Human β-Tryptase: Detection and Characterization of the Active Monomer and Prevention of Tetramer Reconstitution by Protease Inhibitors†

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Received March 15, 2004; Revised Manuscript Received June 4, 2004

ABSTRACT: β-Tryptase is a trypsin-like serine protease stored in mast cell secretory granules primarily as an enzymatically active tetramer. The current study aims to determine whether monomeric β-tryptase also can exhibit enzyme activity, as suggested previously. At neutral pH β-tryptase tetramers in the absence of heparin or dextran sulfate spontaneously convert to inactive monomers. Addition of a polyanion to these monomers at neutral pH fails to convert them back to a tetramer or to an enzymatically active state. In contrast, at acidic pH addition of a polyanion resurrects enzyme activity. Whether this activity is associated with tetramers or monomers depends on the concentration of β-tryptase. Under the experimental conditions employed at pH 6 in the presence of heparin, the monomer concentration at which 50% conversion to tetramers occurs is 193 ng/mL. Activity against tripeptide substrates by monomers is detected at pH 6 but not at pH 7.4, whereas tetramer activity is greater at pH 7.4 than pH 6.0. Active monomers are inhibited by soybean trypsin inhibitor, bovine pancreatic trypsin inhibitor, antithrombin III, and α2-macroglobulin, whereas active tetramers are resistant to these inhibitors. Active monomers form complexes with these inhibitors and cleave both antithrombin III and α2-macroglobulin. These inhibitors also prevent reconstitution of monomers to tetramers, indicating that inactive monomers become active monomers before becoming active tetramers. The ability of tryptase monomers to become active at acidic pH raises the possibilities of expanded substrate specificities as well as inhibitor susceptibilities where the low-pH environments associated with inflammation or poor vascularity are encountered in vivo.

β-Tryptase is the principal protease, on a weight basis, that is stored in the secretory granules of human mast cells (*1*). There it resides as an enzymatically active tetramer in a complex with proteoglycan, presumably heparin (*1*−*6*). Heparin and dextran sulfate (DS)[1] polyanionic polymers, in vitro, facilitate autoprocessing of β-protryptase to β-pro′tryptase and conversion of mature β-tryptase monomers to tetramers (*7*) and also stabilize the tetramer by binding to a cationic groove that spans each dimer of the tetramer (*5*, *6*). At neutral pH and a physiologic ionic strength, in the absence of a polyanionic stabilizer active tetramers spontaneously convert to inactive monomers and remain as such even if heparin is added to the monomers (*3*). However, the intermediate forms of β-tryptase during conversion of active tetramer to inactive monomer have been difficult to determine. Experimental data have suggested that both inactive tetramers (*8*) and active monomers (*9*) might form during the transition to inactive monomers and that intermediate forms of tryptase can be converted back to active tetramer at neutral pH by addition of heparin. An alternative explanation is that heparin stabilized the remaining tetramers that otherwise rapidly converted to inactive monomers when diluted into the assay solution. These experimental data became more difficult to interpret when it was discovered that inactive tryptase monomers placed in an acid pH at physiologic ionic strength convert to active tetramers, a process that is facilitated by, but not totally dependent on, heparin (*10*). Further, the fibrinogenolytic activity of the tryptase tetramer was dramatically higher at acidic than at neutral pH (*11*). Reactivation of β-tryptase monomers was prevented by NaCl concentrations ≥0.5 M, which also stabilized previously formed tetramers. During reactivation at acidic pH, the quaternary state of the enzyme was monitored by gel filtration; enzymatic activity was monitored by cleavage of a synthetic tripeptide at neutral pH; and all enzymatic activity was associated with the tetrameric form of the enzyme (*10*). However, other studies have reported active monomers. In one instance, gel filtration fractions of a monomeric peak assayed at neutral pH were active (*9*). However, the gel filtration running buffer contained 0.3 M NaCl and heparin at pH 6.1, raising the question of whether active tetramer may have formed after elution. In another case, based on a prior study with murine tryptase (*12*), human β-tryptase monomers were isolated by gel filtration that was performed with a pH 6.0 buffer containing heparin at physiologic ionic strength (*13*). These putative monomers were shown to be active against a tripeptide substrate in a pH 6.0 buffer containing heparin at physiologic ionic

† This work was supported by Grant AI20487 from the National Institutes of Health.

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; α2M, α2-macroglobulin; ATIII, antithrombin III; TGPK, tosyl-Gly-Pro-Lys-*p*-nitroanilide; IPR, H-D-Ile-Pro-Arg-*p*-nitroanilide; SBTI, soybean trypsin inhibitor; DS, dextran sulfate; DS500, 500000 Da DS; DS10, 10000 Da DS; DS8, 8000 Da DS; DS5, 5000 Da DS.

10.1021/bi049486c CCC: $27.50
Published on Web 07/31/2004

strength. Again the question of reactivation, particularly under the conditions used for assays, needs to be considered. In favor of active monomers was the observation that the associated enzyme activity was completely inhibited by bovine pancreatic trypsin inhibitor (BPTI) (*13*). However, whether BPTI worked as a classical active site inhibitor or prevented reactivation of inactive monomer to active tetramer was not considered.

The current study explores whether β-tryptase monomers can be active and concludes that active monomers can form at acidic pH in the presence of heparin or DS and that biologic protease inhibitors, such as antithrombin III (ATIII) and α2-macroglobulin (α2M), prevent reconstitution of inactive monomers to active tetramers by inhibiting an active monomeric intermediate.

