The Tetra-aspartate Motif in the Activation Peptide of Human Cationic Trypsinogen Is Essential for Autoactivation Control but Not for Enteropeptidase Recognition*

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The activation peptide of vertebrate trypsinogens contains a highly conserved tetra-aspartate sequence (Asp19–22 in humans) preceding the Lys-Ile scissile bond. A large body of research has defined the primary role of this acidic motif as a specific recognition site for enteropeptidase, the physiological activator of trypsinogen. In addition, the acidic stretch was shown to contribute to the suppression of autoactivation. In the present study, we determined the relative importance of these two activation peptide functions in human cationic trypsinogen. Individual Ala replacements of Asp19–22 had minimal or no effect on trypsinogen activation catalyzed by human enteropeptidase. Strikingly, a tetra-Ala19–22 trypsinogen mutant devoid of acidic residues in the activation peptide was still a highly specific substrate for human, but not for bovine, enteropeptidase. In contrast, an intact Asp19–22 motif was critical for autoactivation control. Thus, single Ala mutations of Asp19, Asp20 and Asp21 resulted in 2–3-fold increased autoactivation, whereas the Asp22→Ala mutant autoactivated at a 66-fold increased rate. These effects were multiplicative in the tri-Ala19–21 and tetra-Ala19–22 mutants. Structural modeling revealed that the conserved hydrophobic S2 subsite of trypsin and the unique Asp218, which forms part of the S3-S4 subsite, participate in distinct inhibitory interactions with the activation peptide. Finally, mutagenesis studies confirmed the significance of the negative charge of Asp218 in autoactivation control. The results demonstrate that in human cationic trypsinogen the Asp19–22 motif per se is not required for enteropeptidase recognition, whereas it is essential for maximal suppression of autoactivation. The evolutionary selection of Asp218, which is absent in the large majority of vertebrate trypsins, provides an additional mechanism of autoactivation control in the human pancreas.

Digestive trypsins are synthesized and secreted by the pancreas as inactive precursors. Physiological activation of trypsinogen takes place in the duodenum, where enteropeptidase (enterokinase) specifically cleaves the Lys23-Ile24 peptide bond (see Refs. 1, 2, and references therein), which corresponds to Lys15-Ile16 in the chymotrypsin-based numbering system (chymo#). The activating cleavage removes a typically 8-amino acid-long activation peptide. In vertebrate trypsinogens, the activation peptide contains a highly conserved tetra-aspartate sequence next to the scissile peptide bond (Fig. 1). Experiments with synthetic peptides and protein substrates indicated that the acidic residues are required for enteropeptidase recognition and cleavage (1–7). Definitive evidence that the Asp19–22 motif of the activation peptide participates in essential substrate interactions with enteropeptidase came from the crystal structure of the bovine enteropeptidase catalytic subunit complexed with an inhibitor analog of the activation peptide, Val-Asp-Asp-Asp-Lys-chloromethane (8). The structure demonstrated that the P2 and P4 Asp residues (corresponding to Asp22 and Asp20 in Fig. 1) formed salt bridges with Lys99 (chymo#), a unique basic exosite on the catalytic subunit of enteropeptidase. In addition, the P3 Asp (Asp21 in Fig. 1) was hydrogen-bonded to the hydroxyl group of Tyr174 (chymo#). The P5 Asp (Asp19 in Fig. 1) was disordered in the structure. In accordance with structural predictions, mutation of Lys99 in the catalytic subunit of enteropeptidase abolished trypsinogen activation. Although the crystal structure suggested an important role for at least 3 of the 4 Asp residues in enteropeptidase recognition, a number of studies using various protein substrates or synthetic peptides indicated that a minimal recognition sequence for enteropeptidase consists of a Lys/Arg at P1 and an Asp/Glu at P2, whereas the P3–P5 acidic residues might enhance activity (1–7). Consistent with the critical role of the P2 Asp was the recent observation that the D22G mutant of human cationic trypsinogen was resistant to activation by bovine enteropeptidase (9).

