Human Cationic Trypsinogen

ROLE OF Asn-21 IN ZYMOGEN ACTIVATION AND IMPLICATIONS IN HEREDITARY PANCREATITIS*

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Mutation Asn-21 → Ile in human cationic trypsinogen (Tg-1) has been associated with hereditary pancreatitis. Recent studies with rat anionic Tg (Tg-2) indicated that the analogous Thr-21 → Ile mutation stabilizes the zymogen against autoactivation, whereas it has no effect on catalytic properties or autolytic stability of trypsin (Sahin-Toth, M. (1999) J. Biol. Chem. 274, 29699-29704). In the present paper, human cationic Tg (Asn-21-Tg) and mutants Asn-21 → Ile (Ile-21-Tg) and Asn-21 → Thr (Thr-21-Tg) were expressed in Escherichia coli, and zymogen activation, zymogen degradation, and trypsin autolysis were studied. Enterokinase activated Asn-21-Tg approximately 2-fold better than Ile-21-Tg or Thr-21-Tg, and catalytic parameters of trypsins were comparable. At 37°C, in 5 mM Ca²⁺, all three trypsins were highly stable. In the absence of Ca²⁺, Asn-21- and Ile-21-trypsins suffered autolysis in an indistinguishable manner, whereas Thr-21-trypsin exhibited significantly increased stability. In sharp contrast to observations with the rat proenzyme, at pH 8.0, 37°C, autoactivation kinetics of Asn-21-Tg and Ile-21-Tg were identical; however, at pH 5.0, Ile-21-Tg autoactivated at an enhanced rate relative to Asn-21-Tg. Remarkably, at both pH values, Thr-21-Tg showed markedly higher autactivation rates than the two other zymogens. Finally, autolytic proteolysis of human zymogens was limited to cleavage at Arg-117, and no digestion at Lys-188 was detected. The observations indicate that zymogen stabilization by Ile-21 as observed in rat Tg-2 is not characteristic of human Tg-1. Instead, an increased propensity to autoactivation under acidic conditions might be relevant to the pathomechanism of the Asn-21 → Ile mutation in hereditary pancreatitis. In the same context, faster autoactivation and increased trypsin stability caused by the Asn-21 → Thr mutation in human Tg-1 might provide a rationale for the evolutionary divergence from Thr-21 found in other mammalian trypsinogens.

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‡ The abbreviations used are: HP, hereditary pancreatitis; Tg, trypsinogen; PAGE, polyacrylamide gel electrophoresis; Asn-21-Tg, wild-type Tg-1; Ile-21-Tg, Tg with the Asn-21 → Ile mutation; Thr-21-Tg, Tg with the Asn-21 → Thr mutation; N-CBZ, N-carbobenzoxy.

Hereditary pancreatitis (HP) is a relatively rare autosomal dominant disorder, characterized by early onset episodes of acute pancreatitis with frequent progression to chronic pancreatitis (1–4). Two mutations in the cationic trypsinogen (Tg) gene, Arg-117 → His and Asn-21 → Ile, have been identified in patients affected by HP worldwide (2–10). It has been proposed that mutation Arg-117 → His eliminates a trypsin-sensitive cleavage site on trypsin and leads to pancreatitis by rendering prematurely activated trypsin resistant to inactivation through autolysis or proteolysis by trypsin-like enzymes (5). Degradation of trypsin by such mechanisms in the pancreas is believed to serve as a fail-safe mechanism against excessive trypsin accumulation (11–13). Although direct biochemical evidence with recombinant human cationic Tg is still lacking, Arg-117 has been shown to be a critical autolysis site in bovine (14) as well as rat (15) anionic trypsin, and mutations of this residue stabilize rat anionic trypsin against autolysis (16–18). Arg-117 is also the primary cleavage site for autocatalytic breakdown of the proenzyme Tg (18, 19), and it was demonstrated that under Ca²⁺-free conditions, the Arg-117 → His substitution significantly stabilizes rat anionic Tg (Tg-2). The observations suggested that in HP the Arg-117 → His mutation may exert its detrimental effect through proteolytic stabilization of both trypsin and Tg (19).

