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Research Article

Tryptase is the major protein constituent of human mast cells, where it is stored within the secretory granules as a fully active tetramer. Two tryptase genes (alpha and beta) are expressed by human mast cells at the level of mRNA and protein, each with a 30 amino acid leader sequence. Recombinant precursor forms of human alpha- and beta-tryptase were produced in a baculovirus system, purified, and used to study their processing. Monomeric beta-protryptase first is shown to be intermolecularly autoprocessed to monomeric beta-pro'tryptase at acid pH in the presence of heparin by cleavage between Arg-3 and Val-2 in the leader peptide. The precursor of alpha-tryptase has an Arg-3 to Gln-3 mutation that precludes autoprocessing. this may explain why alpha-tryptase is not stored in secretory granules, but instead is constitutively secreted by mast cells and is the predominant form of tryptase found in blood in both healthy subjects and those with systemic mastocytosis under nonacute conditions. Second, the NH2-terminal activation dipeptide on beta-pro'tryptase is removed by dipeptidyl peptidase I at acid pH in the absence of heparin to yield an inactive monomeric form of tryptase. Conversion of the catalytic portion of beta-tryptase to the active homotetramer at acid pH requires heparin. Thus, beta-tryptase homotetramers probably account for active enzyme detected in vivo. Also, processing of tryptase to an active form should […]

A Novel Heparin-dependent Processing Pathway for Human Tryptase

Autocatalysis Followed by Activation with Dipeptidyl Peptidase I

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Abstract

Tryptase is the major protein constituent of human mast cells, where it is stored within the secretory granules as a fully active tetramer. Two tryptase genes (α and β) are expressed by human mast cells at the level of mRNA and protein, each with a 30 amino acid leader sequence. Recombinant precursor forms of human α- and β-tryptase were produced in a baculovirus system, purified, and used to study their processing. Monomeric β-protryptase first is shown to be intermolecularly autoprocessed to monomeric β-pro'tryptase at acid pH in the presence of heparin by cleavage between Arg^{-3} and Val^{-2} in the leader peptide. The precursor of α-tryptase has an Arg^{-3} to Gln^{-3} mutation that precludes autoprocessing. This may explain why α-tryptase is not stored in secretory granules, but instead is constitutively secreted by mast cells and is the predominant form of tryptase found in blood in both healthy subjects and those with systemic mastocytosis under nonacute conditions. Second, the NH_2-terminal activation dipeptide on β-pro'tryptase is removed by dipeptidyl peptidase I at acid pH in the absence of heparin to yield an inactive monomeric form of tryptase. Conversion of the catalytic portion of β-tryptase to the active homotetramer at acid pH requires heparin. Thus, β-tryptase homotetramers probably account for active enzyme detected in vivo. Also, processing of tryptase to an active form should occur optimally only in cells that coexpress heparin proteoglycan, restricting this pathway to a mast cell lineage. (*J. Clin. Invest.* 1996. 97: 988–995.) Key words: mast cells • protease • anaphylaxis • mastocytosis • baculovirus

Introduction

Tryptase, a neutral serine protease, is the most abundant protein component of human mast cells, where it is selectively stored with full enzymatic activity in the secretory granules of all types of human mast cells, the major effector cell of allergic reactions (1). When activated mast cells degranulate, they release histamine and tryptase in parallel (2). Because tryptase persists in the circulation far longer than histamine, it has been used as a precise clinical marker of mast cell activation in diseases such as systemic anaphylaxis and asthma (3). Because of its potential to stimulate smooth muscle, fibroblasts, and tissue turnover (4), tryptase enzymatic activity has become a therapeutic target in asthma, where a putative inhibitor of this enzyme appears to show efficacy in a sheep model of human asthma (5).

In humans, one α-tryptase gene and at least one β-tryptase gene reside in the normal haploid genome on chromosome 16 (6–8). mRNAs corresponding to both α- and β-tryptase encode proteins that are 92% identical and are found in almost all preparations of mast cells dispersed from human lung and skin (9). Using immunoassays that distinguish recombinant human (rh)[1] α- and β-tryptase from one another, α-tryptase is the predominant form of tryptase detected in normal serum and is markedly elevated in patients with systemic mastocytosis, whereas β-tryptase is the major form elevated in serum during systemic anaphylaxis (10). A molecular explanation for the apparent constitutive secretion of α-tryptase, and secretory granule storage of β-tryptase, as yet, is lacking.

Like other serine proteases in secretory granules of hematopoietic cells (chymase, mast cell carboxypeptidase A, cathepsin G, granzymes, and elastase) (11), tryptase is synthesized as a precursor that must be converted into its mature form by intracellular proteolysis. Proforms of chymase and cathepsin G, like most of the granzymes, each have an NH_2-terminal activation dipeptide with an acidic residue at position −1 that is removed by dipeptidyl peptidase I (DPPI), a cysteine protease with an acid pH optimum localized to the secretory granules of bone marrow-derived cells (12). In contrast, protryptase has been predicted to contain 12, 10, and 10 amino acid activation peptides for human, murine, and canine forms, respectively (6–8, 13, 14), each beginning with Ala-Pro, a stop sequence for DPPI. For human chymase, Glu^{-1} is critical for the heparin-specific activation by DPPI (15), but each tryptase precursor contains a Gly^{-1}. Involvement of DPPI in the processing of murine tryptase studied pharmacologically in cultures of murine mast cells is controversial (12, 16). Also, unlike the monomeric chymases and cathepsin G, enzymatically active tryptase is a tetramer. Heparin binds to and stabilizes the enzymatically active form of human tryptase (17), but surprisingly does neither to rat tryptase (18), even though both molecules colocalize to rat mast cell secretory granules. Mouse mast cell protease 7, another tryptase, by protein modeling, is predicted to bind to heparin through clustered His residues at acidic but not at neutral pH, this prediction being supported experimentally with recombinant mouse mast cell protease 7

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1. *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; DPPI, dipeptidyl peptidase I; rh, recombinant human; SBTI, soybean trypsin inhibitor; TGPL, tosyl-L-glycine-L-lysine-*p*-nitroanilide; TPCK, *N*-tosyl-phenylalanine-chloromethyl ketone.

precursor protein (19). Thus, tryptase may have unique heparin-dependent activation requirements.