The inhibitory function of the trypsinogen activation peptide on trypsin-mediated trypsinogen activation (autoactivation) was first demonstrated by chemical modification of the Asp residues in the activation peptide, which greatly enhanced autoactivation (10). Subsequently, tryptic digestion of synthetic model peptides indicated that Asp residues are not favored in the P2–P5 positions (11, 12). More recently, biochemical characterization of pancreatitis-associated activation peptide mutations in human cationic trypsinogen confirmed the importance of Asp residues in the activation peptide in autoactivation control. A model peptide with the D22G mutation was cleaved by bovine trypsin at a higher rate compared

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with the wild type activation peptide, and recombinant human trypsinogens carrying the D19A or D22G mutations exhibited markedly increased autoactivation (9). Taken together with previous data, these findings indicated that the tetra-Asp sequence in the mammalian trypsinogen activation peptides has evolved for both efficient inhibition of trypsinogen autoactivation within the pancreas and optimal enteropeptidase recognition in the duodenum. However, to provide further experimental support to this notion, quantitative comparison of enteropeptidase- and trypsin-mediated trypsinogen activation, combined with systematic mutagenesis of the trypsinogen activation peptide, was necessary.

In the present study, the role of the Asp19–22 motif in the dual functionality of the activation peptide was investigated by site-directed mutagenesis in human cationic trypsinogen. This human trypsinogen isoform exhibits an unusually high propensity for autoactivation, to the extent that inborn mutations that moderately increase autoactivation cause hereditary pancreatitis (14–18). Our findings demonstrate that the primary function of the tetra-Asp sequence in the activation peptide of human cationic trypsinogen is suppression of autoactivation. Two inhibitory interactions have been identified: one between Asp19 of trypsinogen and the conserved hydrophobic S2 subsite of trypsin and another between the unique Asp219 exosite and Asp19–21 of the activation peptide. Together, these interactions can suppress the rate of autoactivation by more than 2 orders of magnitude. In contrast, the Asp19–22 sequence is not required for enteropeptidase recognition and confers only a modest catalytic improvement to enteropeptidase-mediated trypsinogen activation in humans.

EXPERIMENTAL PROCEDURES

Materials—N-Benzoylcarbonyl-Gly-Pro-Arg-p-nitroanilide was from Sigma, and ultrapure bovine enterokinase was from Biozyme Laboratories (San Diego, CA). Recombinant human proenteropeptidase was from R & D Systems (Minneapolis, MN). Human proenteropeptidase (0.07 mg/ml stock solution; 64 nM concentration) was activated with 50 nM human cationic trypsin in 0.1 M Tris-HCl (pH 8.0), 10 mM CaCl2, and 2 mg/ml bovine serum albumin (final concentrations) for 30 min at room temperature and diluted 10-fold to obtain a working stock solution of 64 nM enteropeptidase concentration in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl2, and 2 mg/ml bovine serum albumin. The inclusion of bovine serum albumin was essential for the long term stability of enteropeptidase. Soybean trypsin inhibitor was purchased from Fluka and purified to homogeneity on a bovine trypsin affinity column following the manufacturer’s instructions.

Nomenclature—The common names “cationic trypsinogen” and “anionic trypsinogen” are used to denote the two major human trypsinogen isoforms. However, these names reflect only the ionic trypsinogen that is used to denote the two major human trypsinogen isoforms. Note that these names reflect only the specific trypsinogen that is used to denote the two major human trypsinogen isoforms. The names “human cationic trypsinogen” and “human anionic trypsinogen” are used to denote the two major human trypsinogen isoforms. Note that these names reflect only the specific trypsinogen that is used to denote the two major human trypsinogen isoforms.

Calculation of Initial Rates—Progress curve analysis with KINSIM and FITSIM computer programs (21, 22) was used to estimate the second-order rate constant of the “trypsinogen activation” reaction. Initial rates were calculated by multiplying the rate constant with the initial concentrations of the reactants (i.e., 10 nM trypsin and 2000 nM trypsinogen). Under the conditions used in this study, the degradation of cationic trypsinogen during autoactivation was minimal, and this side reaction was ignored in the calculations. Autoactivation of the less stable anionic trypsinogen was always measured in the presence of 10 mM Ca2+, which provides sufficient protection against autoactivation.

