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The B12 Anti-Tryptase Monoclonal Antibody Disrupts the Tetrameric Structure of Heparin-Stabilized β-Tryptase to Form Monomers That Are Inactive at Neutral pH and Active at Acidic pH

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The novel tetrameric structure of human β-tryptase faces each active site into the central pore, thereby restricting access of most biologic protease inhibitors. The mechanism by which the anti-tryptase mAb B12 inhibits human β-tryptase peptidase and proteolytic activities at neutral pH, but augments proteolytic activity at acidic pH, was examined. At neutral pH, B12-β-tryptase complexes are inactive. At acidic pH, B12 (intact and Fab) minimally affects peptidase activity when added to β-tryptase tetramers, but does induce susceptibility to inhibition by soybean trypsin inhibitor and antithrombin III. Surprisingly, B12 Fab-β-tryptase complexes formed at both neutral and acidic pH exhibit the apparent molecular mass of a complex with 1 β-tryptase monomer and 1 Fab by gel filtration. B12 does not compete with heparin for binding to tryptase at either neutral or acidic pH. Thus, B12 directly disrupts β-tryptase tetramers to monomers that are inactive at neutral pH, whereas at acidic pH, are active and more accessible to protein inhibitors and substrates. The Journal of Immunology, 2006, 176: 3165–3172.
of active monomers by potential protein substrates and protease inhibitors is enhanced.

Materials and Methods
Reagents and Abs
MES, HEPES, BSA, heparin from porcine intestinal mucosa, low-molecular-weight heparin from porcine intestinal mucosal (3 kDa), 500-kDa DS, 5-kDa DS, chromogenic substrate TGPK, BPTI, SBTI, human ATIII, human αM, human fibrinogen, mouse IgG1 (MOPC 31C), 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/NBT tablets, p-nitrophenyl phosphate tablets, and goat anti-human fibrinogen (Sigma-Aldrich); alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific), and alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories); alkaline phosphatase-conjugated streptavidin (Roche Molecular Biochemicals); chromogenic substrate S-2288 IPR (H-D-Ile-Pro-Arg-p-nitroanilide); Chromogenx); and protein A-agarose. Using immobilized papain digestion and were purified by protein A-agarose chromatography as described (23). 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. Anti-human tryptase mAbs B2, B12, G3, and G4 (all of these Abs are IgG1 isotype) were prepared as described (23). Fab of mAbs B2, B12, and G4 were prepared using immobilized papain digestion and were purified by protein A-agarose column (Pierce). The apparent molecular mass of the Fab was 50 kDa by nondenaturing SDS-PAGE.

Measurements of β-tryptase activity and protein
Enzymatic activity of β-tryptase was measured by cleavage of TGPK or IPR. One milliliter of 0.1 mM TGPK in 0.05 M HEPES buffer (pH 7.4), containing 0.12 M NaCl, was placed into a 1-ml plastic cuvette. Measurements were initiated by adding up to 40 μl of polyanion-stabilized β-tryptase at room temperature. Released p-nitroanilide was monitored at 405 nm by a Cary 3E UV-Visible spectrophotometer (Varian) for up to 30 min. Initial cleavage rates were determined over a time interval during which <10% of the substrate had been cleaved. Activity measurements at pH 6.0 were performed with IPR. Polyanion-stabilized β-tryptase was preincubated with intact or Fab Ab before the incubation mixture was transferred to 0.9 ml of PBS (10 mM phosphate buffer, pH 6.0, 10 μl of each inhibitor solution was added to the cuvette containing β-tryptase-Ab complexes 5 min after IPR had been added, and the enzymatic reaction was monitored an additional 25 min. The percentage of inhibition of β-tryptase activity was calculated by setting the activity after adding 10 μl of a buffer control to 100%. β-Trypsin protein levels were determined by ELISA using B12 mAb for capture, biotinylated G4 mAb for detection and alkaline phosphatase-conjugated streptavidin (1/2000 dilution) and p-nitrophenyl phosphate solution for color development (24).