MATERIALS AND METHODS

Reagents. Mes, Hepes, EDTA, BSA, heparin (porcine intestine, MW ~6000), low molecular mass heparin (MW ~3000), tosyl-Gly-Pro-Lys-*p*-nitroanilide (TGPK), 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium, bovine trypsin, DFP, leupeptin, soybean trypsin inhibitor (SBTI), BPTI, human α2M, human ATIII, rabbit anti-α2M Ab, rabbit anti-ATIII Ab, different molecular weights of DS, including 500000 (DS500), 10000 (DS10), 8000 (DS8), and 5000 (DS5) (Sigma Chemical Co., St. Louis, MO), and H-D-Ile-Pro-Arg-*p*-nitroanilide (IPR) (S-2288) (Chromogenix, West Chester, OH) were obtained as indicated. Anti-human tryptase mAbs, B2, B12, G3, and G4, were prepared and used as described (*14*). Human β-tryptase was isolated from human lung using sequential B2-Affi-Gel and heparin−agarose chromatography as described (*15*). Purified β-tryptase (100−200 μg/mL) was stored in 10 mM Mes buffer, pH 6.5, containing 0.8 M NaCl and 20% glycerol at −70 °C.

Measurements of β-Tryptase Activity and Protein. Enzymatic activity of β-tryptase was measured by cleavage of TGPK or IPR. To 1 mL of 0.1 mM TGPK in 0.05 M Hepes buffer, pH 7.4, containing 0.12 M NaCl in a 1 mL plastic cuvette was added 10 μL of enzyme. In some experiments, IPR was used to measure tryptase activity in a solution containing PBS (10 mM phosphate, pH 6.0, containing 137 mM NaCl and 2.68 mM KCl) with 0.2 mM IPR. Heparin (25 μg/mL) was included in these two incubation solutions unless stated otherwise. Released *p*-nitroanilide was monitored at 405 nm by a Cary 3E, UV−visible spectrophotometer (Varian, Walnut Creek, CA) for 10−40 min. β-Tryptase protein concentrations were determined by ELISA using the B12 mAb for capture and the biotinylated G4 mAb for detection in a 96-well plate as described (*16*). Absorbance values were measured in a Spectramax plus micro-plate reader (Molecular Devices, Sunnyvale, CA).

SDS−PAGE and Western Blotting. Samples were mixed with an equal volume of sample buffer containing 2% SDS and 2% β-mercaptoethanol and placed in a boiling water bath for 3 min. SDS−PAGE and electrophoretic blotting to nitrocellulose membranes were performed with a Novex electrophoresis system (Invitrogen, Carlsbad, CA). Blotted membranes were blocked with 5% BSA in PBS containing 0.05% Tween 20 and incubated with primary Ab for 1 h at room temperature. Primary Abs included G3 mAb against tryptase (2 μg/mL), rabbit anti-α2M (1:10000), and rabbit anti-ATIII (1:10000). Depending upon the primary Ab, secondary Abs conjugated to alkaline phosphatase included goat anti-mouse Fcγ (Jackson Immuno Research Lab, West Grove, PA) or goat anti-rabbit IgG (Jackson Immuno Research Lab, West Grove, PA) for 1 h at room temperature. Protein bands were detected by staining with 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium solution.

Gel Filtration Analysis. Gel filtration chromatography was conducted with a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden) using a Shimadzu LC-10Avp HPLC system (Shimadzu Corp., Tokyo, Japan). The column was equilibrated with 0.01 M Mes buffer, pH 6.5, containing 1 M NaCl at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected. Molecular mass calibration markers included Blue dextran (2×10^6 Da), β-amylase (200000 Da), alcohol dehydrogenase (150000 Da), BSA (66000 Da), carbonic anhydrase (29000 Da), and cytochrome *c* (12400 Da).

Formation of Tryptase Monomers and Re-Formation of Tryptase Tetramers from Monomers. Tryptase monomers were made by diluting β-tryptase tetramers to 2−3 μg/mL in 10 mM Hepes buffer, pH 7.4, containing 0.12 M NaCl and 0.5 mg/mL BSA, and incubating the mixture at 37 °C for 90 min. Monomer formation was monitored by loss of enzyme activity (TGPK at pH 7.4) and confirmed by gel filtration. Fractions were analyzed by ELISA. To re-form tetramers from monomers, the pH was lowered to 6.0 with one-fifth volume of 0.5 M Mes buffer, pH 6.0, in the presence of either heparin or DS and incubated at room temperature for 60 min. In some experiments, a protease inhibitor was added to the mixture. Tetramer formation was monitored by enzyme activity and confirmed by gel filtration.

RESULTS

Effect of pH and Temperature on the Inactivation of Active β-Tryptase Tetramers and of Heparin and DS on the Reactivation of Inactive β-Tryptase Monomers. β-Tryptase tetramers convert to inactive monomers when they are placed in normal saline at neutral pH without a stabilizing molecule, such as heparin or DS (*3*, *10*). The stability of the active tryptase tetramer at acidic and neutral pH values and at 22 and 37 °C was examined by incubating the active tetramer in the absence of DS or heparin for the times indicated in Figure 1. Enzyme activity was then measured by cleavage of TGPK at pH 7.4 in the presence of heparin. Enzyme activity declined to negligible levels under each incubation condition. In each case the rate of decline fit an exponential decay equation consistent with first-order kinetics. Notably, the enzyme was 7−20-fold more stable at the lower temperature and 2−4-fold more stable at acidic than neutral pH. No inhibition by SBTI was detected under these conditions.

The optimal pH for reactivation with heparin was 5.5−6.0 and with DS500 was 6.0−6.5 (*10*). These observations were confirmed in the current study by enzyme activity and gel filtration (see below). With heparin, 65% of the original enzyme activity was recovered, while without heparin, only 20% was recovered. The effect of the size of heparin and DS on reactivation was further examined. β-Tryptase monomers (2.5 μg/mL) were incubated with 25 μg/mL of heparin or DS at pH 6.0 for 1 h. Figure 2 shows that DS500 and DS10 produced recoveries of about 80% of the initial enzyme