The mechanism of action of the Asn-21 → Ile mutation has been much more elusive. In an attempt to find a common mechanism for both mutations, it was proposed that the Asn-21 → Ile mutation may render the autolysis site at Arg-117 less available for proteolysis by bringing Glu-24 close enough to form a salt bridge (2, 3, 7). An alternative theory suggested that the mutation might change the structure of the activation peptide region and lead to increased autoactivation and excessive trypsin formation (7–9). Surprisingly, when these two theories were tested on recombinant rat Tg-2, neither increased autolytic stability nor increased autoactivation was observed (18). In sharp contrast, the analogous Thr-21 → Ile mutation significantly decreased autoactivation and zymogen degradation without affecting trypsin stability or activity. These findings suggested that unwanted zymogen stabilization by the Asn-21 → Ile mutation might play an important role in HP.

Taken together, biochemical evidence obtained on recombinant rat Tg-2 pointed to a possible unifying scheme for the initiating steps of HP, in which zymogen stabilization by HP-associated mutations is the key pathogenic factor (18, 19). We hypothesized that proteolytic degradation of Tg by trypsin generated during autoactivation might be another important safeguard mechanism of controlling excessive trypsin liberation in the pancreas. Mutations, which stabilize Tg against autoactivation and/or trypsin degradation, would impair the efficacy of this protective mechanism and render the zymogen storage pool more susceptible to widespread activation.

Although the proposed pathomechanism appears to be readily testable on human cationic Tg, problems associated with recombinant expression and purification of this zymogen have hindered progress. In the present study, for the first time,
human Tg-1 was expressed in Escherichia coli into inclusion bodies and successfully refolded in vitro. Wild-type Tg-1 (Asn-21-Tg) and mutants Asn-21→Ile (Ile-21-Tg) and Asn-21→Thr (Thr-21-Tg) were produced, and thezymogens and active trypsins were characterized and compared with properties of the previously studied analogous rat Tg-2 constructs. The results indicate that effects of mutations of amino acid 21 in Tgs from the two species are markedly different and suggest that enhanced autoactivation of Ile-21-Tg under acidic conditions, rather than zymogen stabilization, might play a role in HP. Furthermore, the Asn-21→Thr substitution is shown to alter human Tg-1 in such a manner that might predispose humans to pancreatitis. The findings provide a plausible explanation for the evolutionary divergence from Thr-21 found in all other known mammalian trypsins. Finally, the new availability of recombinant human Tg-1 sets the stage for much needed further studies to elucidate the role of HP-associated Tg mutations in the pathogenesis of HP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ecinope was overexpressed in E. coli BL21 (DE3) as described by Fai et al. (20, 21) and purified to homogeneity using trypsin affinity column. Purified ecinope was immobilized to VCTIGE dil acid resin (Stergene Bioseparations, Carlsbad, CA) as described previously (22). Plasmid pTrp was a generous gift from László Grafi (Eotvos University, Budapest, Hungary). Plasmid pET28c and competent E. coli BL21(DE3) cells were purchased from Newagen, Inc. (Madison, WI). Ultrapure bovine enterokinase was purchased from Biotechnology Laboratories (San Diego, CA). bovine chymotrypsinogen A from Worthington Biochemical Corp. (Lakewood, NJ). N-phenyl glycyl-prolyl-arginine-nitroanilide from Sigma, and Suc-Ala-Ala-Pro-p-nitroanilide from BACH Chem Technology, Inc. (Torrance, CA).

**Construction of a Human Cationic Tg Gene and Mutations at Position 21**—A gene encoding human cationic Tg was constructed by oligonucleotide-directed site-specific mutagenesis of the rat Tg-2 gene. Construction of the autonomous gene of Tg-2 was inserted at the 5′ end of the restriction end, which can facilitate further mutagenesis studies. The two trypsins are 80% identical in their amino acid sequence, and a total of 44 mutations were introduced into the rat Tg-2 gene using a series of “overlap extension” polymerase chain reactions (23). The final polymerase chain reaction product, containing the full-length human Tg-1 gene, was digested with restriction end and ligated into the similarly treated Tg-expression vector pTrp. The DNA sequence of the entire gene was verified by dideoxy-sequencing. Mutations Asn-21→Ile and Asn-21→Thr were introduced by polymerase chain reaction mutagenesis, and the polymerase chain reaction fragments were ligated into pTrp using the BarnHI and EcoRI restriction sites. The expression plasmid pTrp was routinely used to express rat trypsin-1 periplasm of E. coli SM138 via the alkaline phosphatase promoter and leader sequence (15–19, 22, 24). Although expression levels are relatively high, highly purified zymogen preparations can be obtained from periplasmic fractions by conventional ion-exchange (15–17, 24) or affinity chromatography (18, 19, 22). Unexpectedly, when the gene encoding human Tg-1 was cloned into pTrp and transformed SM138 cells were grown, no appreciable amounts of wild-type or mutant Tgs were recovered from the periplasm. Cell fractionation experiments suggested that some of the expressed Tg might be present as inclusion bodies in the cytoplasm. Subsequently, to enhance cytoplasmic expression, an Ncol site was inserted at the 5′ end of the prepeptide coding sequence in pTrp. The Ncol site also introduced a new initiator Met codon (ATG), and the prepeptide sequence was changed to Met-Ala-Phe-Pro-Val-Asp-Lys. Thus, native Tg-1 and its recombinant counterpart differ only at the very N terminus, where the native sequence is Ala-Pro-Phe-Asp-Lys. Finally, the BglII-Ncol region, containing the T7 promoter, lac operator and a Shine-Dalgarno sequence, was excised from the commercial vector pET28c and inserted between the BarnHI and Ncol sites of the modified pTrp (pTrp-T).