Formation of tryptase monomers
Tryptase monomers were formed by diluting purified β-tryptase to 3.5 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. Addition of BSA (0.5 mg/ml) prevented loss of β-tryptase at the low concentrations present during this incubation, but was omitted from certain gel-filtration experiments in which 280 nm absorbance values were continuously monitored. The 3.5 μg/ml concentration of β-tryptase was the highest at which essentially all tetramers converted to monomers under these buffer and temperature conditions. The generated β-tryptase monomers had no TGPK activity at pH 7.4 in the presence of heparin and exhibited an apparent molecular mass of ~30 kDa by Superose 12 chromatography in 10 mM MES buffer (pH 6.5), containing 1 M NaCl (detected by ELISA). To form active monomers, inactive monomers (3.5 μg/ml) were dialyzed, typically to ~70 ng/ml, in PBS (pH 6.0) with heparin (50 μg/ml), and incubated at room temperature for 30 min. Enzyme activity was measured using IPR at pH 6.0.

Gel filtration analysis
Gel filtration was performed with a Superose 12 HR 10/30 column (Pharmacia Biotech) using a Shimadzu LC-10Avp HPLC system at a flow rate of 1 ml/min. The column was equilibrated with the 10 mM MES running buffer (pH 6.5), containing 1 M NaCl or 10 mM HEPES buffer (pH 7.4), containing 1 M NaCl. High concentrations of NaCl (>0.5 M) prevent conversions of tetramer to monomer and monomer to tetramer (18). Protein concentration was monitored by recording the absorbance at 280 nm using a PerkinElmer LC-95 UV/VIS Spectrophotometer Detector. When BSA was included in the running buffer, fractions of 0.5 ml were collected for analysis of tryptase by ELISA or by Western blotting. Molecular mass markers for gel filtration included: blue dextran (200,000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylose (200 kDa), alcohol dehydrogenase (150 kDa), β-tryptase tetramer (~136 kDa), BSA (66 kDa), B12 Fab (50 kDa), β-tryptase monomer (~34 kDa), and carbonic anhydrase (29 kDa) (Sigma-Aldrich). The molecular mass for lung-derived β-tryptase is based upon its diffuse banding pattern after SDS-PAGE, which results from the impact of glycosylation on its 27.5 kDa peptide sequence. Apparent molecular mass values for different forms of β-tryptase and β-tryptase Ab complexes were calculated from the linear relationship of the logmolecular mass to the elution time.

SDS-PAGE and Western blotting
To examine β-tryptase from gel filtration fractions by Western blotting, samples were first precipitated in ice-cold 10% TCA with BSA (50 μg/ml) as a carrier protein, washed with ice-cold acetone, and dissolved and boiled for 3 min in SDS sample buffer containing 1% 2-ME. SDS-PAGE was performed in a 12% polyacrylamide gel (Inviron Life Technologies). Proteins were transferred to a nitrocellulose membrane using a Novex electrophoresis system (Inviron Life Technologies) for 1 h at 50 V. After blocking with PBS (pH 7.4), containing 5% BSA and 0.05% Tween 20 for 1 h, the membranes were incubated with G3 anti-human tryptase mAb (2 μg/ml) for 1 h at room temperature followed by incubations with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific), and alkaline phosphatase-conjugated rabbit anti-goat IgG (1/1000 dilution) for 1 h and then with BCIP/NBT solution (Sigma-Aldrich) for color development.

