Human Tryptases α and β/II Are Functionally Distinct Due, in Part, to a Single Amino Acid Difference in One of the Surface Loops That Forms the Substrate-binding Cleft*

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Tryptases α and β/II were expressed in insect cells to try to ascertain why human mast cells express these two nearly identical granule proteases. In contrast to that proposed by others, residue -3 in the propeptide did not appear to be essential for the three-dimensional folding, post-translational modification, and/or activation of this family of serine proteases. Both recombinant tryptases were functional and bound the active-site inhibitor diisopropyl fluorophosphate. However, they differed in their ability to cleave varied trypsin-susceptible chromogenic substrates. Structural modeling analyses revealed that tryptase α differs from trypase β/II in that it possesses an Asp, rather than a Gly, in one of the loops that form its substrate-binding cleft. A site-directed mutagenesis approach was therefore carried out to determine the importance of this residue. Because the D215G derivative of trypatase α exhibited potent enzymatic activity against fibrinogen and other tryptase β/II-susceptible substrates, Asp215 dominantly restricts the substrate specificity of trypatase α. These data indicate for the first time that tryptases α and β/II are functionally different human proteases. Moreover, the variation of just a single amino acid in the substrate-binding cleft of a trypatase can have profound consequences in the regulation of its enzymatic activity and/or substrate preference.

Mast cells (MCs)1 reside in connective tissue matrices and epithelial surfaces and are important effector cells in acquired and innate immunity. Human MCs express at least four closely related tryptases (designated human tryptases I, β/II, III, and α)2 (1–4), and this family of serine proteases has been implicated in asthma and other allergy-related disorders. Although the amino acid sequences of the varied tryptases are ≥93% identical, there are at least four genes on human chromosome 16 that encode related but distinct tryptases (6, 7). It has been shown recently that the two related tryptases designated mouse MC protease (mMCP)-6 (8, 9) and mMCP-7 (10, 11) are metabolized differently in vivo (12) and have dissimilar substrate specificities (13, 14). Nevertheless, it is presently unclear why human MCs express so many homologous tryptases. Native (15) and recombinant (16) human trypatase β/II can degrade fibrinogen but whether or not human trypatase α is a functional enzyme is controversial. While normal human basophils contain a small amount of trypatase α protein (17) and mRNA (18), substantial numbers of tryptase α+ cells have been found in the blood of patients with asthma, chronic allergies, or adverse drug reactions (19). The level of trypatase α is also elevated in the sera of patients with systemic mastocytosis (20). Thus, whether or not tryptase α is a functional neutral protease in humans is of critical importance.

Using an expression/site-directed mutagenesis approach, we now show that tryptases α and β/II are functional enzymes but that trypatase α exhibits a more restricted substrate specificity due to an alteration in one of the loops that forms its S1, S2, and S3 sites. Thus, like in the mouse, a primordial human trypatase gene duplicated a number of times to generate a family of functionally distinct granule proteases.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Human Tryptases—Using a reverse transcriptase polymerase chain reaction approach, cDNAs were isolated from human lung RNA (CLONTECH) that encode trypatases α and β/II. In each instance, synthesis of the first DNA strand was accomplished using total RNA, random primers, and avian myeloblastosis virus-derived reverse transcriptase. The trypatase α cDNA was amplified from the multitude of lung cDNAs in the preparation using oligonucleotides (5′-ATTGCTGAGGTGCGTTCTG-3′ and 5′-TGACTCTCAGGTTTTTTGGG-3′) that correspond to relatively conserved sequences in the hydrophobic signal peptide and the C terminus of the trypatase transcript. The trypatase β/II cDNA was amplified using oligonucleotides 5′-GGCCAGGATTGCTGAATCTG-3′ and 5′-TGACTCACGGCTTTTTGGG-3′. Each cycle of the polymerase chain reaction consisted of a 1-min denaturing step at 90 °C, a 2-min annealing step at 60 °C, and a 3-min extension step at 72 °C. The polymerase chain reaction products were purified on a 1% low melting point gel and ligated into the TA-cloning vector (Novagen, Milwaukee, WI). After Novablast competent cells were transformed with the plasmids, clones were isolated, and the nucleotide

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† The abbreviations used are: MC, mast cell; DFp, diisopropyl fluorophosphate; EK, enterokinase; mMCP, mouse MC protease; pRNA, p-nitroanilide; PAGE, polyacrylamide gel electrophoresis.
‡ Although the initial publication (2) that described the nucleotide and amino acid sequences of human trypatase α contained a number of errors, the correct sequences (5) have been deposited recently in GenBank® (accession number M30038). Tryptases β (3) and II (4) were independently cloned by different groups. Nevertheless, they are the same MC protease.
quences of their inserts were determined to confirm that the isolated cDNAs encode authentic tryptases \( \alpha \) and \( \beta / \II \).