**Expression and Purification of Recombinant Tgs**—The pTrp-T construct was transformed into BL21(DE3) carrying a chromosomal copy of T7 RNA polymerase under the control of the lacZ promoter. In a typical experiment, 50-ml cultures of BL21(DE3)/pTrp-T in Luria-Bertani medium with 50 μg/ml carbenicillin were grown to an A600 of 0.5, induced with 1 μmol isopropyl-1-thio-β-D-galactopyranoside, and grown for an additional 5 h. Cells were harvested, and inclusion bodies were isolated by sonication and centrifugation. The inclusion body pellet was washed twice with 1.5 ml of 0.1 M Tris-HCl (pH 8.0) and dissolved in 500 μl of 4 M guanidine-HCl/0.1 M Tris-HCl, pH 8.0. Dithiothreitol was added to a final concentration of 30 mM, and Tg was completely reduced at 37 °C for 30 min. Denatured Tg was then rapidly diluted into 50 ml of refolding buffer (0.1 M Tris-HCl, pH 8.0, 30 mM L-cystine, 30 mM L-cysteine), slowly stirred under argon for 5 min at room temperature, and kept at 4 °C overnight. The final guanidine-HCl concentration after the dilution proved to be a critical factor in successful, quantitative refolding. Concentrations below or above 0.8–0.9 M resulted in the appearance of misfolded species, which could be easily detected on nonreducing SDS-PAGE gels by their slower mobility. To remove any aggregates, denatured and refolded protein solutions were adjusted to a final concentration of 30 mM L-cystine, 30 mM L-cysteine, and the solution was diluted 2-fold with 50 ml of 0.1 M Tris-HCl (pH 8.0) and applied directly to a 2-ml elutin affinity column (22). The column was washed with 20 ml Tris-HCl (pH 8.0)/0.2 M NaCl, and elutions were zymogen were eluted with 50 mM HCl. The eluate was homogenous Tg; no other bands were detectable on Coomassie Blue-stained gels. Concentrations of zymogen solutions were estimated from their ultraviolet absorbance using a calculated extinction coefficient of 36160 M⁻¹ cm⁻¹ at 280 nm. In addition, to ensure that identical amounts of zymogens were used in the experiments, serial dilutions of the proteins were electrophoresed on 12% SDS-PAGE gels, and the intensities of the Coomassie Blue-stained bands were compared. Typical Tg yields were 0.8–1.0 mg of purified zymogen per 50-ml culture. Contrasting the anecdotal instability and rapid autoactivation of human Tg-1, recombinant Tg-1 preparations were essentially trypsin-free, and similarly to the rat proenzyme, they were also stable for months on end stored at 4 °C. Interestingly, N-terminal protein sequencing identified the first amino acid as Ala, indicating that most of the N-terminal formyl-Met is removed during expression.

**Assay of Trypsin Activity**—Trypsin activity was determined using the synthetic chromogenic substrate N-CBZ-Gly-Pro-p-nitroanilide. Kinetics of the chromophore release was followed at 405 nm in 0.1 M Tris-HCl, pH 8.0, 1 mM CaCl₂ at 22 °C.

**Activation of Chymotrypsinogen**—Bovine chymotrypsinogen A (final concentration, 3 μM) was activated by catalytic amounts of trypsin (final concentration, 3 nM) at 37 °C, in 0.1 M Tris-HCl, pH 8.0, 1 mM CaCl₂. In a final volume of 800 μl. Chymotrypsinogen concentrations were determined by activity assays using the synthetic chromogenic substrate Suc-Ala-Ala-Pro-p-nitroanilide.

