur after export from the *trans*-Golgi network.

Taken together, our results suggest a crucial role for heparin in the formation of active mMCP-6 tetramers. Hence, heparin proteoglycan appears to be an essential companion to tryptase during most phases of its life-span, including the removal of the activation peptide, tetramer formation, storing, and stabilization after exocytosis.

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