Formation of active monomers from tetrameric human β -tryptase

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Tryptase is a serine protease that is stored at low pH in the mast cell secretory granules in complex with heparin proteoglycan. When mast cells are activated, e.g. during allergic responses, the tryptase/heparin complexes are released together with a variety of other preformed inflammatory mediators. Previous crystallization of human β -tryptase revealed a unique tetrameric structure with all of the active sites facing a central pore that has a limited accessibility both for potential substrates as well as for protease inhibitors. In this study we examined whether human β -tryptase, in addition, could form active monomers. Incubation of recombinant tetrameric human β -tryptase at neutral pH and 37 °C, followed by gel-filtration analysis using a running buffer containing pig mucosal heparin, led to the formation of enzymically active compounds that were of a size compatible with tryptase monomers in complex with heparin. The monomers

were, in contrast to tryptase in the tetrameric form, inhibited by bovine pancreatic trypsin inhibitor. Further, the monomers, but not the tetramers, degraded fibronectin. Formation of active monomers was more pronounced at pH 7.5 than at pH 6.0 and was not detected at room temperature or at high heparin/tryptase ratios. The present findings thus introduce the possibility that human β -tryptase, after mast cell degranulation and exposure to neutral pH in the tissue, may dissociate into active monomers with properties that are distinct from the tetrameric counterpart. Possibly, some of the biological activities of human tryptase may be attributable to active tryptase in its monomeric rather than tetrameric form.

Key words: fibronectin, mast cell, proteoglycan, serine protease, trypsin inhibitor.

INTRODUCTION

Mast cells are effector cells involved in many events with an inflammatory component, for example allergic diseases such as asthma. The mast cells store a number of preformed inflammatory mediators that are released after cellular activation in response to a variety of stimuli [1]. These mediators include histamine, cytokines and various proteases: tryptases, chymases and carboxypeptidase A [2]. Mast cell proteases are stored as active enzymes in the mast cell granules, in complex with serglycin proteoglycans, mainly of the heparin subtype [3]. Human tryptases are further subdivided into α , β , γ and mouse mast cell protease (mMCP)-7-like isoenzymes, where the different β -tryptases appear to be the major type stored in the mast cell granules [4]. A role for mast cell tryptase in inflammation has been suggested by the findings that tryptase itself can provoke an inflammatory response in different animal systems [5–7]. Further, tryptase inhibitors have been shown to block inflammatory responses [8,9]. However, the mechanism of action, i.e. the physiological substrate(s) for this protease *in vivo*, has not been determined.

Early studies indicated that β -tryptase is a tetramer in its active form [10]. Later, crystallization of human β -tryptase showed that the four monomers are situated at the corners of a flat rectangular frame, with the active sites oriented towards the inner face of a central pore [11]. This arrangement explained some particular features of human tryptase, for instance its resistance to most proteinaceous inhibitors [12]. This orientation of the active sites would also restrict the accessibility for various macromolecular substrates, which for steric reasons would be unable to gain access to the tryptase active sites. Indeed, many of the identified substrates for tryptase are small peptides [13]. However, cleavage of a number of large proteins, e.g. fibronectin [14] and fibrinogen [15], has been reported and it is not clear how such large compounds could enter the central pore of the tryptase tetramer.

These apparently conflicting findings led us to investigate whether human β -tryptase can also be active as a monomer. A monomeric tryptase would theoretically have an open access to macromolecular substrates, and thus much less restricted substrate cleavage properties. An active monomer would, in addition, be more accessible to proteinaceous protease inhibitors.

In a previous report we showed that inactive monomers of a mouse tryptase, mMCP-6, could be transferred into active monomers if they were exposed to heparin oligosaccharides of insufficient chain length to induce tetramer formation [16]. However, the conditions required for formation of active tryptase monomers according to the latter mechanism may not be present *in vivo*, e.g. short heparin oligosaccharides are not known to be present in mast cells. Here we investigated whether tryptase that has achieved a tetrameric state can undergo dissociation into lower-molecular-mass active compounds under conditions mimicking those present *in vivo* after mast cell degranulation. Our data indicate that tetrameric human β -tryptase, when incubated at neutral pH and 37 °C, dissociates into lower-molecular-mass components that are active and functionally distinct from the tetrameric enzyme.

