Hereditary Pancreatitis-associated Mutation Asn\textsuperscript{21} → Ile Stabilizes Rat Trypsinogen \textit{in Vitro}\textsuperscript{*}

Miklós Sahin-Tóth\textsuperscript{‡}

From the Department of Physiology, University of California Los Angeles, Los Angeles, California 90095-1662

Mutations Arg\textsuperscript{117} → His and Asn\textsuperscript{21} → Ile in human trypsinogen-I have been recently associated with hereditary pancreatitis (HP). The Arg\textsuperscript{117} → His substitution is believed to cause pancreatitis by stabilizing trypsin against autolytic degradation, while the mechanism of action of Asn\textsuperscript{21} → Ile has been unknown. In an effort to understand the effect(s) of this mutation, Thr\textsuperscript{21} in the highly homologous rat trypsinogen-II was replaced with Asn or Ile, and the recombinant zymogens and their active trypsin forms were studied. Kinetic parameters of all three trypsins were comparable, and the active enzymes suffered autolysis at similar rates, indicating that neither catalytic properties nor proteolytic stability of trypsin are influenced by mutations at position 21. When incubated at pH 8.0, 37 °C, pure zymogens underwent autoactivation with concomitant trypsinolytic degradation in a Ca\textsuperscript{2+}-dependent fashion. Thus, in the presence of 5 mM Ca\textsuperscript{2+}, autoactivation and digestion of the zymogens after Arg\textsuperscript{117} and Lys\textsuperscript{188} were observed, while in the presence of 1 mM EDTA autoactivation and cleavage at Lys\textsuperscript{188} were reduced, and zymogenolysis at the Arg\textsuperscript{117} site was enhanced. Overall rates of zymogen degradation in [Asn\textsuperscript{21}]- and [Ile\textsuperscript{21}]trypsinogens were higher in Ca\textsuperscript{2+} than in EDTA, while [Thr\textsuperscript{21}]trypsinogen demonstrated inverse characteristics. Remarkably, both in the presence and absence of Ca\textsuperscript{2+}, [Ile\textsuperscript{21}]trypsinogen exhibited significantly higher stability against autoactivation and proteolysis than zymogens with Asn\textsuperscript{21} or Thr\textsuperscript{21}. The observations suggest that autocatalytic trypsinogen degradation may be an important defense mechanism against excessive trypsin generation in the pancreas, and trypsinogen stabilization by the Asn\textsuperscript{21} → Ile mutation plays a role in the pathogenesis of HP.

EXPERIMENTAL PROCEDURES

Materials—Ecotin was overexpressed in \textit{Escherichia coli} BL21 as described by Pál \textit{et al.} (9, 10) and purified to homogeneity using a trypsin affinity column. Purified ecotin was immobilized to ACTIGEL ALD resin (Sterogene Bioseparations, Carlsbad, CA) as described previously (11). Plasmid pTrap was a generous gift from László Gráf (Eötvös University, Budapest, Hungary). Ultrapure enterokinase was purchased from Biozyme Laboratories (San Diego, CA), and N-CBZ-Gly-Pro-Arg-p-nitroanilide from Sigma.

Construction of TG Mutants—Mutations were introduced into the rat anionic TG gene (TG-II) by oligonucleotide-directed site-specific mutagenesis, using the “overlap extension” PCR method (12). The PCR fragments were digested with restriction enzymes BamHI-EcoRI (mutants Thr\textsuperscript{21} → Asn and Thr\textsuperscript{21} → Ile), EcoRI-SacI (mutant Arg\textsuperscript{117} → His), or XhoI-SacI (mutant Lys\textsuperscript{188} → Asn) and ligated into the similarly treated TG gene in the expression vector pTrap (13). The DNA sequence of the mutations as well as the entire subcloned PCR fragments were verified by dyeo sequencing.

