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Review

Mast cell tryptase, a still enigmatic enzyme

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Abstract. Tryptases constitute a subfamily of trypsin-like proteinases, stored in the mast cell secretory granules of all mammalian organisms. These enzymes are released along with other mediators into the extracellular medium upon mast cell activation/degranulation. Among the trypsin-like enzymes, tryptases are unique: they are present as active enzymes in the mast cell granules, but display activity only extracellularly, and have a specificity which is much more restricted than trypsin. Tryptases are mostly tetrameric, and in only few organisms (not in humans) are they inhibited by endogenous inhibitors in vitro. The enzymatic and molecular properties of tryptases are far better characterized that any of their plausible biological functions. On the basis of its structural and functional features it could be predicted that tryptase would not degrade a large number of proteins in vivo due to low accessibility to the tetramer central pore where the active sites face inwards. Although their biological function has not yet been clarified, tryptases seem to be involved in a number of mast cell-mediated allergic and inflammatory diseases. In particular, the involvement of tryptase in asthma, an inflammatory disease of the airways often caused by allergy, has been proposed. Here we review the present knowledge on the structure-function relationship of tryptases from different organisms, with special emphasis on human enzymes, and on their role in a variety of pathophsyiological processes.

Key words. Tryptase; mast cell; protease-activated receptor; tryptase inhibitors; allergy; asthma.

Introduction

Tryptases and chymases, the neutral serine proteases with trypsin-like and chymotrypsin-like activity, respectively, are the major protein components of mast cells (MCs) in higher eukaryotes. They are expressed as protein precursors, mostly processed and stored in the secretory granules of mast cells in a catalytically active form, at variance with the pro-enzymes (zymogens) of the pancreatic granules. The acidic pH (5.1) of the mast cell granules maintains the low activity of tryptase and chymase in situ, thus helping to prevent autolysis and degradation of mast cell protein constituents. Mast cells, which are ubiquitous cells located in all mammalian connective tissues or associated with small blood vessels and nerves, play a role in inflammation, immunity and tissue remodelling. These cells derive from haematopoietic precursors [1, 2] and express on their surface the receptors (FcεRI) that bind the Fc portion of immunoglobulin (Ig)E antibody with high affinity [3]. Their activation/degranulation is in fact mediated by IgE-FcεRI binding and antigen recognition, which then leads to receptor crosslinking and aggregation. This results in the rapid release, from cytoplasmic granules to the extracellular milieu, of preformed mediators, including proteases, cathepsin G, carboxypeptidases, histamine and heparin. Mast cells can also be activated by IgE-independent reactions, mediated by drugs or proinflammatory molecules. Tryptase, chymase, cathepsin G and carboxypeptidases are the main protein molecules of the MC_{TC} subtype secretory granules, whereas

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Table 1. Sequence identity percentage of 20 mature tryptases from different organisms.

human β II	**99**	**98**	**92**	**93**	47	46	38	**75**	**77**	**76**	48	38	**77**	54	**77**	**72**	**73**	**76**	**76**
human β I		**98**	**93**	**94**	47	46	38	**76**	**78**	**76**	48	38	**77**	54	**77**	**72**	**73**	**76**	**76**
human β III			**94**	**95**	47	47	37	**76**	**78**	77	47	37	**77**	53	**77**	**73**	**74**	**77**	**77**
human α I				**98**	48	48	38	**73**	**74**	**76**	47	37	**75**	53	**75**	**71**	**73**	**75**	**75**
human α II					48	48	38	**74**	**75**	**76**	48	37	**75**	53	**76**	**72**	**73**	**75**	**75**
human γ I						**97**	36	47	47	46	**71**	38	47	45	48	44	44	47	47
human γ II							37	46	48	46	**71**	38	47	45	48	44	44	47	47
human ε								41	42	40	36	35	40	35	39	42	42	40	40
rat MCT									**89**	71	45	39	**74**	52	**73**	69	**70**	**70**	**70**
mouse MCP-6										71	45	40	**73**	52	**74**	**70**	71	71	71
mouse MCP-7											46	40	71	53	**88**	67	68	**72**	71
mouse TMT												40	47	46	47	46	45	47	48
mouse T4													40	35	40	39	40	39	39
dog														53	72	73	74	76	76
dog MCP-3															53	53	53	52	53
gerbil																68	69	74	74
sheep 1																	96	81	83
sheep 2																		82	82
bovine 1																			96
bovine 2																			

Sequence identities ≥70% between 245-aa tryptases are shown in bold.

only tryptase is present in the secretory granules of MC_T subtype. While MC_{TC} subtype predominates in dermis and other connective tissue, MC_T subtype is present in other sites, such as intestinal mucosa, alveolar interstitium and also bronchi [4].

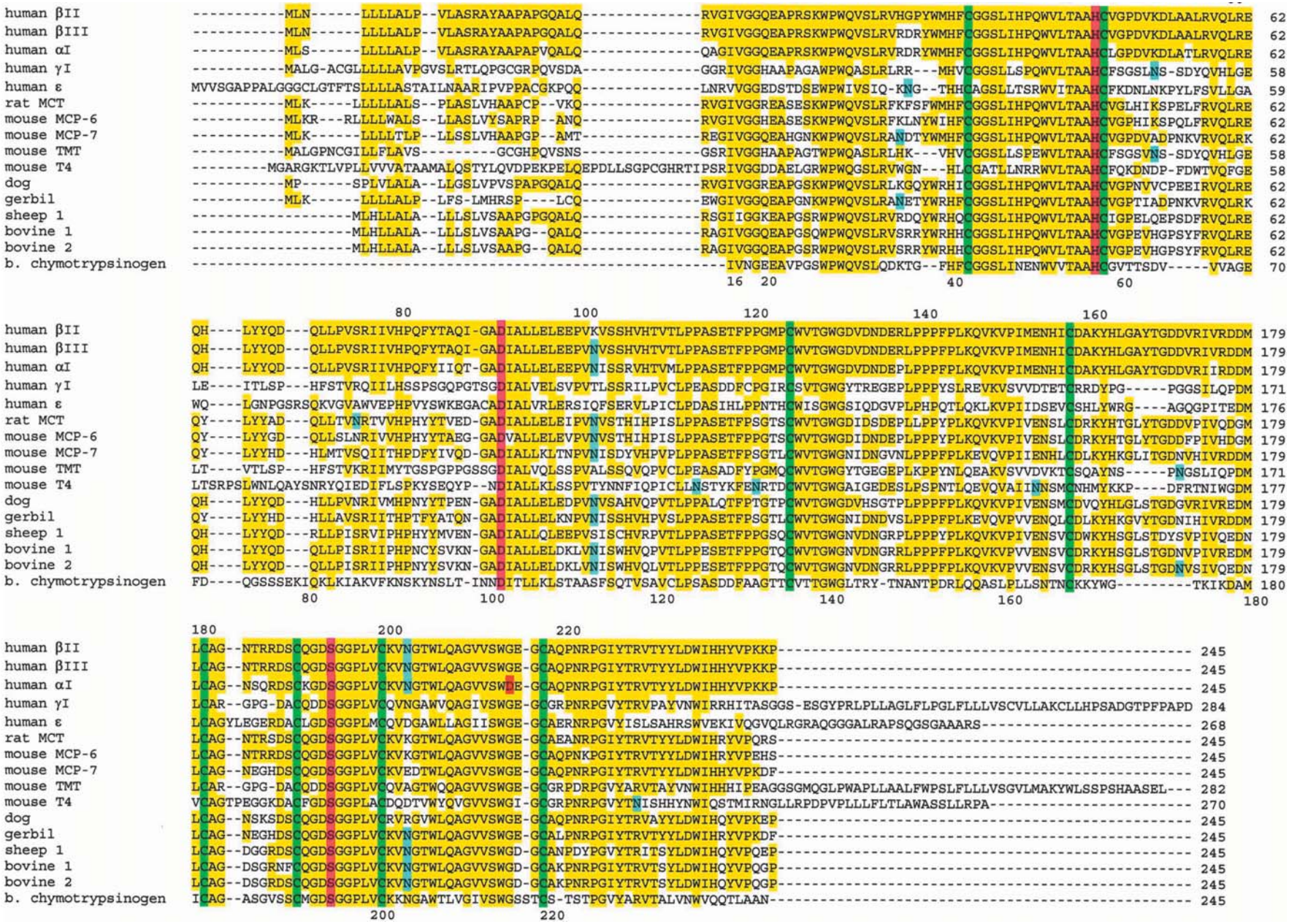
Most tryptases are stored in the secretory granules as catalytically active tetramers made of identical subunits. In humans, tryptase amounts to ~90% of the total protein content of the secretory granules and seems to have an important role in a number of mast cell-mediated allergic and inflammatory diseases, including rhinitis, conjunctivitis and most notably asthma. Moreover, human tryptase is implicated in certain gastrointestinal, dermatological and cardiovascular disorders [5, 6]. However, although a trypsin-like activity in human and dog mast cells was recognized more than 4 decades ago [7], the true biological function of tryptases is not yet clarified. This review will focus on the present knowledge of the structure-function relationships of tryptase from different organisms and on the biological function of this still enigmatic enzyme.