For the current study rhα- and rhβ-tryptase cDNAs encoding the entire 30 amino acid leader peptides and 245 amino acid catalytic portions of each were expressed in a baculovirus/insect cell system, and immunoaffinity purified with the B2 mAb (20). The Ala^{-12} to Gly^{-1} portions of the leader peptides remained in these enzymatically inactive tryptase precursors, referred to as protryptases, which were N-glycosylated on 0, 1, or 2 of the two putative sites on α-tryptase and on 0 or 1 of the single putative site on β-tryptase. The current study shows that the β-protryptase is processed from an inactive monomer to a fully active tetramer at acid pH in the presence of heparin by an autocatalytic cleavage at Arg^{-3}/Val^{-2} and removal of the dipeptide by DPPI, whereas α-protryptase cannot undergo the autoprocessing step and therefore remains an inactive precursor.

Methods

Materials. Mes, 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium–buffered substrate tablet, 2-mercaptoethanol, alkaline phosphatase–conjugated goat IgG anti–mouse IgG (Fcγ specific), TCA, dextran sulfate (500,000 D), acetone, glycerol, glycine, ethylene glycol, pepstatin A, cysteine, E-64, iodoacetamide, EDTA, EGTA, 1,10-*o*-phenanthroline, soybean trypsin inhibitor (SBTI), *N*-tosyl-phenylalanine-chloromethyl ketone (TPCK), aprotinin, diisopropyl fluorophosphate (DFP), 3,4-dichloroisocoumarin, PMSF, BSA, blue dextran (2,000,000 D), catalase, ovalbumin, apoferritin, thyroglobulin, aldolase, and chymotrypsinogen, bromophenol blue (Sigma Chemical Co., St. Louis, MO); *N*-glycosidase F, *O*-glycosidase, tosyl-glycine-L-proline-L-lysine-*p*-nitroanilide (TGPL); bovine DPPI (Boehringer Mannheim Biochemicals, Indianapolis, IN); Coomassie brilliant blue R-250 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden); prestained protein molecular weight standards, HBSS and heparin-agarose ALD (Gibco BRL Life Technologies, Inc., Grand Island, NY); SDS (Bio-Rad Laboratories, Inc., Hercules, CA); and Centricon-10 (Amicon, Inc., Beverly, MA) were obtained as indicated. Antitryptase mAb, G3, was prepared and purified as previously described (21). The human mast cell leukemia cell line, HMC-1, was a gift from Dr. G. Gleich and Dr. J. Butterfield (Mayo Clinic, Rochester, MN) (22). The human basophil leukemia cell line, KU812, was obtained from Dr. G. Nilsson (University of Uppsala, Uppsala, Sweden) (23). The human monocytic leukemia cell line, Mono-Mac-6, was supplied by Dr. H. W. Ziegler-Heitbrock (University of Munich, Munich, Germany) (24). The mouse 3T3 fibroblast cell line was obtained from American Type Tissue Culture (Rockville, MD). Heparin proteoglycan (~ 750,000 D) from rat peritoneal mast cells (25) and porcine heparin glycosaminoglycan (1,200-20,000 D) (Sigma Chemical Co.) (26) were prepared as described. Gly-Phe-CHN_2, a DPPI-specific inhibitor, was prepared as described (16) and kindly provided by Dr. W. Serafin (Vanderbilt University, Nashville, TN). Native tryptase from human skin was purified as described previously by sequential affinity chromatographies on B2 mAb affi-gel and heparin-agarose ALD (27). Rhα-protryptase and rhβ-protryptase were expressed in a baculovirus/insect cells system and were immunopurified by B2 mAb affi-gel as described (20).

Activation of rhα-protryptase and rhβ-protryptase by cell extracts. HMC-1, Mono-Mac-6, KU-812, and mouse 3T3 fibroblast cells, 10^7 each, were washed three times with 1.5 ml of HBSS, sonicated (8 pulses, 50% pulse cycle, 4 power, microtip attachment, Sonicator™ cell Disruptor Model W-225R; Heat System-Ultrasonics. Inc., Planview, NY) on ice with 0.5 ml of 10 mM Mes, pH 6.5, containing 0.1 M NaCl and centrifuged at 12,000 *g* for 5 min at 4°C to remove cell debris. The supernatants were diluted with the same buffer to adjust the protein concentration to 0.5 mg/ml and stored at −75°C.

Rhα-protryptase or rhβ-protryptase (500 ng) was incubated in 100 μl of 0.1 M Mes, pH 6.5, containing 0.1 M NaCl, 0.3 mg/ml of various cell extracts, 0.1 mg/ml of dextran sulfate and 0.5 mg/ml of BSA at 37°C for the times indicated. In some experiments, heparin proteoglycan, heparin glycosaminoglycan, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, or chondroitin sulfate E was substituted for dextran sulfate. One portion of each sample removed from the incubation mixtures was assessed for tryptase enzyme activity based on TGPL hydrolysis, and another portion denatured by adding an equal volume of 5 mM Tris, pH 8.3, buffer containing 2% (wt/vol) SDS, 10% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 38 mM glycine, and 0.2% (wt/vol) bromophenol blue (2 × SDS-PAGE loading buffer). The preparations were boiled for 5 min and were subjected to electrophoresis and Western blotting. For the effects of protease inhibitors on activation of rhβ-protryptase by cell extracts, each protease inhibitor at the concentration described in the text was incubated with 500 ng of rhβ-protryptase for 2 h at 37°C as above, except that each cell extract was preincubated with the inhibitor for 15 min at room temperature. pH profiles of activation of rhβ-protryptase by cell extracts were determined in 0.1 M acetate (pH 4.0–6.0), 0.1 M Mes (pH 6.0–7.0) and 0.1 M Tris-HCl (pH 7.0–9.0) buffers containing 0.1 M NaCl, 0.3 mg protein/ml of cell extracts, 0.1 mg/ml of dextran sulfate, and 0.5 mg/ml of BSA at 37°C for 2 h.

Autocatalytic processing of rhβ-protryptase to rhβ-pro'tryptase. Rhβ-protryptase (0.125–1.25 μg) was incubated in 100 μl of 50 mM acetate, pH 5.5, containing 25 μg/ml of heparin glycosaminoglycan along with 0.15 M NaCl and 0.5 mg/ml of BSA (BSA-saline) at room temperature for the times indicated. In some experiments, 0.125 μg of rhβ-pro'tryptase were incubated with various amounts of immunoaffinity purified rhβ-pro'tryptase or mature rhβ-tryptase in 100 μl of the same buffer described above at room temperature. The reactions were terminated by boiling each sample in an equal volume of 2 × SDS-PAGE loading buffer. Samples were then subjected to SDS-PAGE and Western blotting. To examine the effects of protease inhibitors on autocatalytic processing, 0.5 μg of rhβ-protryptase and each inhibitor were preincubated for 30 min at room temperature under the same conditions described above except that heparin glycosaminoglycan was omitted from the preincubation mix. Then autocatalytic processing was initiated by adding heparin glycosaminoglycan to a final concentration of 25 μg/ml. For the effects of pH on autocatalytic processing of rhβ-protryptase, the buffers described above were used at 50 mM over the same range of pH values, each also containing 25 μg/ml of heparin glycosaminoglycan and BSA-saline, and incubations were performed at room temperature for 1 h.