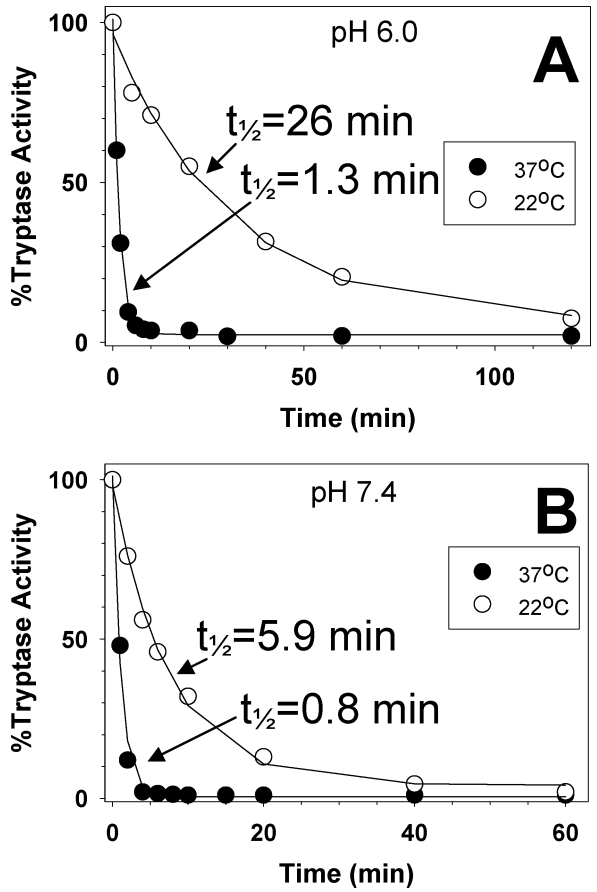

FIGURE 1: Time course for tryptase tetramer inactivation at neutral and acidic pH. Tetrameric tryptase was diluted to 166 ng/mL in 10 mM phosphate buffer, pH 6.0, containing 0.137 M NaCl, 2.68 mM KCl, and 22 μg/mL BSA (A) or in 10 mM Hepes, pH 7.4, containing 0.12 M NaCl and 22 μg/mL BSA (B) and incubated at 22 and 37 °C in the absence of a stabilizing anionic polymer. At different times, enzymatic activity was measured at neutral pH with TGPK in the presence of heparin.

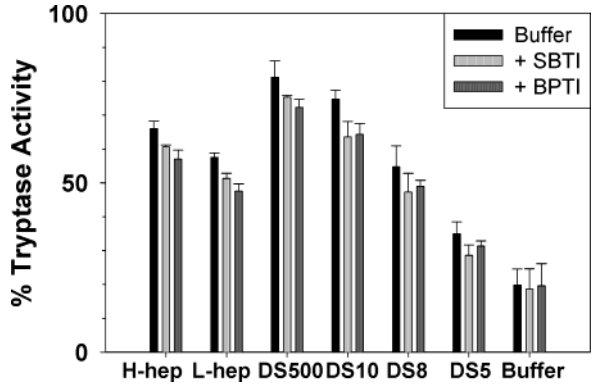

FIGURE 2: Effect of heparin and dextran sulfate on tryptase reactivation. Monomers of tryptase (2.7 μg/mL) that had been formed at pH 7.4 were incubated at pH 6.0 for 1 h at room temperature in the presence of heparin (25 μg/mL), different forms of DS (25 μg/mL), or buffer alone, each mixture also containing BSA (250 μg/mL). Then SBTI (100 μg/mL), BPTI (100 μg/mL), or buffer was added, and each mixture was incubated for 15 min at room temperature. Enzymatic activity was then measured by cleavage of TGPK and reported as a percentage of the initial tetramer activity prior to formation of the monomers.

activity, while 60% was recovered with DS8 and 40% with DS5.

Effect of Protease Inhibitors on the Reactivation Step of β-Tryptase. Neither SBTI nor BPTI (final concentrations of

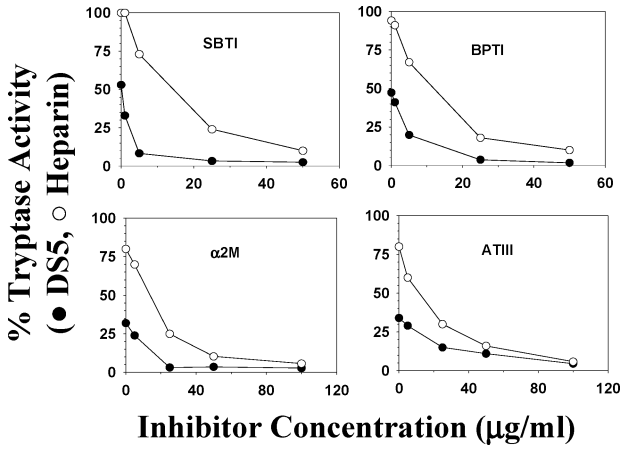

FIGURE 3: Effect of high molecular mass serine protease inhibitors on tryptase reactivation. Tryptase monomers (2.5 μg/mL) formed at pH 7.4 were incubated with different concentrations of various inhibitors in the presence of heparin (25 μg/mL) or DS5 (100 μg/mL) and BSA (250 μg/mL) at pH 6.0 at room temperature for 1 h. Enzyme activity was measured by cleavage of TGPK, and 100% was defined as the activity of the tetramer before formation of monomers. Inhibitors used were SBTI, BPTI, α2M, and ATIII.

100 μg/mL) inhibited reactivated tryptase (Figure 2) under standard conditions, regardless of the size of DS and heparin used for reactivation. Analogous results were obtained with ATIII and α2M. In contrast to the high molecular mass inhibitors that inhibit tryptase reactivation but do not inhibit reactivated or natural tryptase tetramers, certain low molecular mass inhibitors, including leupeptin, PMSF, and DFP (data not shown), inhibit reactivated tryptase tetramers as well as the natural tetramer.

High molecular mass inhibitors of trypsin-like enzymes that fail to inhibit tetrameric β-tryptase were examined for their effects on reactivation of β-tryptase monomers. After active tetramers were converted to inactive monomers at neutral pH as described above, different concentrations of SBTI, BPTI, α2M, or ATIII were added along with heparin or DS5 to monomeric β-tryptase at pH 6. Each mixture was incubated at room temperature for 1 h. As shown in Figure 3, each of these inhibitors prevented reactivation of β-tryptase in a dose-dependent manner in the presence of both heparin and DS5. α2M appeared to be the most efficient inhibitor on a molar basis, followed by ATIII, SBTI, and BPTI.

