The 2.2-Å Crystal Structure of Human Pro-granzyme K Reveals a Rigid Zymogen with Unusual Features*

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Clara Hink-Schauer‡§, Eva Estébanez-Perpiñán‡§, Elke Wilharm‡, Pablo Fuentes-Prior‡, Wolfgang Klinkert‡, Wolfram Bodenmüller‡, and Dieter E. Jenne‡

From the ‡Department of Neuroimmunology, Max-Planck-Institute of Neurobiology and the ¶Department of Structural Research, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18a, Planegg-Martinsried D-82152, Germany

Granzyme K (GzmK) belongs to a family of trypsin-like serine proteases localized in electron dense cytoplasmic granules of activated natural killer and cytotoxic T-cells. Like the related granzymes A and B, GzmK can trigger DNA fragmentation and is involved in apoptosis. We expressed the Ser195→Ala variant of human pro-GzmK in Escherichia coli, crystallized it, and determined its 2.2-Å x-ray crystal structure. Pro-GzmK possesses a surprisingly rigid structure, which is most similar to activated serine proteases, in particular complement factor D, and not their proforms. The N-terminal peptide Met1-Ile4 projects freely into solution and can be readily approached by cathepsin C, the natural convertase of pro-granzymes. The pre-shaped S1 pocket is occupied by the ion paired residues Lys188B-Asp194 and is hence not available for proper substrate binding. The Ser214-Cys220 segment, which normally provides a template for substrate binding, bulges out of the active site and is distorted. With analog to complement factor D, we suggest that this strand will maintain its non-productive conformation in mature GzmK, mainly due to the unusual residues Gly215, Glu219, and Val24. We hypothesize that GzmK is proteolytically active only toward specific, as yet unidentified substrates, which upon approach transiently induce a functional active-site conformation.

Granzymes are chymotrypsin-like serine proteases of cytotoxic T-lymphocytes and natural killer cells that are stored in specialized secretory granules together with the membrane-phile perforin and proteoglycans (1). Upon recognition of antigenically altered target cells or transformed or virally infected host cells, granymes and other granule components are released as high molecular weight complexes and are taken up by the target cell via membrane receptors. Perforin enhances this uptake and appears to contribute to the release of granzymes from endosomal vesicles (2–4).

Granzyme K (GzmK)1 discovered in granules of human lymphokine-stimulated killer cells together with granzyme A (GzmA) as a trypsin-like non-glycosylated serine protease (molecular mass = 28 kDa) that cleaves N-benzoylcarbonyl-L-lysine ethyl ester (BLT); rPSTI, bovine pancreas trypsin inhibitor; DF, complement factor D (adipsin); pro-DF, pro-factor D; FIXa, factor IXa; r.m.s., root mean square.

1 The abbreviations used are: Gzm, granzyme; BLT, N-benzoylcarbonyl-L-lysine thiobenzyl ester; rPSTI, bovine pancreas trypsin inhibitor; DF, complement factor D (adipsin); pro-DF, pro-factor D; FIXa, coagulation factor IXa; r.m.s., root mean square.

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The atomic coordinates and structure factors (code 1MZA and 1MZD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correpondence should be addressed. Tel.: 49-89-8578-3588; Fax: 49-89-8578-3541; E-mail: djenne@neuro.mpg.de.

‡ Both authors contributed equally to this work.