Results
Effect of B12 mAb on enzymatic activity of β-tryptase tetramers and monomers
Among our anti-tryptase mAbs, B12 uniquely inhibits β-tryptase activity at pH 7.4 against both TGPK and fibrinogen (22). In contrast at pH 6.0, B12 had a negligible effect on cleavage of TGPK, while cleavage of fibrinogen was enhanced. To begin to understand whether both Ag binding sites of the intact B12 mAb were needed for these effects, inhibition of β-tryptase peptidase activity by intact and the Fab B12 were compared at pH 7.4 and 6.0 (Fig. 1). As shown in Fig. 1A, both B12 IgG and Fab could completely inhibit the activity of tetrameric β-tryptase at pH 7.4. Intact and Fab forms of B12 were almost equipotent, the ID50 values occurring at Ab (Ag binding sites) to β-tryptase (subunit) molar ratios of 1:1 and 0.9, respectively. On the contrary, no inhibition of tetramer activity was detected at pH 6.0 with intact B12, whereas slight inhibition of ~20% was observed with the Fab (Fig. 1B). As controls, no inhibition of β-tryptase activity at neutral or acidic pH was observed with either intact or the Fab of another anti-tryptase mAb, B2, and of the nonimmune isotype-matched control mAb, MOPC 31C (mouse IgG1, data not shown). As shown in Fig. 1C, active β-tryptase monomers were made and tested at pH 6.0 for their response to B12, but no inhibition of peptide cleavage activity by either intact or Fab B12 was observed. β-Tryptase monomers are inactive at neutral pH, regardless of the presence of a polyanion like heparin (21). Thus, bivalent binding of intact B12 was not critical for the inhibition of the peptide-cleaving activity of tetrameric β-tryptase at neutral pH.
Both intact and Fab B12 made intact or Fab B12 mAb. As shown in Fig. 2, increasing amounts of different concentrations of intact or Fab forms of B12 mAb for 30 min at (50/1H9262). Although tetramers stabilized with heparin or DS at acidic pH and monomers.

Effect of protease inhibitors on B12 mAb-treated (50/1H9252). ml of PBS (pH 6.0), in a cuvette and assessed for IPR cleavage activity.

Materials and Methods

B mixture (40/11025) of the mixture were added to 0.9 ml of PBS (pH 6.0), with heparin (50/11025) in a cuvette and incubated for another 30 min at room temperature to produce active monomers, and assessed for IPR cleavage activity.

Effect of protease inhibitors on B12 mAb-treated β-tryptase tetramers stabilized with heparin or DS at acidic pH

Although β-tryptase tetramers are resistant to protease inhibitors such as SBTI, BPTI, and ATIII at both neutral and acidic pH, active tryptase monomers at acidic pH are sensitive (21). To examine whether B12 alters the protective conformation of the tetramer at acidic pH, the susceptibility of polyanion-stabilized β-tryptase tetramers to SBTI was examined in the presence of intact or Fab forms of B12 mAb. After incubation for 30 min at room temperature, 40 μl of the mixture were added to 0.9 ml of PBS (pH 6.0), with heparin (50/11025) in a cuvette and incubated for another 30 min at room temperature to produce active monomers, and assessed for IPR cleavage activity.

Analysis of the quaternary structure of β-tryptase treated with B12 mAb

For B12 to make β-tryptase accessible to SBTI and ATIII, a conformational change of β-tryptase must occur. Possibilities to consider include formation of an "open" tetramer, monomer, or intermediate quaternary structures. To examine these possibilities, complexes were formed between tetramers or monomers of β-tryptase and the Fab s of B12, B2, or G4 and assessed for their apparent molecular masses by gel filtration. With BSA in the running buffer, β-tryptase tetramer and monomer peaks were detected by ELISA in fractions 24 (~134 kDa) and 28 (~34 kDa), respectively. The B12 Fab (50 kDa) peak was detected in fraction 27 by Western blotting using rabbit anti-mouse IgG F(ab')2. When β-tryptase monomers were incubated with excess B12 Fab at pH 7.4, peak values for the complex were detected by Western blotting with G3 anti-tryptase mAb in fractions 24 and 25 (Fig. 4A), indicating a molecular mass that was less than that of the β-tryptase tetramer but greater than that of B12 Fab. Surprisingly, heparin-stabilized β-tryptase tetramers incubated with B12 Fab at pH 7.4 (Fig. 4B) or at pH 6.0 (data not shown) also were detected in these same fractions. In contrast to B12 Fab, β-tryptase tetramers treated with B2 Fab (Fig. 4C) or with G4 Fab (data not shown) caused it to elute in fractions 21 and 22, indicating a molecular mass substantially larger than that of the β-tryptase tetramer, consistent with four Fab molecules binding to an intact β-tryptase tetramer.