EXPERIMENTAL

Reagents

All studies were performed using recombinant human β I-tryptase (rh- β T) purchased from Promega (Madison, WI, U.S.A.). The expression and purification of the enzyme have been described previously [17]. The enzyme preparation was stored at –70 °C in 2 μ g aliquots at a concentration of 0.1 mg/ml in 2 M NaCl/10 mM Mes, pH 6.1. Pig mucosal heparin [high-molecular-mass heparin (HMM heparin); $\approx$ 15000 Da] and ³H-labelled HMM heparin (25000 c.p.m./ μ g) were gifts from Ulf Lindahl (Uppsala University, Uppsala, Sweden).

Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; HMM heparin, high-molecular-mass heparin; LMM heparin, low-molecular-mass heparin; mMCP, mouse mast cell protease; rh- β T, recombinant human β I-tryptase.

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Low-molecular-mass heparin (LMM heparin) from porcine intestinal mucosa (≈ 3000 Da), human fibronectin and human fibrinogen were from Sigma. Bovine pancreatic trypsin inhibitor (BPTI) was purchased from Roche (Mannheim, Germany). The chromogenic substrate S-2288 (*H*-D-Ile-Pro-Arg-*p*-nitro-anilide) was from Chromogenix (Mölnådal, Sweden). Polybrene (hexadimethrine bromide, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) was obtained from Janssen Chimica (Beerse, Belgium). Since Polybrene is a heterogeneous polycation, with molecular masses ranging from 5000 to 10000 Da, a mean molecular mass of 7500 Da was estimated for calculation of molar concentrations. All buffer salts were provided by Merck Eurolab or Sigma.

Enzymic assays

Enzymic assays were performed in 96-well microtitre plates. Trypsase activities were routinely assayed in a volume of $100\ \mu\text{l}$ of PBS (10 mM phosphate, 137 mM NaCl and 2.68 mM KCl, pH 6.0) in the absence or presence of either HMM or LMM heparin ($10\ \mu\text{M}$). Enzymic activity was recorded after addition of $20\ \mu\text{l}$ of a 2.16 mM solution (in water) of the chromogenic substrate S-2288. The absorbance at 405 nm was monitored at room temperature with a Titertek Multiscan spectrophotometer (Flow Laboratories).

Size-exclusion gel chromatography

Size-exclusion gel chromatography was performed on a Superdex 200 column ($10\ \text{mm} \times 300\ \text{mm}$) using an FPLC system (Amersham Biosciences). A flow rate of $0.5\ \text{ml/min}$ was used routinely. Molecular masses were calculated after calibration of the column with the following molecular-mass standards (all from Sigma): carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa). The void volume of the column was determined using Blue Dextran. Running buffers were PBS (pH 6.0) without heparin, PBS (pH 6.0)/ $10\ \mu\text{M}$ HMM heparin or PBS (pH 6.0)/ $10\ \mu\text{M}$ LMM heparin. Calibration of the column with molecular-mass standards was performed for all of the running buffers used. Before runs, the column was pre-equilibrated with two column volumes of the respective buffer. In each experiment, $2\ \mu\text{g}$ of rh- βT ($2\ \mu\text{g}$ of rh- βT in 2 M NaCl/10 mM Mes, pH 6.1, was diluted with $180\ \mu\text{l}$ of PBS, either pH 6.0 or 7.5) was analysed either directly or after being subjected to the different treatments specified in the Figure legends. Fractions of $500\ \mu\text{l}$ were collected, from which $100\ \mu\text{l}$ samples were analysed for trypsin activity (see above). In the inhibition studies, $90\ \mu\text{l}$ portions of the fractions were transferred to 96-well plates and were treated with either $10\ \mu\text{l}$ of PBS (pH 6.0; control), or $10\ \mu\text{l}$ of PBS (pH 6.0) containing either $10\ \mu\text{g}$ of BPTI (final concentration, $15\ \mu\text{M}$) or $37.5\ \mu\text{g}$ of Polybrene (final concentration, $50\ \mu\text{M}$). After an incubation time of 30 min (BPTI) or 15 min (Polybrene) at room temperature, residual trypsin activity was determined as described above.