Activation of rhβ-pro'tryptase by bovine DPPI. Rhβ-protryptase was incubated at 37°C for 1 h in 100 μl of either 50 mM acetate, pH 5.5, containing BSA-saline and 25 μg/ml of heparin glycosaminoglycan, or 0.1 M Mes, pH 6.5, containing 0.1 M NaCl, 0.5 mg/ml of BSA and 100 μg/ml of dextran sulfate. After the incubation, small portions of DTT at a final concentration of 1 mM and bovine DPPI (0.5-10 μg/ml) were added to the reaction mix which was then incubated at 37°C for varying time periods. Enzyme activity (TGPL hydrolysis) was monitored for each condition.

Purification of rhβ-pro'tryptase and mature rhβ-tryptase. All purification procedures were performed at 4°C unless otherwise stated. Rhβ-protryptase (150 μg) was incubated in 15 ml of 0.1 M Mes, pH 6.5, containing BSA-saline and 0.1 mg/ml of dextran sulfate at 37°C for 1 h in the presence (for generation of mature form) or absence (for generation of pro'-form) of 1 mM DDT and 300 μg of bovine DPPI. After the incubation, enzyme activities of approximately 0.6 and 30 TGPL U/mg of recombinant protein were routinely detected in the preparations of rhβ-pro'tryptase and rhβ-tryptase, respectively. The samples were then cooled on ice, adjusted to 2 M NaCl, and applied to an immunoaffinity column (7 ml bed vol) containing B2 mAb-affigel, which had been known to recognize a conformational determinant present on rh-protryptase and tissue-derived active tryptase that is unavailable on native tryptase converted to inactive monomers (20, 27). After loading, the column was washed with five column volumes of 10 mM Mes, pH 6.5, containing 1 M NaCl and

Table I. Activation of rhβ-Protryptase by Cell Extracts

Tryptase	Dextran sulfate	Specific Activity			
		HMC-1	Mono-Mac-6	KU812	3T3 Fibroblasts
		U/mg			
rhα-pro	+	1.0	0	0	0
	−	0	0	0	0
rhβ-pro	+	31.2	29.3	21.4	1.2
	−	1.0	0.6	0.5	1.0

Immunoaffinity purified rhα- and rhβ-protryptase (0.5 μg) were incubated with cell extracts (0.3 mg protein/ml) in the presence (+) or absence (−) of dextran sulfate (0.1 mg/ml) at 37°C overnight as described in Methods. Specific activity with TGPL is shown as U (μmol TGPL cleaved/min) per mg tryptase, the lower limit under the conditions employed being 0.1 U/mg.

again with 10 mM Mes, pH 6.5, containing 0.1 M NaCl. Rhβ-pro'tryptase and mature rhβ-tryptase bound and were eluted with 10 mM diethanolamine, pH 10.0, containing 0.2 M NaCl and 50% (vol/vol) ethylene glycol as described previously for purification of natural tryptase and rhα- and rhβ-protryptase (20, 27). The eluted fractions were immediately neutralized with 1 M Mes, pH 6.5. Preparations of mature rhβ-tryptase were then subjected to heparin-agarose chromatography as described for purification of tissue-derived tryptase, elution effected with a linear NaCl gradient (0.45 to 2 M) in 10 mM Mes, pH 6.5, containing 20% glycerol. Each chromatographic profile was monitored by TGPL hydrolysis and OD280. To assess purity, samples were subjected to SDS-PAGE (8 cm long 12% polyacrylamide SDS gels; Novex, San Diego, CA) in a Novex Xcell II TM Mini-Cell system. The gels were then stained with Coomassie brilliant blue R-250, destained, and scanned.

Western blotting. Samples were subjected to SDS-PAGE (8 cm long 12% polyacrylamide SDS gels) under reducing conditions, and transferred electrophoretically to a supported precut nitrocellulose membrane (Novex). The membrane was then soaked in 3% BSA to block nonspecific protein binding, and incubated overnight at 4°C with 0.5 μg/ml of the G3 mAb (21). Bound antibodies were detected using goat IgG anti–mouse Fcγ conjugated with alkaline phosphatase and 5-bromo-4-chloro-3-indoyl phophate/nitroblue tetrazolium buffered substrate tablets for color development. Prestained protein molecular weight standards were used as molecular weight markers.

Analytical techniques. The NH_2-terminal amino acid sequences of immunoaffinity purified rhβ-pro'tryptase and mature rhβ-tryptase (150 pmol of each) were determined by Commonwealth Biotechnologies Corporation (Richmond, VA) using a 470 Gas Phase Sequencer with dedicated Model 120A PTH-Amino Acid Analyzer (Applied Biosystems Inc., Foster City, CA). Protein concentrations were measured with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) using BSA as a standard (28). Rates of hydrolysis of TGPL were determined by adding of 5–20 μl of enzyme preparations to 1.0 ml of 50 mM Hepes, pH 7.4, containing 0.15 M NaCl and 0.1 mM TGPL in a quartz cuvette at 25°C unless indicated otherwise. The amount of *p*-nitroanilide liberated from the substrate was monitored by absorbance at 405 nm on a Cary 2200 spectrometer (Varian Associates, Palo Alto, CA). Kinetic constants were determined by Lineweaver-Burke analyses from initial velocities of substrate cleavage at five substrate concentrations during a time period when no more than 10% of the substrate had been cleaved. Activity is expressed in U, where one U of enzyme cleaves 1 μmol of substrate per minute. A mol wt of 27,458, calculated from the predicted amino acid sequence of the β-tryptase monomer (7), was used to calculate Kcat values. HPLC was performed with a IsoPure LC system using the Series 410 BIO LC Pump solvent delivery system (Perkin Elmer, Corp., Foster City, CA) and a Superose 12 HR 10/30 size-exclusion column (1 cm × 30 cm; Pharmacia LKB, Uppsala, Sweden).