The tryptase family

The tryptase family is very large and still growing. New mast cell isoenzymes with tryptic-like activity have been discovered in different species and characterized, mainly by cloning their complementary DNAs (cDNAs) and genes. Tryptases have been found in a number of mammalian organisms, including human, dog, mouse, rat, gerbil, sheep and cow.

Human tryptase (E.C.3.4.21.59) was first isolated from human lung mast cells in 1981 and later directly from tissues such as pituitary, lung and skin [5]. Eight-nine human tryptases are known so far. The best studied among them are the α-type and β-type tryptases, which are quite similar in their primary structure (see fig. 1 and table 1). The cDNAs encoding human tryptases I [8], II or β [8, 9] and III [8] were obtained from skin [8] and lung [9]; these three tryptases were then considered as β-type tryptases and named βI, βII and βIII. αI tryptase cDNA was cloned for the first time from lung [10], while a gene for a second α-type tryptase (αII tryptase) was more recently identified [11]. β-Tryptases are abundantly expressed in mast cells (up to 35 pg per mast cell in human lung), where they are stored as mature proteins before release; in contrast, α-tryptases are predominant in basophils [12] and are constitutively released as pro-forms from mast cells (see later), where they are expressed in small amounts.

Probing the GenBank expressed-sequence tag (EST) databases with known tryptase cDNAs, new partial tryptases were identified; amplification of lung cDNA with proper primers resulted in the isolation of complete cDNAs encoding novel tryptases, whose sequences were in agreement with those deduced by genomic sequencing. These new members of the tryptase family were named γ-tryptases I and II [13] and were found at the RNA level in a variety of human tissues and in the human mast cell line HMC-1/subclone 5C6 [14], where they are also expressed at the protein level. At variance with α and β tryptases and most tryptases from other organisms, γ tryptases contain an extended C-terminal hydrophobic domain, which is considered important in anchoring the protease to the membrane, and a small cytoplasmic tail. γ-Tryptase I is almost identical (98–99%) to human TMT tryptase (transmembrane tryptase), described by


human βII
human βIII
human αI
human γI
human ε
rat MCT
mouse MCP-6
mouse MCP-7
mouse TMT
mouse T4
dog
gerbil
sheep 1
bovine 1
bovine 2
b. chymotrypsinogen

Stevens and co-workers [15, 16]; these authors isolated and characterized the *hTMT* gene and the gene for the mouse ortholog mTMT. Immunological evidence suggests that γ tryptases/hTMT are expressed in lung mast cells. It has been proposed that due to the membrane-spanning C-terminal domain, after mast cell degranulation, hTMT is retained on the mast cell surface, thus interacting with nearby cells [16].

Recently, expression at the RNA level of the so called δI and δII tryptases in multiple human tissues was reported [17]. The cloned cDNAs contained a premature termination codon relative to that of other tryptases. The corresponding protein(s) was immunologically detected in the mast cells of some tissues using a polyclonal antibody raised against a peptide having 50% identity with other tryptases. Genes corresponding to δ tryptases were originally characterized and named human mouse mast cell protease 7 (hmMCP-7)-like I and II genes [11], for the resemblance of their putative exon 5 with the corresponding exon of mMCP-7. However, these genes were then considered as pseudogenes by the same authors [18] in agreement with independent results by Schwartz and coworkers [19].

Finally, by screening the EST database and through a PCR approach, a novel human tryptase, named ε tryptase, was identified [20], which is abundantly expressed at the messenger RNA (mRNA) level in epithelial cells, in adult trachea and esophagus and in phetal lung. However, the same authors reported that ε tryptase transcript was not detected in a number of mast cell-rich tissues and the enzyme level is below detection in cultured HMC-1 mast cells. Thus, this enzyme is considered a normal product not of mast cells, but rather of airway epithelial cells.

In a milestone paper on chromosome location and characterization of known and novel human tryptase genes, Caughey and co-workers [11] identified and mapped to chromosome 16p13.3 two human, bacterial artificial chromosome (BAC) clones containing large, overlapping and homologous, but not identical, portions of the tryptase multigene cluster, in a region close to the telomere of chromosome 16 short arm. Their results indicate that the human genome contains three loci of transcribed and translated mast cell tryptase genes encoding α, βI, βII, βIIII and γ/transmembrane tryptases. The locus accommodating α tryptase also accommodates βI tryptase. The latter is the βIa version, having Glu at position 99, while a βIb version, with Lys in the same position, is also transcribed in HMC-1 cells. βII and βIII tryptase genes are only found in the adjacent locus, and γ/transmembrane tryptases are accommodated in the third locus. δ tryptase pseudo-genes are situated more centromerically [18]. The tryptase ε gene also resides on chromosome 16p13.3, within the same 2.5-Mb region of tryptase genes [20]. The finding that α and βI alleles compete at one locus led to the hypothesis that some individuals may have a ββββ genotype, with no α-tryptase genes [18]. In fact, it was found that genetic α-tryptase deficiency is surprisingly common (29% human population) and that individuals with no α-tryptase genes inherited two βI genes at locus α/βI [21].

Four murine tryptases have been described so far, named mMCP-6 (mast cell protease-6 or mouse tryptase 1), mMCP-7 (mast cell protease-7 or mouse tryptase 2), mTMT (mouse transmembrane tryptase) and mT4 (mouse tryptase 4). mMCPs-6 and -7 were identified by cDNA and gene cloning [22–24]. mMCP-6 mRNA is present in connective tissue mast cells and not in mucosal mast cells, whereas the mMCP-7 transcript has been detected only in immature bone-marrow-derived mast cells (BMMCs), where it is selectively and transiently expressed, but not in more mature mast cells. Substantial amounts of mMCP-6 and mMCP-7 are contained in the secretory granules of mice tongue, skin, spleen and heart mast cells. mTMT encodes for a transmembrane tryptase, containing an unusual C-terminal hydrophobic extension, also present in hTMT; transcription of mTMT is regulated in BALB/c mouse in a tissue-dependent manner, which is independent of mMCP-6 and mMCP-7 expression [15]. Mouse tryptase 4 (mT4) was identified by probing the GenBank EST database with mMCP-6, mMCP-7, mTMT cDNAs and proper amplification [25]. Full-length transcript shows the presence of a short hydrophobic extension at the C-terminus in the translated protein, which is a membrane-anchored protease, similar to mTMT. However, at variance with other members of its family, mT4 mRNA is below detection in mast cell-rich tissues; large amounts of the transcript were found in interleukin (IL)-5-dependent mouse eosinophils, as well as in ovaries and testes of

Figure 1. Comparison of the preprosequences of human tryptases and of tryptases from different organisms. The sequences shown are: human tryptases βII [8], βIII [8], αI [10, 63], γI [13], ε [20], rat MCT [30], mouse MCP-6 [23], mouse MCP-7 [24], mouse TMT [15], mouse T4 [25], dog tryptase [35], gerbil tryptase [38], sheep tryptase 1 [39] and bovine tryptases 1 [44] and 2 [46]. Multiple sequence alignment was performed using the Clustal W program. Numbering above the sequences refers to tryptase precursors having 245-residue catalytic domains and begins at the first residue of all the mature proteins. Numbering below the sequences refers to the standard numbering of chymotrypsinogen A sequence (shown at the bottom). Identities between βII tryptase and other tryptases are shaded in yellow; the catalytic triad residues, the cystein residues (forming disulphide bridges in βII and αI tryptase structures) and the Asn residues of the putative N-glycosylation sites are shaded in fuchsia, green and turquoise, respectively. The unique Asp 216 residue (chymotrypsinogen numbering), mainly responsible for the inactivity of α tryptase (see text), is shaded in red.

adult mice. *mMCP-6*, *mMCP-7*, *mTMT* and *mT4* genes are all members of the chromosome 17 family of mouse tryptases [15, 25, 26].