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Crystallization, Data Collection, and Processing—Crystals suitable for diffraction analysis were grown by the sitting drop vapor-diffusion method. One microliter of the protein solution (10 mg/ml) was mixed with 1 μl of the reservoir solution (3.2 mM sodium formate, pH 8.4) and concentrated against 300 mM sodium chloride. A second complete data set to 2.9 Å resolution was recorded at room temperature from a single crystal mounted in a thin-walled glass capillary. A second complete data set to 2.23 Å was collected at 100 K from a flash-cooled crystal that had previously been cryo-equilibrated in a cryo-protectant (3.9 mM sodium formate, pH 8.4, 15% (v/v) glycerol). Both data sets were recorded “in house” on a 300-mm MAR Research image plate detector, using monochromated CuKα radiation from a RIGAKU rotating anode x-ray generator. The crystals of pro-GzmK Ser195 → Ala did not experience appreciable radiation decay over the 2 days that they were exposed to the x-ray beam at room temperature. This feature seems to be related to the relatively low solvent content of the crystals (39%, corresponding to a Matthews coefficient of 2.02). The crystals belong to space group P2_12_12_1 and contain one molecule per asymmetric unit.

Diffraction data sets were evaluated with MOSFLM (20), reduced, and scaled without applying a sigma cut-off, using programs supported by the Collaborative Computational Project No. 4 (21). Crystal structures were solved by molecular replacement with AMoRe (available at www.ccp4.ac.uk/dist/html/amore.html) using 15.0- to 3.5-Å resolution crystals of a second crystal grown using the same conditions as the first. Molecular replacement was carried out using the program AMoRe (24). Significant improvements were noted when the refined model of the third domain of turkey ovomucoid inhibitor (1PPF) was used as the search model. The S1 pocket and surrounding loops were modeled in the refined electron density map. Refinement was done on a Silicon Graphics Indigo 2 workstation using MAIN (24). The S1 pocket and surrounding loops were modeled in the refined electron density map. Refinement was done on a Silicon Graphics Indigo 2 workstation using MAIN (24). The S1 pocket and surrounding loops were modeled in the refined electron density map. Refinement was done on a Silicon Graphics Indigo 2 workstation using MAIN (24). The S1 pocket and surrounding loops were modeled in the refined electron density map. Refinement was done on a Silicon Graphics Indigo 2 workstation using MAIN (24). The S1 pocket and surrounding loops were modeled in the refined electron density map. Refinement was done on a Silicon Graphics Indigo 2 workstation using MAIN (24). The S1 pocket and surrounding loops were modeled in the refined electron density map. Refinement was done on a Silicon Graphics Indigo 2 workstation using MAIN (24). The S1 pocket and surrounding loops were modeled in the refined electron density map.
Residues Gly 89 to Phe 94 comprising the reactive-site loop of the second bikunin domain were taken from the reported crystal structure (1BIK) (31) and adjusted to the model of active GzmK according to the main-chain trace in the above-mentioned complexes. The side chains of Lys192 of GzmK and Phe94 of bikunin D2 were rotated to avoid clashes between the two moieties (inter atom distances below 3 Å). The structure of the complex was finally minimized with CNS, and the quality of the model was controlled with PROCHECK version 3.5.4 (28). Additional atomic coordinates for serine proteases used in this work were obtained from the Structural Bioinformatics Protein Data Bank (PDB) with the accession codes 2PTC, 1TPA, 1PPF, 1BIK, 1FDP, 1DIC, 1HFD, 1BIO, 1DFP, 1DSU, 1DST, 1JPF, 1KLT, 1CGH, 1FQ3, 2PKA, 2CGA, 1FDP, 1FAX, and 1TGB.

RESULTS AND DISCUSSION

Overall Structure of Pro-granzyme K—In this report we describe and discuss the first three-dimensional structure of one of thezymogens that are converted by cathepsin C to an active serine protease in lymphocytes, mast cells, and granulocytes during granule biosynthesis. We have chosen human pro-GzmK, because the natural zymogen starts with a Met residue, lacks glycosylation sites, and can be expressed at high levels in E. coli and refolded from inclusion bodies. The catalytic Ser195 was replaced by an Ala residue to avoid any autocatalytic cleavages in highly concentrated solutions during crystallization. Complete data sets were collected independently for two crystals at room temperature and at 100 K after flash cooling in liquid nitrogen, respectively. Both Fourier maps were essentially equivalent and allowed continuous tracing of the polypeptide chain from Gly19 to Ser92, and from Ser100 to Pro244, whereas the first N-terminal residues between Met14 and Gly18, seven residues of the 99 loop between Arg93 and Gln99, and the four residues of the C terminus (Pro245-Asn248) were found disordered. The temperature independence of this flexibility indicates that these segments are statically rather than thermally disordered (32).