Expression and Purification of Recombinant TGs—Wild-type and mutant TGs were expressed to the periplasm in \textit{E. coli} SM138 as described previously (13, 14). In a typical experiment, 2-liter cultures of SM138/pTrap in Luria-Bertani medium with 100 \(\mu\)g/ml ampicillin were grown to saturation overnight, and periplasmic fractions were isolated by osmotic shock. Tris-HCl (pH 8.0) and NaCl were added to a final concentration of 20 mM and 0.2 M, respectively, and the approximately 200-ml periplasm was applied directly to a 2-ml ecotin affinity column (11). The column was washed with 20 mM Tris-HCl (pH 8.0), 0.2 M NaCl, and zymogens were eluted with 50 mM HCl. The pH of the eluate was

\textsuperscript{*} The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{‡} To whom correspondence should be addressed: HHMI/UCLA, 5-748 MacDonald Research Laboratories, Box 951662, Los Angeles, CA 90095-1662. Tel.: 310-206-5055; Fax: 310-206-8623; E-mail: miklos@hhmi.ucla.edu.
RESULTS

Construction of Mutants—Rat anionic TG (TG-II) is probably the best characterized and most widely used recombinant TG/trypsin model system. Homology with the human cationic TG (TG-I) is extensive (60% identity), and the N-terminal regions are particularly well conserved between the two proteins (15). Interestingly, one of the very few differences is found at position 21, where the rat species contains a Thr. In an attempt to explore the significance of the amino acid side chain at position 21, and to understand the effect(s) of the Asn21 → Ile change observed in the TG-I of HP patients, we replaced Thr21 in rat TG-II with Asn or Ile, and the recombinant zymogens and their active trypsin forms were studied. PCR mutagenesis was carried out as described under “Experimental Procedures,” and mutant TG genes were cloned into the expression vector pTrAp (13) behind the alkaline phosphatase promoter and signal sequence. E. coli SM138 was transformed with verified clones, and TG was expressed into the periplasmic space in a constitutive fashion. Zymogens were purified to homogeneity from periplasmic extracts with a one-step affinity procedure using immobilized ecotin (11) as detailed under “Experimental Procedures.” Due to the relatively low levels of expression, the rapid purification technique and the acidic conditions used for elution, remarkably pure zymogen preparations without any detectable trypsin activity were obtained.

Enterokinase Activation of Zymogens—Time courses of enterokinase-catalyzed conversion of TG to trypsin were followed by SDS-PAGE and Coomassie Blue staining. As shown in Fig. 1A, in the presence of 5 mM Ca2+ at 22 °C, enterokinase activated zymogens with Thr21 or Ile21 at comparable rates, while activation of [Asn21]TG was at least 2-fold accelerated. Although not shown, no other degradation products were observed on the stained gels. In a different experiment, enterokinase activation of zymogens was followed by continuously monitoring trypsin activity 37 °C, in the presence of 1 mM Ca2+. Under these conditions the initial appearance of trypsin activity reflects predominantly enterokinase action, without significant interference from autocatalytic activation. Fig. 1B demonstrates that rates of trypsin generation from [Asn21]TG were approximately 2-fold higher than those from [Thr21]TG and [Ile21]TG. No trypsin activity was detected in the absence of enterokinase.

Catalytic Properties and Autolysis of Mutant Trypsins—Zymogens were activated by enterokinase and catalytic parameters of the active enzymes were determined using the chromogenic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide (Table I). No significant differences were detected in the kcat/Km values of trypsins with Thr, Asn, or Ile at position 21, indicating that catalytic properties of trypsin are not influenced by mutations at this position.

It has been proposed that the Asn21 → Ile mutation may decrease the accessibility of the autolytic site at Arg21 and cause increased autolytic stability, in a manner that is similar to the effects of the Arg117 → His mutation (3–5). To verify this suggestion, autolytic degradation of trypsins with Thr21, Asn21, or Ile21 were characterized together with the Asn21/Arg117 → His double mutant. When solutions of trypsin were incubated at 37 °C, enzyme activity was gradually lost as a function of time (Fig. 2). Surprisingly, rates of autolytic degradation showed no appreciable difference when trypsins with different
substitutions at position 21 were compared, while stability of the Asn21\textsuperscript{21}Arg117\textsuperscript{21} His mutant was significantly increased. As described previously (7), 5 mM Ca\textsuperscript{2+} afforded significant protection against autolysis (compare Fig. 2, A and B); however, it had no effect on the relative degradation patterns of the trypsin mutants. The results indicate that autolytic stability is not altered by mutations at position 21 and suggest essential mechanistic differences between the effects of the Asn21\textsuperscript{21} Thr21\textsuperscript{21} His mutations in HP.