Rat tryptase was purified from peritoneal mast cells [27] and skin [28], and localized in mast cell secretory granules [29]. Rat tryptase (rMCT) cDNA was cloned and sequenced, and its mRNA was found in lung and tongue peritoneal mast cells [30]. Two additional cDNAs encoding rat tryptases, named rMCP-6 and rMCP-7, were cloned from connective tissue mast cells of rat peritoneum and ears [31]. Recombinant rMCP-7 has recently been expressed in *Pichia pastoris* [32]. The rMCP-6 cDNA sequence differs in few positions from that of rMCT cDNA, resulting in two to three different residues in the deduced 274 aa protein precursor sequences.

Dog tryptase was isolated and characterized from dog mastocytoma cells [33] and from dog mast cells [34]. Its full protein precursor sequence was deduced from cloned cDNA obtained from a dog mastocytoma cDNA library [35]. In the same paper, the primary structure of an unknown serine protease (50% identical to dog tryptase) is reported, as deduced from a second dog mastocytoma cDNA. This protease, named dog MCP-3, was then purified and characterized [36]. The protein shares some features with mast cell tryptases, and immunochemical evidence indicates that it is expressed in mast cells and neutrophils [37].

cDNA encoding a trypsin-like enzyme was cloned from the small intestine of the Mongolian gerbil [38]; the deduced protein sequence is 88% identical to that of mMCP-7 (see table 1 and fig. 1). The enzyme exists as a functionally active protein in the mast cells of intestinal mucosa.

Two highly similar cDNA sequences encoding sheep tryptases (sheep tryptases 1 and 2) were isolated from sheep cultured BMMCs and from abomasal tissue [39]. In the same paper, a significant expression of tryptase transcript is reported occurring in sheep skin and lung. However, the corresponding protein has not been definitely identified. Quite interestingly, a sheep mast cell proteinase 1, sMCP-1, which is expressed only in mucosal mast cells, was found to have both chymotrypsin-like and trypsin-like activity [40], similarly to what has been reported for bovine duodenase [41].

The presence of tryptase in bovine cutaneous and tumour mast cells was demonstrated by cytochemical and immunohistological methods [42]. Bovine tryptase (BLCT, bovine tryptase 1) was initially isolated from bovine liver capsule (Glisson capsule) [43]. Its close association within the same mast cell granules, with bovine pancreatic trypsin inhibitor (BPTI or aprotinin), expressed in the same cells [44], was demonstrated [45]. Bovine tryptase 1 primary structure and cDNA cloning and sequencing were also reported [44]. More recently, the cDNA encoding a new bovine tryptase isoform (BLT, bovine tryptase 2) was cloned and sequenced from lung tissue [46]. Bovine tryptase 2 sequence was determined by mass fingerprinting on the tryptic digest of the isolated protein, and the tissue specific expression of both bovine tryptases was established. In lung only bovine tryptase 2 is expressed, whereas in liver capsule only bovine tryptase 1 was found. Both enzymes are present in similar amounts in heart and spleen.

Isolation of tryptase from guinea pig [47], from cynomolgus monkey lung [48], from pig lung [49] and from equine mastocytoma [50] has been also reported.

Primary structure

The monomer sequences of most tryptase precursors reported so far were deduced from their cDNAs. In many cases, the N-terminal sequence of the purified proteins, isolated from mast cells or from mast cell-rich tissues has also been reported. Only in the case of βII tryptase [51] and bovine tryptases [44, 46], have independent data on the primary structure of the isolated proteins been reported. Figure 1 shows a comparison of the primary structures of the most representative tryptase precursors from different organisms. The sequence identity percentage of twenty mature tryptases is reported in table 1.

Processing of tryptase precursors

Although the activation site of tryptases from their precursors is unknown, it is likely that activation occurs in the Golgi apparatus before packaging in the mature secretory granules. IVGG- is the N-terminal sequence conserved in all mature tryptases, except in ε tryptase (VVGG-) and in sheep 1 and 2 tryptases (IIGG-). Similarly to other mammalian proteases, tryptases are expressed as precursors, with a prepro-sequence of different length and preponderance of hydrophobic residues in the signal sequence. The most likely signal peptidase cleavage site, predictable according to the updated method of von Hejine and co-workers [52], suggests that the residual propeptide is 12 residues long in human β and α tryptases and in sheep tryptases, while it is 10 residues long in dog tryptase (as first proposed in [35]), rat tryptase (see also [30]), mMCP-6, mMCP-7 and bovine tryptases. Likewise, the most likely cleavage site predicts an 18-aa prosequence in γ/hTMT protryptase [16] and a 17-aa prosequence in ε protryptase [20].

As to the activation process, termination of the propeptide of dog mast cell tryptase with a Gly residue, present in the majority of tryptases (see fig. 1), first suggested [35] that the prosequence is removed by one or more steps and that most tryptases should be activated by proteases different from those activating pancreatic or other gran-

ule-associated serine proteases, where the hydrolysis site is a basic or an acidic residue, respectively. As a matter of fact, in vitro experiments with recombinant human β protryptase indicate that two proteolytic steps are required for activation [53]. According to the authors, the first step appears to be autocatalytic, intermolecular processing with cleavage at the Arg-3/Val-2 bond of the proenzyme, giving a precursor form containing a residual prodipeptide Val-Gly-. Both precursor forms could participate in the processing. However, why this processing is not active against other protein substrates remains unclear. In the second step, the prodipeptide is removed by dipeptidyl peptidase I (DPPI) also known as cathepsin C, a lysosomal cysteine protease present in the mast cells. Both autoprocessing and DPPI processing occur at acidic pH and would require heparin. However, data on the in vivo role of DPPI in human tryptase activation in cultured mast cells have not been reported yet. In mice, the role of DPPI appears different. By enzyme histochemistry, Caughey and co-workers [54] showed that tryptase activity in BMMCs of DPPI–/–mice is only 25% less than that found in DPPI+/– BMMC lysates. The authors reached the conclusion that DPPI regulates, by a still unknown mechanism, the total amount of active tryptase within mast cells, but is not essential for tryptase activation, unlike chymase activation.

A similar activation mechanism including two or more steps could be shared by other protryptases containing residues Arg-3 and Gly-1, such as human β-type tryptases, rMCT, mMCP-6 and mMCP-7, dog tryptases, and sheep and bovine tryptases, whereas other mechanisms must be considered for the activation of human α tryptases, γ/TMT, ε tryptases, mTMT, mT4 and gerbil tryptase. In the case of both γ protryptases, termination of the propeptide with an Arg-1 residue (as occurs in ε tryptase, mTMT and mT4) suggested [13] that activation by a tryptic protease might generate the catalytic domain of these enzymes in one step. It has also been proposed that mature γ tryptases might be two-chain proteins, with a disulphide linkage between Cys-12 in the propeptide region and Cys-108 of the catalytic domain. Whether or not the covalently attached propeptide prevents rapid inactivation of γ tryptase/hTMT by other proteases present in the mast cell secretory granules remains to be determined [16].