By analogy with trypsinogen and pro-complement factor D
(pro-DF) and because of the weak proteolytic activity of activated GzmK, the pro-GzmK structure was expected to be quite flexible as in trypsinogen (33) and DF (34). To our surprise, the crystal structure analysis, however, revealed a rigid zymogen with a preformed active-site cleft (see below). Especially the activation domain and the S1 specificity pocket are well-ordered. The pro-GzmK molecule has the shape of an oblate ellipsoid with principal axes of about 35 and 50 Å (Fig. 1). As in other chymotrypsin-like serine proteases, the single GzmK polypeptide chain folds into two β-barrels each comprising six antiparallel strands labeled 1 to 6, and 7 to 12 (Fig. 2), which are strapped together by the domain-linking segments Val22-Pro28, Leu121-Ser129, and Thr229-Lys232A (Fig. 2). The residues of the catalytic triad, His57, Asp102, and Ser195, are located at the junction of the two β-barrels. The preformed active-site cleft (see below) runs perpendicular to this junction along the surfaces of both barrels. Three helical elements are found on the surface of the molecule, a single 310 turn (Ala55 to Gln59, 1), an “intermediate” α-helix (Ser164 to Asn169, 2), and a long C-terminal α-helix extending from Lys232A to Leu243 (3). The optimal alignment to bovine chymotrypsinogen A required insertions of one tetrapeptide (60A to 60D), three dipeptides (170A and 170B; 173A and 173B; 188A and 188B), and two single amino acid residues (223A and 232A), and deletions of three single residues (36, 127, 218) and of the tetrapeptide segment between 203 and 206 (see Fig. 2).

A direct topological comparison between pro-GzmK and the available enzyme-zymogen pairs deposited with the Protein Data Bank (35) revealed a better superposition with the active enzymes than with their respectivezymogens. The best fit was obtained with the six PDB entries for active human complement factor D (DF), 1DIC, 1HFD, 1BIO, 1DFP, 1DSU, and...
1DST. Considering only α carbon atom pairs having Cα-Cα distances of 1.5 Å or less, 181 residues turned out to be topologically equivalent between pro-GzmK and DF, showing an average root mean square (r.m.s.) deviation of 0.72 Å (Fig. 1B). This topological similarity extends into many structural details (see below). By contrast, pro-GzmK and the four independent pro-DF molecules in the asymmetric unit of 1FDP possess only 157 topologically equivalent residues, with a slightly higher r.m.s. deviation of 0.81 Å. Second and third on the score ranking list are two groups of active serine proteases, comprising human mast cell chymase (1JPJ and 1KLT) (36, 37) and human cathepsin G (1CGH) (38), followed by GzmB (1FQ3) (22) and porcine kallikrein (2PKA) (39). From the two latter pairs 162 and 161 α-carbon atoms, respectively, can be superimposed on pro-GzmK with r.m.s. deviations of 0.74 and 0.76 Å. The topological fit with chymotrypsin and trypsin was worse, but still better than for theirzymogens, chymotrypsinogen and trypsinogen.

Mast cell chymase, cathepsin G, and GzmB are slightly more distant in agreement with their evolution and chromosomal clustering. These three proteases and GzmH lack the disulfide bond Cys154-Cys152 and are clustered together on chromosome 14q11.2 within 130 kb (4), whereas granzyme-related members with four disulfide bonds are distributed over two separate loci on chromosome 5q11.2 (GzmA and GzmK) and chromosome 19p13.3 (GzmM, azurocidin 1, proteinase 3, neutrophil elastase, and complement factor D).