To determine whether this inhibition was associated with prevention of tetramer formation, mixtures of BPTI or SBTI and inactive tryptase monomers were subjected to reactivation conditions and were then analyzed by gel filtration on Superose 12 equilibrated with 10 mM Mes buffer, pH 6.5, containing 1 M NaCl. Collected fractions were analyzed for tryptase by ELISA. As shown in Figure 4A, in the absence of an inhibitor and in the presence of heparin, as expected, monomers (2.5 μg/mL) converted almost completely to tetramers. If DS5 was substituted for heparin, two peaks were detected, one corresponding to tetramer and another broad peak overlaying a putative monomer peak. Thus, DS5 was not as efficient as heparin at facilitating conversion of monomers to tetramers. When SBTI or BPTI was added to the reactivation mixture, in the presence of DS5 as well as heparin (not shown), a single peak was observed that coincided with tryptase monomers. The peak of tryptase with SBTI corresponded to a slightly higher molecular mass than

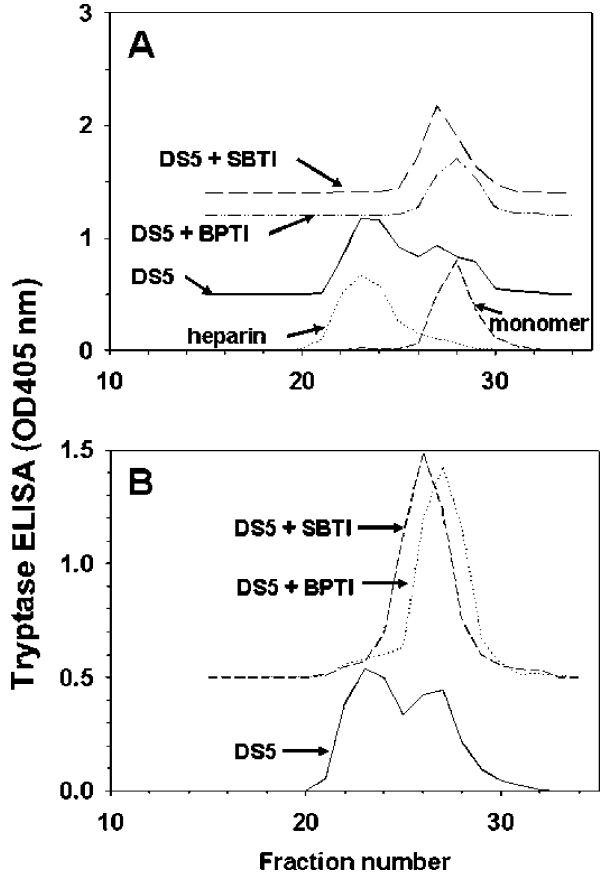

FIGURE 4: Size analysis of reactivated tryptase. In (A), tryptase monomers (2.5 μg/mL) formed at pH 7.4 were assessed directly or after incubation at pH 6.0 for 1 h at room temperature in the presence of heparin (25 μg/mL) or of DS5 (100 μg/mL) or of DS5 together with SBTI (75 μg/mL) or BPTI (75 μg/mL). No enzymatic activity was detected in the SBTI−tryptase and BPTI−tryptase mixtures after the 1 h incubations. In (B), DFP-labeled tryptase monomers (2.5 μg/mL) made from DFP-labeled tryptase tetramers at pH 7.4 were incubated at pH 6.0 for 1 h at room temperature in the presence of DS5 (100 μg/mL) together with buffer alone, SBTI (75 μg/mL), or BPTI (75 μg/mL). Superose 12 chromatography was performed in 10 mM Mes buffer, pH 6.5, containing 1 M NaCl at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected. Tryptase protein content was measured by ELISA.

tryptase with BPTI and tryptase monomer alone, presumably because the complex formed by tryptase monomers (~30000 Da) and SBTI (20100 Da) was measurably larger than with BPTI (6500 Da). Thus, BPTI and SBTI prevent conversion of tryptase monomers to tetramers.

To determine whether tryptase proteolytic activity was required for these inhibitors to prevent tetramer formation, tryptase monomers were first formed from tetramers that had been inactivated with DFP. Interestingly, under reactivation conditions these DFP−tryptase monomers formed tetramers that were enzymatically inactive, tetramer formation again being less efficient with DS5 (Figure 4B) than heparin (not shown). Furthermore, SBTI and BPTI inhibited formation of tetramers from DFP−tryptase monomers (Figure 4B), similar to their effect on free tryptase monomers. As before, SBTI-treated tryptase eluted at a slightly higher apparent molecular mass than BPTI-treated tryptase, indicating that tryptase−SBTI complex formation did not require active enzyme.

Whether tryptase monomers become transiently active when their reactivation is inhibited by ATIII was examined