The case of human α protryptase, which appears not to be processed to the mature enzyme, is particularly interesting and noteworthy. In fact, the destiny of β and α tryptases is very different. Besides showing that β protryptase monomers form an enzymatically active tetramer upon monomer activation, Schwartz and coworkers [53] found that recombinant α protryptase cannot be processed for the presence of a Gln rather than an Arg residue at position –3 of the prosequence. This was in agreement with data indicating that α tryptase is not stored in the mast cell granules but is constitutively secreted as an inactive precursor. On the basis of these findings and to evaluate the tryptase serum level, two monoclonal antibodies were prepared, one recognizing only recombinant human β tryptase, and the other recognizing both β tryptase and α protryptase [55]; since β tryptase is not detectable in the serum of healthy subjects, the total tryptase level (α protryptase level) was said to reflect the total body burden of mast cells, while elevated levels of it were indicative of systemic mastocytosis; on the other hand, the serum level of β tryptase reflects mast cell activation and rises dramatically after anaphylaxis. However, as pointed out in [21], in the serum of individuals with no α-tryptase genes, α protryptase is obviously absent, and the serum tryptase level may underestimate the mast cell burden and also alter the diagnosis of systemic mastocytosis. These and other issues raised in connection with laboratory tests in individuals with total or partial α-tryptase genetic deficiency [21] were examined in a very recent paper by Schwartz and co-workers [56]. The authors measured spontaneous tryptase secretion in cultured cells expressing β-tryptase mRNA or expressing preferentially α-tryptase mRNA, and in skin-derived mast cells which contain both tryptase mRNAs. It was found that protryptases, either α- or β-type, are spontaneously secreted by mast cells at rest, whereas mature tryptase(s) is mostly retained in the mast cell granules and released upon activation. From their data, the authors reached the conclusion that total tryptase levels in plasma are not different in healthy subjects with or without the gene for α tryptase, in accordance with the spontaneous secretion of both α and β tryptases as pro-forms. However, whether or not the diagnosis of systemic mastocytosis or associated haematological disorders, based on the levels of α tryptase relative to β tryptase, is reliable in patients with α-tryptase genetic deficiency, remains to be elucidated.

Tryptase catalytic domain

The tryptase catalytic domain (fig. 1) is made of 245 residues for tryptases sharing a sequence identity higher than 70% with human β and α tryptases, and is 22 residues longer than that of bovine trypsin. Mature β tryptases are 98–99% identical: βII tryptase has a K residue at position 102, while in βI and βIII tryptase the same position is occupied by a N residue. Further, βIII tryptase contains residues RDR at positions 21–23, while in βI and βII tryptases the same positions are occupied by residues HGP (fig. 1). The fact that residues 21–23 are α-like in βIII tryptase suggests that there may have been genetic exchange between β and α genes [11]. Mature α and β tryptases are 92–95% identical, while αI and αII tryptases have a 98% identity. Human γ/TMT and ε tryptases, mTMT and mT4 have C-terminal extensions of varying length. All tryptase sequences shown in figure 1

contain the residues of the so-called serine protease catalytic triad, H44, D91 and S194 (H57, D102 and S195 in the standard chymotrypsinogen numbering) present in trypsin, chymotrypsin and in all trypsin-like enzymes, and many other identical sequence regions. An aspartic acid residue is present at position 188 in all tryptases with the exception of bovine tryptase 1 (see below). This residue corresponds to Asp 189 (chymotrypsinogen numbering), present at the bottom of the primary specificity pocket in trypsin and all trypsin-like enzymes [57]. Eight cysteine residues are highly conserved in all tryptases and align with those of trypsin and chymotrypsin, where they form four disulphide linkages as in βII and αI tryptases [51, 58] and presumably in all tryptases (see later). Extra Cys residues (up to four in γ-tryptase/hTMT and dMCP3) are present in some tryptases.

All tryptases contain putative N-glycosylation sites, which are in part conserved among species (fig. 1). In fact, human, rat, dog and bovine tryptases contain N-linked glycans, as shown by the small drop in their apparent molecular weight upon treatment with N-glycosidase F [28, 59, 60–62]. Variable N-glycosylation likely contributes to size and charge heterogeneity of tryptases from different tissues [59, 62]. Other sequence features, common to many tryptases, will be discussed later.

Gene structure

The gene structure and organization of human β and α tryptases [8, 11], mouse tryptases mMCP-6 and mMCP-7 [23, 24], and bovine tryptases 1 and 2 [46] show common features. Except for the *mMCP-7* gene, which consists of five exons and four introns, all these genes (1.8–2.3 kb) have a six exon/five intron organization, with highly conserved intron phase and size (~80–200 nt), and intron/exon boundary position. The first described α-tryptase gene [11], named αII tryptase gene, has an 11-bp deletion in intron 4 compared with the β-tryptase genes; it also shows few differences in the exon 5 sequence when compared with the sequence of the previously reported αI tryptase cDNA [10, 63], corresponding to three different residues between αI and αII tryptases. Intron I (~200 nt) is in a peculiar and unusual position, characteristic of tryptases, being inserted in phase between the 5′ untranslated region and the translation initiation codon; thus, exon 1 consists entirely of the 5′ untranslated region. In the *mMCP-7* gene [24], a point mutation at the intron 1 acceptor splice site gives rise to a large 5′ untranslated region that contains the unspliced intron and is thus longer than in mMCP-6 and other tryptases. Moreover, intron 3 (~0.8 kb) is remarkably longer than the corresponding intron 4 of other tryptases. Position and phase of the four introns within the translated region of the tryptase genes mentioned above are similar to those of trypsin and glandular kallikrein genes, but different from other serine protease genes. Therefore, assuming a common ancestor for trypsin and tryptase genes, it was suggested that the latter gene diverged from the trypsin gene more recently than from other granule-associated serine protease genes [8].

Human γ/hTMT, human ε tryptases, mouse mTMT and mT4 have a different gene structure. As mentioned above, hTMT and γ tryptase genes are very similar, the corresponding proteins being 98–99% identical, except for an exon less in the *hTMT* gene [15], which probably corresponds to the small exon 2 of γ tryptases. Caughey and co-workers [13] pointed out that the six exon/five intron organization, with extended introns, of γ-tryptase 1 and 2 genes, is more similar to that of human prostasin gene [64], a serine protease considered a potential serum marker for ovarian cancer [65], than to the more compact α- and β-tryptase genes. Not only do γ-tryptase genes share the position, phase and size of the first two introns with prostasin gene, but both prostasin and γ-tryptase genes encode for a C-terminal extension, which could behave as a putative transmembrane segment. Thus, it has been proposed that γ tryptases are phylogenetically more related to prostasin than to α and β tryptases. Alternatively, the ancestors of prostasin and α/β-tryptase genes might have contributed through various gene recombination, gene conversion and exon-shuffling mechanisms to the γ-tryptase genes [13]. In this perspective, it is interesting to observe that the position and phase of hTMT introns 2 and 3 [15] are identical to those of the corresponding introns 3 and 4 of α and β human tryptases. Tryptase ε gene is also made of six exons and five introns, as found by aligning tryptase ε cDNA against the related sequence of chromosome 16.p13.3 [20].

The *mTMT* gene contains five exons and four introns, the last exon also encoding for a C-terminal extension corresponding to the transmembrane segment [15]. Phase and position of introns 2–4 are identical to those of the same introns of mMCP-7 and of the corresponding introns 3–5 of mMCP-6, α and β tryptases. The gene encoding mT4 contains six exons and five introns, with a very extended intron 4 (~2,7 kb) [25].

In all tryptase genes described so far, codons for the catalytic triad residues are located in separate exons, namely exons 3, 4 and 6 (corresponding in *mMCP-7* and *mTMT* genes to exons 2, 3 and 5).

Several recognition sequences for positive transcription factors are present in the promoter regions of tryptase genes. In the case of mMCP-6 gene expression, the important role of a basic helix-loop-helix (bHLH) transcription factor (MITF), encoded by the *mi* locus in mice, has been demonstrated [66]. The authors showed that the transactivation effect of MITF on the mMCP-6 promoter occurs through binding/recognition of specific sequence motifs (E boxes).

Native structure and stability

Several studies have described the stabilizing role of heparin on the tetrameric structure and activity of ß-type tryptases [67, 68]. In connection with the in vivo storage of tryptases, it was suggested that within the mast cell granules, and after secretion, tryptase is bound predominantly to heparin proteoglycans [69, 70]. However, it is worth mentioning that immunoelectron microscopy studies on human skin mast cells showed that high amounts of tryptase are localized primarily over crystalline substructures within the granules, whereas chymase and cathepsin G are found over amorphous granule regions. This was taken as an evidence of tryptase self-assembly and packaging in a crystalline network, which could be responsible for the enzyme stabilization, independent of binding to heparin [71]. Moreover, it was shown [72] that substantial amounts of enzymatically active mMCP6 are present in the BMMCs of a transgenic mouse that cannot express fully sulphated heparin, whereas chymase is practically absent. These findings suggest that the role of heparin (and other glycosaminoglycans) in the posttranslational regulation of tryptase levels within the granules could depend on the specific mast cell populations studied [73, 74]. In any case, this point will require further investigation.