Activation of S200A Trypsinogen Mutants—Trypsinogen (2 μM concentration) carrying the S200A mutation were activated with 10 nM human cationic trypsin, 50 ng/ml (0.45 nM) bovine enteropeptidase, or 14 ng/ml (0.13 μM) human enteropeptidase at 37°C in 0.1 M Tris-HCl (pH 8.0) with 1 mM CaCl2, and pH 5.0, Na-MES at pH 6.0, Na-HEPES at pH 7.0, and Tris-HCl at pH 8.0. As inert protein, 2 mg/ml bovine serum albumin was included in the autoactivation mixtures. The albumin was extensively dialyzed against distilled water before use. Because commercial albumin preparations slightly inhibit human anionic trypsin, but not cationic trypsin (20), in the autoactivation experiments with anionic trypsinogen, bovine serum albumin was omitted. Autoactivation reactions were initiated by the addition of 10 nM trypsin (final concentration), and reaction mixtures were incubated at 37°C in the presence of wild-type and mutant cationic trypsinogen) or 10 nM CaCl2 (wild-type and mutant anionic trypsinogen). These Ca2+ concentrations were previously found optimal for autoactivation of the two trypsinogen isoforms (20). At given times, 2-μl aliquots were removed for trypsin activity assay. Trypsin activity was determined using the synthetic chromogenic substrate, N-benzoyloxycarbonyl-Gly-Pro-Arg-p-nitroanilide (0.14 mM final concentration) in a 200-μl volume. One unit of trypsinogen was defined as the amount of p-nitroanilide release followed at 405 nm in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl2, at room temperature using a SpectraMax Plus 384 microplate reader (Molecular Devices).

RESULTS

Zymogen Activation with Human and Bovine Enteropeptidase—To study the significance of the Asp19–22 residues (Fig. 1) in zymogen activation, individual Ala and Glu replacements were carried out in recombinant human cationic trypsinogen, yielding single Ala mutants D19A, D20A, D21A, and D22A and single Glu mutants D19E, D20E, D21E, and D22E. In addition,
FIG. 1. Activation of single alanine mutants in the Asp<sup>19–22</sup> motif by enteropeptidase. The activation peptide sequence of wild-type human cationic trypsinogen is indicated, with the mutated positions highlighted. Activation of wild-type (wt) and D19A, D20A, D21A, and D22A trypsinogens (2 μM concentration) was carried out with 50 ng/ml (0.45 nM) bovine or 14 ng/ml (0.13 nM) human enteropeptidase (EP) at 37 °C, in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl<sub>2</sub>, and 120 μM soybean trypsin inhibitor (final concentrations). Samples were precipitated with trichloroacetic acid and analyzed by 13% reducing SDS-polyacrylamide gels and Coomassie Blue staining. The relevant portions of representative gels (n = 3–5), demonstrating the trypsinogen → trypsin mobility shift, are shown. The calculated activation rates by 0.13 nM human enteropeptidase were 85 nM/min for wild-type, D19A, D20A, and D21A trypsinogens and 70 nM/min for the D22A mutant. Using 0.45 nM bovine enteropeptidase, these values were 96 and 4 nM/min, respectively. Note that the wild-type and mutant trypsinogens all contained the inactivating S200A mutation; therefore, autoactivation did not interfere with the assay.

FIG. 2. Activation of the tri-alanine (A<sub>3D</sub>) and tetra-alanine (A<sub>4</sub>) mutants in the Asp<sup>19–22</sup> motif by enteropeptidase (EP). The mutated activation peptide sequences are indicated. The mutants also carried the S200A mutation to prevent autoactivation. Reaction conditions are given in Fig. 1. Samples were resolved under nonreducing conditions on 21% SDS-polyacrylamide gels and stained with Coomassie Blue. Representative experiments (n = 5) are shown; note the longer time course in B. The activation rates by 0.13 nM human enteropeptidase were 84 nM/min for A<sub>3D</sub> and 36 nM/min for A<sub>4</sub> trypsinogen. Activation of the A<sub>4</sub> mutant by 0.45 nM bovine enteropeptidase exhibited a rate of 51 nM/min.

The data also raise the possibility that, in fact, acidic residues within the activation peptide are entirely dispensable for activation by human enteropeptidase and do not explain the strong evolutionary conservation of this sequence. The data also raise the possibility that, in fact, acidic residues within the activation peptide are entirely dispensable for enteropeptidase recognition and cleavage. To address this question, mutant A<sub>3D</sub>, in which only Asp<sup>22</sup> is present as a single acidic residue, and mutant A<sub>4</sub>, which is completely devoid of Asp residues, were activated with enteropeptidase. A number of technical difficulties hindered these experiments. First, removal of the Asp residues from the activation peptide abolished the mobility shift routinely observed on reducing SDS-polyacrylamide gels, and trypsinogen and trypsin migrated at identical positions. To visualize the activation reaction, activated trypsinogens were resolved on 21% gels, under nonreducing conditions. As shown in Fig. 2, in this electrophoresis system, the mobility shift becomes apparent again. Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, we have confirmed that the gel shift was caused by the removal of the activation peptide (not shown). Second, mutant A<sub>4</sub> was extremely trypsin-sensitive, and even minute amounts of trypsin(ogen) contamination originating from the affinity column or labware could result in significant trypsin-mediated activation. This problem was overcome by inclusion of soybean trypsin inhibitor in the activation reactions, and experiments were carried out with 0.12, 0.5, 1, and 2 μM inhibitor concentrations, with identical results. Fig. 2A demonstrates that both
**Trypsinogen Activation Peptide**