Recombinant human tryptases \( \alpha \) and \( \beta / \II \) were expressed as pseudoglobulins in baculovirus-infected High Five™ insect cells (Invitrogen, San Diego, CA), as described previously for mMCP-6 (14) and mMCP-7 (13). The cDNAs encoding the human tryptases were inserted in the pALTER®-1 vector (Promega, Madison, WI). Site-directed mutagenesis was performed using the Altered Sites® II mutagenesis system (Promega), according to the manufacturer's instructions. The mutagenic oligonucleotide 5'-TTCAGCTGGGGCGAGGGCTGT-3' (corresponding to nucleotides 724–744 in the isolated tryptase \( \alpha / \II \) cDNA) was used to convert Asp9 to Gly9.

A similar site-directed mutagenesis approach was used to evaluate whether or not Arg3 was essential for the proper folding and/or activation of recombinant tryptase \( \beta / \II \) in our expression system. In these latter experiments, the mutagenic oligonucleotide 5'-CAGGGCCG-TGAGAAGTGGGC-3' was used to convert Arg3 to Gln3 in the tryptase \( \beta / \II \) pseudoglycomogin.

Human Mast Cell Tryptases—MC tryptases bind to heparin in a conformation-dependent manner due to the alignment of a number of positively charged residues on the surface when the serine proteinase is properly folded (23). Analogous to that carried out on recombinant pro-MCP-7 (23), heparin-Sepharose chromatography was therefore used to evaluate whether or not recombinant tryptase \( \beta / \II \), tryptase \( \alpha \), and the D215G mutant of tryptase \( \alpha \) were properly folded. Samples were treated with Coomassie Blue or placed in a Bio-Rad immunoblotting apparatus. The samples were incubated for 60 min at 37 °C. SDS-PAGE loading buffer was added (33 μl), and each sample was boiled for 5 min before subjecting the [3H]DFP-labeled proteins to SDS-PAGE analysis. The resulting gel was treated with EN'HANCE (NEN Life Science Products), dried, and exposed to x-ray film. A duplicate gel was stained with Coomassie Blue to confirm that similar amounts of protein were present in each lane.

Tryptase \( \alpha \), the D215G mutant of tryptase \( \alpha \), tryptase \( \beta / \II \), and the R-3Q mutant of tryptase \( \beta / \II \) were incubated at room temperature or 37 °C for up to 24 h to determine the stability of each recombinant protease. At various times, samples of the digest were evaluated for the ability of the tryptase to cleave the trypan-susceptible \( \beta \)-nitroanilide (pNA) chromogenic substrates Ile-Phe-Lys-tosyl-Gly-Pro-Phe-Arg-pNA, N-benzoyl-Ile-Glu-Ala-Arg-pNA, N-benzoyl-Lys-Ala-Arg-pNA, N-benzoyl-Phe-Val-Arg-pNA, N-benzoyl-Pro-Phe-Arg-pNA, tosyl-Gly-Pro-Lys-pNA, tosyl-Gly-Pro-Arg-pNA (Sigma), and H-D-PRO-Arg-pNA (American Diagnostica Inc., Greenwich, CT), as described previously for recombinant mouse tryptases (13, 14, 26). One unit is defined as the amount of enzymatic activity that induces an 0.091 change in optical density at 405 nm/5 min when tosyl-Gly-Pro-Arg-pNA is used as a substrate.