Heparin Binding Regions of Human Pro-GzmK—Compared with other serine proteases, the percentage of polar residues in GzmK is not particularly high. The 6 glutamate and 11 aspartate residues, however, do not compensate for the positive charges of the 8 arginine and 23 lysine residues, rendering pro-GzmK very basic in accordance with a calculated isoelectric point of 10.2. The basic amino acid residues are unevenly distributed on the surface and form several clusters of positively charged surface patches (Fig. 3). A particularly large basic region extends from the intermediate helix toward the C-terminal helix running along the upper rim (in the standard representation, Fig. 3A). This patch comprises residues Lys128, Arg131, Arg156, Lys166, Lys178, Lys202A, Lys231, and Lys239 and shows clear topological similarities with the heparin binding site (also called anion binding exosite II) of α-thrombin (40). Other positively charged surface patches comprise residues Lys36, Lys57, Lys107, and Lys113 on the front side (Fig. 3A) and Lys20, Arg27, Lys135, Lys137, and Lys202 on the back side of the molecule (Fig. 3B). In and around the S1-pocket, the lysines (Lys188B, Lys192, and Lys202) and arginines (Arg150, Arg156, and Arg162) and the catalytic His175 are charge compensated by aspartates (Asp195, Asp201, Asp215, and Asp219) and glutamates (Glu172B and Glu179) (Fig. 3A).

In line with these structural features, we were able to demonstrate strong interactions of both pro-GzmK and mature GzmK with a heparin matrix, from which they were eluted at high salt concentrations (0.8 M NaCl). Using the same buffer system, pro-GzmK also bound to a cation-exchange column (S15 Sepharose; Amersham Biosciences) but eluted at 0.6 M NaCl, suggesting that heparin-like ligands specifically interact with pro-GzmK with high affinity in vivo. This latter notion is also supported by our observations that heparin affects proteolytic activity of both murine and human GzmK. In activity assays using 0.5 unit/ml heparin, the activities of human and mouse GzmK were elevated by 50 and 30%, respectively, as compared with measurements in the absence of heparin (data not shown).

The fact that esterolytic activity is not reduced by heparin indicates that heparin and small substrates bind to separate regions on the surface of the molecule. This heparin binding (exo)site could thus contribute to the recognition of macromolecular substrates, as shown e.g. for α-thrombin, resulting in a highly selective function in vivo. Alternatively, the heparin-binding region of GzmK may play a role in the binding and uptake of GzmK by the target cells during killer cell attack. In this regard, all three mammalian homologs of GzmK are devoid of carbohydrates and thus cannot be transported into endosomal vesicles via the mannose 6-phosphate receptor during target cell killing, as recently shown for GzmB (41).

Pro-GzmK, a Rigid Zymogen with a Preformed Active-site Cleft—Fig. 1 illustrates the preformed active-site cleft of pro-GzmK, which runs across the front side in standard view. The floor of this cleft is made by segments Ser213-Cys220, Gly178, and the inner part of the 145 loop. The “upper” rim (addressed from right to left) is mainly formed by the 37 (β1β2) loop, the 60 (β3β4) loop, the 99 (β5β6) loop, and the 175 (α2β9) loop (loops are named after the central residue of the strands/helices flanking the loop, respectively). The 37 loop projects out of the molecule in a DF-like manner, with the side chains of Ser22 and His46 connected by a hydrogen bond. The 60 loop of pro-GzmK, four residues longer than in chymotrypsinogen but...
The active site region of pro-GzmK is shown in standard orientation, with residues displayed as a ball-and-stick model. The oxygen atoms are highlighted in red, the nitrogens in blue, and the Cys191-Cys220 disulfide bridge in yellow color. The 214–220 segment, the basement of the preformed S1 pocket, the residues of the unusual zymogen triad of pro-GzmK, Lys188B, Asp194, and Ser190, and His197 are completely defined. The figure was generated with Bobscript version 2.5 (60, 61).