In their native state, most tryptases are tetramers with molecular mass ~140 kDa, made of four identical glycosylated subunits (245 aa) of ~30–36 kDa, as revealed by gel electrophoresis and gel filtration under denaturing and nondenaturing conditions. Bovine tryptase 1 (native molecular mass 420 kDa ± 20, made of 33–36 kDa glycosylated subunits) appears to be in an association state larger than a tetramer [61], as it occurs in guinea pig tryptase that is made of 38-kDa subunits and has a native molecular mass ~860 kDa ± 100 [47]. However, it is likely that the high apparent molecular mass of the bovine enzyme might be due to an aggregation form and/or to the presence of some heparin or other proteoglycans very tenaciously bound and thus resistant to high ionic strength concentration.

Molecular models of several tryptase monomers were proposed [75–77] based on the sequence alignment with bovine trypsin (~40% identity) and on its crystal structure [78]. It was suggested [75] that a group of conserved tryptophan residues and a proline-rich region make on the tryptase monomer surface two hydrophobic patches that are involved in tetramer formation. According to the same authors, heparin binding to native human tryptase is mediated by a large number of His residues positioned on the tetramer surface and by clustered Arg/Lys residues. However, all the proposed monomer models could not predict the real tetramer assembly, which was clarified in 1998 [51] when the crystal structure of human lung βII-tryptase in a complex with the inhibitor 4-amidinophenyl pyruvic acid, was solved at 3 Å resolution (fig. 2). In the tryptase tetramer, the monomer structure, stabilized in its active conformation by interaction with the other monomers, is similar to that of trypsin- or chymotrypsin-like proteases, and consists of two distinct β barrels each made of six β sheets, two small α helices and a number of polypeptide loops. Moreover, the free α-amino group of the highly conserved Ile+1 residue is inserted into the so-called Ile pocket, forming a salt bridge with the carboxyl group of Asp 193 (Asp 194 in the chymotrypsinogen numbering). This salt bridge is inaccessible to the solvent and is present in practically all mature trypsin-like serine proteinases. An acidic cluster, made by Asp residues 132, 134 and 136 (Asp 143, 145 and 147 in the chymotrypsinogen numbering), is close to the Ile+1 cleft and may act as an anchor for the α-amino-terminal group, thus competing with Asp 193 and favouring a zymogen-like structure. The residues of the catalytic triad are in the region joining the two β barrels, while the active site cleft is located perpendicularly to the same region. Similar to trypsin, the primary specificity pocket S1 accommodates the basic side chain of the substrate P1 (Arg or Lys) residue whose carbonyl group belongs to the scissile peptide bond. At the bottom of the S1 pocket, an Asp 188 residue (corresponding to trypsin Asp 189, in the chymotrypsinogen numbering) is present in all tryptases and

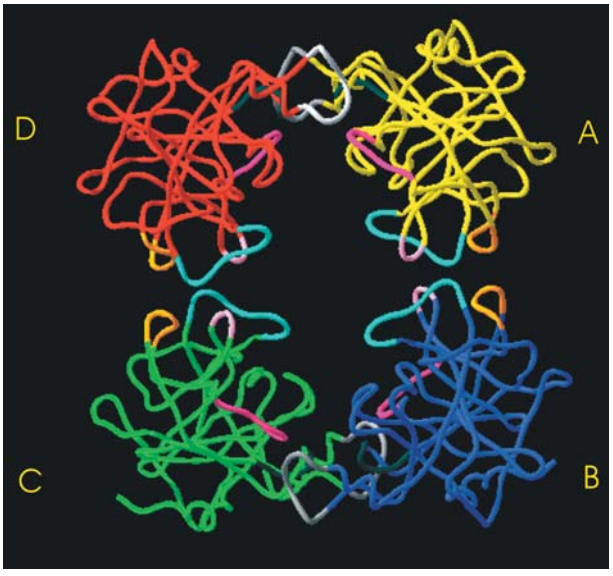


Figure 2. Schematic representation of human lung βII tryptase tetramer structure in complex with the inhibitor 4-amidino phenylpyruvic acid (APPA, not shown) (PDB ID code 1A0L [51]). The four identical monomers A–D are arranged in a square flat ring, with the four active sites facing inside toward the oval central pore. The six loops forming two different interface contacts between adjacent monomers (A/B, C/D and B/C, D/A) are highlighted by different colours. The entrances to the central cavity are in part obstructed by two identical loops (shown in turquoise) belonging to opposite monomers and responsible for the restricted accessibility of macromolecular substrates and inhibitors [51].

contributes to the trypsin-like specificity by binding the basic side chain of substrates or inhibitors. The only known exception is bovine tryptase 1, where an Asn residue replaces Asp 188 without changing substrate specificity [44].

The four identical monomers A–D, which are only quasi-equivalent within each βII tryptase tetramer, are arranged in a square flat ring, with the four active sites facing inwards, toward an oval central pore (40 Å × 15 Å); the entrances to this central cavity are in part obstructed by two loops belonging to opposite monomers and thus responsible for the restricted accessibility for macromolecular substrates and inhibitors. Each monomer contacts two adjacent monomers through two distinct interfaces. Three loops per monomer (identical in the two interacting monomers) are involved in each interface. Most of the 22 extra residues present in tryptase relative to trypsin contribute to these six loops that are arranged around the active site of each monomer and, compared with those present in trypsin and other serine proteases, have different conformation and length. The large interface between monomers A and D (or B and C) is stabilized by hydrophobic interactions, hydrogen bonds and salt bridges, whereas the much smaller interface area between monomers A and B (or C and D) involves a number of Tyr and Pro side chains and is exclusively hydrophobic. The in vitro stabilizing effect of high ionic strength on human tryptase has been ascribed to the strengthening of the hydrophobic interaction at the A/B (and C/D) monomer interfaces [68]. Furthermore, heparin fractions [67] of Mr greater than 5700 [79] prevent inactivation/dissociation into inactive monomers of the enzymatically active tetramer [80]. Pereira and co-workers [51] proposed that stabilization could occur through tight electrostatic binding of an extended heparin chain to the clustered positively charged residues at the periphery of the A–B and C–D homodimers. In any case, the spontaneous inactivation of human β tryptase at low ionic strength or in the absence of heparin is a complex process; whether it occurs (i) via relaxation of the active tetramer to a zymogen-like, inactive tetrameric conformation, with the salt bridge between Ile+1 and Asp 193 disrupted, and then dissociation into inactive monomers or (ii) through direct tetramer dissociation into 'active' monomers which then rapidly assume a zymogen-like conformation leading to inactivation, is a question still open to debate [68, 81–84]. It is worth recalling that formation of 'active' monomers from tetrameric human β tryptase was examined and proposed to occur in the presence of heparin [85].

The 2.2-Å crystal structure of recombinant human αI tryptase was solved recently [58] and explains the inactivity of this tryptase isoform. The overall trypsin-like monomer folding and tetramer assembly is essentially similar to βII tryptase, although packing of the tetramers in the crystal is different. The four monomers, interacting through six loops similar to those in βII tryptase, are located at the corner of a flat rectangular frame, and form a central pore of similar dimension. Moreover, the crystal structure has revealed the main reason for αI tryptase inactivity: residues 215–219 (chymotrypsinogen numbering) follow a different track than in βII tryptase, being bent for the presence of Asp 216 side chain; the observed distortion is incompatible with substrate binding. In the βII enzyme, where residues 215–219 form the template for substrate binding, residue 216 is Gly, as in all other tryptases (fig. 1). As a matter of fact, the importance of Asp 216 for the inactivity of tetrameric α tryptase was recognized by site-directed mutagenesis and structural modelling analyses, prior to resolution of the crystal structure. Recombinant α tryptase was reported to react with diisopropylfluorophosphate, similar to other serine proteases, while the catalytic activity toward peptide substrates was found to be very restricted and could be restored in part by replacing Asp 216 with Gly [86]. Other authors later showed that recombinant α tryptase is enzymatically silent, and that, when residues Asp 216 or Lys 192, or both, are replaced by the βII-like residues (Gly and Gln, respectively), the mutated proteins gain proteolytic activity as well as good catalytic efficiency, which in the double mutant is only twofold less than that of recombinant βII tryptase [83]. Another important difference between mature α and β tryptases is that heparin is not required for α tryptase tetramer stability [58, 83]. As a matter of fact, α-tryptase crystal structure has revealed that as for βII tryptase, the periphery surface of A-B dimer (and of C-D dimer) possesses clustered positive charges, suitable for interaction with heparin chains. However, few amino acid substitutions were considered important in weakening the heparin affinity; in particular, the presence of Asn 102, which is Lys in βII tryptase, led to the decrease of the positive surface charges and to the formation of a new putative N-glycosylation site (fig. 1). It was reasoned that upon glycosylation, this site could make negatively charged heparin binding more difficult [58]. Quite interestingly, many other tryptases have the same Asn residue at position 102, which participates at a putative N-gycosylation site (see above). Among them, bovine tryptases 1 and 2 were found practically insensitive to heparin, which does not affect their in vitro activity or stability [43, 44, 46, 87].