The ability of recombinant tryptase \( \alpha \), the D215G mutant of tryptase \( \alpha \), tryptase \( \beta / \II \), and the R-3Q mutant of tryptase \( \beta / \II \) to degrade the more physiologic substrate fibrinogen was also evaluated. Human fibrinogen (Sigma) (40 μg), 50 μl of digestion buffer (25 mM sodium phosphate, 1 mM EDTA, pH 7.4), and 10 μl of mature tryptase \( \beta / \II \) (2 μg) were mixed and incubated for 1–2 h at 37 °C. The resulting digests were subjected to SDS-PAGE analysis, as described previously for the evaluation of the mMCP-7-mediated digestion of mouse fibrinogen (13).

RESULTS

Recombinant tryptase \( \alpha \), tryptase \( \beta / \II \), and the R-3Q mutant of tryptase \( \beta / \II \) were generated in insect cells to determine whether residue 3 was critical for the three-dimensional folding of their zymogens, as well as to compare the substrate specificities of these tryptases in their mature forms. Insect cells could be induced to express large amounts (up to 10 μg/ml) of 0.4 units of recombinant EK (New England Biolabs) was added, and the mixture was incubated at room temperature for 17 h to allow EK to activate each tryptase pseudozymogen.

Immunoblot and N-terminal Amino Acid Analysis—Samples from insect cell-conditioned medium infected with varied recombinant baculovirus pseudoglycomogins were analyzed by SDS-PAGE using a 10% polyacrylamide gel in a Bio-Rad Mini-PROTEAN® III cell, and proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk and 0.02% Tween 20 in TBST buffer, the protein blot was incubated for 1 h at room temperature in Tris-buffer saline containing 5% nonfat milk and 0.02% Tween 20 (TBST buffer). After three washes with TBST buffer, the protein blot was incubated with a 1:500 dilution of affinity-purified anti-human tryptase immunoglobulin (Ig) (–1.5 μg/ml final concentration) (25) (Chemicon, Temecula, CA) or with mouse anti-FLAG M2 Ig (–5 μg/ml final concentration) (Sigma) in TBST buffer for 1 h at room temperature. After three washes with TBST buffer, the protein blot was incubated in a 1:1000 dilution of affinity-purified mouse anti-heparin antibodies (Oncogene Science, Mancheter, MA) for 1 h. Bound antibody was detected by using 1:1000 diluted in TBST buffer, the protein blot was incubated for 1 h at 37 °C. SDS-PAGE loading buffer was added (33 μl), and each sample was boiled for 5 min before subjecting the [3H]DFP-labeled proteins to SDS-PAGE analysis. The resulting gel was treated with EN'HANCE (NEN Life Science Products), dried, and exposed to x-ray film. A duplicate gel was stained with Coomassie Blue to confirm that similar amounts of protein were present in each lane.

The secreted recombinant protein was attempted. When the D215G mutant of tryptase \( \alpha / \II \) was subcloned into the pALTER®-1 vector (Promega, Madison, WI), site-directed mutagenesis was performed using the Altered Sites® II mutagenesis system (Promega), according to the manufacturer's instructions. The mutagenic oligonucleotide 5'-TTCAGCTGGGGCGAGGGCTGT-3' (corresponding to nucleotides 724–744 in the isolated tryptase \( \alpha / \II \) cDNA) was used to convert Asp9 to Gly9. A similar site-directed mutagenesis approach was used to evaluate whether or not Arg3 was essential for the proper folding and/or activation of recombinant tryptase \( \beta / \II \) in our expression system. In these latter experiments, the mutagenic oligonucleotide 5'-CAGGGCCG-TGAGAAGTGGGC-3' was used to convert Arg3 to Gln3 in the tryptase \( \beta / \II \) pseudozymogom.