**Table I**

| Source/Reference | $K_m$ (µM) | $k_{cat}/K_m$ (s⁻¹·M⁻¹) |
|------------------|-----------|----------------------|
| Human enteropeptidase and human cationic trypsinogen* | 1.4 ± 0.3 | 2.5 x 10⁷ |
| Human enteropeptidase and A₄ mutant trypsinogen* | 2.1 ± 0.3 | 5.3 x 10⁸ |
| Human enteropeptidase and human cationic trypsinogenb | 1.5 ± 0.2 | 5.9 x 10⁶ |
| Human enteropeptidase and human cationic trypsinogen | 7.2 | 3.3 x 10⁵ |
| Bovine enteropeptidase and bovine trypsinogen⁵ | 5.6 ± 0.9 | 7.1 x 10⁹ |
| Bovine enteropeptidase and bovine trypsinogen⁴ | 1.2 ± 0.3 | 5.7 x 10⁶ |

* 37 °C, 100 mM Tris-HCl (pH 8.0), 1 mM CaCl₂, 0.12 µM soybean trypsin inhibitor, 0.13 nM human enteropeptidase.  
⁴ 37 °C, 100 mM Tris-HCl (pH 8.0), 1 mM CaCl₂, 0.12 µM soybean trypsin inhibitor, 0.45 nM bovine enteropeptidase.  
⁵ 25 °C, 28 mM sodium succinate (pH 5.6), 10 mM CaCl₂.  
⁶ 21 °C, 50 mM sodium citrate (pH 5.6), 1 nM bovine enteropeptidase.  
⁷ 37 °C, 25 mM Tris-HCl (pH 8.4), 10 mM CaCl₂ 40 µM ovomucoid, 0.3 nM bovine enteropeptidase.

**Fig. 3.** Autoactivation of single alanine mutants D19A, D20A, D21A, and the tri-alanine mutant A₃D. Trypsin-mediated trypsinogen activation was measured at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂, and 10 nM trypsin initial concentrations. Trypsin activity was expressed as percentage of potential maximal activity, which was determined by activation with human enteropeptidase. The rates of autoactivation calculated from progress curve analysis were as follows: wild type (open circles), 1.7 nM/min; D19A (triangles), 3.4 nM/min; D20A (squares), 5.4 nM/min; D21A (inverted triangles), 2.9 nM/min; A₃D (diamonds), 22 nM/min.

**Fig. 4.** Activation of the D22A and D22E trypsinogen mutants by trypsin. Activation of wild type (wt)/S200A (open circles), D22A/S200A (diamonds), and D22E/S200A (triangles) cationic trypsinogens (2 µM) with 10 nM trypsin was carried out in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ at 37 °C. The open and filled symbols represent independent experiments. Samples were analyzed by 13% reducing SDS-PAGE, Coomassie Blue staining, and densitometry. The intensity of the trypsin band was expressed as percentage of the sum of the trypsin and trypsinogen bands. The initial rates were 1.4 nM/min for wild type; 6.4 nM/min for D22E, and 93.2 nM/min for D22A.

human and bovine enteropeptidase activated A₃D trypsinogen. When compared with activation of wild-type trypsinogen (cf. Fig. 1), the rate of A₃D trypsinogen activation by human enteropeptidase was identical, whereas activation by bovine enteropeptidase was 2-fold decreased. This observation was consistent with the findings in Fig. 1, indicating that the Asp₉₋₂₁ triplet is not required for recognition by enteropeptidase from either species. Strikingly, A₄ trypsinogen was also activated by human enteropeptidase with a rate that was 2-fold decreased relative to wild-type trypsinogen. In contrast, bovine enteropeptidase did not cleave A₄ trypsinogen to any detectable degree (Fig. 2B). Kinetic analysis of A₄ trypsinogen activation by human enteropeptidase revealed a slightly increased $K_m$ (2.1 µM) and an ~3-fold decreased $k_{cat}$ (11.2 s⁻¹; Table I). Therefore, A₄ trypsinogen is still a specific enteropeptidase substrate, which is cleaved with high catalytic efficiency ($k_{cat}/K_m = 5.3 \times 10^6$ M⁻¹ s⁻¹). Taken together, the results shown in Figs. 1 and 2 clearly demonstrate that the Asp₉₋₂₁ motif per se in human cationic trypsinogen is not essential for recognition and cleavage by human enteropeptidase.