Based on the crystal structure of βII tryptase [51], homology models of human γI-tryptase monomer [13] and human ε-tryptase monomer [20], which have 47 and 41% identity with βII tryptase, respectively, were reported. In the case of ε tryptase, both modelling and functional studies on the recombinant protein revealed that substrate specificity is different from that of β-type tryptases, and that formation of tetramers similar to βII tryptase is unlikely due to the absence of Tyr- and Pro-rich domains in the structure. Comparative modelling analyses and stud-

ies on recombinant mMCP-6 and mMCP-7 [88] have shown that these proteins are enzymatically active as tetramers and that they do not require heparin for the maintenance of their quaternary structure and activity. Furthermore, it was shown that the Trp domain in mMCP-7 is essential for tetramer formation, whereas N-linked glycans only contribute to the thermal stability of the enzyme. In contrast with these findings, other authors have reported that heparin (or similar highly charged polysaccarides) of sufficient chain length is indeed required for tetramer formation of recombinant mMCP-6, while short (8–10 units) heparin oligosaccarides (never found in mast cells) are able to generate 'active' monomers [89].

Biological functions of tryptase

The enzymatic and molecular properties of tryptases are far better characterized that any of their plausible biological functions; on the basis of their structural and functional features, it could be predicted that tryptases would not degrade a large number of proteins in vivo due to low accessibility to the tetramer central pore where the active sites are faced inwards. Recently, with the aim of predicting potential physiological substrates containing suitable sequences for human β-type tryptase cleavage, the substrate specificity of recombinant enzymes was investigated using differently constructed, positional scanning combinatorial tetrapeptide libraries and a phage display peptide library [90, 91]. Utilizing these methods, the optimal sequences were found to have Lys or Arg in P1 and P3 positions and a more broad specificity in P2 or P4 positions with some preference for Pro residue. In some cases, the cleavage site of proposed physiologically relevant substrates of β tryptases show consistency with the specificity determined in those studies (see below).

In broad terms, the physiological and/or pathological consequences of tryptase's proteolytic activity in vivo are strictly linked to the complex distribution of mast cells throughout the body. Among the plethora of proinflammatory, vasoactive, chemotactic and growth-promoting mediators synthesized and released by mast cells upon stimulation, tryptase seems to play a pivotal role in inflammatory and allergic responses (table 2). In particular, the involvement of tryptase in asthma, an inflammatory disease of the airways often caused by allergy, has been claimed by many authors. Asthma is characterized by airway eosinophilia, mucus hypersecretion and hyper-responsiveness to both inhaled allergens and nonspecific stimuli. Mast cells are important in the pathogenesis of asthma, with release of inflammatory mediators such as histamine, leukotrienes, cytokines and proteases. Among the last, tryptase has unique features as it is resistant to circulating inhibitors (see above) and remains active for a longer period after release than other proteases. The well-known clinical evidence concerning the increased level of tryptase in the bronchoalveolar lavage (BAL) fluid of patients with bronchial asthma or interstitial lung disease [92] suggests that tryptase is released along with histamine from activated airway mast cells in atopic asthma, thus further increasing the magnitude and sensitivity of histamine-induced bronchoconstriction. Most of the action of tryptase demonstrated in vitro on peptides, proteins, cells and tissues has been related to asthma symptoms. Indeed, important mediators with regulatory functions of the bronchial tone and airway responsiveness may be degraded or produced by the proteolytic activity of tryptase. In fact, a mechanism for tryptase-mediated enhancement of bronchoconstriction in asthmatic patients was proposed at the beginning of the nineties [93].

Table 2. Potential biological functions of human tryptase.

Substrate or target	Activity	Biological effect	References
Peptides	degradation of VIP, PHM, CGRP	decrease of endogenous bronchodilatatory activity in lung	93
Proteins	generation of kinins	vascular permeability enhancing	114
	activation of pro-MMP-3 and pro-urokinase	tissue remodelling and tumour invasion	115, 116
	degradation of fibronectin and collagen	tissue remodelling	84, 121
	inactivation of fibrinogen	clotting prevention	117, 118
Cells (through dependent and/or independent PAR-2 activation)	proliferation of fibroblasts, smooth muscle cells and epithelial cells	fibrosis, tissue remodelling	104–106, 113
	release of cytokines from endothelial and epithelial cells, eosinophils and airway smooth muscle cells	recruitment of eosinophils, neutrophils and mast cells	94, 95, 108, 109, 112, 126
Tissues	Promotion of capillary tube formation (angiogenesis)	solid tumour growth, wound healing	122, 123

VIP, vasoactive intestinal peptide; PHM, peptide histidine-methionine; CGRP, calcitonin gene-related peptide; pro-MMP-3: pro-matrix metalloprotease-3.

The authors demonstrated by in vitro experiments that tryptase degrades neuropeptides with broncodilating activity such as vasoactive intestinal peptide (VIP), peptide histidine-methionine (PHM) and calcitonin gene-related peptide (CGRP). Relevant functional implications of these findings were obvious given the anatomic relationships of mast cells and nerve fibers in the airway tissue. More recently, a further compelling link to the activity of tryptase and acute and chronic allergic inflammation was proposed, relying on the ability of this enzyme to stimulate mast cell degranulation as well as eosinophil and neutrophil chemotaxis in vitro. Injection of human lung tryptase into the skin of guinea pigs or sheep provokes mast cell activation with a consequent microvascular leakage and massive accumulation of inflammatory cells such as eosinophils and neutrophils [94]. A further mechanism by which the release of tryptase may amplify the response of mast cells to the initial stimulus is based on the ability of protease to stimulate the expression of ICAM-8 (intercellular adhesion molecule-8) and IL-8, a potent neutrophil chemoattractant, in endothelial and epithelial cells [95]. Lactoferrin and myeloperoxidase (both neutrophil proteins) are inhibitors of tryptase proteolytic activity in vitro by displacement of stabilizing heparin (see below) [96, 97]. This finding suggests the existence of a mechanism of tryptase regulation in vivo subsequent to the recruitment of inflammatory cells to a site of inflammation. It is worthy noting that tissue eosinophilia as well as airway hyper-responsiveness to antigens and vascular leakage in the airways of sheep sensitized with *Ascaris suum* are reduced by the administration of the synthetic tryptase inhibitor APC366 [98] (see below).

The mechanism by which tryptase interacts and stimulates inflammatory cells seems mainly related to its ability to cleave and activate a member of the proteinase-activated receptor, namely PAR-2, present on the surface of such cells. This is a member of a family of G-protein-coupled receptors that are activated by either proteolytic release of a tethered ligand from the N-terminus of the extracellular domain of the receptor, or by direct interaction of the receptor with peptides that mimic the newly generated N-terminus [99]. PAR-2 is expressed in a variety of inflammatory cells, such as mast cells, eosinophils and neutrophils, and on other cell types, such as fibroblast, smooth muscle cells, epithelial cells, endothelial cells, myocytes and nerve fibers in the respiratory tract [100]. Indeed, whether PAR-2 agonists protect or promote airway inflammation is currently under discussion. PAR-2 activation seems to induce an eicosanoid-dependent bronchodilation in rats, mice and guinea pigs [101], and human bronchi relaxation by inducing nitric oxide (NO) release from the epithelium [102]. On the other hand, it has been demonstrated that the use of genetically modified mice overexpressing PAR-2 exacerbates both the infiltration of eosinophils cells into the lumen and the hyperactivity of the airway. Accordingly, deletion of PAR-2 diminishes inflammatory cell infiltration and reduces airway hyperreactivity [103]. Apart from the acute inflammatory actions mentioned before, tryptase induces more long-term effects contributing to airway and vascular tissue remodelling by PAR-2 triggering and subsequent mitogenic response initiation. Tryptase is a known mitogen for dog tracheal smooth muscle cells [104], human smooth muscle cells [105], and human lung and dermal fibroblasts [106]. Both smooth muscle cell hyperplasia and fibrotic changes contribute to the 'basement membrane thickening' (i.e. subepithelial fibrosis) seen in the airway mucosa of many persons with asthma. Many studies using cells that express PAR-2 have demonstrated that tryptase induces a concentration-dependent increase in cytosolic calcium and inositol phosphate, effects that were blocked by tryptase inhibitors [107]. These observations are consistent with the initiation of a phosphotidylinositol hydrolysis cascade, leading to the downstream activation of protein-kinase C, a key regulator of gene expression. In connection with mast-cell-eosinophil cross-talk, the relevance of this interaction has been highlighted [108] by showing that tryptase induces IL-6 and IL-8 production and release in human peripheral blood eosinophils. The mechanism is probably mediated by PAR-2 cleavage, which involves the mitogen-activated protein kinase (MAPK) and the activator protein-1 (AP-1).