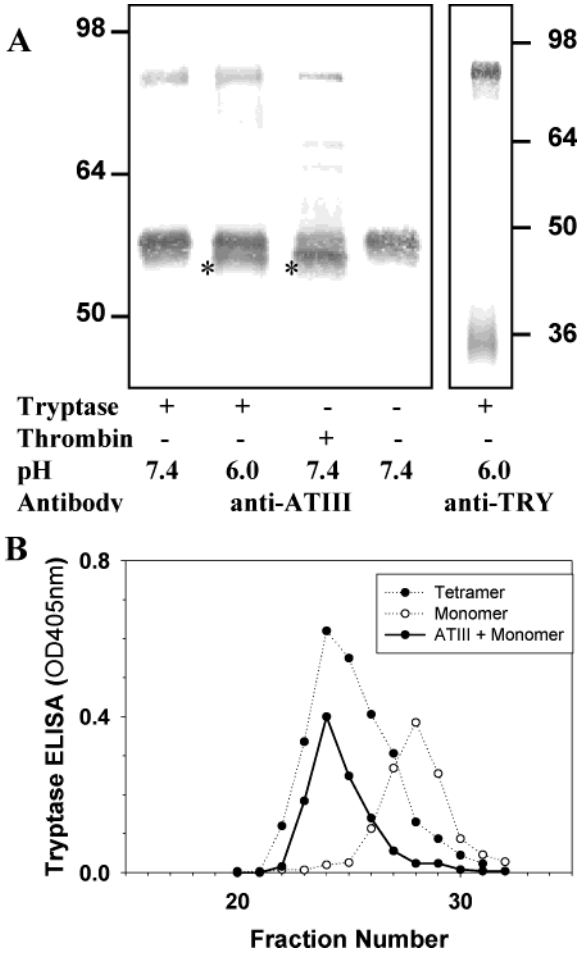

FIGURE 5: Effect of tryptase reactivation on ATIII. (A) Western blots. In the left panel tryptase monomers (2.5 μg/mL) formed at pH 7.4 were incubated at 37 °C for 30 min with ATIII (5 μg/mL) in the presence of heparin (25 μg/mL) at pH 7.4 or at pH 6.0. A positive control experiment used thrombin (4 μg/mL) and ATIII with heparin at pH 7.4. A negative control was performed with ATIII at pH 7.4. Samples were subjected to electrophoresis in an 8% polyacrylamide gel under denaturing and reducing conditions, blotted onto a nitrocellulose membrane, and labeled with rabbit anti-ATIII Ab. Molecular mass markers (kDa) are shown to the left of the gel panel. Asterisks mark the degraded ATIII fragment in lanes 2 and 3. In the right panel tryptase monomers (2.8 μg/mL) were incubated at 37 °C for 30 min with ATIII (40 μg/mL) in the presence of heparin (25 μg/mL) at pH 6.0. The incubation mixture was then subjected to SDS−PAGE under reducing conditions in a 10% polyacrylamide gel and Western blotted using the G3 mAb. Molecular mass markers (kDa) are shown to the right of the gel panel. (B) Gel filtration. Incubation mixtures of tryptase monomers and ATIII prepared at pH 6.0 as in (A) were subjected to Superose 12 chromatography as in Figure 4. Elution patterns of tryptase monomers and tryptase tetramers are also shown. Fractions were analyzed for tryptase by ELISA.

as follows. ATIII was incubated for 30 min at 37 °C with monomeric β-tryptase in a 1:1 molar ratio in the presence of heparin at pH 6.0 and at pH 7.4 and was incubated with thrombin at pH 7.4. Incubation mixtures were then analyzed by Western blotting with anti-ATIII Ab. As shown in Figure 5A (left panel), tryptase monomers, only at pH 6.0, cleaved ATIII, yielding a product that comigrated with the cleavage product produced by thrombin at pH 7.4. Analysis of these incubation mixtures by gel filtration revealed elution of tryptase at an apparent molecular mass near 100000 Da, indicating that tryptase probably formed a complex with its inhibitor (Figure 5B). Examination of these complexes by

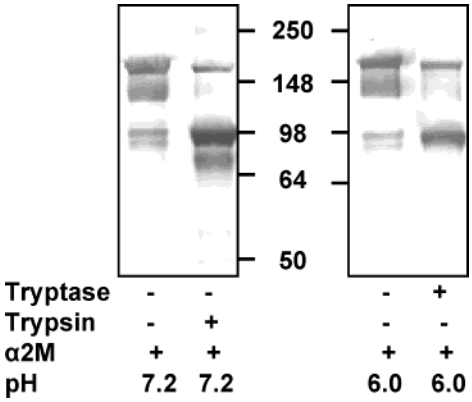

FIGURE 6: Effect of β-tryptase reactivation on α2M. Inactive monomers of tryptase (2.5 μg/mL) formed at pH 7.4 were incubated in the presence of α2M (25 μg/mL) at room temperature for 1 h in Mes buffer containing heparin (25 μg/mL) at pH 6.0. Control experiments were done using α2M (25 μg/mL) incubated alone or with trypsin (2 μg/mL) for 1 h in Hepes buffer containing heparin (25 μg/mL) at pH 7.2. Each lane was loaded with 0.18 μg equivalent of α2M and analyzed by Western blotting using 12% polyacrylamide gels under reducing and denaturing conditions and rabbit anti-α2M Ab for labeling. Lanes: 1, α2M alone at pH 7.2; 2, trypsin and α2M at pH 7.2; 3, α2M alone at pH 6.0; 4, tryptase monomers and α2M at pH 6.0.

anti-tryptase Western blotting revealed a tryptase-positive band near 90000 Da, indicating that a covalent complex between ATIII and tryptase monomer formed under these conditions (Figure 5A, right panel).

Whether active tryptase monomers can be detected by cleavage of α2M during reactivation of inactive β-tryptase monomers also was examined by Western blotting with anti-α2M Ab. As shown in Figure 6, trypsin incubated with α2M at pH 7.2 and tryptase monomers incubated with α2M and heparin at pH 6.0 each yielded a predominant cleavage product with an apparent molecular mass of 98 kDa by SDS−PAGE under reducing conditions, as predicted when the bait region of α2M is cleaved. This cleavage product was not observed when a polyanion was omitted from the mixture of tryptase and α2M and was not observed when the tryptase tetramer was incubated with α2M at either neutral or acidic pH (not shown). Thus, under reactivation conditions tryptase monomers incubated with α2M form covalent complexes with the inhibitor, cleave α2M, and are prevented from forming tetramers.