**Autoactivation of Trypsin**—Autoactivation of trypsin was followed by residual activity measurements (16, 18). Tgs (final concentration, 2.5 μM) were activated with 200 ng/ml enterokinase (final concentration) for 60 min at 22 °C in 0.1 M Tris-HCl (pH 8.0)/5 mM CaCl₂. Trypsin solutions were then incubated at 37 °C without any further additions (i.e. in the presence of 5 mM Ca²⁺) or after addition of K-EDTA (pH 8.0) to a final concentration of 10 mM. At the indicated times 2.5-μl samples were withdrawn for trypsin activity determination.

**Acutization of Trypsin**—Trypsinogen to (final concentration, 2.5 μM) were incubated at 37 °C in 0.1 M Tris-HCl (pH 8.0) or 0.1 M sodium acetate buffer (pH 5.0) in the presence of 5 mM CaCl₂ or 1 mM K-EDTA, in a final volume of 100 μl. At the indicated times, 2.5-μl aliquots were removed for trypsin activity assays. Alternatively, reactions were terminated by trichloroacetic acid precipitation, proteins were separated by 12% reducing SDS-PAGE, and bands were visualized by Coomassie Blue staining.

**RESULTS**

**Enterokinase Activation**—Wild-type human Tg-1 (Asn-21-Tg) was activated by bovine enterokinase approximately 2-fold faster than mutants Ile-21-Tg and Thr-21-Tg at pH 8.0, 37 °C, in the presence of 5 mM Ca²⁺ (Fig. 1A). Because trypsin-catalyzed Tg activation may alter the enterokinase activation kinetics, mutants were also compared at dilute zymogen concentrations, where autoactivation is less likely to interfere. As shown in Fig. 1B, Asn-21-Tg clearly exhibits enhanced activation rates relative to the other two mutants under these conditions as well.

**Catalytic Properties and Autolytic Stability of Trypsins**—Enzyme kinetic parameters of trypsins were determined on the synthetic small peptide N-CBZ-Gly-Pro-p-nitroanilide at 22 °C, pH 8.0, in the presence of 1 mM Ca²⁺. Wild-type and mutant trypsins exhibited essentially identical Kₘ and kₗₚ values.
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values (Table I), which were also comparable to those found with rat trypsin (16). In addition, to test a protein substrate in which the P subsites also participate in interactions, the ability of human trypsins to activate bovine chymotrypsinogen A to chymotrypsin was also compared. As shown in Table I, wild-type and mutant trypsins activated chymotrypsinogen with comparable efficiencies. The findings clearly support the notion that mutations at Asn-21 do not alter catalytic activity of human cationic trypsin.

It is well documented that mammalian trypsins lose activity over time due to autocatalytic proteolysis (see Ref. 16 for references). In the absence of Ca$^{2+}$, autolytic degradation is rapid, whereas millimolar concentrations of Ca$^{2+}$ protect against autolysis. When incubated at 37 °C, pH 8.0, human cationic trypsin was completely stable in 5 mM Ca$^{2+}$ up to 24 h, and more than 50% activity was detectable after 5 days. Mutations at position 21 (Ile-21- or Thr-21-trypsins) had no appreciable effect on trypsin stability in Ca$^{2+}$. In contrast, in the absence of Ca$^{2+}$ trypsin suffered relatively rapid autolysis, and under these conditions, Thr-21-trypsin exhibited significantly increased stability relative to Asn-21- and Ile-21-trypsins (Fig. 2). Importantly, no difference was apparent in the autolysis kinetics of Asn-21-trypsin and Ile-21-trypsin, indicating that the Asn-21 → Ile mutation does not alter autolytic stability of human cationic trypsin.

**Autoactivation of Zymogens**—Previous studies on rat Tg-2 demonstrated that replacement of Thr-21 with Ile resulted in significantly higher zymogen stability against autoactivation and concomitant zymogenolysis (18, 19). In contrast, when human Tg-1 mutants were incubated at 37 °C, in the presence of 5 mM Ca$^{2+}$, pH 8.0, autoactivation kinetics of Asn-21-Tg and Ile-21-Tg were practically indistinguishable, whereas Thr-21-Tg showed significantly faster autoactivation (Fig. 3). Reducing SDS-PAGE gels indicated the appearance of 2 degradation products generated during autoactivation of zymogens (bands A and B). N-terminal protein sequencing revealed that band A is a mixture of two proteins, containing the N- and C-terminal fragments of Tg cleaved after Arg-117. The N-terminal zymogen fragment is converted to band B by trypsino-lytic cleavage at Lys-15 in the activation peptide, and at the 2 h time point, bands A and B represent trypsin cleaved at Arg-117. In contrast to the rat Tg-2 (18), no cleavage whatsoever was observed at Lys-158 in human Tg-1.