To more precisely assess the apparent molecular mass of Fab-β-tryptase complexes, elution patterns from a Superose 12 gel filtration column were monitored continuously at 280 nm (in the absence of BSA). G4 mAb was used as a control mAb that binds well to both monomeric and tetrameric forms of β-tryptase and does not appear to modify or inhibit β-tryptase activity. As shown in Fig. 5A, G4 Fab forms a complex with tetrameric β-tryptase that

FIGURE 1. Effect of B12 mAb on the activity of β-tryptase tetramers and monomers. A, β-tryptase tetramers (3.5 μg/ml) stabilized with heparin (50 μg/ml) in 50 mM HEPEs buffer (pH 7.4), containing 0.12 M NaCl, were incubated with equal volumes of different concentrations of intact or Fab forms of B12 mAb for 30 min at room temperature. The incubation mixture (40 μl, 70 ng of β-tryptase) was assayed for TCKP cleavage activity at pH 7.4. B, β-tryptase tetramers (3.5 μg/ml) stabilized with heparin (50 μg/ml) in PBS (pH 6.0), were incubated with equal volumes of different concentrations of intact or Fab forms of B12 mAb for 30 min at room temperature. Mixtures (40 μl, 70 ng of β-tryptase) were added to 0.9 ml of PBS (pH 6.0), in a cuvette and assessed for IPR cleavage activity. C, β-tryptase monomers (3.5 μg/ml) were made at pH 7.4 as described in the Materials and Methods and mixed with an equal volume of different concentrations of intact or Fab forms of B12 mAb. After incubation for 30 min at room temperature, 40 μl of the mixture were added to 0.9 ml of PBS (pH 6.0), with heparin (50 μg/ml) in a cuvette and incubated for another 30 min at room temperature to produce active monomers, and assessed for IPR cleavage activity.

Effect of protease inhibitors on B12 mAb-treated β-tryptase tetramers stabilized with heparin or DS at acidic pH

Although β-tryptase tetramers are resistant to protease inhibitors such as SBTI, BPTI, and ATIII at both neutral and acidic pH, active tryptase monomers at acidic pH are sensitive (21). To examine whether B12 alters the protective conformation of the tetramer at acidic pH, the susceptibility of polyanion-stabilized β-tryptase tetramers to SBTI was examined in the presence of intact or Fab B12 mAb. As shown in Fig. 2, increasing amounts of both intact and Fab B12 made β-tryptase susceptible to inhibition by SBTI irrespective of different stabilizing polyanions. This surprising effect first became apparent at a molar ratio of intact or Fab B12 to tryptase at unity. The IC50 molar ratio was between 2:1 and 1:1 in each case. Minor differences were apparent between the different polyanions. For example, the activity of the 500-kDa DS-stabilized β-tryptase was enhanced by ~47% with intact B12 (8:1 molar ratio) and by ~41% with Fab B12 (4:1 molar ratio). The activity of low-molecular-weight heparin-stabilized β-tryptase diminished by 32% with intact B12 (8:1 molar ratio) and by 40% with Fab B12 (4:1 molar ratio). The activity of heparin-stabilized β-tryptase decreased by 24% with Fab B12 (4:1 molar ratio). β-Tryptase activity was not appreciably altered by intact B12 when stabilized with 5-kDa DS or heparin, or by Fab B12 when stabilized by 5-kDa DS. Thus, univalent binding of B12 to β-tryptase tetramers at acidic pH increases accessibility of SBTI to the active sites.