Results

Activation of rhα-protryptase and rhβ-protryptase by cell extracts. To search for processing enzyme activity, cell extracts of the human leukemic cell lines HMC-1 (mast cell), Mono-Mac-6 (monocytic), and KU812 (basophil), and mouse 3T3 fibroblasts were combined with immunoaffinity purified rhα- and rhβ-protryptase precursors in the presence of heparin, dextran sulfate, chondroitin sulfate subtypes or buffer alone over a pH range of from 4.0 to 9.0 at 37°C in 0.15 M NaCl. Enzymatic activity against TGPL was strongly detected for rhβ-tryptase when dextran sulfate was present with each cell extract except that from the mouse 3T3 fibroblasts (Table I). Heparin glycosaminoglycan or proteoglycan, when substituted for dextran sulfate, also facilitated the activation of rhβ-protryptase, whereas chondroitin sulfates A, B, C, and E did not. Activation of rhβ-protryptase was optimal at pH 5.0–6.5 and was inhibited by both cysteine (20 μM E-64 and 0.1 mM iodoacetamide) and serine protease (50 μg/ml of aprotinin, 100 μg/ml of SBTI and 1 mM TPCK) inhibitors, and by Gly-Phe-CHN_2 (20 μM) a DPPI-specific inhibitor (16). Negligible inhibition was observed with pepstatin (3.6 mM), EDTA (1 mM), EGTA (1 mM), and 1-10-*o*-phenanthroline (2 mM). TPCK inhibits serine proteases with chymotryptic substrate specificity and DPPI (29). These data suggested that at least two types of enzymes process rhβ-preprotryptase, DPPI, a cysteine dipeptidase, and a serine protease.

Evidence for proteolytic processing of rhβ-protryptase, but not rhα-protryptase is shown in Fig. 1. Note that rhα-protryptase starting material exhibits two major and one minor bands, which appear to correspond, from top to bottom, to rhα-protryptase with two, one, or zero N-linked glycosylation sites being occupied (20). Rhβ-protryptase also exhibits heterogeneity, showing two major bands corresponding to N-glycosylated (30.7 kD) and nonglycosylated (30.2 kD) species (20). The apparent molecular weights of these bands were determined empirically based on their electrophoretic mobilities relative to those of commercial standards. Minor bands in the rhβ-protryptase preparation migrating with the autoprocessed products also can be discerned (< 10% of total tryptase by band densitometry). A shift in the electrophoretic mobilities of the two major rhβ-protryptase proteins, corresponding to an apparent loss of less than 500 D from each, consistent with limited proteolytic processing, was detected by SDS-PAGE/Western blotting in all cases where enzyme activity was observed

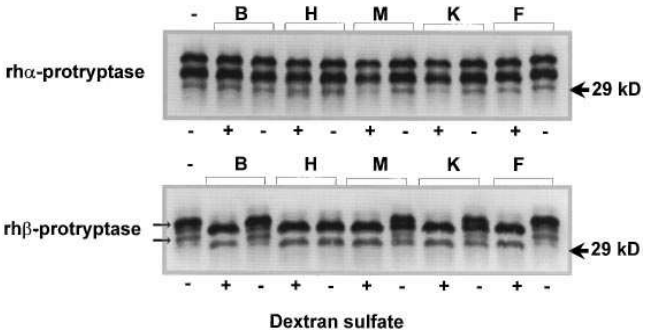

Figure 1. Analysis of the processing of rhβ-protryptase and rhα-protryptase in buffer (*B*) and in extracts of HMC-1 (*H*), Mono Mac-6 (*M*), KU812 (*K*), and 3T3 fibroblasts (*F*) in the presence (+) and absence (−) of dextran sulfate. Lanes labeled at the top as (−) show starting material. Incubations were for 12 h at 37°C. Tryptase in each reaction mixture (50 ng/lane) was denatured, reduced, and subjected to Western blotting with the G3 mAb as described in Methods. A 29-kD mol wt standard is shown to the right of each blot. Two small arrows to the left of the lower blot point to glycosylated and nonglycosylated bands of rhβ-protryptase. The less intense bands just below each of these presumably correspond to rhβ-pro'tryptase.

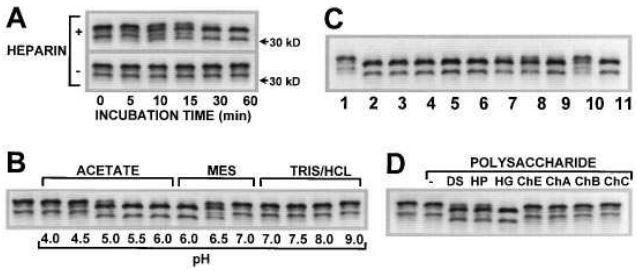

Figure 2. Autoprocessing of rhβ-protryptase to rhβ-pro'tryptase. Autoprocessing was analyzed by Western blotting using the G3 anti-tryptase mAb (21). The time course (*A*), pH optimum (*B*), protease inhibitor profile (*C*), and polysaccharide requirements (*D*) are shown. In each case rhβ-preprotryptase (5 μg/ml) was incubated in 50 mM buffer containing BSA-saline at room temperature for 1 h or the times indicated. Polysaccharides were tested at 25 μg/ml. The time course in *A* used 50 mM acetate, pH 5.5. In *B* and *C* lane *1* shows a rhβ-protryptase unincubated control. In *C* rhβ-protryptase was preincubated alone (lane *2*) or with a protease inhibitor as follows: pepstatin A (10 μM) (lane *3*), E-64 (20 μM) (lane *4*), iodoacetamide (1 mM) (lane *5*), EDTA (1 mM) (lane *6*), DFP (1 mM) (lane *7*), 3,4-dichloroisocoumarin (250 μM) (lane *8*), SBTI (100 μg/ml) (lane *9*), aprotinin (50 μg/ml) (lane *10*) or TPCK (1 mM) (lane *11*) in acetate buffer, pH 5.5, containing BSA-saline at room temperature for 60 min. After the preincubation, autoprocessing was initiated by adding heparin to each sample and incubating the mixtures at room temperature for 60 min. Lane numbers are shown under the blot. By themselves, the solvents used for dissolving the inhibitors did not affect autoprocessing (data not shown). In *D*, dextran sulfate (*DS*), porcine chondroitin sulfate A (*ChA*), porcine chondroitin sulfate B (*ChB*), shark chondroitin sulfate C (*ChC*), rat serosal mast cell heparin proteoglycan (*HP*), heparin glycosaminoglycan (*HG*), and murine chondroitin sulfate E (*ChE*) were used as indicated.

(Fig. 1). Proteolytic processing also was observed in the presence of heparin and either the fibroblast extract or buffer alone, conditions that did not result in enzymatic activity (Fig. 1). Proteolytic processing in the presence of HMC-1 extract without exogenous heparin also was observed, even though enzymatic activation was not apparent. This might be attributable to small amounts of endogenous tryptase and heparin present in the HMC-1 extracts (see below). No detectable activation or proteolytic processing of rhα-protryptase precursor was observed by incubation with the cell extracts under any of the conditions.