**Autoactivation of Mutant Zymogens in the Presence of Ca\textsuperscript{2+}**

One of the prevailing theories to explain the effects of the Asn21\textsuperscript{21} Thr21\textsuperscript{21} His substitutions at position 21 was that altered accessibility of the activation site may lead to excessive trypsin formation through increased autoactivation (3–5). To examine this possibility, a series of experiments autoactivation of zymogens was characterized by activity assays and SDS-PAGE. When incubated at 37 °C in the presence of 5 mM Ca\textsuperscript{2+}, pure zymogens underwent relatively rapid autoactivation and proteolytic degradation (Fig. 3). Remarkably, [Ile\textsuperscript{21}]TG exhibited significantly higher stability than [Asn\textsuperscript{21}]TG, while [Thr\textsuperscript{21}]TG had intermediate characteristics. Thus, after 1-h incubation approximately 50% of [Asn\textsuperscript{21}]TG was converted to trypsin and proteolytic products, and at 1.5 h no more intact zymogen was detectable on gels (Fig. 3A). In contrast, [Thr\textsuperscript{21}]TG and [Ile\textsuperscript{21}]TG were almost completely stable at least up to 1 and 1.5 h, respectively. Rapid autoactivation of the Asn\textsuperscript{21}-zymogen was also evident from the early appearance of trypsin activity. Differences were most pronounced at 1 h, when more than 20-fold higher trypsin activity was detectable in [Asn\textsuperscript{21}]TG samples relative to [Ile\textsuperscript{21}]trypsin activity. It is important to note that maximal activity achieved during autoactivation was only 40–50% of the potential maximal value, as determined after entero激ase activation of zymogens. This apparent loss of activity is presumably due to the significant proteolytic degradation also observed on gels (Fig. 3A). On the routinely used 12% gels four major stable degradation products were identified by Coomassie Blue staining (bands I–IV in Fig. 3A). Appearance of bands I and III in all three zymogen variants coincided with the

---

**TABLE I**

*Kinetic parameters of wild-type (Thr\textsuperscript{21}) and mutant trypsins (Asn\textsuperscript{21}, Thr\textsuperscript{21} \rightarrow Asn, Ile\textsuperscript{21}; Thr\textsuperscript{21} \rightarrow Ile) on synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide at 37 °C*

|       | $k_{cat}$ (1/min) | $K_m$ ($\mu$M) | $1/K_m \times min$ |
|-------|------------------|-----------------|---------------------|
| Thr\textsuperscript{21} | $1.7 \times 10^{-5}$ | $1.3 \times 10^{-5}$ | $1.2 \times 10^{10}$ |
| Asn\textsuperscript{21} | $1.7 \times 10^{-5}$ | $1.3 \times 10^{-5}$ | $1.2 \times 10^{10}$ |
| Ile\textsuperscript{21} | $1.4 \times 10^{-4}$ | $1.3 \times 10^{-5}$ | $1.1 \times 10^{10}$ |

---

**Fig. 2. Autoactivation of trypsin mutants.** Aliquots of trypsinogens (1.36 $\mu$m final concentration; Asn\textsuperscript{21}, mutant Thr\textsuperscript{21} \rightarrow Asn; Thr\textsuperscript{21}, wild-type rat anionic trypsinogen; Ile\textsuperscript{21}, mutant Thr\textsuperscript{21} \rightarrow Ile; His\textsuperscript{117}, double mutant Thr\textsuperscript{21} \rightarrow Asn/Arg\textsuperscript{117} \rightarrow His) were activated by enterokinase for 30 min in the presence of 5 mM CaCl\textsubscript{2}, as described in the legend to Fig. 1A. Autolytic inactivation of trypsinogens was then followed at 37 °C without any further additions (upper panel) or after addition of K-EDTA (pH 8.0) to a final concentration of 15 mM (lower panel). Residual activities were expressed as a percentage of trypsin activity measured immediately after enterokinase activation.
emergence of the trypsin band, indicating that proteolysis of zymogens is due to the trypsin generated by autoactivation. No differences were observed in the overall proteolytic pattern among the three zymogens studied. Rat TG-II migrates at an anomalously high molecular weight in SDS-PAGE (11), therefore reliable estimation of fragment sizes by comparing to molecular mass standards was not feasible. However, changes in the intensities of bands I–IV during the investigated time course suggested that band I may get converted to band II, and cleavage of band III may give rise to band IV (see Fig. 3A). In turn, the relatively higher amounts of trypsin resulted in massive digestion at Arg 117 (band III). The overall effect was a surprisingly rapid zymogen degradation with little trypsin liberation (Fig. 5A). Monitoring trypsin activity during autoactivation in 1 mM EDTA revealed a pattern consistent with those observed on gels (Fig. 5B). Only [Thr 21]TgI exhibited significant activity, but even at its maximum it was below 20% of the total potential activity, as determined on enterokinase activated zymogen samples. Trypsin liberated from [Asn 21]TgI was minimal, while [Ile 21]TgI samples did not yield any measurable signal above background over the 3-h time period.