High Five™ insect cells (Invitrogen) were cultured in serum-free, Xpress medium (BioWhittaker, Walkersville, MD). General days later, the conditioned medium was centrifuged at 1500 × g for 15 min at room temperature before purification of the secreted recombinant protein was attempted. Asp9 resides in one of the loops predicted to form the substrate-binding cleft of tryptase \( \alpha \) based on the crystallographic structures of pancreatic trypsin (21) and tryptase \( \beta / \II \) (22). To evaluate the functional role of this amino acid, the bioengineered cDNA that encodes the pseudozymogen form of the tryptase was subcloned into the pALTER®-1 vector (Promega, Madison, WI). Site-directed mutagenesis was performed using the Altered Sites® II in vitro mutagenesis system (Promega), according to the manufacturer's instructions. The mutagenic oligonucleotide 5'-TTCAGCTGGGGCGAGGGCTGT-3' (corresponding to nucleotides 724–744 in the isolated tryptase \( \alpha / \II \) cDNA) was used to convert Asp9 to Gly9. A similar site-directed mutagenesis approach was used to evaluate whether or not Arg3 was essential for the proper folding and/or activation of recombinant tryptase \( \beta / \II \) in our expression system. In these latter experiments, the mutagenic oligonucleotide 5'-CAGGGCCG-TGAGAAGTGGGC-3' was used to convert Arg3 to Gln3 in the tryptase \( \beta / \II \) pseudozymogom.

Human Mast Cell Tryptases—An immunoaflinity column (24) (International Biotechnol. Inc., New Haven, CT) was used to purify recombinant tryptase \( \alpha \), the D215G mutant of tryptase \( \alpha \), tryptase \( \beta / \II \), and the R-3Q mutant of tryptase \( \beta / \II \) from the insect cell-conditioned medium. Each column (2 ml) was equilibrated in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4. After application of 200 ml of the insect cell-conditioned medium, the column was washed with the pH 7.4 buffer. Bound recombinant protein was eluted with 0.1 M glycine, pH 4.0. The final eluate was collected into tubes that contained 0.1 M Tris-HCl, pH 7.0, to minimize acid-mediated denaturation of the recombinant pseudozymogen. The protein concentration of the eluate was estimated using a Bio-Rad colormetric assay or by measuring its enzymatic activity at 280 nm. Purified pseudozymogen (~100 μg placed in 100 μl buffer containing 0.1% Triton X-100, with or without 50 μg of heparin glycosaminoglycan. The heparin (Sigma) used in the study had been subjected to a guanidination HCl/CsCl density-gradient centrifugation step (14) to denature and remove any trace protein contaminants that might be present in the commercial preparation of this glycosaminoglycan. In most cases, a 1-μl solution containing
conditioned medium) of pseudozymogen forms of tryptase \(a\) (Fig. 1A), tryptase \(\beta/II\) (Fig. 1B), and the tryptase \(\beta/II\) mutant (data not shown) that, in each instance, contained an EK-dependent site in its propeptide and the FLAG peptide in its C terminus. The recombinant pseudozymogens were secreted into the conditioned media and were recognized by an anti-human tryptase antibody. Because the recombinant tryptases also bound to an anti-FLAG immunofluorescence column (Figs. 1A and 1B) and a heparin-Sepharose column in a conformation-dependent manner (data not shown), all three expressed pseudozymogens appeared to be properly folded.

After EK treatment, the activated recombinant tryptase monomers exhibited the appropriate molecular mass \(a\), an SDS-PAGE gel (Fig. 1C). The mature forms of tryptases \(a\) and \(\beta/II\) also possessed the expected N-terminal amino acid sequence of Ile-Val-Gly-Gly-Gln-Glu-Ala-Pro-Arg. The initially expressed mutated tryptases \(\beta/II\) zymogen secreted into the insect cell culture media also possessed the expected N-terminal amino acid sequence of Ala-Pro-Ala-Pro-Gly-Gln-Ala-Leu-Gln-Gln-Val-Gly which corresponds to residues \(-12\) to \(-1\). Mature tryptase \(a\) (Fig. 2) and \(\beta/II\) (data not shown), but not the inactive pseudozymogens, readily bound the active site inhibitor DFP. Recombinant tryptase \(\beta/II\) and its R-3Q mutant were both able to degrade tosyl-Gly-Pro-Lys-pNA and tosyl-Gly-Pro-Arg-pNA, but not Ile-Phe-Lys-pNA, N-acetyl-Ile-Glu-Ala-Arg-pNA, N-benzoyl-Ile-Glu-Gly-Arg-pNA, N-benzoyl-Phe-Val-Arg-pNA, or N-benzoyl-Pro-Phe-Arg-pNA. Thus, residue \(-3\) is not essential for generating active enzyme in this \(in\) \(vitro\) system. Recombinant mature tryptase \(a\) was able to cleave H-b-HHT-Ala-Arg-pNA and was able to cleave tosyl-Gly-Pro-Arg-pNA, but only if the digestion reaction was carried at room temperature for at least 4 h. The observation that tryptase \(a\) cleaves H-b-HHT-Ala-Arg-pNA much less efficiently than trypsin and cleaves tosyl-Gly-Pro-Arg-pNA much less efficiently than tryptase \(\beta/II\) suggests that tryptase \(a\) possesses a substrate specificity that is even more restricted than that of tryptase \(\beta/II\).