To analyze complex formation between tryptase (2.5 μg/mL) and α2M (100 μg/mL) during reactivation with DS5 at acidic pH, gel filtration was performed. However, compared to the two peaks of tryptase detected by ELISA in the absence of α2M, negligible tryptase was detected in the presence of α2M (Figure 7). This result suggests that tryptase monomers are trapped within the α2M tetramer and thereby unavailable to bind to the capture and detector mAbs used for the ELISA. A similar result was observed when heparin was substituted for DS5, whereas no decrease in tryptase levels was observed in the absence of a stabilizing polyanion. To further examine α2M-treated tryptase, each column fraction was precipitated with ice-cold 10% TCA, washed with ice-cold acetone, dried, dissolved in SDS sample buffer containing 5% β-mercaptoethanol, and placed in a boiling water bath for 3 min. Samples were then analyzed by Western blotting with the G3 anti-tryptase mAb. Tryptase reactivated in the absence of α2M was detected in the same fractions that were positive by ELISA (corresponding to monomeric and tetrameric tryptase) and exhibited an apparent molecular mass by electrophoresis under denaturing and reducing conditions of 30−40 kDa (not shown). In contrast, tryptase reactivated in the presence of excess α2M and DS5 was detected by Western blotting in column fractions associated with a much higher molecular mass than tryptase monomers or tetramers. Indeed, these are the fractions where α2M elutes. Furthermore, denatured and reduced tryptase in these fractions migrated electrophoretically in bands associated with molecular masses of 140 kDa and higher. These results indicate that, under reactivation conditions, tryptase monomers form covalent complexes with α2M that survive denaturation and reduction.

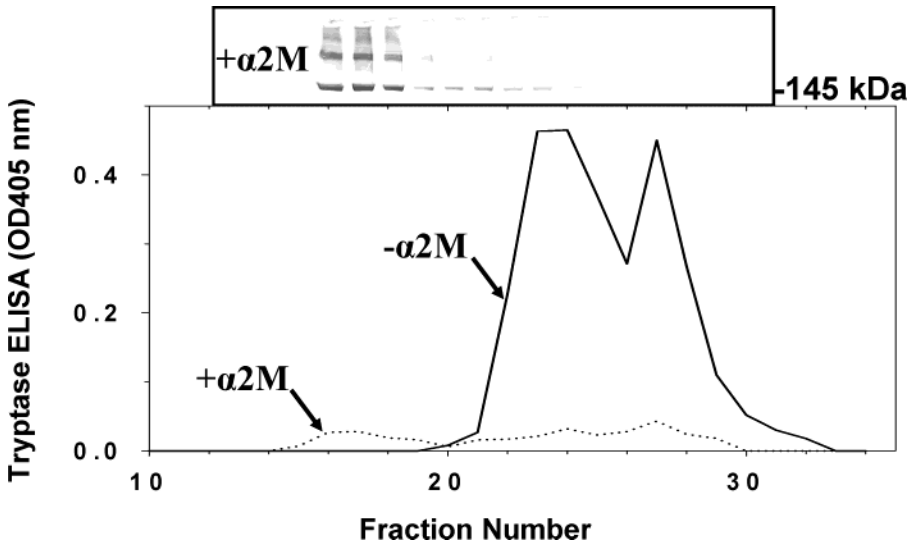

FIGURE 7: Size analysis of reactivated tryptase in the presence of α2M. Tryptase monomers (2.5 μg/mL) formed at pH 7.4 were incubated at pH 6.0 for 1 h at room temperature with DS5 (100 μg/mL) in the absence or presence of α2M (100 μg/mL) and then were subjected to Superose 12 chromatography. Tryptase content in the column fractions was measured by ELISA. Portions of the fractions from the +α2M condition (and the −α2M condition, not shown) were then subjected to electrophoresis on a 12% polyacrylamide gel under reducing and denaturing conditions, blotted onto a nitrocellulose membrane, and labeled with G3 anti-tryptase mAb. Stained band patterns and associated molecular mass markers are placed over their respective column fractions.

Detection of Active Tryptase Monomers at Acidic pH. The data with both α2M and ATIII suggest that inactive tryptase monomers become active monomers before becoming active tetramers. One key biochemical difference between active monomers and tetramers is their susceptibility to inhibition by such protease inhibitors. To analyze the balance of active monomer to active tetramer at pH 6.0, tryptase preparations were incubated at pH 6.0 in PBS to study the concentration (Figure 8A) and time (Figure 8B) dependence. The effect of excess SBTI on IPR cleavage was then measured at pH 6.0 to determine the portions of active monomer (SBTI sensitive) to active tetramer (SBTI resistant). As shown in Figure 8A, the portion of active tryptase that was resistant to inhibition by SBTI increased as the concentration of the monomer increased, indicating that reconstitution of tetramer was more efficient at higher concentrations. Inhibition by SBTI of 20% of the tryptase activity was observed at a calculated tryptase concentration range of 500−600 ng/mL, 50% inhibition at a calculated concentration of 193 ng/mL, and 80% inhibition at a concentration range of 100−150 ng/mL. If 166 ng/mL tryptase tetramers (without polyanion) were incubated as above, total enzyme activity measured at pH 6 with heparin and IPR decreased by 60−70% by about 8 h, and most of this activity became sensitive to SBTI (Figure 8B). To confirm the monomeric character of the active tryptase that was sensitive to SBTI, inactive β-tryptase monomers (83 ng/mL) were incubated at 22 °C for 30 min with heparin or DS5 as in Figure 8A and subjected to Superose 12 chromatograhy (Figure 8C). Nearly all of the tryptase eluted as the monomer. In contrast, incubations of inactive β-tryptase monomers at concentrations higher than 1 μg/mL with heparin or DS5 coincided with the elution pattern of the tryptase tetramer (not shown). Thus, by both gel filtration and susceptibility to inhibtion by SBTI, polyanion-stabilized tryptase at low concentrations was mostly monomeric at acidic pH.