A novel mechanism by which tryptase may induce fibroblast proliferation has been proposed in [109]. The authors showed that PAR-2 activation by tryptase leads to the increased expression of cyclooxygenase 2 (COX2), a key enzyme in the biosynthesis of prostaglandins. Subsequent cell proliferation is mediated by the enhanced synthesis of prostaglandin J2 (PGJ2) that acts via the nuclear peroxisome proliferator-activated receptor γ (PPARγ). Interestingly, this pathway seems to be involved in the fibrotic change documented in testes of infertile men. Generally speaking, the hallmark of fibrotic disease is the progressive accumulation of extracellular matrix collagen. Tryptase, beyond the stimulation of fibroblast proliferation, induces an increase in mRNA for collagen production in human skin and lung fibroblasts [110, 111]. A further role for tryptase in the remodelling process observed in asthma was recently reported [112]. The finding that human airway smooth muscle cells (HASMCs) are able to stimulate accumulation of mast cells also seems to involve the activation of PAR-2. The receptor is expressed on the surface of HASMCs, and its proteolysis by tryptase stimulates the synthesis and secretion of the chemotactic cytokine tumour growth factor β1 (TGF-β1), in an auotoactivation loop involving mast cells, HASMCs and chemotactic cytokines. At variance with the plethora of proposed mechanisms for PAR-2 involvement in cell activation, it was recently reported that the mitogenic effects of tryptase in human airway smooth muscle cells

might be mediated by nonproteolytic mechanisms (i.e. not through PAR-2 activation). The authors suggest that proliferation may occur upon the binding of glycosylated tryptase to cell surface mannose receptors [113].

As to direct action of secreted tryptase on soluble extracellular proteins, relatively few macromolecular substrates are known: tryptase prefers peptides as substrates (such as the neuropeptides mentioned above) rather than proteins. As a matter of fact, the central pore is just large enough for elongated peptides of the diameter of an α helix to thread through and to interact with the active sites. Accordingly, the mechanism by which tryptase was shown to activate the kallikrein-kinin system through prekallikrein processing with subsequent generation of kinins is mediated by cooperation with neutrophil elastase (NE) [114]. NE cleaves kininogen, and the fragments generated are small enough to enter the central pore of tryptase tetramer that releases bradikinin, an enhancing vascular permeability peptide. Docking studies with some of the protein substrates known to be activated by tryptase, such as pro-matrix-metalloprotease-3 (pro-MMP-3) and pro-urokinase (see below) [115, 116], suggest that proteinaceous substrates must extend cleavable surface loops to interact with an active site [68]. Alternatively, the residual and broad trypsin-like specific activity of tryptase monomers was considered to be responsible for such cleavages, as shown by data indicating that only the monomeric form of β tryptase degrades fibronectin [85]. Although the interaction mechanism among large proteins and tryptase remains undefined, fibrinogen is currently proposed as one of most relevant physiological substrates for human β [117] and mouse (MCP-7) [118] tryptases. Tryptase has been shown to cleave both α and β chains of fibrinogen, making it unclottable by thrombin. Fibrin(ogen) also interacts with cells and contains a single RGD motif near the carboxyl terminus of the chain that binds to integrin $\alpha_v\beta_3$ on endothelial cells, and the removal of such a motif on the fibrinogen molecule by tryptase reduces its binding to endothelial cells. Interestingly, mast cells also express $\alpha_v\beta_3$ integrins, and Johnson and co-workers [117] suggested that tryptase might serve to free mast cells from fibrin and extracellular matrix proteins with cleavable RGD sites. Accordingly, although tissue edema can be quite pronounced during a mast cell-mediated inflammatory reaction, large amounts of fibrin/platelet clots in affected tissues are generally absent. Thus, tryptase probably plays an important role in preventing the accumulation of clots that would physically hinder lymphocyte and granulocyte extravasation into the inflamed tissue. A further link between tryptase activity and its contribution to vascular permeability has been evoked in the pathogenesis of multiple sclerosis (MS). The mast cell protease was found to be upregulated in acute MS plaques [119] and increased in cerebrospinal fluid of patients with MS [120]. These data were linked to the presence of perivascular fibrinogen/fibrin deposits in inflammatory MS lesions and correlated to the fibrinogenolytic activity of tryptase.

From a functional point of view, the anticoagulant activity of tryptase and its ability in modulating cellular interactions would complement tryptase activation of the matrix metalloproteinase and plasminogen systems proposed by earlier studies. The cascade of matrix metalloproteases activation is likely initiated through the cleavage of prostromelysin (proMMP-3) by tryptase [115]. Once activated, MMP-3 can degrade proteoglycans, fibronectin and laminin as well type IV and type IX collagen. As a result, tryptase may function in a number of pathological conditions where MMP-3 activity and cartilage degradation are involved, including arthritis, periodontitis and sclerosis. A further proteolytic activity of tryptase was demonstrated on single-chain urinary-type plasminogen activator (scu-PA or pro-urokinase) whose activation by tryptase, which generates the active two-chain form, tcu-PA [116], may be an alternative to the well-known action of other serine proteases such as plasmin and kallikrein. tcu-PA has been found in association with numerous invasive and metastatic cells. Thus, the increased presence of mast cells, both intact and degranulated, in the vicinity of tumours, suggested a further role for tryptase in the initiation and/or amplification of proteolysis activity occurring in the aberrant degradation and remodelling of extracellular matrix during tumour invasion. More recent data report that human β tryptase may have a more direct role in the extracellular matrix-degrading processes: indeed it acts in a similar fashion to the previously described gelatinase (e.g. MMP-2 and -9) in that it binds to and degrades denatured collagen (gelatin) [121].

The contribution of tryptase to tissue remodelling by promoting capillary tube formation (angiogenesis) has been suggested by Gruber and co-workers [122]. In pathological conditions such as solid tumour growth and rheumatoid arthritis, and in normal physiological processes, such as wound healing and placental development, the growth of new blood vessels is critical. Tryptase may play a significant role in such processes owing to its angiogenic activity. The authors performed experiments in which degranulation of a human mast cell line (HMC-1) in the presence of human dermal microvascular endothelial cells (HDMEC) led to rapid differentiation of these cells and maturation into vascular tubes. Such an effect could be augmented with purified tryptase and attenuated, by up to 80%, by pretreatment with tryptase inhibitors. This previous finding is in keeping with more recent evidence that outlines the parallel increase between tryptase-positive mast cells and neovascularization in cervical cancer, pulmonary adenocarcinoma and malignant lymphoma [123].

The emerging concept of a central role of mast cells in natural immunity, as well as their significant contribution

to acquired immunity [124, 125], goes beyond the detrimental action of these cells after their activation that occurs in pathological conditions. In keeping with these currently accepted opinions, some authors demonstrated the functional importance of tryptase in innate immunity in a murine experimental model. mMCP-6 and human tryptase βI have the ability to regulate neutrophil extravasation into the peritoneal cavity and lung [126]. Moreover, tryptase instilled into the lung does not alter airway reactivity, while it seems to have an antimicrobial effect, selectively recruiting neutrophils and rescuing the pulmonary antibacteria deficiency of mast cell-deficient W/W^v mice challenged with *Klebsiella pneumoniae*. Such activity does not seem to be mediated by the tryptase activation of PAR-2, although neutrophils express the receptor [127].