Determination of cleavage sites in fibronectin

rh- βT ($2\ \mu\text{g}$) was diluted with PBS (pH 7.4; total volume, $200\ \mu\text{l}$) and incubated for 30 min at 37°C . Then the sample was further diluted by adding $900\ \mu\text{l}$ of PBS (pH 6.0) containing $10\ \mu\text{M}$ HMM heparin. Next, $50\ \mu\text{g}$ of human fibronectin was added and the mixture was incubated for 40 h at room temperature. Subsequently the sample was concentrated at 4°C to a final volume of $50\ \mu\text{l}$ by using Microcon YM-10 centrifugal filter

devices (Millipore, Bedford, MA, U.S.A.) following the instructions provided by the manufacturer. Samples were mixed with $16.7\ \mu\text{l}$ of $4\times$ SDS sample buffer (without reducing agent) and subjected to SDS/PAGE under non-reducing conditions in a Laemmli system, using a 6% polyacrylamide gel. Proteins were transferred electrophoretically to a PVDF membrane (Pall Corporation; previously equilibrated for $\approx 10\ \text{min}$ in methanol) in 25 mM Tris buffer containing 192 mM glycine and 20% methanol, using a BlueBlot Wet/100 transfer system (Serva) at 4°C . After transfer (1 h, 200 mA) the membrane was stained with Coomassie Brilliant Blue for 10 min, destained in 50% methanol/10% acetic acid and allowed to dry on Whatman paper. N-terminal sequence analysis of recovered fragments was carried out at the Protein Analysis Center (Karolinska Institute, Stockholm, Sweden).

RESULTS

Isolation of active trypsin tetramers and monomers

Size-exclusion gel chromatography of rh- βT on a Superdex 200 column revealed a single peak of enzymic activity at $\approx 200\ \text{kDa}$ in the absence of heparin in the running buffer (Figure 1A), indicating that the active trypsin is exclusively tetrameric under these conditions. When $10\ \mu\text{M}$ HMM heparin was included in the running buffer the tetramer peak was accompanied by an additional peak with an apparent molecular mass of $\approx 95\ \text{kDa}$ (Figure 1B). The lack of active compound in this position when heparin was excluded from the running buffer (Figure 1A) clearly indicates that this form of trypsin is dependent on heparin for activity under these conditions, suggesting interaction of the

Figure 1 Size-exclusion gel chromatography analysis of rh- βT under different conditions

rh- βT ($2\ \mu\text{g}$; in $20\ \mu\text{l}$ of 10 mM Mes, pH 6.1/2 M NaCl) was diluted 10-fold in PBS (without heparin), pH 6.0 (B) or pH 7.4 (A, C–F). Samples were either analysed directly (B, C) or analysed after incubation at 37°C for 30 min (A, D, F) or 3 h (E). Samples were analysed on a Superdex 200 column, eluted at $0.5\ \text{ml/min}$ with PBS (pH 6.0) without (A) or with $10\ \mu\text{M}$ HMM heparin (B–E) or $10\ \mu\text{M}$ LMM heparin (F). Fractions of $0.5\ \text{ml}$ were collected and, after chromatography, $100\ \mu\text{l}$ samples from the fractions were assayed for trypsin activity using the chromogenic substrate S-2288. T and M indicate tetramer and monomer peaks, respectively.

Figure 2 Effect of BPTI and Polybrene on rh- β T tetramers and monomers

(A, B) rh- β T (2 μ g; in 20 μ l of 10 mM Mes, pH 6.1/2 M NaCl) was diluted 10-fold in PBS (pH 7.4), incubated for 30 min at 37 °C and subjected to size-exclusion gel chromatography on Superdex 200 using PBS (pH 6.0; containing 10 μ M HMM heparin) as running buffer. After chromatography, 90 μ l samples from the fractions collected were mixed with 10 μ l of PBS (pH 6.0) without inhibitor (■) or with 10 μ l of PBS (pH 6.0) containing either 37.5 μ g of Polybrene (A, Δ) or 10 μ g of BPTI (B, $\square$). After incubation at room temperature for 15 min (Polybrene) or 30 min (BPTI), residual trypsin activity was measured with S-2288. (C) rh- β T (2 μ g; in 20 μ l of 10 mM Mes, pH 6.1/2 M NaCl) were diluted 10-fold in PBS (pH 7.4), incubated for 30 min at 37 °C and subjected to size-exclusion gel chromatography on Superdex 200 using PBS (pH 6.0; containing 10 μ M LMM heparin) as running buffer. LMM heparin was used to obtain maximal separation of tetramers and monomers (see Figure 1F). Fractions corresponding to tetramers (●) and monomers (○) were pooled and 90 μ l samples from each pool were incubated at room temperature for 30 min in the absence or presence of different concentrations of BPTI, followed by measurement of residual trypsin activity with S-2288. Results are expressed as means $\pm$ S.D. ($n = 3$).