**DISCUSSION**

The present study is the first attempt of an in-depth biochemical investigation into the effects of the HP-associated Asn 21 → Ile mutation in human cationic TG. Since high yield recombinant expression and purification protocols for the human trypsinogens are not readily available yet, we used the homologous rat TG model system and replaced Thr 21 with Asn or Ile, and properties of the three zymogens and their active trypsin forms were compared. More specifically, we tested two theories providing speculative explanations while this mutation leads to the HP phenotype (3–5, 8). (i) In analogy to the effects of the Arg 117 → His mutation, it was suggested that the Asn 21 → Ile mutation may indirectly decrease autolytic cleavage at Arg 117 and causes increased autolytic stability of trypsin. (ii) Alternatively, the relative proximity of the Asn 21 → Ile
mutation to the activation peptide region prompted the hypothesis, that enhanced autoactivation may increase trypsin liberation. A common feature of both models that HP-associated mutations are supposed to lead to uncontrolled, excessive trypsin activity and tissue autodigestion. Surprisingly, our observations demonstrate that mutations at position 21 have no effect whatsoever on autolytic stability of trypsin and autoactivation is not increased in [Ile21]/TG. In sharp contrast, the primary effect of the Asn21 → Ile mutation appears to be stabilization of the activation peptide region, resulting in significantly decreased autoactivation rates (Figs. 3 and 5). While the exact mechanism of this stabilization is not clear yet, structural rearrangements in the activation peptide (5) are likely to be responsible for the altered proteolytic accessibility and/or cleavage efficiency of the Lys15 site. This notion is also supported by the reduced rates of activation by entokerinase in [Ile21]/TG relative to [Asn21]/TG (see Fig. 1).

One important consequence of the decreased autoactivation rates in [Ile21]/TG is the delayed onset of autocatalytic zymogen degradation (see Figs. 3 and 5). Proteolytic inactivation of TG by trypsin generated during autoactivation appears to be highly efficient, and it is intriguing to speculate that this might function as a “failsafe” mechanism of controlling excessive trypsin liberation in the pancreas. In the presence of Ca2+ only about 50% of the total potential trypsin activity can be produced from [Asn21]/TG by autoactivation, while in the absence of Ca2+ this number is well below 10%. We found that cleavage of TG occurs first at Arg117 and Lys188. Digestion of the Lys188, Asp189 bond in bovine trypsin has been shown to significantly impair activity (16), while cleavage of the Arg117,Val118 bond per se does not inactivate trypsin (6); however, it destabilizes the autolysis loop and leads to widespread trypsinolysis in this region (7). Cleavage at the Lys15 and Arg117 sites are inversely controlled by Ca2+, which enhances proteolysis at Lys15 and inhibits at Arg117. Remarkably, as best demonstrated by the behavior of [Thr21]/TG, the combination of these two opposing actions of Ca2+ appears to determine the overall reaction kinetics of zymogen degradation. Thus, in the absence of Ca2+, inhibition of proteolysis at Lys15 is less pronounced in [Thr21]/TG than in [Asn21]/TG or [Ile21]/TG, and relatively higher levels of trypsin are liberated, which in turn can more efficiently attack the sensitized Arg117 site. From these observations it is also apparent, that the amino acid side chain at position 21 is an important determinant of the effect of Ca2+ on the activation site. Further studies are required to clarify whether amino acid 21 influences the Ca2+ binding affinity of the activation peptide or modulates the effects of the bound Ca2+.

It is generally believed that in pancreatitis pathologic trypsin generation is initiated inside the acinar cells, presumably in a low Ca2+ environment (17). We hypothesize that rapid and efficient reduction of the “potentially hazardous” zymogen storage pool under these conditions can be critical in preventing widespread autoactivation. Overall rates of zymogen degradation in different TG species are primarily determined by amino acid 21. With the exception of human TG-I and -II, mammalian trypsinsogens carry a Thr residue at this position, which appears to allow for rapid zymogenolysis in a low Ca2+ environment. The presence of Asn and Ile in human TG-I and -II, respectively, are unique and so far not observed in any other species. While the evolutionary rationale behind these changes is not yet understood, both substitutions lead to slower zymogen degradation in the absence of Ca2+, which may weaken an important defense mechanism against uncontrolled trypsin generation and autodigestion. In HP caused by the Asn21 → Ile mutation of TG-I, the highly stable Ile21-zymogen is overproduced, causing a significant increase in the risk of pancreatitis. The increased stability against autoactivation of human TG-II relative to TG-I has been described previously (18), and based on our results this may be attributed solely to the Asn versus Ile difference at position 21.