Autocatalytic processing of rhβ-protryptase to rhβ-pro'tryptase. Based on the above findings, two sequential steps of proteolytic processing of rhβ-protryptase were postulated; a heparin-dependent autocatalytic step and a DPPI-dependent step. The autocatalytic step is demonstrated in Fig. 2. After 1 h of incubation autoprocessing of rhβ-protryptase occurs only in the presence of heparin, as reflected by a decrease in apparent size of the two major bands (Fig. 2 *A*). Autoprocessing in the presence of heparin exhibits an acid pH optimum of 5.5–6.0 (Fig. 2 *B*). In the presence of dextran sulfate the pH optimum is 6.0–6.5 (not shown). Autoprocessing was inhibited best by aprotinin (~60% inhibition by densitometry), weakly by diisopropyl fluorophosphate, and not at all by cysteine, aspartic acid, or metallo protease inhibitors (Fig. 2 *C*). Of biologic interest, chondroitin sulfates A, B, C, and E could not substitute for heparin and facilitate autoprocessing of tryptase (Fig. 2 *D*).

Autocatalytically produced rhβ-protryptase was purified by B2 mAb immunoaffinity chromatography and subjected to five cycles of amino acid sequencing. A single sequence of Val-Gly-Ile-Val-Gly was obtained, indicating the autocatalytic cleavage site is Arg^{-3}/Val^{-2}, consistent with tryptase having a specificity for basic residues. This probably explains why the rhα-tryptase precursor, which contains Gln^{-3}/Val^{-2} is not autocatalytically processed in vitro. The calculated size of the released peptide is 1,170 D, somewhat greater than the apparent loss in mol wt observed by SDS-PAGE.

Autocatalytic processing of rhβ-protryptase appears to be inter rather than intramolecular, because the relative rate of conversion, as measured by the time to 50% cleavage, increased as the concentration of rhβ-protryptase was increased, being 50, 25, 12, and 6 min at 1.25, 2.5, 5, and 12.5 μg/ml, respectively, as shown in Fig. 3 *A*. Intramolecular processing should display kinetics that are independent of enzyme concentration, as shown for hedgehog (30) and cathepsin B (31) precursor proteins. Whether tryptase autoprocessing activity is due to rhβ-protryptase, contaminant putative rhβ-pro'tryptase, or both also was considered. Approximately 10% of the total tryptase by Western blotting with G3 mAb migrates with rhβ-pro'tryptase, and yields the amino acid sequence found for rhβ-pro'tryptase (see below), suggesting that processing to rhβ-pro'tryptase can occur at a slow rate in the insect cell system. Nevertheless, rhβ-protryptase preparations expressed no detectable enzymatic activity using TGPL (< 0.1 U/mg tryptase). Thus, the substrate specificity of rhβ-protryptase is quite restrictive. As shown in Fig. 3 *B*, incubation of purified rhβ-pro'tryptase (1.25 μg/ml) with rhβ-protryptase (1.25 μg/ml) at pH 5.5 accelerated autoprocessing to about the same extent as when rhβ-protryptase was increased to 2.5 μg/ml, suggesting that both precursor forms could participate in autoprocessing. Mature rhβ-tryptase was very potent at processing rhβ-protryptase (1.25 μg/ml), 0.01 μg/ml of the mature enzyme decreasing the time to 50% cleavage from 50 to 8 min at pH 5.5. Interestingly, in spite of enzymatic activity against TGPL being optimal at neutral pH for both mature and rhβ-pro'tryptase, the rate of autoprocessing was at least 10-

fold faster at the acidic pH (Fig. 3 *B*). Commercial bovine trypsin also appeared to catalyze conversion of rhβ-protryptase to rhβ-pro'tryptase (Fig. 3 *C*), but this reaction was heparin-independent, occurred better at neutral than acidic pH, and required at least 10-fold greater amounts of trypsin than tryptase to achieve the same rate.

Activation of rhβ-pro'tryptase by bovine DPPI. DPPI, based on preliminary experiments with extracts of bone marrow–derived leukemic cells, was assessed directly for its ability to remove the dipeptide (Val^{-2}-Gly^{-1}) present at the NH_2 terminus of β-pro'tryptase and yield active tryptase. As shown in Fig. 4 *A*, DPPI, in the presence of dextran sulfate, converted rhβ-pro'tryptase into enzymatically active rhβ-tryptase, which exhibited a sp act (31.1±2.9 U/mg of tryptase; mean±SD, n = 4), Kcat (1,016 min^{-1}) and K_m (1.3 mM) for TGPL in Hepes buffer, pH 7.4, containing 0.15 M NaCl, that were similar to those observed for immunoaffinity purified, skin-derived tryptase (31 U/mg, 972 min^{-1}, and 1.4 mM, respectively). Active rhβ-tryptase also formed after addition of rhβ-protryptase under the same conditions, but with a slight lag period due to the time of autoprocessing (accelerated processing probably occurred as fully active tryptase formed). As shown in Fig. 4 *B*, rhβ-pro'tryptase exhibits trace enzymatic activity against TGPL at neutral pH (~ 0.7 U/mg); no detectable activity against this synthetic substrate was detected at pH 5.5 (not shown). In the presence of aprotinin TGPL-cleaving activity of rhβ-pro'tryptase was > 80% inhibited at pH 7.4, in contrast to the relative resistance of the native tryptase and rhβ-tryptase to this inhibitor. Rhβ-protryptase exhibited no detectable enzyme activity at pH 7.4 (Fig. 4 *B*) or pH 5.5 (not shown). Although heparin was not necessary for the DPPI-catalyzed removal of the NH_2-terminal dipeptide as determined by NH_2-terminal amino acid sequencing, production of enzymatically-active rhβ-tryptase did require heparin. Furthermore, as in the autoprocessing step heparin glycosaminoglycan and proteoglycan could replace dextran sulfate, whereas chondroitin sulfates A, B, C, and E could not (data not shown).