compound with heparin. Interaction of a trypsin monomer with HMM heparin (≈ 15000 Da) would result in a complex of ≈ 45 kDa. However, heparin chains are extended in solution due to repulsion between their negatively charged sulphate/carboxyl groups, and thus are eluted considerably earlier on gel-filtration columns compared with globular proteins of a similar size. Analysis of 3 H-labelled HMM heparin (10000 c.p.m.) on the Superdex 200 column with the same conditions as those used for chromatography of trypsin (running buffer, PBS, pH 6.0/10 μ M HMM heparin) showed a broad elution profile of the gly-

Figure 3 Kinetics for formation of active monomers of rh- β T: BPTI present during incubation

rh- β T (2 μ g; in 20 μ l of 10 mM Mes, pH 6.1/2 M NaCl) was incubated for 30 min at room temperature in the absence (■, □) or presence of 0.2 μ g (▲, △) or 2 μ g (●, ○) of HMM heparin. Next, samples were diluted 10-fold in PBS (containing 250 μ g/ml BSA) of pH 6.0 (closed symbols; A, C) or pH 7.5 (open symbols; B, C) and incubated further at 37 °C (A, B) or room temperature (C). The incubations were performed in either the absence (solid lines) or presence (dashed lines) of 20 μ g of BPTI. Every 30 min, 5 μ l samples (equivalent to 50 ng of trypsin) were removed, mixed with 95 μ l of PBS (pH 6.0; without heparin) and assayed for trypsin activity with the chromogenic substrate S-2288. Results are expressed as means $\pm$ S.D. ($n = 3$). Note that the pH values indicated in the Figure correspond to the pH used during the different incubations, whereas the activity assays in all cases were performed at pH 6.0.

cosaminoglycan, with the peak eluting at a position corresponding to ≈ 65 kDa as compared with protein standards (results not shown). The size of the retarded trypsin peak, ≈ 95 kDa, is thus compatible with a trypsin monomer (≈ 30 kDa) in complex with HMM heparin. We therefore propose that the identified additional peak of trypsin activity corresponds to active monomeric trypsin. Therefore, in the following this form of trypsin will be referred to as a monomer. However, we cannot completely exclude that the ≈ 95 kDa peak may correspond to trypsin in a form other than monomeric, e.g. dimeric. Prolonged incubation of rh- β T at pH 7.5 (37 °C) resulted in a gradual increase of the monomer peak, with $\approx 50\%$ of the activity recovered in the monomer fraction after 180 min of incubation (Figures 1C–1E). When LMM heparin (≈ 3000 Da), instead of HMM heparin, was included in the running buffer the

Figure 4 Kinetics for formation of active monomers of rh- β T: BPTI absent during incubation

rh- β T ($2 \mu\text{g}$; in $20 \mu\text{l}$ of 10 mM Mes, $\text{pH } 6.1/2 \text{ M}$ NaCl) was incubated for 30 min at room temperature in the absence ($\blacksquare$, $\square$) or presence of $0.2 \mu\text{g}$ ($\blacktriangle$, $\triangle$) or $2 \mu\text{g}$ ($\bullet$, $\circ$) of HMM heparin. Next, samples were diluted 10-fold in PBS (containing $250 \mu\text{g/ml}$ BSA) of $\text{pH } 6.0$ (closed symbols; **A**, **C**) or $\text{pH } 7.5$ (open symbols; **B**, **D**) and incubated further at 37°C . Every 30 min, $5 \mu\text{l}$ samples (equivalent to 50 ng of trypsin) were removed and were mixed with $85 \mu\text{l}$ of PBS ($\text{pH } 6.0$) without (**A**, **B**) or with (**C**, **D**) $10 \mu\text{M}$ HMM heparin. After 5 min, $10 \mu\text{l}$ of PBS ($\text{pH } 6.0$), $10 \mu\text{M}$ HMM heparin with (dashed lines) or without (solid lines) 500 ng of BPTI were added and residual trypsin activities were measured after 2 min using S-2288. Results are expressed as means $\pm$ S.D. ($n = 3$). Note that the pH values indicated in the Figure correspond to the pH used during the different incubations, whereas the activity assays in all cases were performed at $\text{pH } 6.0$.

tetramer peak was, similarly to runs using HMM heparin in the running buffer, accompanied by a retarded peak of enzymic activity. However, the retarded peak was eluted later (at $\approx 30 \text{ kDa}$) than the corresponding peak obtained in the presence of HMM heparin, clearly indicating an active monomer (Figure 1F).