To examine whether other high molecular weight inhibitors would behave in a similar manner, B12-treated tetramers were exposed to ATIII and α3M. As shown in Fig. 3, the enzymatic activity of heparin-stabilized β-tryptase tetramers treated with B12 Fab was inhibited by SBTI and ATIII, but not by α3M. In contrast, α3M effectively inhibited the reconstitution of β-tryptase tetramers from monomers, indicating its potency. When the activity of trypsin ensnared by α3M (α3M-(trypsin)2) was examined for inhibition by SBTI and BPTI, negligible inhibition was observed by SBTI (20.1 kDa), whereas marked inhibition occurred with the smaller BPTI (6.5 kDa). However, β-tryptase activity present in a mixture of heparin-stabilized β-tryptase, B12 Fab and α3M were readily inhibited by SBTI (data not shown), indicating that the β-tryptase-B12 Fab complex was not entrapped by α3M. Neither B2 anti-tryptase mAb (which binds to β-tryptase without inhibiting its activity at neutral and acidic pH) nor MOPC 31C (mouse IgG1) mAb rendered β-tryptase susceptible to these inhibitors (Fig. 3).
elutes with a calculated molecular mass of 297 kDa (10.6 min), consistent with the data in Fig. 4C. The complex formed between G4 Fab and 
β-tryptase monomers eluted with a calculated molecular mass of 88 kDa (12.9 min) (Fig. 5B). Protein elution patterns of G4 Fab (50 kDa), 
β-tryptase tetramers (∼134 kDa), and 
β-tryptase monomers (∼34 kDa) are presented in Fig. 5, C–E. 

Contrary to these expected elution patterns for G4 Fab-β-tryptase complexes, B12 Fab-β-tryptase complexes formed with either β-tryptase monomers (Fig. 6A) or tetramers (Fig. 6B) at pH 7.4 or with tetramers at pH 6.0 (Fig. 6C) eluted with an 
calculated molecular mass of 96 kDa (12.7 min), consistent with the Western blotting data shown in Fig. 4, A and B. To analyze the stoichiometry of the B12 Fab-β-tryptase complex, different molar ratios of B12 Fab to 
β-tryptase (calculated based on 
β-tryptase monomers) were incubated and assessed by gel filtration (Fig. 7). With a molar ratio of 0.5, most of the 
β-tryptase appeared to migrate as tetramers (Fig. 7A). No protein eluted at a higher apparent molecular mass; a small portion migrated at a lower apparent molecular mass; but no free Fab peak was detected. At a molar ratio of 1:1, the major protein peak at 12.7 min, between those of the 
β-tryptase tetramer and Fab, corresponded to a molecular mass of 96 kDa (Fig. 7B). At molar ratios of 2:1 and 4:1 (Fig. 7, C and D, respectively), essentially all of the protein appeared in two peaks, one of which corresponded to B12 Fab (50 kDa, 14 min) and the other to the B12 Fab-β-tryptase complex (12.7 min) previously noted.

Effect of B12 mAb on the interaction of 
β-tryptase with heparin

The interaction of human 
β-tryptase with heparin is weaker at pH 7.4 than at pH 6.0 (25, 26). Whether B12 mAb could interfere with heparin binding at neutral but not acidic pH, and thereby indirectly inactivate the enzyme was examined. 
β-Tryptase tetramer and monomer and B12-treated 
β-tryptase were subjected to chromatography on heparin-Sepharose. As shown in Fig. 8, all samples of 
β-tryptase-B12 Fab bound to heparin when loaded in PBS at either pH 7.4 or pH 6.0. When eluted with a linear gradient of NaCl, B12 Fab-β-tryptase eluted at 0.25–0.4 M NaCl (column fractions 7 and 8) at both pH values. This result is comparable to the salt concentration that elutes 
β-tryptase monomers at neutral pH, and is considerably less than the salt concentration at which 
β-tryptase tetramers elute at pH 7.4 (0.7–0.8 M NaCl, column fractions 10 and 11) and pH 6.0 (∼0.8 M NaCl, data not shown). Thus, B12 Fab-β-tryptase complexes bind to heparin at both neutral and acidic pH conditions in PBS, and separate from heparin at NaCl concentrations comparable to those for Fab-free 
β-tryptase. It seems unlikely that B12 inhibits 
β-tryptase by blocking the binding of heparin.
To Western blotting with G3 anti-tryptase mAb as in TCA, washed with acetone, dissolved in SDS sample buffer, and subjected proteins in column fractions (0.5 ml/tube) were precipitated with 10% TCA, treated with a 4-fold molar excess of B12 Fab as described in Materials and Methods, and applied to a Superose 12 column equilibrated with 10 mM HEPES running buffer (pH 7.4), containing 1 M NaCl. B, β-Tryptase monomers (1 µg) incubated with 6.7 µg of B2 Fab were subjected to Superose 12 chromatography as described. C, Elution pattern of incubated with 6.7 µg of B4 Fab were subjected to Superose 12 chromatography as described. D, Elution pattern of each mixture showing a retention time of 11.9 min. E, Elution pattern of β-Tryptase monomers (1 µg) showed a retention time of 14.8 min. Peak OD values for each major peak were 4.4 × 10⁻³, 4.9 × 10⁻³, 5.7 × 10⁻³, 1.4 × 10⁻³, and 0.4 × 10⁻³ (A–E, respectively). Retention times of various standards were as follows: thyroglobulin (8.9 min, 669 kDa), apoferritin (10.7 min, 443 kDa), β-amylase (11.6 min, 200 kDa), alcohol dehydrogenase (12.3 min, 150 kDa), BSA (13.0 min, 66 kDa), and carbonic anhydrase (14.7 min, 29 kDa).