**Zymogen Activation by Trypsin (Autoactivation)**—Individual Ala mutations in all positions of the Asp₉₋₂₁ motif stimulated autoactivation. At pH 8.0, 1 nM CaCl₂, and 37 °C, mutations D19A and D21A increased the autoactivation rate about 2-fold, whereas mutation D20A enhanced the rate more than 3-fold (Fig. 3). When the combined effect of these three mutations was tested with A₃D trypsinogen, the autoactivation rate was 13-fold increased, indicating that the inhibitory effects of the Asp residues within the Asp₉₋₂₁ triplet are multiplicative. Remarkably, the D22A mutation stimulated autoactivation to such an extent that it was impossible to follow by activity assays. Therefore, trypsin-mediated activation of D22A trypsinogen was analyzed with SDS-PAGE and densitometry using the catalytically deficient D22A/S200A double mutant (Fig. 4). Relative to wild-type trypsinogen, mutant D22A exhibited a drastic increase in trypsin-mediated activation, which was 66-fold higher at pH 8.0. Although data are not shown, activation of the tetra-Ala A₃D/S200A mutant by trypsin was ~500-fold accelerated relative to wild-type (S200A) trypsinogen.

The significance of the negative charges within the Asp₉₋₂₁ motif was also assessed by conservative Glu substitutions. Individual replacements of Asp₉, Asp₁₀, and Asp₂₁ by Glu had minimal, mostly inhibitory effects on autoactivation. Using 2 µM trypsinogen and 10 nM trypsin concentrations, at pH 8.0, autoactivation rates of 1.7, 1.8, 0.9, and 0.9 nM/min were determined for wild-type, D19E, D20E, and D21E trypsinogens, respectively (not shown). However, conservative Glu replacement of Asp₂₂ in mutant D22E resulted in a 5-fold increased autoactivation rate (9.8 nM/min). Similarly, trypsin-mediated activation of the D22E/S200A mutant showed a 5-fold increased rate relative to S200A trypsinogen (Fig. 4). The results not only identify Asp₂₂ as the critical determinant of autoacti-
A negative charge at position 218 is required for inhibition of autoactivation—First, Asp\textsuperscript{218} was replaced with Tyr (mutant D218Y), because human anionic trypsinogen and most other mammalian trypsinogens carry a tyrosine residue at this position. Because protonation of Asp residues might affect the electrostatic repulsion between Asp\textsuperscript{218} and Asp\textsuperscript{21}, rates of autoactivation were measured over the pH range from 4.0 to 8.0. Autoactivation of wild-type and D218Y trypsinogen was essentially identical at pH 4.0 and pH 5.0; however, in the pH 6.0–8.0 range, autoactivation of D218Y-trypsinogen was markedly stimulated, whereas wild-type cationic trypsinogen exhibited only a marginal increase (Fig 6, A and B). At the pH 7.0 optimum, the difference in the rates of autoactivation between wild-type and mutant D218Y trypsinogens amounted to 11-fold (32.3 versus 2.9 nM/min). For comparison, the pH dependence of autoactivation was also determined for the single Ala mutants D19A, D20A, and D21A as well as the tri-Ala mutant A3D (Fig. 6C). Interestingly, the single Ala mutations caused less pronounced changes (2–3-fold increase at the pH 7.0 optimum) than the D218Y mutation, suggesting that not only Asp\textsuperscript{21} but the full Asp\textsuperscript{19–21} sequence is required for the optimal inhibitory interaction with Asp\textsuperscript{218}. Consistent with this interpretation, removal of the entire Asp\textsuperscript{19–21} triplet in the A3D mutant resulted in an autoactivation profile that was essentially identical to that of the D218Y mutant. Taken together, these results support the predicted electrostatic inhibitory interaction between Asp\textsuperscript{218} and the trypsinogen activation peptide.