The S1 specificity pocket is preformed in pro-GzmK. Stereo view of the final electron density map around the preformed S1 pocket of pro-GzmK. The active site region of pro-GzmK is shown in standard orientation, with residues displayed as a ball-and-stick model. The oxygen atoms are highlighted in red, the nitrogens in blue, and the Cys191-Cys220 disulfide bridge in yellow color. The 214–220 segment, the basement of the preformed S1 pocket, the residues of the unusual zymogen triad of pro-GzmK, Lys188B, Asp194, and Ser190, and His197 are completely defined. The figure was generated with Bobscript version 2.5 (60, 61).

Crystal Structure of Human Pro-granzyme K

The "lower" boundary of the pro-GzmK active-site cleft (described from left to right) is mainly formed by the 222 (β11/β12) loop, the Cys191-Gly215 S1 base, the well-defined compact 145 residue loop chain does not cover the catalytic Asp102 side chain and is hence unable to shield the "catalytic hydrogen bond" between His197 Nδ1 and Asp102 Oδ2 from bulk solvent molecules. With respect to position 94, GzmK resembles DF, which also carries a small residue, a serine, at this position (42). Adjacent to the flexible 99 loop of pro-GzmK lies the well-ordered 175 loop. This loop is two residues longer than in chymotrypsinogen and intrudes, in particular with its Pro174 pyrrolidine ring, deeply into the active-site cleft (Fig. 1).

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**Fig. 5.** A comparison of the active site regions of pro-GzmK and related proteases/zymogens. Stereo views of the S1 pocket of pro-GzmK (yellow) superimposed with: A, complement factor D (red); B, pro-DF (dark blue); and C, chymotrypsinogen A (light blue). The same colors have been used to designate selected amino acid residues except for pro-GzmK, which is labeled in black. The stereo images were generated with Molscript (62).
ulation factor VII (44), but the adjacent main chain segments are more flexibly arranged than in pro-GzmK.

Stability of the Inactive Zymogen—Stabilization of catalytically inactive conformations in multidomain serine proteases is achieved in several ways, whereas serine protease precursors with short propeptides can be stabilized by a hydrogen bond network between Ser32, His40, and Asp194, known as the "zymogen triad," because its first description in trypsinogen and chymotrypsinogen (45). These three residues are conserved in human, rat, and mouse GzmK but do not form a trypsinogen-like triad in pro-GzmK.

By contrast, the inactivity of pro-GzmK is ensured by a number of other structural features. The pre-shaped S1 pocket is occupied by the Lys188B-Asp194 ion pair, which although leaving space for substrates with small P1 residues, is not suited to bind a substrate correctly. In all pro-GzmK homologs sequenced so far (Fig. 2), both residues Lys188B and Asp194 are conserved. This suggests that the zymogen-stabilizing salt bridge Asp194-Lys188B is a common feature among all GzmK homologs, and in turn points to a higher stability of this ionic interaction compared with the zymogen triad. More important, the template segment Ser214-His217 is far from allowing peptide substrates to correctly align to the active site. Finally, the His57 imidazoyl side chain is pushed out of its functional site by the uncommon Gly215 (Figs. 4 and 5).

Conversion of Granzyme Precursors by Cathepsin C—Cathepsin C, a papain-like dipeptidyl-aminopeptidase, seems to be the genuine activator of several granule-targeted serine proteases produced by mast cells, natural killer cells, and activated lymphocytes (17), including pro-GzmK (13). Our crystallographic study of pro-GzmK was also aimed at understanding zymogen recognition by cathepsin C. In particular, we explored the possibility that exosites on the cathepsin C surface, distinct from its catalytic center, contribute to this high recognition specificity and cleavage efficacy via docking experiments between human cathepsin C (46) and pro-GzmK. We found that all four substrate binding regions of the cathepsin C tetramer are freely accessible. Any monomer of the tetrameric cathepsin C molecule can productively bind to the exposed N-terminal Met14-Gly18 segment of pro-GzmK through subsites S2 to S3 without the imminence of clashes between the two approaching molecules. In particular, the deep S2 specificity pocket of cathepsin C appears to be well-suited to accommodate the side chain of the N-terminal Met14 of pro-GzmK. Subsequent cleavage can occur between Glu15 (P1) and Ile16 (P1'). We could not discern any additional complementary surfaces in vicinity to the N terminus of pro-GzmK and the substrate binding region of cathepsin C.