We have previously shown that polycationic compounds, such as protamine and Polybrene, are potent inhibitors of both human lung trypsin and mouse trypsin [18]. To investigate whether monomeric rh- β T was sensitive to polycations we incubated each fraction, recovered after separation of tetramers from the monomers by size-exclusion gel chromatography, for 15 min with $50 \mu\text{M}$ Polybrene, followed by measurement of residual enzymic activities. Results shown in Figure 2(A) indicate that both the monomeric and tetrameric forms of rh- β T are inhibited by Polybrene.

To provide functional proof that the observed compound corresponds to monomeric active trypsin we investigated if the enzymic activity was sensitive to inhibition by BPTI, an inhibitor that would be too large to enter the central pore of tetrameric trypsin. Indeed, BPTI was completely unable to inhibit the tetrameric form of trypsin (Figure 2B) but gave a complete abolition of the monomer peak (Figure 2B), indicating that the active sites in this trypsin form are less restricted in steric terms. Trypsin monomers, produced in the presence of LMM heparin, were equally susceptible to inhibition by BPTI as were the monomers prepared with HMM heparin (results not shown). In a dose-response experiment performed on pooled fractions of separated trypsin tetramers and monomers (prepared in the presence of LMM heparin to achieve maximal separation of

peaks) it was observed that the tetramer was completely resistant to inhibition by BPTI even at a large excess of inhibitor over trypsin, whereas the monomers were readily inhibited (Figure 2C). The inhibition of the trypsin monomers by BPTI was rapid, with maximal inhibition achieved within 60 s (results not shown). Taken together, these results indicate strongly that the observed enzymic activity corresponds to active monomers of rh- β T that are functionally distinct from the corresponding tetramers, and rule out that the monomer peak could correspond to trypsin that has been re-tetramerized after the chromatography step.

Stability of trypsin monomers and tetramers

Studies were performed to investigate the stability of trypsin tetramers and monomers. To this end, trypsin tetramers and monomers were separated by size-exclusion gel chromatography using HMM heparin in the running buffer. Fractions corresponding to tetramers and monomers were pooled and incubated for various times at both room temperature and 4°C followed by measurement of residual activities. Both the tetramers and monomers gradually lost activity, with no difference in stability observed between the monomers and tetramers (50% activity lost within 120 min at room temperature). Loss of activity was more rapid (≈ 1.5 -fold) at room temperature than at 4°C (results not shown).

Kinetics of formation of active trypsin monomers

Experiments were performed to study the kinetics of active monomer formation (Figure 3). To discriminate between the

Figure 5 Cleavage of macromolecular substrates by tryptase tetramers and monomers

(A) rh- β T (2 μ g; in 20 μ l of 10 mM Mes, pH 6.1/2 M NaCl) was diluted 10-fold in PBS (pH 7.4), incubated at 37 °C for 30 min and subjected to size-exclusion gel chromatography as described in the Experimental section using PBS (pH 6.0) containing 10 μ M HMM heparin as running buffer. Fractions containing the highest levels of activities corresponding to tetramers and monomers were recovered and 100 μ l-samples from these fractions were mixed with 3 μ g of human fibronectin, followed by incubation at room temperature in the absence or presence of 10 μ g of BPTI. As a control, fibronectin was incubated with running buffer in the absence or presence of 10 μ g of BPTI. Samples (30 μ l) from the tryptase/fibronectin incubation mixtures were removed after the times indicated, mixed with 10 μ l of 4 $\times$ SDS sample buffer (without reducing agent) and subjected to SDS/PAGE (8% gels) under non-reducing conditions. Proteins were visualized after silver staining. (B) Tryptase tetramers and monomers were isolated as in (A) and 100 μ l samples from the tetramer or monomer fractions were mixed with 3 μ g of human fibrinogen, followed by incubation at room temperature. As a control, fibrinogen was incubated with running buffer. Samples (30 μ l) from the tryptase/fibrinogen incubation mixtures were removed after 10 min, 3 h and 20 h, and were subjected to SDS/PAGE under reducing conditions (8% gels). Proteins were visualized by silver staining.