To characterize further the side chain requirement at position 218 for suppression of autoactivation, Asp\textsuperscript{218} was mutant to serine, histidine, or glutamate. These side chains also occur naturally at position 218 (e.g. in bovine cationic trypsin (Ser), in human mesotrypsin (His), or in a snake trypsin (Glu)). As shown in Fig. 7, a marked increase in autoactivation was observed with both the D218S and D218H mutations, indicating that the Ser and His side chains, together with Tyr, are responsible for the documented hydrophobic S2 preference of trypsin (26). Clearly, a negatively charged Asp residue is unfavorable in this environment, whereas the small hydrophobic Ala in the D22A mutant would be readily accommodated. The 5-fold increased autoactivation of the D22E mutant is probably explained by the larger rotamer repertoire of the longer Glu side chain, which might mitigate the conflict between the negative charge and the hydrophobic S2 subsite.

Introduction of Asp\textsuperscript{218} into Anionic Trypsinogen Suppresses Autoactivation—Human anionic trypsinogen carries a Tyr resi-
due at position 218 and exhibits a pH profile of autoactivation that is similar to the D218Y cationic trypsinogen mutant (Fig. 8; compare with Fig. 6). Because the activation peptide sequence of anionic trypsinogen is identical to that of cationic trypsinogen, it seemed reasonable to assume that replacement of Tyr218 with Asp should reconstitute the same interaction that operates in cationic trypsinogen. Indeed, the Y218D mutation reduced autoactivation of anionic trypsinogen almost 6-fold at pH 7.0 (5.4 versus 0.9 nM/min; Fig. 8), indicating that the inhibitory repulsion between Asp218 and the tetra-aspartate tract of the activation peptide has been successfully engineered.

**DISCUSSION**

The most surprising finding of this study is that the highly conserved tetra-aspartate sequence in the activation peptide of human cationic trypsinogen is not required for enteropeptidase-mediated activation. Thus, not only were single Ala mutants D19A, D20A, D21A, and D22A all activated normally by human enteropeptidase, but the tetra-Ala replacement mutant A4, which is completely devoid of any acidic residues in the activation peptide, was also activated with somewhat reduced but still remarkable efficiency. Therefore, the tenet that human enteropeptidase recognizes its physiological substrate through the Asp19–22 motif appears to be wrong. Instead, enteropeptidase recognition seems to be determined by so far uncharacterized distant subsite interactions, in which the heavy chain of enteropeptidase might play a significant role. Interestingly, however, the presence of the P2 Asp22 in the activation peptide was an almost absolute requirement for autoactivation of wild-type (open symbols) and D218Y (solid symbols) human cationic trypsinogen. A, time courses of autoactivation were followed at 37 °C in 0.1 M Na-HEPES (pH 7.0), 1 mM CaCl2, and 2 mg/ml bovine serum albumin. Initial trypsinogen and trypsin concentrations were 2 μM and 10 nM, respectively. B, effect of pH on autoactivation of wild-type and D218Y trypsinogen. C, for comparison, the pH dependences of autoactivation of the activation peptide mutants D19A, D20A, D21A, and A4D are also shown (for time courses at pH 8.0, see Fig. 3). Initial rates were calculated from time courses of autoactivation using progress curve analysis, as described under “Experimental Procedures.” The buffers used were sodium acetate, (pH 4.0 and pH 5.0), Na-MES (pH 6.0), Na-HEPES (pH 7.0), and Tris-HCl (pH 8.0).

**FIG. 6.** Autoactivation of wild-type (open symbols) and D218Y (solid symbols) human cationic trypsinogen. A, time courses of autoactivation were followed at 37 °C in 0.1 M Na-HEPES (pH 7.0), 1 mM CaCl2, and 2 mg/ml bovine serum albumin. Initial trypsinogen and trypsin concentrations were 2 μM and 10 nM, respectively. B, effect of pH on autoactivation of wild-type and D218Y trypsinogen. C, for comparison, the pH dependences of autoactivation of the activation peptide mutants D19A, D20A, D21A, and A4D are also shown (for time courses at pH 8.0, see Fig. 3). Initial rates were calculated from time courses of autoactivation using progress curve analysis, as described under “Experimental Procedures.” The buffers used were sodium acetate, (pH 4.0 and pH 5.0), Na-MES (pH 6.0), Na-HEPES (pH 7.0), and Tris-HCl (pH 8.0).

**FIG. 7.** Effect of different amino acid side chains at position 218 on trypsinogen autoactivation. See Fig. 6A for experimental conditions. The rates of autoactivation calculated from progress curve analysis were as follows: wild type (open circles), 2.9 nM/min; D218Y (solid circles), 32.3 nM/min; D218S (triangles), 16.1 nM/min; D218H (squares), 31.2 nM/min; D218E (inverted triangles), 2.7 nM/min.