Seven loops form the substrate-binding cleft of each tryptase (Fig. 3A). When the substrate-binding cleft of tryptase \(a\) was compared with that of tryptases \(I, \beta/II,\) and III, 9 amino acid differences were seen in loops \(A, B, C, 1,\) and \(2\) (Fig. 3A). The discovery that a novel charged residue in loop 2 dominantly controls the substrate specificity of granzyme B, coupled with discovery that most MC tryptases cloned from different species have a noncharged Gly at residue 215 (Fig. 3B), raised the possibility that Asp\(^{215}\) restricts the substrate specificity of tryptase \(a\). A site-directed mutagenesis approach was therefore carried out to convert Asp\(^{215}\) in tryptase \(a\) to Gly. The mutant was expressed in insect cells, and after EK treatment, it was able to cleave tosyl-Gly-Pro-Lys-pNA and tosyl-Gly-Pro-Arg-pNA (Fig. 4), but not Ile-Phe-Lys-pNA, N-acetyl-Ile-Glu-Ala-Arg-pNA, N-benzoyl-Arg-pNA, N-benzoyl-Ile-Glu-Gly-Arg-pNA, N-benzoyl-Phe-Val-Arg-pNA, or N-benzoyl-Pro-Phe-Arg-pNA. Using tosyl-Gly-Pro-Arg-pNA as the substrate in reactions carried out at room temperature, the estimated \(K_m, k_{cat}\), and \(k_{cat}/K_m\) values\(^3\) of the D215G mutant of tryptase \(a\) were

\[ K_m = \frac{V_{max}}{k_{cat}} \]

\[ k_{cat}/K_m = \frac{V_{max}}{K_m} \]

\[ k_{cat} = \frac{V_{max}}{[S]} \]

where \([S]\) is the substrate concentration and \(V_{max}\) is the maximum reaction rate.

\(^3\) In these comparative studies, similar amounts (as measured by the Bio-Rad protein bioassay) of tryptase \(\beta/II\) pseudozymogen and the D215G mutant of tryptase \(a\) pseudozymogen were exposed to EK. The processed recombinant tryptases were then examined for their relative ability to cleave tosyl-Gly-Pro-Arg-pNA. Even when comparing tryptases whose amino acid sequences are 93% identical, such as these two proteases, obtained \(K_m, k_{cat}\), and \(k_{cat}/K_m\) values can only be estimated, because a number of assumptions have to be made. Studies carried out
120 μM, 1.9 s⁻¹, and 0.016 μM⁻¹ s⁻¹, respectively, whereas the corresponding values of tryptase β/II were 49 μM, 21 s⁻¹, and 0.43 μM⁻¹ s⁻¹, respectively. Because tryptase β/II cleaved tosyl-Gly-Pro-Arg-pNA ∼25-fold better than the D215G mutant of tryptase α, it is likely that pocket residues 21, 22, 23, 46, 84, 85, 88, and/or 91 (Fig. 3) contribute to the different substrate specificities of the two human tryptases.

Kinetic experiments revealed that the enzymatic activity of the D215G mutant was maintained for at least 24 h if heparin was present (Fig. 4). In addition, there was no evidence of autolysis of the recombinant protein by SDS-PAGE analysis (Fig. 1C). Wild type tryptase β/II and its R-3Q mutant were able to cleave the α chain of fibrinogen (Fig. 5B).