Monomers formed at neutral pH were inactive against TGPK and IPR at pH 7.4 in the presence and absence of heparin, similar to previous results (*3*, *10*). When low concentrations (<100 ng/mL) of inactive β-tryptase monomers were incubated under reactivation conditions, they remained mostly monomeric but exhibited enzymatic activity against IPR and TGPK. In contrast to monomeric tryptase, tetrameric tryptase exhibited greater activity at neutral than acidic pH. Tetramers at both neutral and acidic pH and monomers at acidic pH cleaved IPR 2−3-fold faster than TGPK under comparable assay conditions. With each substrate, the activity of tetramers was about 5-fold higher at pH 7.4 than at pH 6.0. At pH 6.0 with IPR, tryptase monomer activity was ~30% of tetramer activity. Thus, the specific activity of individual tryptase molecules for cleavage of small synthetic substrates is greater for tetramers than monomers, but monomers are only active at acidic pH.

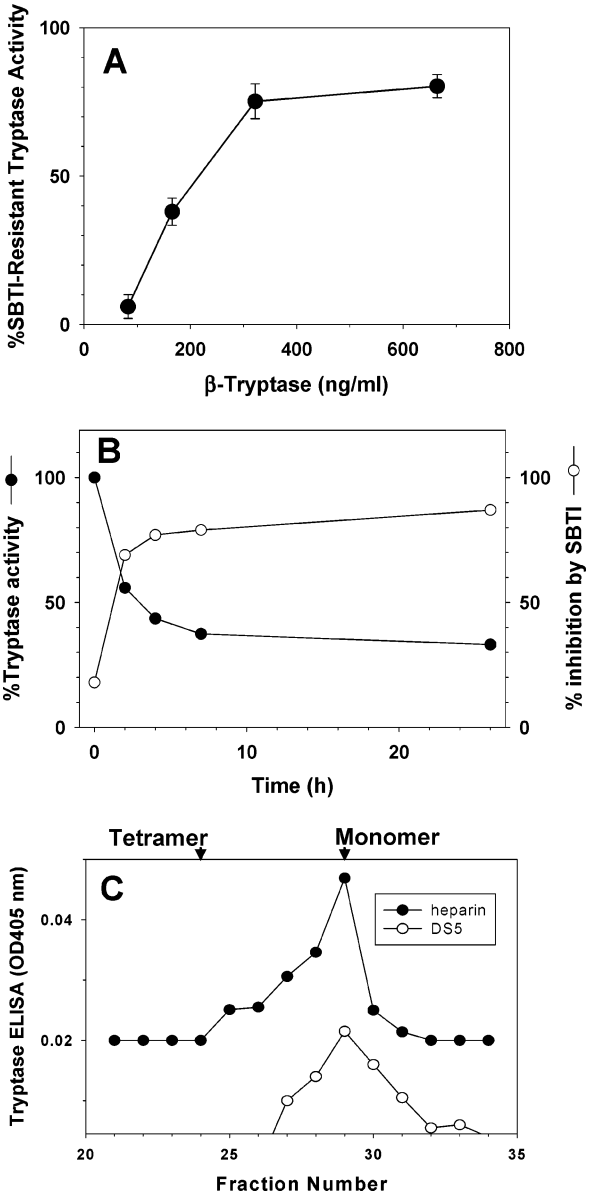

FIGURE 8: Formation and characterization of active tryptase monomers. (A) Effect of tryptase concentration on the formation of tryptase monomer at acidic pH. Different concentrations of tryptase monomers that had been formed at pH 7.4 were incubated at pH 6.0 for 60 min in the presence of heparin (100 μg/mL) at 22 °C. Enzyme activity measurements were then conducted on the incubated tryptase samples with IPR. After 5 min of monitoring activity, SBTI (50 μg/mL) or buffer alone was added, and activity was monitored for another 10 min. In the absence of SBTI, activity was linear for 15 min in each case. The percentage of activity not inhibited by SBTI (reflecting active tetramer) was then calculated at each tryptase concentration. (B) Time course of tryptase monomer formation. Tryptase tetramer (166 ng/mL) was incubated without heparin for the indicated time intervals, and then activity was measured with IPR in the presence of heparin at pH 6.0. The percentages of initial tryptase activity are shown in solid circles, while the percentages of residual tryptase activity that were inhibited by SBTI (reflecting active monomer) are shown in open circles. (C) Size analysis of putative tryptase monomers incubated with heparin or DS5. Tryptase monomers (83 ng/mL) formed at pH 7.4 were incubated with heparin (50 μg/mL) or DS5 (50 μg/mL) at pH 6.0 for 30 min at 22 °C, during which time most of the enzyme activity became sensitive to SBTI, and then were subjected to chromatography on Superose 12 equilibrated with 10 mM Mes buffer, pH 6.3, containing 1 M NaCl. Tryptase content in the fractions was measured by ELISA. Elution positions for tryptase tetramers and monomers are shown for comparison.

DISCUSSION

The current study demonstrates that mature β-tryptase monomers, complexed with heparin or DS, are enzymatically active at acidic pH but are inactive at neutral pH. Four principal findings support this conclusion. First, at concentrations of β-tryptase monomers less than 100 ng/mL, most heparin- or DS-dependent enzymatic activity was associated with monomers by gel filtration chromatography and was

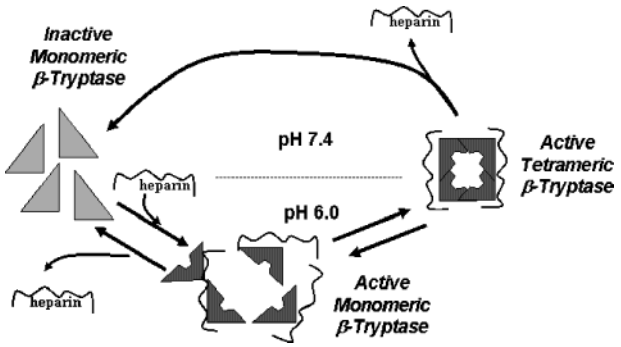

FIGURE 9: Pathway for reactivation of tryptase monomers. Active tetramer is converted to inactive monomers in the absence of heparin at pH 7.4. Inactive monomers become active at pH 6.0 in the presence of heparin, and then, depending in part on their concentration, convert to active tetramers.