enzymic activity of monomers and tetramers the observed susceptibility of active monomers (but not tetramers) to inhibition by BPTI was used (see above). Thus the appearance of BPTI-susceptible activity would correspond to formation of active monomers. Tryptase was incubated without heparin or together with HMM heparin in different amounts. Incubations were carried out in the absence or presence of BPTI and after various time points, residual tryptase activities were measured (Figure 3). Note that BPTI in this experiment was present continuously throughout the incubations, to ensure rapid inhibition of any active monomers that are formed. In the presence of 2 μ g of heparin, tryptase activity was fairly constant throughout the incubation period, with no detectable loss of activity observed after 180 min at pH 6.0 (37 °C; Figure 3A) and only $\approx$ 10 % loss of activity after 180 min at pH 7.5 (37 °C; Figure 3B). Very little BPTI-sensitive activity was observed when incubations were carried out in the presence of 2 μ g of heparin (or 20 μ g heparin; results not shown). When 0.2 μ g of heparin (corresponding to a molar tryptase tetramer:heparin ratio of $\approx$ 1:1) was present during incubations at 37 °C, the decay in total activity occurred more rapidly than at higher heparin concentrations at both pH 6.0 (Figure 3A) and pH 7.5 (Figure 3B). Under these conditions (0.2 μ g of heparin) a rapid increase in BPTI-sensitive activity was observed at pH 7.5 (Figure 3B), but not at pH 6.0 (Figure 3A). When heparin was absent from the incubation mixtures, loss of total tryptase activity was even more rapid. Moreover, in the absence of heparin, a large fraction of the residual activities at both pH 6.0 (Figure 3A) and pH 7.5 (Figure 3B) was sensitive to BPTI. To address whether the incubation

temperature was an important factor for the formation of active monomers, assays similar to those described above were performed at room temperature. The data displayed in Figure 3(C) clearly show that no (pH 6.0) or very little (pH 7.5) BPTI-sensitive activity was formed when tryptase was incubated without heparin at room temperature, in sharp contrast with the rapid formation of BPTI-sensitive activity generated at 37 °C. Taken together, the results indicate that formation of active monomers of tryptase is strongly promoted when heparin is absent or present in low amounts. Active monomer formation is also favoured by neutral pH and is strikingly temperature-dependent.

In the experiments described above, BPTI was present together with tryptase during the incubation periods to ensure rapid binding to any active monomers that are formed. In another experimental set-up, tryptase was incubated alone (i.e. without BPTI) at various conditions, followed by subsequent addition of BPTI and measurement of residual activities (Figure 4). When experiments were performed in this way it was clear that BPTI had no effect on tryptase that had been incubated under any of the conditions tested (with or without heparin, pH 6.0 or 7.5; Figures 4A and 4B). In similar experiments tryptase was first incubated alone (without BPTI) under various conditions. Subsequently, the tryptase samples were mixed with buffer containing heparin, followed by the addition of BPTI and measurement of residual activities. Results displayed in Figures 4(C) and 4(D) show that heparin caused a marked reactivation of tryptase that had been incubated at pH 6.0 in the absence of heparin (compare ■ connected by solid lines, Figures 4A and 4C), and of tryptase

Figure 6 Determination of a tryptase cleavage site in fibronectin

(A) rh- β T ($2 \mu\text{g}$; in $20 \mu\text{l}$ of 10 mM Mes, $\text{pH } 6.1/2 \text{ M}$ NaCl) was diluted 10-fold in PBS ($\text{pH } 7.4$; total volume, $200 \mu\text{l}$), incubated at 37°C for 30 min and further diluted with $900 \mu\text{l}$ of PBS ($\text{pH } 6.0$) containing $10 \mu\text{M}$ HMM heparin to generate active monomers. Next, $50 \mu\text{g}$ of fibronectin was added, followed by 40 h of incubation at room temperature. As a control, $50 \mu\text{g}$ of fibronectin was incubated without tryptase. After concentration ($\approx 20\times$), the samples were subjected to SDS/PAGE (non-reducing conditions), followed by transfer on to PVDF membranes and visualizing of proteins with Coomassie Brilliant Blue. Intact fibronectin (FN) dimers ($2 \times \approx 250 \text{ kDa}$), connected by an S–S bond, are indicated. Major fibronectin cleavage products (fragments 1–4) were recovered and subjected to N-terminal sequence analysis. (B) Schematic representation of the human fibronectin dimer and location of the identified cleavage site for the tryptase monomer. The sequence shown in italics represents the N-terminal sequence found in fragments 1–4. The dashed arrow in parentheses indicates the approximate position of an additional unidentified cleavage site that would lead to the observed major decrease in apparent molecular size after cleavage by active tryptase monomers. The schematic presentation indicates the modular organization of fibronectin and shows the locations of binding regions for various known fibronectin ligands. Roman numerals (I, II and III) refer to fibronectin modules types I–III. There are 12 type I, two type II and 15 type III modules, indicated with Arabic numerals. There are several sites of alternative splicing of fibronectin mRNA. Two result in the insertion of extra type III domains, EDA and EDB, after modules III-11 and III-7, respectively. IIICS refers to another alternatively spliced region inserted between III-14 and III-15.