In the absence of Ca$^{2+}$ (i.e. in 1 mM EDTA), autoactivation of human zymogens was drastically suppressed; however, when followed over a longer period of time, enhanced autoactivation of Thr-21-Tg relative to Asn-21-Tg and Ile-21-Tg became apparent (Fig. 4). SDS-PAGE indicated greatly enhanced proteolysis of the zymogens at Arg-117 (i.e. band A is prominent). In agreement with activity assays, trypsin formation was only significant in Thr-21-Tg, whereas a trypsin band was hardly detectable in lanes with Asn-21-Tg or Ile-21-Tg.

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**FIG. 1.** Activation of zymogens with enterokinase. A, approximately 2.5 μM Tgs (final concentration) (Asn-21, wild-type human Tg-1; Ile-21, mutant Asn-21 → Ile; Thr-21, mutant Asn-21 → Thr) were incubated with 200 ng/ml enterokinase (final concentration) at 37 °C, in 0.1 M Tris-HCl, pH 8.0, and 5 mM CaCl$_2$ in a final volume of 100 μl. Aliquots of 2.5 μl were withdrawn from reaction mixtures at the indicated times, and trypsin activity was determined with the synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. Trypsin activity was expressed as a percentage of full activity. B, approximately 6 nM Tgs (final concentration) were mixed with 50 ng/ml enterokinase (final concentration) in a cuvette containing 400 μl of 0.1 M Tris-HCl, pH 8.0, 1 mM CaCl$_2$, and 200 μM synthetic trypsin substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. The activation reaction was followed at 37 °C by continuous monitoring of p-nitroanilide release at 405 nm as a measure of trypsin activity. Tg samples without added enterokinase or enterokinase alone exhibited no detectable changes in their absorbance readings over the time period shown.

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**FIG. 2.** Autolysis of trypsins. Aliquots of Tgs (final concentration, 2.5 μM) (Asn-21, wild-type human Tg-1; Ile-21, mutant Asn-21 → Ile; Thr-21, mutant Asn-21 → Thr) were activated by enterokinase for 60 min at 22 °C in the presence of 5 mM CaCl$_2$. Autocatalytic inactivation of trypsins were then followed at 37 °C without any further additions (open symbols) or after addition of K-EDTA (pH 8.0) to a final concentration of 10 mM (solid symbols). Residual activities were expressed as a percentage of trypsin activity measured immediately after enterokinase activation.

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**TABLE I** Kinetic parameters of wild-type and mutant trypsins on synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide at 22 °C and on bovine chymotrypsinogen A at 37 °C. S.E. values (n = 3) are also indicated.

|                   | 1/s  | Km  | 1/M × s |
|-------------------|------|-----|---------|
| N-CBZ-Gly-Pro-Arg-p-nitroanilide |      |     |         |
| Asn-21*           | 99 ± 5 | 2.7 ± 0.3 × 10$^{-5}$ | 3.7 × 10$^5$ |
| Ile-21*           | 90 ± 4 | 2.0 ± 0.2 × 10$^{-5}$ | 4.5 × 10$^5$ |
| Thr-21*           | 96 ± 6 | 2.2 ± 0.3 × 10$^{-5}$ | 4.3 × 10$^5$ |
| Chymotrypsinogen A|      |     |         |
| Asn-21*           | 2.3 ± 0.08 | 2.1 ± 0.2 × 10$^{-5}$ | 1.1 × 10$^5$ |
| Ile-21*           | 2.4 ± 0.09 | 2.1 ± 0.2 × 10$^{-5}$ | 1.2 × 10$^5$ |
| Thr-21*           | 2.5 ± 0.08 | 2.1 ± 0.2 × 10$^{-5}$ | 1.2 × 10$^5$ |