detected at acidic but not neutral pH with tripeptide substrates. In contrast, heparin- or DS-stabilized β-tryptase tetramers were active at both pH values with such substrates, but more so at neutral than acidic pH. Second, BPTI, SBTI, ATIII, and α2M inhibited β-tryptase monomer-associated but not tetramer-associated activity, regardless of whether natural tetramers or those created in vitro from monomers were examined. These inhibitors prevented tetramer formation by both native monomers and DFP monomers; i.e., proteolytic activity was not needed for protease inhibitors to block tetramer formation. Because BPTI, SBTI, and ATIII are each apparently too large to fit into the core of the tryptase tetramer, their binding to tryptase monomers presents a physical barrier to tetramer formation. In contrast to our results with human β-tryptase, those reported for mouse mast cell protease-6 indicate BPTI but not SBTI inhibits the active monomer (*12*). Third, monomeric tryptase forms complexes with SBTI and ATIII only in the presence of heparin or DS. The elution patterns of these tryptase complexes shift to a molecular mass that reflects a 1:1 tryptase:inhibitor molar ratio. Fourth, inhibition of heparin- or DS-stabilized monomeric β-tryptase by both ATIII and α2M was associated with their cleavage, similar to what was observed with thrombin and trypsin, respectively. Because tetrameric β-tryptase formation is prevented by these inhibitors, active monomeric tryptase must have been responsible for their cleavage. Also, as illustrated in Figure 9, active monomeric tryptase must be an intermediate in the pathway whereby inactive monomers convert to active tetramers at acidic pH in the presence of heparin.

With heparin at pH 6.0, a high concentration of inactive β-tryptase monomers (3.5 μg/mL) converted predominantly to active tetramers, while a low concentration of these inactive monomers (80 ng/mL) became active monomers. The efficiency of tetramer formation from inactive monomers diminished as the size of either heparin or DS decreased. For example, although DS500 and DS10 showed similar efficiencies, DS8 and DS5 were progressively less efficient. This size dependency is analogous to previous biochemical (*17*) and crystallographic (*5*) data that a heparin length of ≥20 sugars, corresponding to a molecular mass ≥5500 Da, is optimal to stabilize the tetramer. After reactivation of high concentrations of tryptase monomers to active tetramers, no significant inhibition of reactivated β-tryptase by SBTI or BPTI was observed, regardless of the size of the polyanion and efficiency of reactivation. Thus, active monomers that are sensitive to biologic protease inhibitors are short-lived transients during re-formation of the tetramer at these high tryptase concentrations. At low tryptase concentrations, monomers were active only at acidic pH when bound to heparin or DS5 and were sensitive to protease inhibitors such as SBTI, BPTI, ATIII, and α2M.

ATIII (58 kDa) is a single-chain glycoprotein of 432 amino acids that inactivates serine proteases, particularly those involved in blood coagulation, and uses heparin as a cofactor. Its concentration in normal plasma is about 125 μg/mL. Inactivation of protease is based on the formation of a 1:1 complex between the reactive Arg-393 of mature ATIII to the active site serine residue of the protease (*18*). Transient covalent thrombin−ATIII complexes have been reported previously (*19*). A similar 1:1 complex between ATIII and tryptase monomers stabilized with heparin or DS was found in the current study and was associated with cleavage of ATIII. Tryptase tetramers failed to cleave ATIII.

α2M, a protease inhibitor found in blood with a broad specificity for endoproteinases, is composed of four identical 185 kDa subunits. Pairs of these subunits are linked covalently through disulfide bonds, and two of these dimers associate noncovalently to form the α2M tetramer. Generally, cleavage of or binding to the α2M bait region by the targeted protease causes a conformational change in α2M that traps the protease in a cage-like structure (*20*). α2M formed a complex with β-tryptase monomers in the presence of heparin at acidic pH in the current study. These β-tryptase−α2M complexes eluted in the high molecular mass fractions after Superose 12 chromatography. By Western blotting, tryptase in these column fractions appeared to be covalently associated with α2M, because it was detected in high molecular mass bands after being denatured, reduced, and subjected to SDS−PAGE. In the absence of heparin or DS, tryptase incubated with α2M eluted from a Superose 12 column in fractions corresponding to the tryptase monomer. For both α2M and ATIII, complex formation as well as cleavage of the inhibitor by the β-tryptase monomer was dependent on the presence of a stabilizing polyanion. Presumably, the polyanion causes a conformational change in tryptase that opens the active site to inhibitors as well as to potential substrates.

Complexes of tryptase with either BPTI, SBTI, or ATIII were detected by the total tryptase ELISA. In contrast, α2M, when it forms a complex with monomeric β-tryptase, blocks detection of tryptase by this ELISA. For such complexes to form in vivo with released β-tryptase tetramers, the enzyme would need to dissociate from heparin, convert to monomers, find an acidic pH environment, reattach to heparin, and then encounter one of the natural inhibitors of serine proteases. Whether such complexes between either mature tryptases or pro/pro′tryptases and protease inhibitors form naturally in vivo remains to be assessed. If they do, a portion of that tryptase would be masked from detection by ELISA.

Previous reports showed the presence of active human (*13*) and mouse (mMCP6) (*12*) tryptase monomers by gel filtration at pH.6.0 in the presence of 10 μM heparin. The concentration of human tryptase at which active monomers formed was not precisely determined (*13*). BPTI could inhibit monomer activity, while SBTI, tested only on mMPC-6, did not inhibit the monomer. However, because heparin was present, conversion of the inactive monomer to the active

tetramer at this stage was a possibility that was not excluded. Whether BPTI was simply blocking conversion of monomer to tetramer also was not considered. Finally, potential biologic inhibitors of tryptase were not examined in these studies. The current study extends the findings of these earlier reports to unambiguously show that active tryptase monomers exist, to further characterize their formation and properties, and to show their susceptibility to protease inhibitors relevant to their existence in vivo. Further, reactivation of inactive tryptase monomers at acidic pH proceeds through an active monomer intermediate.

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BI049486C