**DISCUSSION**

Using a bioengineering approach, pseudozymogen forms of human tryptases α and β/II were expressed in insect cells that could be activated and studied immediately (Fig. 1). Although Mirza et al. (31) were able to obtain enzymatically active tryptase α in transiently transfected COS cells, Sakai et al. (32) concluded that this human MC protease probably is not active in this cellular model. On the other hand, the pseudozymogen forms of human tryptases α and β/II expressed in insect cells were activated and studied immediately (Fig. 1). This suggests that the percentage of pseudozymogen in each preparation which is converted by EK into active enzyme is the same. It is therefore assumed that the rate of conversion of inactive monomer to active tetramer and then back to inactive monomer is the same for both tryptases. Finally, it is assumed that each tryptase tetramer has the same number of active sites.

**FIG. 3.** Comparison of amino acid sequences of human tryptases α, β/II, and III. A, the amino acid sequences of human tryptases α (2), I (4), β/II (3, 4), and III (4) are compared. The seven loops (A, B, C, D, E, F, and G) predicted to form the substrate-binding cleft of each tryptase based on the structure of bovine pancreatic trypsin (21) and human tryptase β/II (22) are indicated in bold. The first amino acid in each mature tryptase is indicated (¥). B, the amino sequences of loop 2 are compared in human (hTryptase-α (2), I (4), β/II (3, 4), and III (4)), mouse (mMCP-6 (8, 9) and mMCP-7 (10, 11)), rat (rMCP-6 (27, 28) and rMCP-7 (28)), dog (dTryptase (29)), and gerbil (gTryptase (30)) tryptases. The Asp residue that is only found in human tryptase α is indicated (¥).
functional in vivo. The latter investigators noted that the propeptide of tryptase α differs from that of mouse, rat, and other human tryptase zymogens at residue −3. Based on their inability to obtain active enzyme in an insect cell expression system, Sakai et al. (32) concluded that all tryptases probably undergo an unusual multistep activation process in MCs that is exquisitely heparin-dependent. To explain their in vitro findings, these investigators proposed that a conformation-dependent change occurs when a tryptase zymogen initially binds to heparin, which in turn causes a partial autocatalytic event resulting in the removal of the first 8 residues of the propeptide. It was then proposed that dipeptidyl peptidase I removes the remaining 2 residues of the zymogen in the granule to create the mature enzyme. Unexplained in the Sakai et al. (32) mechanism of tryptase activation is how a heparin-bound tryptase zymogen can partially activate itself yet still fail to cleave varied low molecular weight substrates.

We have noted that certain recombinant mouse MC chymases denature spontaneously during their expression in insect cells. Because the propeptide often is needed for the proper folding of a serine protease (33), we examined whether or not Arg−3 is essential for the proper folding of tryptases in insect cells. Using a site-directed mutagenesis approach, Arg−3 in tryptase β/II was converted to Gln−3. Analogous to wild type tryptase β/II, the R-3Q derivative of the pseudozymogen could be readily activated in our in vitro system. While this finding alone does not prove that tryptase α is post-translationally processed into a functional enzyme in human MCs, a tryptase has been cloned from gerbil jejunal MCs (30), which is enzymatically active (34) even though its zymogen possesses a Glu at residue −3. In addition, Wang et al. (35) recently generated active tryptase β/II in insect cells using a fusion protein approach in which the natural propeptide of human tryptase β/II was replaced by that which encodes ubiquitin followed by a short EK-susceptible peptide. More definitive data relevant to this issue have been obtained recently during analysis of the transgenic mouse we created which is unable to express heparin due to targeted disruption of the N-deacetylase/N-sulfotransferase-2 gene. Although the MCs developed from these heparin-null mice are unable to store the chymase mMCP-5 and the exopeptidase mMC-CPA in their granules, they do contain substantial amounts of enzymatically active tryptase.5 The cumulative findings from all of these studies now indicate that heparin is not essential for tryptase expression in MCs and that Arg−3 is not essential for the proteolytic activation of tryptase zymogens. Because we concluded that Mirza et al. (31) are probably correct in their conclusion that human tryptase α is a functional enzyme in vivo, we expressed this tryptase and tryptase β/II in our insect cell expression system to try to address why human MCs produce so many nearly identical neutral proteases.