Pro-chymase, one of the known cathepsin C substrates, has been reported to be resistant to cathepsin C in the absence of heparin. To explain this finding, favorable intramolecular electrostatic interactions between the Glu15 carboxylate group and a basic patch on the surface of pro-chymase and their disruption by heparin have been postulated by some investigators (47) but not proven (47, 48). The crystal structure of pro-GzmK now disapproves this postulated role for Glu15. Consistent with our structural findings are recent investigations showing that heparin and other sulfated polysaccharides with high negative charge density do not stimulate the activation of pro-chymase, but rather inhibit its cleavage by cathepsin C at physiological salt concentrations. Increasing ionic strength counteracts the inhibitory effect of heparin, but also leads to some inhibition of the conversion reaction, suggesting that the N terminus of granzyme precursors may be less accessible in heparin/pro-

![Putative structure of active GzmK bound to a substrate/inhibitor.](https://www.msi.com)
granzyne complexes and at high salt concentrations (48). Alternatively, binding of heparin-like proteoglycans tozymogens might induce allosteric changes that impair cathepsin C binding. Such mechanisms could explain the inhibitory role of heparin in the pro-chymase conversion process and would not be at variance with our finding of a highly flexible N terminus in pro-GzmK.

A Structural Model for Mature GzmK—The conformational rearrangements that occur after cathepsin C conversion can be predicted with some confidence (Fig. 6) on the basis of structures available for several zymogen/enzyme pairs and in particular by analogy with pro-DF (1FDP) and DF (1DFP) (34, 49). After removal of the N-terminal dipeptide Met-Glu, Ile16-Gly19 rotates around Gly18-Gly19, dives into the pre-shaped Ile16 pocket between Asp185 and Ala143, and forms a salt bridge with the Asp194 side chain. This change must be preceded or accompanied by disruption of the salt bridge between Lys188 and Asp194 and rotation of the Asp194 side chain around its Ca-Cβ bond (from χ1 = 75° to about −70°). Competition of the newly formed N-terminal segment with the Lys188 side chain for forming a charged hydrogen bond with Asp194 would possibly require some support by a fitting substrate.

The N-terminal Ile16 ammonium group, however, can easily form a second hydrogen bond with the carbonyl oxygen of Ala143, which by contrast to most other zymogens is already in an “active” conformation in pro-GzmK (Fig. 6). The following residue, Ile17, most likely interacts with Asp189 via two inter main chain hydrogen bonds, which necessitates a C=O bond rotation of Asp189 and a complete rearrangement of the preceding segment Asp185-Asp189. The conformational changes associated with an intruding N terminus would thus affect the structure of pro-GzmK at multiple sites. The mutual flipping of Lys188 and Asp189 would result in the exposure of the Lys188 side chain to the bulk solvent, and in the “correct” positioning of the Asp189 side chain at the bottom of the S1 pocket. These conformational rearrangements would also affect segment Ser190-Gly193 and the Ser214-Pro225 loop, including the connecting Cys191-Cys220 disulfide bridge (Fig. 6).