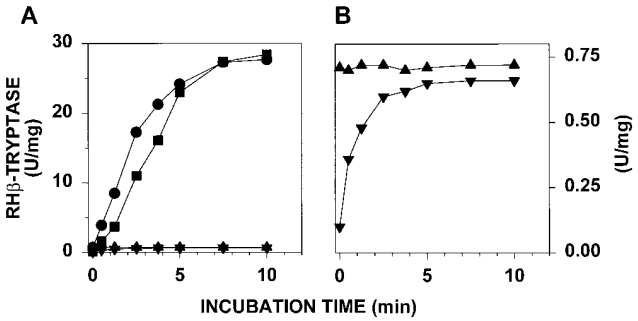

Figure 4. Activation of rhβ-pro'tryptase to mature rhβ-tryptase by DPPI. In *A* immunoaffinity purified rhβ-pro'tryptase (0.5 μg) was incubated in the presence (●) or absence (▲) of 1 μg of bovine DPPI at 37°C in 100 μl of Mes buffer, pH 6.5, containing BSA-saline, 100 μg/ml of dextran sulfate, 1 mM dithiothreitol. Also shown is the time course for processing 0.5 μg of rhβ-protryptase under the same conditions in the presence (■) and absence (▼) of 1 μg of DPPI. At the times indicated, portions of each incubation mixture were removed and immediately added to a cuvette containing TGPL as described above to determine initial reaction velocities. In *B* the y-axis is expanded 250-fold to show the data in *A* for rhβ-pro'tryptase (▲) and rhβ-protryptase (▼) in the absence of DPPI.

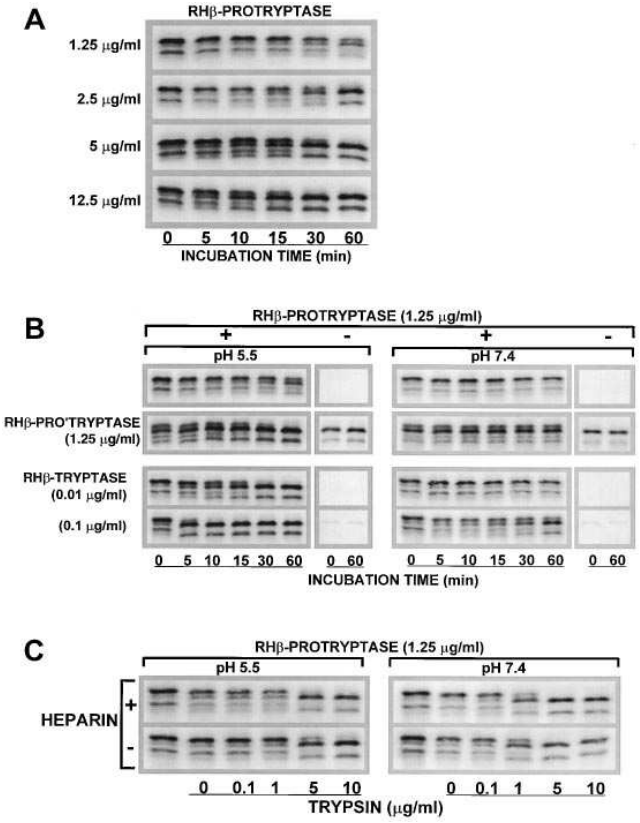

Figure 3. Intermolecular autoprocessing of rhβ-protryptase to rhβ-pro'tryptase. Autoprocessing was analyzed as detailed in Figure 2. *A* shows the effect of varying the concentration of rhβ-protryptase (1.25–12.5 μg/ml) on the rate of autoprocessing in acetate buffer, pH 5.5, containing BSA-saline and heparin (25 μg/ml), at room temperature for up to 60 min. *B* shows the effects of adding rhβ-pro'tryptase (1.25 μg/ml) or mature rhβ-tryptase (0.01 and 0.1 μg/ml) on autoprocessing of rhβ-protryptase (1.25 μg/ml) at pH 5.5 as in *A* or at pH 7.4 in Hepes buffer containing BSA-saline and heparin (25 μg/ml), in each case at room temperature. Panels labeled (−) lack rhβ-protryptase and contain only the added rhβ-pro'tryptase or rhβ-tryptase, while those labeled (+) contain all reaction components. *C* shows the ability of bovine trypsin (0–10 μg/ml) to stimulate autoprocessing of rhβ-protryptase (1.25 μg/ml) at pH 5.5 and pH 7.4 during a 10-min incubation at room temperature in the presence or absence of heparin (25 μg/ml). Lane *1* shows an unincubated rhβ-protryptase control at both pH 5.5 and pH 7.4.

Heterogeneity in the staining patterns of rhα- and rhβ-tryptase species after SDS-PAGE diminished after treatment with N-glycosidase F (Fig. 5 *A*). For rhα-preprotryptase, the digestion product yielded one major band as described (20), consistent with most molecules having either one or two potential N-glycosylation sites occupied before deglycosylation. For rhβ-protryptase, rhβ-pro'tryptase, and rhβ-tryptase the digestion products for each also yielded a single major band that migrated with the lower mol wt band present before digestion, indicating that the single putative N-glycosylation site of these species is either occupied or unoccupied before digestion.

In Fig. 5 *B* the relative binding affinities of rhβ-protryptase, rhβ-pro'tryptase, rhβ-tryptase, human skin-derived tryptase, and bovine trypsin to heparin-agarose were compared at pH 5.5 and pH 7.4. At 0.15 M NaCl each form of tryptase, but not