that had been incubated at $\text{pH } 7.5$ with or without $0.2 \mu\text{g}$ of heparin (compare $\triangle$ and $\square$ by solid lines in Figures 4B and 4D). Tryptase that had been incubated with higher amounts of heparin ($2 \mu\text{g}$) or tryptase that had been incubated with a low amount of heparin ($0.2 \mu\text{g}$) at $\text{pH } 6.0$ did not show any reactivation by subsequent addition of heparin. Interestingly, addition of BPTI to samples that had been reactivated with heparin showed that a major part of the activities was sensitive to inhibition. Taken together, these data indicate that active

tryptase that is produced by reactivation of inactive enzyme is predominantly monomeric.

Cleavage of protein substrates by tryptase monomers and tetramers

The ability of tryptase monomers and tetramers to degrade macromolecular substrates was tested. Previous reports have

shown that fibronectin [14] and fibrinogen [15] are degraded by human tryptase. Here we explored whether these proteins were substrates for both tetrameric and monomeric rh- β T. Incubation of fibronectin with tetrameric rh- β T did not result in any detectable degradation of the protein, even after prolonged times of incubation. In contrast, fibronectin was degraded into two major bands of ≈ 220 and ≈ 195 kDa by the monomeric form (produced in the presence of HMM heparin) of rh- β T (Figure 5A). The degradation of fibronectin was abolished in the presence of BPTI. Tryptase monomers, prepared in the presence of LMM heparin, showed similar fibronectin-degrading properties as the counterparts prepared with HMM heparin (results not shown). In contrast to fibronectin, fibrinogen was readily degraded by both tetrameric and monomeric rh- β T (Figure 5B).

To determine the cleavage sites in fibronectin, excess fibronectin was incubated with rh- β T that had been treated under conditions that generate active monomers. Cleavage products were separated and were subjected to N-terminal sequence analysis. Four cleavage products were recovered (≈ 220 , ≈ 195 , ≈ 175 and ≈ 160 kDa) and in all of these fragments the N-terminal sequence AAVYQ was found (Figure 6). This indicates that active tryptase monomers initially cleave fibronectin at the Arg²⁹⁰–Ala²⁹¹ bond, followed by additional cleavages near the C-terminal end of the protein.

DISCUSSION

In a previous study we showed that heparin was required for the assembly of active tetramers of the mouse tryptase mMCP-6 [19], and it was subsequently shown that heparin acts by binding simultaneously to at least two mMCP-6 monomers, thereby bridging them and thus promoting tetramer formation [16]. When heparin oligosaccharides that were too short for binding to two monomers simultaneously were added to the inactive tryptase monomers, it was observed that active monomers were formed [16]. Although the latter study gave proof that active monomers of mouse tryptase could be formed from inactive monomers, it did not provide a mechanism by which active monomers could be formed from tryptase that is already tetramerized, as during storage in the secretory granules. Another important issue was to establish whether not only rodent but also human tryptase could be active in monomeric form. Moreover, in the mMCP-6 study the active monomers were induced in the presence of LMM heparin of < 20 monosaccharide units. It is questionable if heparin fragments of such sizes may be formed intracellularly in the mast cells. Consequently, it is not certain if the conditions that are required for formation of the active monomers according to the latter mechanism ever are present *in vivo*. Here we show that active monomers can be formed from tetrameric active human β -tryptase. Importantly, we show that the active monomers can be formed in the presence of HMM heparin, i.e. heparin in a form that closely resembles the polysaccharide present *in vivo*. Moreover, we demonstrate that the formation of active monomers is strongly promoted at neutral pH and 37 °C, i.e. the conditions that tetrameric tryptase is exposed to after mast cell degranulation.