**FIG. 8.** Effect of replacement of Tyr218 with Asp (Y218D, solid symbols) on the autoactivation of human anionic trypsinogen (wild type, open symbols). A, autoactivation of 2 μM trypsinogen was initiated with 10 nM trypsin (final concentration), and time courses were followed at 37 °C in 0.1 M Na-HEPES (pH 7.0) and 10 mM CaCl2. B, pH dependence of autoactivation was determined as described under “Experimental Procedures” and in the legend to Fig. 6B. Note that autoactivation experiments with anionic trypsinogen were always performed in 10 mM Ca2+ and in the absence of bovine serum albumin for maximal stability and activity (20).
activation by bovine enteropeptidase (see Fig. 1). This observation suggests that the importance of the tetra-Asp motif in enteropeptidase recognition might be species- and isoform-specific. Because only bovine and human cationic trypsogens have been characterized in detail, it is difficult to generalize the results to other vertebrate trypsogens. We favor the hypothesis that the diminished significance of Asp\textsubscript{19–22} in enteropeptidase-mediated activation of human cationic trypsogen might indicate a recent evolutionary change, and the acidic residues in the activation peptide are necessary for enteropeptidase recognition in the majority of vertebrate trypsogens.

The critical role of the P2 Asp\textsubscript{22} in activation by bovine enteropeptidase is also in agreement with the available crystallographic data and mutational analysis indicating an essential interaction between Lys\textsuperscript{99} (chymo#) of the bovine enteropeptidase catalytic subunit and the P2 Asp (Asp\textsubscript{22} in Fig. 1) of the activation peptide (8). However, the crystal structure also shows that the P4 Asp (Asp\textsubscript{20}) participates in a salt bridge with Lys\textsuperscript{99} (chymo#), and the P3 Asp (Asp\textsubscript{21}) is hydrogen-bonded to the hydroxyl group of Tyr\textsuperscript{174} (chymo#). It is not readily apparent why in our study no functional role could be demonstrated for the P3 and P4 Asp residues in trypsogen activation by bovine or human enteropeptidase. One possible explanation is that the trypsogen activation peptide interacts differently with the enteropeptidase catalytic subunit (used in the crystallization studies) and the enteropeptidase holoenzyme (used here). Alternatively, the interactions observed in the crystal structure might be redundant and might not translate to better catalytic efficiency.

Our results indicate that the conserved Asp\textsubscript{19–22} motif has been maintained in human cationic trypsogen for reasons that are unrelated to enteropeptidase recognition and suggest that autoactivation control is the primary function of this acidic sequence. Indeed, experiments with single and multiple Ala mutants confirmed that each Asp residue plays a role in autoactivation control, and their effects are synergistic in a multiplicative manner. The contribution of Asp\textsubscript{22} is the most significant, as indicated by the 60-fold increased autoactivation of the D22A mutant, whereas mutants D19A, D20A, and D21A exhibited 2–3-fold increased rates of autoactivation. Combination of the D19A, D20A, and D21A mutations in the tri-Ala mutant A\textsubscript{3}D resulted in 13-fold increased autoactivation, whereas the tetra-Ala mutant A\textsubscript{4} was activated by trypsin 500-fold more rapidly than wild-type trypsogen. Structural modeling revealed two distinct inhibitory interactions between the Asp\textsubscript{19–22} motif of the activation peptide and cationic trypsogen. Asp\textsubscript{22} is oriented toward the conserved hydrophobic S2 subsite of trypsogen, formed by His\textsuperscript{63} (chymo# His\textsuperscript{57}), Leu\textsuperscript{104} (chymo# Leu\textsuperscript{99}), and Trp\textsuperscript{216} (chymo# Trp\textsuperscript{215}), resulting in an unfavorable subsite interaction that explains the large effect of the D22A mutation. Furthermore, the unique Asp\textsubscript{218} surface residue, which forms part of the S3-S4 subsite on trypsogen, appears to participate in an inhibitory electrostatic interaction with the P3 Asp\textsubscript{21}. Mutagenesis of Asp\textsubscript{218} clearly confirmed that this acidic exosite is essential for inhibition of autoactivation, and removal of the negative charge at position 218 can result in an 11-fold increase. Although modeling suggested that Asp\textsubscript{218} interacts with the P3 Asp\textsubscript{21}, we remain tentative about this interaction, because single Ala mutation of Asp\textsubscript{21} (D21A) stimulated autoactivation only 2-fold, and triple Ala mutation of the Asp\textsubscript{19–22} sequence was necessary to achieve the same degree of autoactivation stimulation as with the D218Y mutant. To reconcile the structural and functional data, a plausible explanation is that an intact tetra-Asp sequence is required to position Asp\textsubscript{21} for optimal repulsion with Asp\textsubscript{218}. In single Ala mutants D19A, D20A, or D21A, the rearrangement of the remaining Asp residues can maintain a partial inhibitory interaction with Asp\textsubscript{218}, whereas the combined tri-Ala mutant A\textsubscript{3}D exhibits full relief from the Asp\textsubscript{218}-dependent inhibition. An uninterrupted tetra-Asp sequence also appears to be essential for Ca\textsuperscript{2+} binding to the activation peptide. Although data are not shown, autoactivation of wild-type cationic trypsogen was stimulated by Ca\textsuperscript{2+}, whereas no effect was observed with mutants D19A, D20A, D21A, and D22A. It is likely that Ca\textsuperscript{2+}-mediated stimulation is important for physiological zymogen activation in vertebrates and represents an additional selective pressure for the evolutionary conservation of the intact tetra-Asp motif in the activation peptide.