The proposed pathomechanism of HP described above provides an attractive working model, which should be also readily testable. However, given the complexity of the pancreatic zymogen synthesis, storage, and secretion, we cannot rule out that the Asn21 → Ile mutation affects other processes along this pathway, too. One such possibility is that the mutation may decrease affinity for trypsin inhibitors found in the pancreas, e.g. the pancreatic secretory trypsin inhibitor. Although we have not tested this idea in detail in the present study, inhibition experiments with bovine pancreatic trypsin inhibitor (aprotinin) showed no significant differences between the inactivation profiles of trypsins with Asn21, Thr21, or Ile21 (not shown).

Finally, we need to remember that an obvious caveat to the conclusions of the present study is the use of the rat anionic TG/trypsin model system rather than the human cationic TG.

![Fig. 5. Autoactivation and zymogenolysis of mutant trypsins in the absence of Ca2+](image-url)
Although the rodent enzyme is 80% identical to the human isoform, this does not guarantee that the Asn → Ile mutation will have the exact same effect in the two species. Therefore, further experiments to confirm the present findings will be mandatory as human recombinant enzymes become available.

Acknowledgments—Special thanks to Ron Kaback for his patience and support. János Botvánszky and Zsolt Lengyel are gratefully acknowledged for their help in initially documenting some of the properties of the trypsinogen mutants. Thanks are due to László Gráfrózniak, László Sziágyi, Gábor Pál (Department of Biochemistry, Eötvös University, Budapest, Hungary), and Miklós Tóth (Department of Medical Chemistry, Semmelweis University of Medicine, Budapest, Hungary) for helpful discussions and suggestions. Protein sequencing was carried out by Audree Fowler at the UCLA Protein Sequencing Facility.

REFERENCES
1. Perrault, J. (1994) Gastroenterol. Clin. N. Am. 23, 743–752
2. Whitcomb, D. C., Gorry, M. C., Preston, R. A., Furey, 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
3. Gorry, M. C., Gabbaizedeh, D., Furey, W., Gates, L. K., Jr., Preston, R. A., Aston, C. E., Zhang, Y., Ulrich, C., Ehrlich, G. D., and Whitcomb, D. C. (1997) Gastroenterology 113, 1063–1068
4. Teich, N., Mossner, J., and Reim, V. (1998) Hum. Mutat. 12, 39–43
5. 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
6. Maroux, S., and Desmoule, P. (1969) Biochim. Biophys. Acta 181, 59–72
7. Váralyai, E., Pál, G., Patthy, A., Sziágyi, L., and Gráfrózniak, L. (1998) Biochem. Biophys. Res. Commun. 243, 56–60
8. Whitcomb, D. C. (1999) Pancreas 18, 1–12
9. Pál, G., Sziágyi, L., and Gráfrózniak, L. (1996) FEBS Lett. 385, 165–170
10. Pál, G., Sprengler, G., Patthy, A., and Gráfrózniak, L. (1994) FEBS Lett. 342, 57–60
11. Lengyel, Z., Pál, G., and Sahin-Tóth, M. (1988) Protein Expression Purif. 12, 291–294
12. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
13. Gráfrózniak, L., Craik, C. S., Patthy, A., Roczniak, S., Fletterick, R. J., and Rutter, W. J. (1987) Biochemistry 26, 2616–2623
14. Gráfrózniak, L., Jancsó, A., Sziágyi, L., Hegyi, G., Pintér, K., Náray-Szabó, G., Hepp, J., Medzhhradszký, K., and Rutter, W. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4961–4965
15. Ryppiewsáki, W. R., Perrakis, A., Vergias, C. E., and Wilson, K. S. (1994) Protein Eng. 7, 57–64
16. Smith, R. L., and Shaw, E. (1969) J. Biol. Chem. 244, 4704–4712
17. Steer, M. L. (1998) Pancreas 17, 31–37
18. Colomb, E., Figarella, C., and Guy, O. (1979) Biochim. Biophys. Acta 570, 397–405