* Wild-type.
* Asn-21 → Ile.
* Asn-21 → Thr.
Trypsin-catalyzed Activation of Zymogens—Although mutations of Asn-21 do not alter catalytic activity of trypsin on small peptide substrates or on chymotrypsinogen, the ability to activate Tg may be affected differently. To test this possible scenario, human Asn-21-, Ile-21-, and Thr-21-Tgs were activated with Asn-21-, Ile-21-, or Thr-21-trypsin. For these experiments, trypsins were generated through autoactivation of the respective zymogens at 37 °C, in 5 mM Ca\(^{2+}\), pH 8.0, for 3 h. Fig. 5 demonstrates activation of human Tgs with Asn-21-trypsin at an approximately 1:10 trypsin-to-Tg ratio, at 37 °C, in 5 mM Ca\(^{2+}\). As anticipated from autoactivation experiments (see above), Thr-21-Tg was activated approximately 2-fold faster than Asn-21-Tg or Ile-21-Tg. Note that the reaction was linear up to 10 min, indicating that autoactivation by the generated trypsin does not contribute to the activation process within the time period studied. Essentially identical results were obtained when Tgs were digested with Ile-21-trypsin or Thr-21-trypsin. The observations indicate that mutations of Asn-21 affect autoactivation by altering the “substrate” properties of Tg rather than the enzymatic activity of trypsin.

Autoactivation of Human Tg Mutants under Acidic Conditions—Previous studies reported that human Tg-1 was particularly susceptible to spontaneous activation under acidic conditions (25, 26). To explore the significance of pH in autoactivation, human Tg-1 mutants were incubated in 0.1 M sodium acetate buffer (pH 5.0) at 37 °C in the presence or absence of Ca\(^{2+}\). Activity was expressed as a percentage of potential total activity, as determined on similar zymogen samples activated with enterokinase. Bottom, reducing SDS-PAGE analysis of samples in 5 mM Ca\(^{2+}\) (see Fig. 4 for gel pictures of EDTA incubates). Reactions were terminated at the indicated times (1, 1.5, and 2 h) by precipitation with trichloroacetic acid, electrophoresed on 12% gels, and stained with Coomassie Blue. Tr, trypsin; A and B denote stable proteolytic fragments.

Trypsin-catalyzed Activation of Zymogens—Although mutations of Asn-21 do not alter catalytic activity of trypsin on small peptide substrates or on chymotrypsinogen, the ability to activate Tg may be affected differently. To test this possible scenario, human Asn-21-, Ile-21-, and Thr-21-Tgs were activated with Asn-21-, Ile-21-, or Thr-21-trypsin. For these experiments, trypsins were generated through autoactivation of the respective zymogens at 37 °C, in 5 mM Ca\(^{2+}\), pH 8.0, for 3 h. Fig. 5 demonstrates activation of human Tgs with Asn-21-trypsin at an approximately 1:10 trypsin-to-Tg ratio, at 37 °C, in 5 mM Ca\(^{2+}\). As anticipated from autoactivation experiments (see above), Thr-21-Tg was activated approximately 2-fold faster than Asn-21-Tg or Ile-21-Tg. Note that the reaction was linear up to 10 min, indicating that autoactivation by the generated trypsin does not contribute to the activation process within the time period studied. Essentially identical results were obtained when Tgs were digested with Ile-21-trypsin or Thr-21-trypsin. The observations indicate that mutations of Asn-21 affect autoactivation by altering the “substrate” properties of Tg rather than the enzymatic activity of trypsin.

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activity was determined with the synthetic substrate CBZ-Gly-Pro-Arg-CBZ, and trypsin with enterokinase. Total activity, as determined on similar zymogen samples activated by cathepsin B, was significantly enhanced at pH 5.0 relative to pH 8.0. At both pH values, Thr-21-Tg autoactivated considerably faster than Asn-21-Tg or Ile-21-Tg. Surprisingly, at pH 5.0, Ile-21-Tg exhibited enhanced autoactivation relative to Asn-21-Tg. Finally, although data are not shown, at pH 5.0, all three trypsins were comparably stable, and approximately 50% activity was retained after 24 h incubation at 37 °C, both in the absence and in the presence of 5 mM Ca2+.

**DISCUSSION**

Mutation Asn-21 → Ile of the human cationic Tg is the second most frequent Tg mutation associated with HP. The mechanism by which the mutation causes pancreatitis has puzzled researchers since its discovery. In contrast to Arg-117, for which a role in trypsin autolysis and/or zymogenolysis is clearly supported by biochemical evidence (14–19), nothing is known about the possible structural/functional significance of Asn-21. The first biochemical investigations into the possible mechanism of the Asn-21 → Ile mutation in HP were carried out using recombinant rat anionic Tg (Tg-2) as a model (18, 19). Thr-21 in the rat Tg-2 was replaced with Asn or Ile, and the purified recombinant zymogens and their active trypsin forms were characterized. The results clearly indicated that the presence of an Ile at position 21 in the rat Tg-2 significantly decreases autoactivation and autocatalytic zymogen degradation without affecting trypsin stability or activity. On the basis of these observations, it was suggested that (i) zymogen degradation is an important defense mechanism in the pancreas against excessive trypsin liberation, and (ii) unwanted zymogen stabilization by the HP-related mutations might play a role in the pathogenesis of HP.