The present study also provides a molecular explanation for the previously described unique ability of human cationic trypsogen to autoactivate at acidic pH (27). This is clearly due to the electrostatic repulsion between Asp\textsubscript{218} and the acidic activation peptide, which becomes prominent above pH 5.0 and suppresses autoactivation. As a result, despite the typical pH-dependent stimulation of trypsin activity, autoactivation remains essentially unchanged between pH 5.0 and pH 8.0 (see Fig. 6). In contrast, bovine trypsogen (28) or human anionic trypsogen (20) (see also Fig. 8) autoactivate much better at pH 8.0 than at pH 5.0.

In evolutionary terms, the selective advantage of Asp\textsubscript{218} seems to lie in protection against premature trypsogen autoactivation at neutral or alkaline pH, which prevails in the pancreatic ducts. However, this mechanism of autoactivation control appears to be rare among vertebrate trypsogens. This position (chymo# 217) can carry a variety of residues (Tyr, Ala, Ser, His, Ile, Asp, and Glu), but there is a strong preference for Tyr, particularly in mammalian trypsogens (25). Besides human cationic trypsogen, Asp is found in rat trypsogen (26), but in this minor isoform, the tetra-Asp sequence of the activation peptide is disrupted by an Asn residue, suggesting that the electrostatic repulsion might not be optimal. In the recently released genomic sequence of the beta T-cell receptor locus from the rhesus macaque monkey (Macaca mulatta), the try9 and try13 trypsogen isoforms contain Asp\textsubscript{218} (GenBank\textsuperscript{TM} entry AC149201). Finally, Glu was identified at this position in a partial trypsogen cDNA of the snake Bothrops jararaca (GenBank\textsuperscript{TM} entry AF190273) and in a genomic trypsogen sequence of the green pufferfish Tetraodon nigroviridis (GenBank\textsuperscript{TM} entry CAG00064).

A possible explanation why Asp\textsubscript{218} is conspicuously missing from most vertebrate trypsogens is that the true selective advantage is not suppression of autoactivation per se but regulation of the pH dependence of autoactivation in such a manner that ensures essentially identical autoactivation over the pH range from pH 5.0 to pH 8.0. This could offer the obvious physiological benefit of enhanced zymogen activation in the duodenum when acidic gastric output lowers the pH transiently. It is noteworthy that trypsogen activation by enteropeptidase has a similarly broad pH optimum, suggesting a case of convergent evolution to cope with the inhibitory effect of gastric acid. We speculate that in most species enteropeptidase-mediated trypsogen activation is sufficient to achieve rapid and complete trypsogen activation in the duodenum, whereas in humans, and possibly in a handful of other species, trypsogen autoactivation is also required for full zymogen activation.

The same mechanism that ensures efficient zymogen activation in the duodenum might have significant pathological consequences as well. Autoactivation of cationic trypsogen in the acidic secretory compartment can lead to premature intracellular trypsogen activation, even in the absence of cathepsin B activity (27). The situation is further aggravated in hereditary pancreatitis, in which inborn mutations increase the pro-
pensity of cationic trypsinogen to autoactivate (14–18). This notion is also supported by the fact that genetic variants of human anionic trypsinogen, which cannot autoactivate under acidic conditions, have not been found in association with hereditary pancreatitis or other forms of human pancreatitis (29, 30).

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