Insect cell-derived recombinant tryptases α and β/II have all of the features of biologically active serine proteases (Fig. 3A). DFP binds to each mature tryptase but, importantly, not to their pseudozymogens (Fig. 2). These observations indicate that DFP is not binding to either tryptase in a nonspecific manner. In vitro and in vivo studies carried out in mice with recombinant mouse tryptases (13, 14) have confirmed earlier structural (11, 22) and biochemical studies (36), which had suggested that mouse MC tryptases possess substrate specific-

4 C. Huang and R. L. Stevens, unpublished results.
5 D. E. Humphries and R. L. Stevens, unpublished results.
FIG. 5. Digestion of human fibrinogen by recombinant human tryptases. A, fibrinogen was placed in buffer alone (lane 1), or with mature tryptase β/II (lane 3), or the R-3Q mutant of tryptase β/II (lane 2). After a 2-h incubation at 37 °C, the resulting samples were subjected to SDS-PAGE analysis. Molecular mass standards are shown on the right. The solid and open arrows on the left indicate the α and β chains of fibrinogen, respectively. B, in a similar manner, fibrinogen was incubated in buffer alone (lane 1) or buffer containing EK (lane 4), mature tryptase α (lane 3), or the D215G mutant of tryptase α (lane 2). The solid arrow on the right indicates the α chain of fibrinogen.

ities that are considerably more restricted than that of trypsin. The physiologic function of tryptase α remains to be determined. However, the in vitro studies carried out in the present investigation now suggest that tryptase α also has a restricted substrate specificity and one that is different from that of tryptase β/II.

Fibrinogen is a major protein constituent of blood. Although tissue edema can be quite pronounced during a MC-mediated inflammatory reaction, one generally does not see the deposition of large amounts of fibrin/platelet clots in affected tissues. Fibrinogen is a physiologic substrate of mMCP-7 (13), and both native (15) and recombinant tryptase β/II (16) can cleave fibrinogen in vitro. Thus, tryptase β/II probably plays an important role in preventing the accumulation of clots that would physically hinder lymphocyte and granulocyte extravasation into the inflamed tissue. Although the amino acid sequences of tryptase α and β/II are 93% identical, recombinant tryptase α was unable to degrade human fibrinogen in vitro (Fig. 5). Seven loops form the substrate-binding cleft of each serine protease (37). Although the loops that comprise the substrate-binding cleft of a human tryptase represent only ~20% of the mature enzyme, 9 of the 18 amino acid differences between tryptase α and tryptase β/II reside precisely in this small portion of the protease (Fig. 2A).

Because the substrate-binding cleft was selectively mutated during the evolution of these two human proteases, we reasoned that it probably occurred to generate functionally distinct tryptases. Based on the crystallographic structure of tryptase β/II (22), loop 2 (corresponding to amino acid residues 211–218) contributes substantially to the enzyme's S1, S2, and S3 sites. A comparative analysis of their amino acid sequences revealed that tryptase α has an Asp at residue 215 rather than Gly, which is found in mouse, rat, dog, gerbil, and other human tryptases (Fig. 3D). This observation raised the possibility that human tryptases α and β/II might be functionally different, in part, due to this amino acid substitution. To explore this possibility, a site-directed mutagenesis approach was carried out to change Asp215 in tryptase α to Gly (Fig. 1). Like wild type tryptase β/II, the resulting D215G mutant of tryptase α was able to effectively cleave the chromogenic substrate tosyl-Gly-Pro-Lys-pNA (Fig. 4). The mutant also was able to cleave the α chain of human fibrinogen (Fig. 5). It is well known that loop 2 impacts the substrate specificities of other serine proteases. For example (38), the presence of an Arg in loop 2 causes granzyme B to prefer substrates which have an Asp at the P1 site rather than a hydrophobic residue preferred by many other members of the chromosome 14 family of serine proteases. Although the bulky negatively charged amino acid at residue 215 explains the restricted substrate specificity of tryptase α relative to that of tryptase β/II, it is likely that, in vivo, tryptase α still prefers substrates that have a positively charged amino acid at their P1 sites.

Like mMCP-6 and mMCP-7 in the mouse, human tryptases α and β/II are functional proteases that differ in their substrate specificities. It now appears that these two neutral proteases evolved so that human MCs can degrade different extracellular proteins during an inflammatory reaction.

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Human Tryptases \( \alpha \) and \( \beta/II \) Are Functionally Distinct Due, in Part, to a Single Amino Acid Difference in One of the Surface Loops That Forms the Substrate-binding Cleft

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