The present study utilized recombinant human cationic Tg to reexamine the validity of the conclusions obtained on the rat Tg-2 model. Similar to the rat model (18), the Asn-21 → Ile mutation had no effect on the catalytic activity or autolytic stability of human cationic trypsin. In contrast to the rat proenzyme, at pH 8.0, autoactivation of human Tg-1 was not altered by the mutation, and zymogen stabilization was not observed to any extent whatsoever. Furthermore, at pH 5.0, autoactivation of Ile-21-Tg became enhanced relative to Asn-21-Tg. The observations indicate that effects of Ile-21 with respect to autoactivation are strongly context-dependent: inhibition of autoactivation was observed in rat Tg-2; no effect was apparent in human Tg-1 at pH 8.0, and enhancement of autoactivation was detected in human Tg-1, at pH 5.0. There are at least two explanations for the phenomenon. (i) Amino acid 21 may alter the proteolytic accessibility of the propeptide region through intramolecular interactions with other residues in Tg. These interactions can vary at different pH values and/or in different Tgs, leading to the variety of observed effects. (ii) Alternatively, amino acid 21 might directly or indirectly participate in one or more of the subite interactions important for efficient substrate binding and catalysis. These intermolecular interactions can also vary at different pH values and/or with different trypsins, resulting in diverse effects on autoactivation.

The results also suggest that enhanced autoactivation of Ile-21-Tg in an acidic environment might play a role in the pathogenesis of HP. In this context, co-localization of zymogen granules and lysosomes in the acinar cells has been proposed as one of the key initiating steps of pancreatitis (27). Co-localization would expose Tg to an acidic milieu and to the cysteine-protease cathepsin B (26, 28), which has been implicated as the initiator of the activation process under these conditions. It is reasonable to speculate that the increased autoactivation propensity of Ile-21-Tg can intensify a cathepsin B-initiated and/or -mediated activation cascade. Comparison of the activation of human Asn-21-Tg and Ile-21-Tg by human cathepsin B could provide additional support to such a pathomechanism.

Another noteworthy difference from the rat Tg-2 is the apparent lack of efficient zymogenolysis during autoactivation in human Tg-1. Rat Tg-2 is digested after Arg-117 and Lys-188 during autoactivation (18). Cleavage at Arg-117 per se does not inactivate trypsin; however, immediate further proteolysis of the C-terminal fragment, possibly at Lys-188, results in the formation of inactive protein. Due to zymogenolysis, autoactivation of rat Tg-2 only yields approximately 50–60% of the total potential trypsin activity in 5 mM Ca2+, whereas this number is only around 20% in EDTA (18, 19). In contrast, human Tg-1 autoactivates practically to 100% of its potential trypsin activity in 5 mM Ca2+, and only proteolytic cleavage after Arg-117 is observed. Both the N-terminal and C-terminal fragments of the Arg-117-cleaved Tg/trypsin are readily visible on gels, indicating that unlike its rat counterpart, the C-terminal fragment is not proteolyzed to any further extent. This might be due to the inaccessibility of Lys-188, because no cleavage at this site was detectable in human Tg-1 under any of the conditions studied. The physiological significance of zymogenolysis, or the lack of it, is unclear at the moment. Because human Tg-1 appears to be highly stable, zymogen stabilization by any of the HP-associated mutations, as previously proposed (19), is unlikely to play a role in the pathomechanism.

Unexpectedly, the most dramatic effects were observed with the Asn-21 → Thr Tg mutant in this study. Autoactivation of Thr-21-Tg was markedly increased relative to Asn-21-Tg or Ile-21-Tg, under all conditions studied, at pH 5.0 or 8.0, in 5 mM Ca2+ or in 1 mM EDTA. In addition, Thr-21-trypsin exhibited significantly increased stability against autolysis in EDTA.
Unlike all other known Tgs, human cationic Tg contains Asn at position 21. With the exception of human anionic Tg, which carries an Ile at position 21, all other mammalian Tgs sequenced to date contain Thr-21 (29). Recently, it has been proposed that the evolutionary divergence from Thr-21 found in other mammalian trypsins is the result of a positive selection process “endowing an as yet unknown advantageous effect” (30). The present observations provide tangible biochemical support for this theory. Current thinking views both increased Tg activation and increased trypsin stability as potential pathogenic factors of pancreatitis. Consequently, the presence of Thr-21 in human cationic Tg would significantly increase susceptibility to pancreatitis, and evolutionary selection of Asn-21 alleviates this problem.