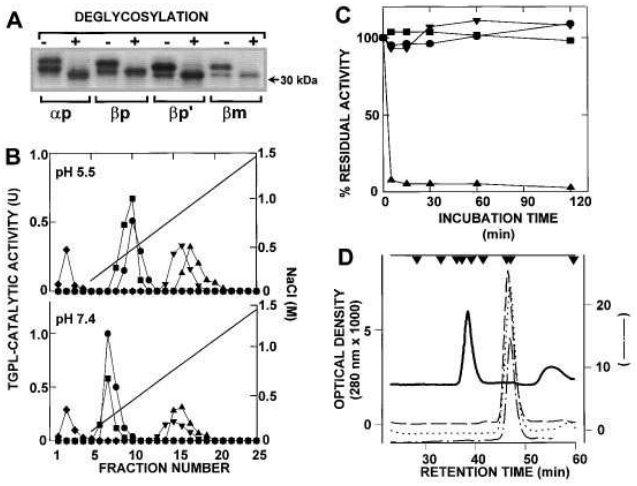

Figure 5. Comparative properties of different forms of rh-tryptase. In *A* immunoaffinity purified and denatured rhα-protryptase (*αp*), rhβ-protryptase (*βp*), rhβ-pro'tryptase (*βp'*) and mature rhβ-tryptase (*βm*) (2 μg of each) were treated with N-glycosidase F (+) according to the manufacturer's instructions or with buffer alone (−), subjected to SDS-PAGE, and stained with Coomassie brilliant blue R-250. The position of the 30,000 kD marker (carbonic anhydrase) is shown to the right. In *B* heparin-agarose chromatography profiles for immunoaffinity purified rhβ-protryptase (●), rhβ-pro'tryptase (■), rhβ-tryptase (▲), and human skin-derived tryptase (▼) (50 μg of each), and 20 μg of bovine trypsin (◆) are shown. Samples were loaded in 1 ml of 50 mM acetate, pH 5.5, or 50 mM Hepes, pH 7.4, both containing BSA-saline and 20% (vol/vol) glycerol, at 4°C onto 1.5 ml (bed volume) of heparin-agarose equilibrated with loading buffer. After washing with 3 ml of loading buffer, elution of bound material was performed with a 25 ml linear gradient of 0.15–1.5 M NaCl at the same pH as the loading buffer. Fractions (1.2 ml) were collected and assayed for NaCl concentration (—) by conductivity measurements and for TGPL-cleaving activity for rhβ-tryptase, human skin-derived tryptase and bovine trypsin. For detection of rhβ-protryptase and rhβ-pro'tryptase in column fractions, TGPL-cleaving activity was measured after conversion to enzymatically active rhβ-tryptase. The stability of rhβ-tryptase was assessed in *C.* Rhβ-protryptase (●), rhβ-pro'tryptase (■), and rhβ-tryptase (▲) (50 μg/ml of each) were incubated in 50 mM Hepes, pH 7.4, containing BSA-saline at 37°C for up to 2 h. Rhβ-tryptase also was incubated in the same buffer containing 250 μg/ml of heparin (▼). TGPL-catalytic activities, expressed as the percent residual activity compared to the 0-min control, were measured directly for rhβ-tryptase and after processing to mature tryptase for rhβ-protryptase and rhβ-pro'tryptase. The quaternary structures of immunoaffinity purified rhα-protryptase and rhβ-tryptase subtypes were assessed by gel filtration chromatography in *D.* Rhα-protryptase (2 μg) (·····), rhβ-preprotryptase (5 μg) (-·-), rhβ-pro'tryptase (1.5 μg) (---), rhβ-tryptase (0.7 μg) (—) in 200 μl of 10 mM Mes, pH 6.5, containing 1.0 M NaCl, were applied to a Superose 12 HPLC column equilibrated with the same buffer. A flow rate of 0.3 ml/min was used, and fractions of 0.24 ml were collected. Mol wt standards (*arrowheads*) from left to right included dextran blue (2×10^6 D), thyroglobulin (669,000 D), apoferritin (443,000 D), catalase (232,000 D), aldolase (158,000 D), BSA (66,000 D), ovalbumin (43,000 D), chymotrypsinogen (25,000 D), and acetone (58 D). Elution of standards and tryptase species were monitored by absorbance at 280 nm.

trypsin, bound to heparin-agarose. Using a linear salt gradient for elution at pH 5.5, rhβ-protryptase and rhβ-pro'tryptase emerged at 0.46 M and 0.48 M NaCl, respectively, whereas mature rhβ-tryptase eluted at 0.99 M NaCl, indicating a stronger affinity for heparin than the precursors, but similar to that of native skin-derived tryptase, which eluted at 0.82 M NaCl. At pH 7.4, elution of each form of tryptase occurred at a NaCl concentration ~ 0.1 M lower than at pH 5.5.

The stability of rhβ-tryptase was compared to that of rhβ-tryptase precursors in Fig. 5 *C.* Rhβ-tryptase was stable in the presence of heparin over the 2-h incubation, but in the absence of heparin, almost all of its enzymatic activity was lost by 5–10 min. This behavior is similar to that reported for tissue-derived tryptase (17). Rhβ-protryptase and rhβ-pro'tryptase, unlike the mature enzyme, were stable when incubated at pH 7.4 in the absence of heparin or dextran sulfate, because both could then be processed to active enzyme. Rhβ-tryptase in the presence of heparin was sensitive to DFP (98% inhibition) and PMSF (66% inhibition), and relatively resistant to SBTI (< 10% inhibition) and aprotinin (30% inhibition), and to aspartic, cysteine, and metallo protease inhibitors (< 10% inhibition), essentially identical to the behavior of native tryptase. However, it is also of interest to note that the autoprocessing step was sensitive to aprotinin and was only slightly inhibited by SBTI and DFP (~ 10% by densitometry). The difference in susceptibility to aprotinin may reflect conformational differences between the mature active tetrameric enzyme and the monomeric precursor (see below).

The quaternary structures of tryptase at various stages of processing were examined by Superose 12 FPLC as shown in Fig. 5 *D.* Rhα-protryptase, rhβ-protryptase, and rhβ-pro'-tryptase each migrated as a monomer at an apparent mol wt of 25,000 D. In contrast, mature, enzymatically active rhβ-tryptase migrated at an apparent mol wt of 107,000 D, consistent with a tetrameric structure. Rhβ-tryptase, like native tryptase, migrated as a monomer both after inactivation by incubation in physiologic buffer without heparin and also after processing by DPPI in the absence of heparin (not shown).

Discussion

The two proteolytic steps involved in the processing of recombinant human tryptase are summarized in Fig. 6. The dependence of both autoprocessing and formation of active tetramer after DPPI processing on either heparin glycosaminoglycan, heparin proteoglycan or dextran sulfate indicates that, like stabilization of the enzymatically active tetramer (32), negative charge density rather than carbohydrate sequence determines efficacy. The requirement for acid pH at each processing step is not dictated by a lack of binding to heparin at neutral pH, because human tryptase precursors like the mature enzyme bind to heparin at neutral and acidic pH when the ionic strength corresponds to 0.15 M NaCl. Thus, unlike mouse mast cell protease 7 (19), histidine residues in human β-protryptase are not critical for binding to heparin. For autoprocessing catalyzed both by mature tryptase and by precursors, acid pH may influence either the conformation of the signal peptide substrate, or the substrate specificity of the catalytic enzyme. For conversion of rhβ-pro'tryptase to the active enzyme, acid pH is optimal in general for DPPI, and also may be critical for conversion of monomer to tetramer.