Effect of B12 mAb and protease inhibitors on fibrinogenolysis by β-tryptase

The ability of β-tryptase to cleave fibrinogen is higher at acidic than at neutral pH; and B12 inhibits fibrinogenolysis at neutral pH and augments this activity at acidic pH (22). As shown in Fig. 9, B12-β-tryptase tetramer (500-kDa DS stabilized) and monomer complexes cleave fibrinogen at acidic pH (Fig. 9, lanes 4 and 9, respectively), and this activity is inhibited by SBTI (Fig. 9, lanes 3 and 8), and ATIII (Fig. 9, lanes 1 and 6), but not by αM (Fig. 9, lanes 2 and 7). Tryptase tetramers in the absence of B12 were not inhibited by any of these protease inhibitors (data not shown).

Analysis of tetrameric and monomeric β-tryptase preparations. A–C, β-Tryptase tetramers (1 µg) incubated with 1.5 µg of heparin were incubated with 6.7 µg of 4-fold molar excess in 250 µl of PBS (pH 7.4), at room temperature for 1 h and applied to a Superose 12 column equilibrated with 10 mM HEPES running buffer (pH 7.4), containing 1 M NaCl. B, β-Tryptase monomers (1 µg) incubated with 6.7 µg of 4-fold molar excess in 250 µl of PBS (pH 7.4), at room temperature for 1 h and applied to a Superose 12 column equilibrated with 10 mM HEPES running buffer (pH 7.4), containing 1 M NaCl. B, β-Tryptase monomers (1 µg) incubated with 6.7 µg of B4 Fab were subjected to Superose 12 chromatography as described. C, Elution pattern of incubated with 6.7 µg of B4 Fab (6.7 µg) showed a retention time of 14.6 min. D, Elution pattern of β-Tryptase tetramers (1 µg) showed a retention time of 11.9 min. E, Elution pattern of β-Tryptase monomers (1 µg) showed a retention time of 14.8 min. Peak OD values for each major peak were 4.4 × 10⁻³, 4.9 × 10⁻³, 5.7 × 10⁻³, 1.4 × 10⁻³, and 0.4 × 10⁻³ (A–E, respectively). Retention times of various standards were as follows: thyroglobulin (8.9 min, 669 kDa), apoferritin (10.7 min, 443 kDa), β-amylase (11.6 min, 200 kDa), alcohol dehydrogenase (12.3 min, 150 kDa), BSA (13.0 min, 66 kDa), and carbonic anhydrase (14.7 min, 29 kDa).

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Interaction of B12-treated tryptase with heparin-Sepharose.

However, fibrinogenolysis by β-tryptase monomers in the absence of B12 was inhibited by each of these three protease inhibitors (Fig. 9, lanes 10–12). Generation of the D-fragment of fibrinogen was only seen in the presence of B12 (Fig. 9, lanes 2, 4, 7, and 9, asterisks). The apparent monomeric conformation of β-tryptase induced by B12 is inactive at neutral pH. In contrast, at acidic pH this conformation is highly active against fibrinogen, but is susceptible to inhibition by SBTI and ATIII while resistant to inhibition by α2M.