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REFERENCES
1. Perrault, J. (1994) Gastroenterol. Clin. North Am. 23, 743–752
2. Whitcomb, D. C. (1999) Pancreas 18, 1–12
3. Whitcomb, D. C. (1999) Gut 45, 517–522
4. Whitcomb, D. C. (1999) Gastroenterol. Clin. North Am. 28, 525–541
5. Gates, L. K., Jr., Ulrich, C. D., and Whitcomb, D. C. (1999) Surg. Clin. North Am. 79, 711–722
6. Whitcomb, D. C., Garry, M. C., Preston, R. A., Purvey, W., Sossenheimer, M. J., Ulrich, C. D., Martin, S. P., Gates, L. K., Jr., Amann, S. T., Toskes, P. P., Liddle, R., McGrath, K., Uomo, G., Post, J. C., and Ehrlich, G. D. (1996) Nat. Genet. 14, 141–145
7. Garry, M. C., Gabbaizedeh, D., Purvey, W., Gates, L. K., Jr., Preston, R. A., Axton, C. E., Zhang, Y., Ulrich, C., Ehrlich, G. D., and Whitcomb, D. C. (1997) Gastroenterology 113, 1063–1068
8. Teich, N., Messner, J., and Keim, V. (1998) Hum. Mutat. 12, 39–43
9. Nishimori, I., Kamakura, M., Fujikawa-Adachi, K., Morita, M., Onishi, S., Yokoyama, K., Makino, I., Ishida, H., Yamamoto, M., Watanabe, S., and Ogawa, M. (1999) Gut 44, 259–263
10. Creighton, J., Lyall, R., Wilson, D. I., Curtis, A., and Charnley, R. (1999) Lancet 354, 42–43
11. Rinderknecht, H., Renner, I. G., Abramson, S. B., and Carmack, C. (1984) Gastroenterology 86, 681–692
12. Rinderknecht, H., Adham, N. P., Renner, I. G., and Carmack, C. (1988) Int. J. Pancreatol. 3, 33–44
13. Rinderknecht, H. (1986) Dig. Dis. Sci. 31, 314–321
14. Maroux, S., and Desneulle, P. (1989) Biochem. Biophys. Acta 181, 59–72
15. Kaslik, G., Pattby, A., Bålint, M., and Graf, L. (1995) FEBS Lett. 370, 179–183
16. Várallyay, E., Pál, G., Pattby, A., Szilágyi, L., and Graf, L. (1998) Biochem. Biophys. Res. Commun. 243, 56–60
17. Li, X. F., Nie, X., and Tang, J. G. (1988) Biochem. Biophys. Res. Commun. 250, 235–239
18. Sahin-Toth, M. (1999) J. Biol. Chem. 274, 29699–29704
19. Sahin-Toth, M., Graf, L., and Toth, M. (1999) Biochem. Biophys. Res. Commun. 264, 505–508
20. Pál, G., Szilágyi, L., and Graf, L. (1996) FEBS Lett. 385, 165–170
21. Pál, G., Sprenger, G., Pattby, A., and Graf, L. (1994) FEBS Lett. 342, 57–60
22. Lengyel, Z., Pál, G., and Sahin-Toth, M. (1998) Protein Expression Purif. 12, 291–294
23. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 51–59
24. Graff, L., Craik, C. S., Pattby, A., Rozenbak, S., Fletterick, R. J., and Rutter, W. J. (1987) Biochem. Biophys. Acta 26, 2616–2623
25. Colomb, E., Figarella, C., and Guy, O. (1979) Biochem. Biophys. Acta 250, 397–405
26. Figarella, C., Miszczuk-Jamska, B., and Barrett, A. J. (1988) Biol. Chem. Hoppe-Seyler 369, (suppl.) 293–298
27. Steer, M. L. (1998) Pancreas 17, 31–37
28. Greenbaum, L. M., Hirshkowitz, A., and Shoichet, I. (1959) J. Biol. Chem. 234, 2885–2890
29. Rypniewski, W. R., Perrakis, A., Vorgias, C. E., and Wilson, K. S. (1994) Protein Eng. 7, 57–64
30. Chen, J.-M., and Ferec, C. (2000) Hum. Genet. 106, 125–126