Several previous reports have shown that human tetrameric tryptase can dissociate into monomers, in particular when stabilizing heparin is not present [10,20,21]. However, in contrast to the present report, these monomers have generally been considered as being non-active, although one previous report has provided limited evidence supporting the presence of active monomers of human β -tryptase [22]. However, since the latter study did not show any functional distinction between the active monomers and tetramers, the validity of the data has been

questioned [21]. In this study we demonstrate that the active tetramers and monomers of human β -tryptase show dramatic functional differences. Firstly, opening up of the constrained tetrameric organization of tryptase into monomers would theoretically result in a much less restricted substrate cleavage profile than the tetramers, in particular as regards large proteins. Indeed, we show here that the monomeric β -tryptase degrades fibronectin, whereas fibronectin is totally resistant to tetrameric tryptase. Further studies will reveal whether also other potential tryptase substrates are differentially susceptible to cleavage by tryptase in its tetrameric or monomeric forms. Interestingly, it appears as though fibrinogen, despite its large size, is able to enter the central cavity of tetrameric tryptase. The reason for this is not clear, but it has been suggested that protrusions from various large proteins could be 'extracted' into the central pore (see [13] for a discussion). Secondly, active monomers of human β -tryptase would theoretically be more susceptible to inhibition by macromolecular protease inhibitors. We show here that the tetrameric tryptase is completely resistant to inhibition by BPTI even at high inhibitor concentrations, whereas β -tryptase in its monomeric form is readily inhibited. These findings provide further firm evidence that the lower-molecular-mass forms of active tryptase identified here have an open access to macromolecular compounds, clearly consistent with tryptase in monomeric form. The dissociation into active monomers could thus, in addition to changing the substrate-cleavage properties of tryptase, also affect its regulation after mast cell degranulation. In contrast to the tetramer, the active monomer would be highly susceptible to inhibition by protease inhibitors that are present in the tissue. Hence, the active monomers are likely to be relatively short-lived after formation.

In most previous studies, active tryptase has been reported to be exclusively in tetrameric form. The reason why active lower-molecular-mass human tryptase has escaped detection previously is not entirely certain. However, we may note that size-exclusion chromatography of tryptase is usually performed at high NaCl concentration and in the absence of heparin included in the running buffer [21,23,24], whereas according to this study it appears necessary to include the glycosaminoglycan in the running buffer to detect the active lower-molecular-mass compounds. It is interesting to note that in the only previous study where the presence of active monomers was suggested, heparin was indeed included in the gel-filtration running buffer [22].

From the findings presented here it is apparent that the formation of active monomers is promoted at neutral pH and is also strongly favoured by body temperature and low concentrations of heparin. Tryptase is stored in complex with heparin proteoglycan in the mast cell granules. The mast cell secretory granules have an acidic pH (≈ 5.5). In line with the present study, these conditions should prevent the dissociation into active monomers, and tryptase should thus predominantly be in the active tetramer form during storage. This may be important in protecting the mast cell from self-degradation, which otherwise may occur. When the mast cells degranulate, the tryptase tetramer–heparin complexes are released into an environment of higher pH. According to the present findings, the higher pH in combination with body temperature and the dilution of the mast cell heparin that will occur after degranulation may lead to at least partial dissociation of the tetramers into active monomers.

Our findings indicate that the active monomers may be formed by two different pathways. In the experiment when tryptase was incubated together with BPTI under selected conditions (see Figure 3), it was found that residual activities were much lower if BPTI had been present. This indicates that active monomers were formed directly during tetramer dissociation and that BPTI

binds rapidly to the active sites of the newly formed monomeric tryptase. In contrast, when BPTI was not present continuously, but added later, it had no inhibitory effect (Figures 4A and 4B). This indicates that the directly formed active monomers lose activity rapidly. When heparin was used to reactivate tryptase it was found that a major part of the reactivated enzyme was inhibited by BPTI, suggesting that the reactivated tryptase was predominantly monomeric. Thus active monomers can be formed not only directly but also through a pathway involving reactivation of inactive enzyme. In fact, heparin-induced reactivation of inactive tryptase monomers to active monomers was suggested in a previous study, although no direct evidence for this pathway was presented [22]. *In vivo*, reactivation into active monomers could be achieved by the heparin proteoglycan that is secreted by the mast cells during degranulation. Alternatively, reactivation could be accomplished through contact of inactive tryptase monomers with the various proteoglycan species that are present in the connective tissue or on cellular surfaces. In this context it is important to note that tryptase activation may be achieved not only by mast cell heparin, but also by other structurally related polysaccharides [16].

In summary, this report indicates that active monomers can be formed from human tryptase tetramers, and that this occurs under conditions that closely resemble those present *in vivo* after mast cell degranulation. Thus contrary to the current belief, active tryptase may after mast cell degranulation be not exclusively tetrameric but also monomeric. These findings may have important consequences in terms of understanding the physiological role of tryptase, and may be of relevance when designing tryptase inhibitors for treatment of mast cell-related diseases.

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