Discussion

The B12 mAb has been previously shown to inhibit β-tryptase-catalyzed cleavage of the peptide TGPK at neutral pH in a non-competitive manner (22). The current study showed that this inhibitory activity was exhibited by both bivalent B12 IgG and univalent B12 Fab, with each having similar IC50 values. Thus, cooperative binding of intact B12 to the tryptase tetramer is probably not involved in the mechanism of inhibition. Nevertheless, the noncompetitive kinetic pattern noted previously suggests B12 might induce a conformational change in tryptase. In contrast to the inhibition observed at neutral pH, at acidic pH intact and Fab forms of B12 had negligible effects on the peptidase activities of enzymatically active tetrameric and monomeric forms of β-tryptase. This finding cannot be explained by lack of Ab binding because tryptase binds to B12-agarose under the pH and ionic strength conditions used (data not shown).

Evidence for a B12-induced conformational change in β-tryptase at acidic pH was first suggested by the observation that both Fab and intact forms of B12 made tetrameric β-tryptase susceptible to inhibition by SBTI and ATIII. Both intact and Fab forms of B12 increased susceptibility to SBTI at a similar Ab to tryptase molar ratio, regardless whether low- or high-molecular-weight DS or heparin was used to stabilize enzyme activity. One type of B12-induced conformational change to explain these findings would be a repositioning of each subunit within the tetramer such that the small pore of the tetramer no longer constrains access to the active sites. Such a change implies the formation of new associations between the subunits, and is highly speculative. Another possibility would be disruption of the tetrameric conformation and formation of trimers, dimers, or monomers. This latter possibility could easily accommodate the inhibitor data. Disruption of the tetrameric structure would remove the major barrier toward access of SBTI to the active sites. However, neither IPR cleavage activity nor fibrinogenolysis was inhibited by α2M. This might be explained by the B12-β-tryptase complexes being too large to be enveloped by α2M. In support of this possibility was the observation that SBTI as well as BPTI effectively inhibited the tryptase activity found in a mixture of β-tryptase, B12 Fab, and α2M. In contrast, trypsin activity of α2M-(trypsin)3 complexes was inhibited much better by the 6.5 kDa BPTI than the 20.1 kDa SBTI, consistent with α2M, which envelopes trypsin, acting as a sieve that
produces a more effective barrier against the larger inhibitor (27, 28).

Superose 12 chromatography was used to assess the quaternary structure of B12, B2, and G4 Fab-tryptase complexes. Both B2 and G4 mAb bind to tetrameric tryptase at acidic and neutral pH, but do not inhibit enzyme activity and do not make β-tryptase susceptible to inhibition by SBTI. The molecular mass of β-tryptase tetramers mixed with B2 or G4 Fabs was calculated to be ~295 kDa, which is most consistent with the predicted molecular mass of ~336 kDa. The molecular mass calculated for the elution position of β-tryptase monomers that had combined with G4 mAb was 88 kDa, which most closely approximates the predicted molecular mass of 84 kDa for one β-tryptase monomer bound to one Fab. In contrast, complexes formed between B12 Fab (50 kDa) and either monomeric (~34 kDa) or tetrameric (~134 kDa) forms of β-tryptase, regardless whether the running solution was buffered at neutral or acidic pH, eluted at the same position, a 12.7 min retention time with a calculated molecular mass of 96 kDa. This result corresponds best to a β-tryptase monomer-B12 Fab complex. Thus, it is likely that B12 inhibits β-tryptase at neutral pH by disrupting the tetramer into inactive monomers, as illustrated in Fig. 10. At acidic pH, this β-tryptase monomer-B12 Ab complex exhibits enzymatic activity that is dependent upon the presence of a polyanion such as heparin or DS, consistent with the previously reported behavior of free β-tryptase monomer at acidic pH (21).

The possibility that B12 acts on β-tryptase by attenuating the binding of heparin also was considered. Because heparin appears to bind to β-tryptase more strongly at acidic than neutral pH (25, 26), attenuation of heparin binding might be of greater consequence at neutral than acidic pH. However, B12 did not appear to alter the binding or elution of β-tryptase to heparin-Sepharose at either neutral or acidic pH. In each case, β-tryptase eluted at a salt concentration comparable to that observed with B12-free β-tryptase monomers at neutral pH. Thus, B12 does not appear to act on β-tryptase by attenuating heparin binding and stabilization.