Not exactly predictable is, of course, the shape of the Ser214, His217 segment, whose extended conformation is critical for productive substrate binding, and the conformation of the segment His217-Cys220, which is important for the correct formation of a trypsin-like S1 pocket. In analogy with observations in several independent structures for DF (49), which also possesses a non-aromatic residue at position 215, we assume first, that segment Ser214-His217 and in particular Gly216 maintain their projecting pro-GzmK conformations and remain exposed after activation cleavage, and second, that the His57 imidazoyl side chain cannot move to its mechanistically correct position. Third, and in analogy to coagulation factor FIXa (1FAX) (50), Glu219 does not adopt the “high energy” trypsin-like conformation. Fourth, a trypsin-like S1 entrance frame is not opened up. Finally, formation of a functional catalytic triad is disfavored by the lack of an aromatic side chain at position 94 as in DF.

The non-productive resting-state conformation, which we postulate for mature GzmK most likely originates from three atypical residues in human GzmK, namely Gly215, Glu219, and Val224 for the following reasons. In trypsin and related typical active serine proteases, the side chain of the aromatic residue 215 fixes the exposed β11 template segment to the underlying strand β12. Simultaneously, this residue provides the basis for subsites S2 and S4. Certainly, and as supported by the DF structures, Gly215 of GzmK cannot substitute for an aromatic residue and, rather, might push His57 out of its catalytic position.

Gly215 in functional serine proteases with trypsin-like structure and specificity, i.e., with a kinked entrance frame, adopts a main-chain conformation that would be of high energy when replaced by any other amino acid. Upon substrate occupation of the S1 pocket, the carbonyl group of Gly215 forms a hydrogen bond with the distal ammonium or guanidyl group of a P1 lysine or arginine residue, respectively. A non-glycine residue at position 219 would have to adopt a high energy main chain conformation to fulfill an equivalent function, but Glu219 of pro-GzmK (Lys219 in the mouse and rat GzmK, see Fig. 2)
adopts a relaxed conformation ($\phi = -100^\circ; \psi = 126^\circ$) instead. The extremely low affinity of GzmK for benzamidine (in the millimolar range (13)) seems to suggest such a cryptic, i.e. suboptimally widened S1 pocket. We had previously advanced a similar explanation for the weak activity of coagulation factor IXa toward chromogenic substrates (49), one of the very rare examples for a non-glycine residue at position 219 in trypsin-like enzymes. Another example is the mannan-binding lectin (mannose-binding protein)-associated serine protease 3, which can interact with mannan-binding lectin complexes (51). With regard to the factor IX-related, but much more active human coagulation factor Xa, we have shown that the substitution of Gly$^{219}$ by Glu in factor Xa (Gly$^{219}$ → Glu mutation) lowers its activity about 1000-fold (52), thus proving the detrimental effect of a Glu residue in this position.

Finally, the typical Tyr/Ph e residue 94 is replaced by the shorter aliphatic Val in GzmK. Normally, an aromatic side activity about 1000-fold (52), thus proving the detrimental BLT than wild type DF (53). This interpretation is consistent with experimental data—

eled residues Gly89 to Phe 94 of the reactive-site loop of the machinery.

and the terminal guanidyl group of the P1 Arg residue. His217 has been shifted away by bikunin subunit D2 into the hypothetical S4 to S2 complex (54), which was taken as the template (see the main chain conformation of Glu219 had to be changed to to be switched from a

characterized by a non-functional substrate-interacting tem-

plate and a blocked S1 pocket) (56). All these proteases are characterized by a non-functional substrate-interacting tem-

pl ate, incompatible with productive substrate binding and processing. Interestingly, these proteases appear to develop their full proteolytic potential by diverse means. Binding of coagulation FIXa to coagulation cofactor VIIa generates a complex (intrins ic X-ase) with proteolytic activity toward coagulation FX, its specific macromolecular substrate. Similarly, DF efficiently cleaves and activates factor B upon binding to the C5b-factor B complex (57). On the other hand, degP seems to behave proteolytically active toward partially unfolded proteins upon large conformational changes at elevated temperatures. Advantages for such a mechanism would be high specificity for macromolecular substrates, inactivity toward short peptides, and dispensability of high affinity inhibitors.

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