Tryptase inhibitors

Reversible, low molecular weight, synthetic inhibitors of trypsin-like serine proteases, such as benzamidine, leupeptin and antipain, are all effective but not specific in inhibiting human β tryptases as well as all tryptases from different species tested so far. In recent years, much effort has been directed toward the identification and synthesis of a variety of new low molecular weight compounds with potential inhibitory activity and appropriate selectivity toward human β tryptases. Such inhibitors may be useful in investigating the physiological role of these enzymes, as well as in providing opportunities for the discovery of drugs to be used in the treatment of tryptase-mediated diseases. Many of these compounds, such as benzamidine derivatives and amidino-phenyl-pyruvic acid [128] contain one basic group (amino or benzamidino group) which establishes electrostatic interactions with Asp 188 at the bottom of the S1 pocket of the enzyme. Recently, a great number of new monobasic inhibitors with good tryptase affinity and selectivity have been synthesized [129]. Among the monofunctional compounds, gabexate mesylate (p-hydroxybenzoic acid ethyl ester-6-guanidinoexanoate, monomethansulphonate salt) was found highly active at nanomolar concentrations and selective [130], being ~150- and 800-fold selective for human tryptase over thrombin and trypsin, respectively. Gabexate mesylate is a synthetic drug (Foy) used in the treatment of pancreatitis and disseminated intravascular coagulation and as a regional anticoagulant for haemodialysis. Moreover, gabexate mesylate prevents inflammation, erosion and ulceration of the skin and mucosae.

The side chains of Asp 132 and 136 present in βII tryptase and in many other tryptases form a cluster of negative charges that can establish electrostatic interactions with the second basic moiety of dibasic synthetic inhibitors [51], which in fact have a good affinity toward tryptases [6, 128, 131]. The first dibasic compound described with a potent inhibitory activity toward human and dog tryptases [132] is bis-(5-amidino-2-benzimidazolyl) methane (BABIM), which is about 100-fold selective for human tryptase over trypsin. However, the crystal structure of the trypsin-BABIM complex has revealed [133] that besides the interaction of one amidino group with Asp 189 in the S1 pocket, the inhibitory activity of BABIM and related compounds toward trypsin and other trypsin-like proteases is mediated by the presence of zinc ion, even at subphysiological levels. Zinc ion was found tetrahedrically coordinated to two nitrogens of BABIM benzimidazoles and two residues of the catalytic triad, His 57 and Ser 195. When the structure of βII tryptase became available [51], the rational design of new inhibitor molecules led to the synthesis of dibasic compounds with different scaffold and appropriate spacer length and rigidity, capable of interacting with the active sites of two adjacent monomers A and D or B and C (33 Å distant) [134–136]. Ki values down to 10–18 nM were found for compounds with two aminomethyl-phenylalanine moieties separated by a spacer with optimized length [136]. Mol-6131 is a bifunctional compound [137] with a potent and specific inhibitory activity toward tryptase (24-fold and 525-fold selective over trypsin and thrombin, respectively, and more than 40,000-fold over factors VIIa, Xa and XIa, urokinase and plasma kallikrein); this molecule reduces some features of allergic airway inflammation in a murine model of asthma. As to the clinical use of tryptase inhibitors, APC-366, a derivative of the N-(hydroxy-naftoyl)-arginyl-prolylamide, attracted pharmacological interest due to its effect on late-phase allergic reactions in sheep, and it is the first molecule that has advanced to clinical trials for the treatment of asthma [6, 98]. More recently, phase II studies on APC-2059, a bifunctional guanidine-containing inhibitor, have been completed for ulcerative colitis [138].

Another class of tryptase inhibitors, known as heparin antagonists, has been described. As mentioned above, these inhibitors are polycationic proteins, such as lactoferrin and myeloperoxidase, apparently competing with the binding of tryptase to heparin, which is essential in maintaining the native structure of the human enzyme.

In contrast with virtually all serine proteinases, human β tryptase is not inhibited by the known natural protease inhibitors [67, 139] and maintains its activity in plasma. This is likely due to the narrow size of the tetramer central pore, which restricts accessibility of large protein inhibitors to the active sites. So far, the only protein inhibitor known for human tryptase is LDTI (leech derived tryptase inhibitor), a small protein (46 residues) isolated from the leech *Hirudo medicinalis*. LDTI inhibits ~50% tryptase activity at nanomolar concentrations, similar to those inhibiting trypsin and chymotrypsin [140]. Other serine proteases, such as tissue and plasma kallikrein, thrombin and

cathepsin G, are not inhibited by LDTI. Binding is favoured by electrostatic interactions of the two N-terminal lysine residues with Asp 132 and 136 of the tryptase monomer, as suggested by the inhibitory activity of several LDTI variants [141]. Modelling studies have shown that only two LDTI molecules can be docked at the active centre of opposite monomers [51], in agreement with LDTI tight binding corresponding to 50% inhibition. Micromolar concentrations of the inhibitor are required to obtain complete tryptase inhibition [68]. Thus, as found previously for the endogenous BPTI (56 residues) binding to bovine tryptase 1 [142], interaction between human tryptase and LDTI follows a multiphasic kinetics, suggesting the presence of different affinity sites in these enzymes for binding of macromolecular inhibitors.

At variance with human tryptase, some tryptases of other species are inhibited by endogenous protein inhibitors. Trypstatin (61 residues), a fragment of the inter-α-trypsin inhibitor light chain, which is synthesized in the liver and taken up into mast cells [143], was initially isolated from rat peritoneal mast cells [27, 144]. Its inhibitory activity toward rat tryptase was found to be quite potent. Moreover, as mentioned above, a close association between bovine tryptase 1 and BPTI within the same granules of liver capsule mast cells was demonstrated [45]. In fact, BPTI is able to inhibit bovine tryptase 1 in vitro [45, 142] with a multiphasic process (~8 nM BPTI inhibits 50% activity, while an inhibitor concentration of ~45 μM is required for total inhibition), as found later for the interaction of LDTI with βII tryptase (see above), suggesting a physiological role for BPTI in the regulation of bovine tryptase activity. BPTI similarly inhibits bovine tryptase 2 [46] and reduces the activity of dog tryptase [33] and rat tryptase [27, 28]. Quite interestingly, LDTI interacts with bovine tryptase as a purely noncompetitive inhibitor, with a simple binding behaviour [145]. Finally, the major fragment (106 residues) of calf thymus histone H2A, obtained from H2A by treatment with bovine tryptase 1, behaves as a noncompetitive, reversible and highly specific inhibitor of bovine tryptase 1 itself [146].

Once more, this variety of behaviour of protein inhibitors stresses the role of the geometry/size of the tryptase central pore, which can be different in tryptases from different species, in modulating the accessibility of large inhibitory molecules and protein substrates. It is obvious that care should be taken in using in vitro systems and animal models to validate tryptases as viable targets for inhibitor molecules to be used for therapy in inflammatory and allergic conditions in humans.

Concluding remarks

The structural features of tryptases have been studied in detail, particularly for the human β enzymes. The subunit assembly of tryptase tetramer leaves an oval central pore which accounts for the restricted accessibility of only specific substrates (or inhibitors). However, in spite of the variety of plausible substrates identified in vitro for tryptases, their true biological role(s) has not yet been clarified, and the main natural target(s) remains unknown. Even if it is currently accepted that β tryptase plays a role in inflammation and tissue remodelling, many aspects regarding the function of this enigmatic enzyme have yet to be discovered. Evidence suggests that activation of PAR-2 by tryptase is a paramount function of the enzyme, though it seems that the effect of tryptase on cells could also be elicited via a mechanism independent of PAR-2 activation. In connection with tryptase expression and processing, the precursor activation process has not been completely clarified. Further, promoter sequences of many tryptase genes are now available, but only a few studies in mice have been reported on the regulation of tryptase gene expression. A tissue-specific expression of homologous tryptases has been recognized in various organisms, and a distinct physiological role has been proposed for various isoforms. But the significance of multiple tryptases in the same organism is not yet understood, and the importance of tryptase glycosylation is unknown. Forthcoming results will certainly clarify many of these points and will help develop new approaches for treating tryptase-mediated mast cell diseases.

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