The crystallographic structure of β-tryptase tetramer was previously solved (11). The four monomers were designated subunit A, B, C, and D and positioned at the corners of a rectangular planar frame, surrounding a central pore. Subunit A contacts subunits B and D through two different interaction surfaces of 500 and 1100 Å², respectively. In this model, the orientations of subunit A are equivalent to that of subunit C, whereas those of subunits B and D are equivalent to one another. The A:B and C:D subunit pairs each interact through three different loops, involving hydrophobic linkages using a remarkable number of Tyr and Pro side chains, but lack hydrogen bonding and ionic linkages. The A:B and C:D outer surfaces have several positively charged residues that cluster along a groove. These grooves, each with an overall length of ~100 Å, allow tight electrostatic binding of a heparin glycosaminoglycan of ~5.5 kDa, which further stabilizes these dimers as well as the tetramer. The monomers of A:D and B:C dimers interact through other loops that stabilize the dimers through both ionic and hydrophobic bonds. Based on the current observation that B12 converts heparin-stabilized tetramers to monomers, we speculate that B12 disrupts both the polyanion-stabilized A:B and C:D interactions and the D:A and B:C interactions, but does not interfere with polyanion binding. Delineating the precise molecular mechanism will require x-ray crystallography of the B12-β-tryptase complex, which is beyond the scope of the current study. Presumably B12 binds to the β-tryptase in its tetrameric as well as monomeric state, and this in turn induces a conformational change that weakens subunit to subunit interactions. An alternative possibility is that tryptase monomers and tetramers are normally in equilibrium, and B12 binds only to the monomer, thereby trapping the enzyme in its monomeric form. However, because prolonged incubations of polyanion-stabilized tetramers with protease inhibitors such as SBTI and α3M (that inhibit tryptase monomers) fail to inhibit the tetramer, even after overnight incubations, this latter possibility seems unlikely.

B12 was previously observed to enhance the rate at which β-tryptase cleaves fibrinogen at acidic pH and to result in the generation of the anticoagulant product, Fragment D (22). These observations were confirmed in the current study, which showed enhanced fibrinogenolysis after B12 Fab had been added to either β-tryptase monomers or tetramers at acidic pH, in each case resulting in the production of one of the subunits of Fragment D. Presumably, this enhanced fibrinogenolytic activity at acidic pH is due primarily to conversion of tetramer to monomer because fibrinogen can better access the active site when β-tryptase is in its monomeric form. Active β-tryptase monomers in the absence of B12 cleaved fibrinogen but did not yield Fragment D. Furthermore, the fibrinogenolysis by active-free β-tryptase monomers was inhibited by ATIII, α3M and SBTI, whereas fibrinogenolysis by complexes of B12 and active β-tryptase, analogous to peptidase activity, was inhibited by SBTI and ATIII, but not by α3M.

In conclusion, we showed that B12 anti-tryptase Ab induces formation of β-tryptase monomers that exhibit polyanion-dependent activity at acidic pH, but are inactive at neutral pH. The substrate repertoire of tetrameric β-tryptase is limited by restricted access to the active sites. In contrast, active β-tryptase monomers (21) and B12-β-tryptase monomers, compared with β-tryptase tetramers, have a broader repertoire and enhanced activity against protein substrates at acidic pH, potentially of great importance in vivo, particularly at sites of inflammation like the airway in asthma patients (29) and at sites of poor vascularity such as the margins of solid tumors (30). Whether natural modulators of β-tryptase, including autoantibodies, exist in vivo is under investigation. Nevertheless, concomitant with enhanced access of substrates to the active site of monomeric β-tryptase is enhanced susceptibility to biologic inhibitors. Thus, active β-tryptase monomers would be regulated both by pH, in that they would become inactive once they diffuse outside of an acidic environment, and by biologic protease inhibitors.

Disclosures
The authors have no financial conflict of interest.

References