Although human tryptase is considered primarily to function in the extracellular space near neutral pH, its autoprocessing activity at acid pH indicates an intracellular enzymatic function as well. Whether this activity might extend to other precursor proteins or to the metabolism of released activation

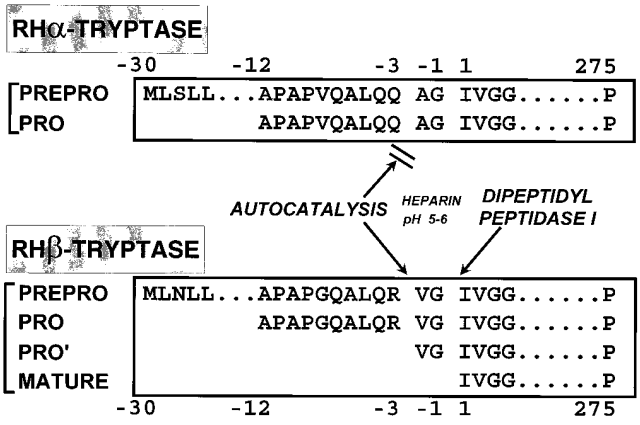

Figure 6. Summary of the processing of human α- and β-tryptase. Three to five cycles of NH_2-terminal amino acid sequence analyses were performed on immunoaffinity purified rhα-protryptase, rhβ-protryptase, rhβ-pro'tryptase, and rhβ-tryptase. Sequencing was performed by Commonwealth Biotechnology Laboratory, Inc. (Richmond, VA). The scheme shown is based on this data, on the sequences predicted from the corresponding cDNA molecules, and the previously determined sequence for tissue-derived tryptase.

peptides should be considered. Another point of interest is the low level of rhβ-pro'tryptase activity found against the synthetic peptide substrate. This property is compatible with chymotryptic proenzymes from granulocytes that exhibit low levels of activity against small synthetic substrates but do not cleave protein substrates (12). Of further interest is the finding that rhβ-pro'tryptase activity against TGPL at neutral pH is sensitive to inhibition by aprotinin, in contrast to the active tetramer. The polar residues in the activation dipeptides of most chymases and granzymes inhibit penetration of the NH_2-terminal amino acids of the mature portion of the enzyme into the hydrophobic interior, where a salt bridge between NH_2-terminal Ile and the carboxylate side chain of an Asp residue near the active site form to obtain optimal enzymatic activity, analogous to chymotrypsin (33). The tryptase activation dipeptide contains hydrophobic residues, but these still should prevent ion binding of Ile^1 to Asp^{193}.

The intracellular site(s) for processing of tryptase precursors, though not directly addressed in the current study, can be considered. Tryptase precursors should be stable until they associate with heparin, permitting autoprocessing to occur in an acid pH environment, presumably in the *trans*-Golgi network, secretory pregranules, and secretory granules (34). Heparin, analogous to chondroitin sulfate subtypes, is expected to be present in the Golgi apparatus, because xylosylation of core protein leading to formation of chondroitin sulfate proteoglycans begins in the endoplasmic reticulum, and continues in the Golgi where chain elongation and sulfation also occur (35, 36). Mast cells, like other bone marrow–derived granulocytes, store DPPI inside post-Golgi secretory granules (12, 16), where the activation dipeptides for cathepsin G and elastase (37, 38), like tryptase, are removed. Any β-protryptase arriving in the secretory granule should be processed rapidly by active tryptase already in residence. The current study shows that 0.1 μg/ml of active tryptase completely converts 1.25 μg/ml of rhβ-protryptase to the dipeptide pro'enzyme within ~ 10 min at pH 5.5; however, the concentration of active tryptase in secretory granules of tissue mast cells is estimated to be at least 100 mg/ml. The higher potency of rhβ-tryptase relative to its precursors raises the possibility that minor contamination of precursor tryptase preparations with mature tryptase or with another processing enzyme could account for the apparent autoprocessing activity of the precursor forms of tryptase. To address this point, heparin-agarose chromatography of rhβ-pro/pro'tryptase was performed under conditions that clearly separate precursor from mature active tryptase (Fig. 5 *C*). The rates of autocatalytic processing of rhβ-pro/pro'tryptase before and after heparin-agarose chromatography were not substantially different, making it likely that autocatalytic processing by tryptase precursors was occurring.

Trimming of tryptase signal peptides in Sf-9 cells to Ala^{-12}-Pro^{-11} suggests intrinsic DPPI-like activity that is stopped by Pro in the P1 position. However, the lack of heparin proteoglycan in these cells, even if the precursor passes through an acid pH milieu, should prohibit further meaningful processing. Whether proteases other than tryptase can process β-protryptase to β-pro'tryptase remains to be seen; trypsin, for example, has only a limited capacity to carry out this step. Cells that might be induced to express tryptase mRNA and protein in the absence of heparin, like the experimental Sf-9 cells, would not be expected to produce active enzyme. In Fig. 1, proteolytic processing of rhβ-protryptase by an HMC-1 extract occurred in the absence of exogenous heparin or dextran sulfate, but without tryptase enzymatic activation. Most likely, endogenous heparin-stabilized tryptase and DPPI removed the propeptide, but insufficient heparin was available to permit formation and stabilization of tetrameric rhβ-tryptase. In vivo, human basophils, which express small amounts of tryptase mRNA and protein, should not be able to efficiently process the enzyme unless heparin synthesis also could be induced. Although the NH_2 terminus of mature β-tryptase (Ile^1-Val^2-Gly^3-Gly^4-Gln^5-Glu^6 . . .) does not contain a stop sequence for DPPI (P1 Pro or P2 Arg), digestion ceases at the 1 position, suggesting a conformational change occurs immediately after dipeptide removal to protect the NH_2 terminus.

The current mechanism of autoprocessing yields an active homotetramer of rhβ-tryptase. This is likely to be the exclusive form of active tryptase in humans. However, it remains possible that allelic variations or multiple genes of β-tryptase exist. The model for tryptase processing predicts that the rhα-tryptase precursor, with an Arg^{-3}/Gln^{-3} substitution, cannot be processed by tryptase, and therefore will not become enzymatically active unless a different processing mechanism exists. Murine and canine tryptases contain Arg^{-3}, suggesting they undergo a similar processing pathway to human β-tryptase. Thus, the association of heparin and tryptase in mast cell secretory granules in different species probably relates to a dependence on heparin for processing of tryptase at acid pH. The requirement for heparin to stabilize the active enzyme at neutral pH pertains primarily to human tryptase.

Human α-tryptase is the predominant form of the enzyme detected by immunoassay in serum from normal subjects and is the predominant form that is elevated in the serum from subjects with systemic mastocytosis (10). In contrast, β-tryptase is the predominant form found by immunoassay in the serum from subjects undergoing severe systemic anaphylactic reactions, and is the predominant form detected in tissue mast cells. The inability to autoprocess α-tryptase precursor may explain this data indicating that α-tryptase is not stored in secretory granules but instead is constitutively secreted (10). We hy-

pothesize that failure to process the propeptide of the α-tryptase precursor diverts it from trafficking to the secretory granules to a pathway leading to its constitutive secretion.

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