Two Mutations in Rat Trypsin Confer Resistance against Autolysis

Éva Várallyay,* Gábor Pál,†-1 András Patthy,* László Szilágyi,† and László Gráf *†
†Department of Biochemistry, Eötvös University, Budapest, Hungary; and †Institute for Biochemistry and Protein Research, Agricultural Biotechnology Center, Gödöllő, Hungary

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Due to autodigestion the activity of dissolved trypsin successively decreases. Autolysis leads to proteolytic cleavages of some arginyl and lysyl peptide bonds of the trypsin structure. Three important autolysis sites have been reported for bovine trypsin: Lys61-Ser62, Arg117-Val118 and Lys145-Ser146. Out of these three sites only the first two exist in rat trypsin, an enzyme that has been the target of protein engineering for more than ten years. In this work Lys61 and Arg117 were replaced by Asn via site directed mutagenesis to transform the corresponding peptide bonds to trypsin resistant ones. Kinetic parameters of K61N, R117N and the double mutant K61N/R117N are practically identical with those of the wild-type enzyme. By contrast, the rate of autolysis of each singly-substituted species is substantially slower than with the parent trypsin. In particular, the double mutant shows dramatically increased stability against autolysis and decreased sensitivity to Ca²⁺. The process of autolysis has been followed by N-terminal sequence determination. We propose a model to explain why these two positions play a key role in autolysis and how Ca²⁺ can influence this process. In addition, our in vitro results strongly support the recently proposed model of human hereditary pancreatitis.

Mammalian trypsins are synthesized in the pancreas as zymogens and activated in the duodenum by enterokinase. During this activation the propeptide of the enzymes (different in length in various species) is released by the proteolysis of Lys15-Ile16 (conventional chymotrypsin numbering is used), resulting in single chain β-trypsin. Since this cleavage occurs at a trypsin sensitive site, trypsin can autocatalytically activate its own zymogen form. In vitro studies on bovine β-trypsin have shown that trypsin can be further cleaved between Lys145 and Ser146, within the so called autolysis loop, generating fully active double chain α-trypsin (1). Detailed studies of Maroux et al (2) showed that two other cleavages of β-trypsin can also take place at peptide bonds Arg117-Val118 and Lys61-Ser62. These cleavages were reported to exert different effects on the activity of the enzyme. They generate fully active or low activity enzymes, respectively. By means of protein engineering for more than ten years we have been studying the structural basis of substrate specificity (3-6), zymogen activation (7) and stability against proteolysis (5-7) of serine proteinases. Our model proteins are recombinant rat trypsin and chymotrypsin. Till recently we have been interested in the structural rather than functional aspects of the above three phenomena, substrate specific catalysis, activation and autolysis which, we believe, are regulated by the same structural unit of these enzymes (7,8).

Rat anionic trypsin does not have trypsin sensitive sites in the so called autolysis loop: Lys145 of bovine trypsin is replaced by Leu in the rat enzyme. For rat trypsin the initial autolysis site has been proposed to be Arg117-Val118 (7). Arg117 is located within a peptide segment that connects the two globular domains of the trypsin molecule. This region protrudes from the surface of the molecule, making it highly accessible to the solvent. The rate of autolysis of trypsin has long been known to depend on Ca²⁺ concentration. Although the structure of the Ca²⁺-binding site of trypsin has been identified (9), the mechanism by which Ca²⁺ binding affects autolysis is still unknown. In this study Asn residues were introduced in place of Lys61 and Arg117 by site directed mutagenesis in the recombinant rat trypsin. The main goal of this work was to investigate the role of these positions in the process of autolysis. We compared the rate of autolysis of the wild type, K61N, R117N and K61N/R117N (double) mutant enzymes in the presence and absence of Ca²⁺ and identified the individual autolytic cleavage sites. These data

†To whom correspondence should be addressed: Department of Biochemistry, Eötvös University, Puskin u.3., H-1088, Budapest, Hungary. Fax:(36) 1 266 7830. E-mail: pal@udens.elte.hu.
Abbreviations: AMC, 7-amino-4-methylcoumarin; pNA, p-nitroanilide; MUGB, 4-methylumbelliferyl p-guanidinobenzoate.
allowed us to propose a simple model that may explain why Lys61 and Arg117 play crucial roles in autolysis and how Ca$^{2+}$ can interfere with this process. Finally, our stable variants of trypsin may prove useful in experiments frustrated by autolysis of the wild-type enzyme.

MATERIALS AND METHODS

Site-directed mutagenesis/construction of mutant enzymes. Site-directed mutagenesis was performed as described by Kunkel (10) with a slight modification (11) starting with M13 template containing the coding sequence of rat trypsinogen. The following oligonucleotides were used (mismatched bases are underlined):

K61N: CAC-TGC-TAT-AA
R117N: GTG-AAA-CTC-AAT

Mutations were confirmed by dideoxy DNA sequencing.

Expression and isolation of mutant enzymes. Wild type and mutant proteins were expressed constitutively and secreted into the periplasmic space of E. coli, purified to homogeneity and activated as described (3). The homogeneity of the enzymes was assessed by SDS polyacrylamide gel electrophoresis.

Enzyme kinetics. Enzyme assays were carried out in 20mM Tris, 0.1M NaCl, 5 mM CaCl$_2$, pH 8.0 buffer at 37°C. The photometric substrate Z-Gly-Pro-Arg-pNA was used, and the resulting pNA concentration was measured. Stock solutions of the substrate were prepared in dimethylformamide. Concentration of liberated pNA was determined photometrically at 410 nm ($e_{410} = 8480$ M$^{-1}$ cm$^{-1}$). Active trypsin concentration was determined fluorimetrically by active site titration with MUGB (12). Kinetic parameters were calculated by the computer program ENZFITTER.

Following the autolysis by residual activity measurement. Wild type and mutant trypsins were incubated at 37°C in 0.1M Tris-HCl buffer pH 8.0 in the presence of 20mM CaCl$_2$ or 1mM EDTA. The enzyme concentration was 10$^{-6}$ M. At incremental time intervals aliquots were withdrawn and autolysis was stopped by decreasing the pH to 3.0 with 1M HCl. Residual activity of each sample was measured fluorimetrically using Suc-Ala-Ala-Pro-Arg-AMC as substrate.

Mutations Increase Stability of Trypsin against Autolysis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (1/min)</th>
<th>$K_M$ (μM)</th>
<th>$K_{cat}/K_M$ (1/M min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT TRYPsin</td>
<td>4400</td>
<td>8.6</td>
<td>5.1 x 10$^8$</td>
</tr>
<tr>
<td>K61N RAT TRYPsin</td>
<td>5470</td>
<td>12.9</td>
<td>4.2 x 10$^8$</td>
</tr>
<tr>
<td>R117N RAT TRYPsin</td>
<td>10000</td>
<td>12.5</td>
<td>5.8 x 10$^8$</td>
</tr>
<tr>
<td>K61N/R117N RAT TRYPsin</td>
<td>7280</td>
<td>17.7</td>
<td>4.1 x 10$^8$</td>
</tr>
</tbody>
</table>

was stopped by adding 300μl 1M HCl to 100μl of the sample. These samples were loaded onto an AQUAPORE OD-300 column (Applied Biosystem) and eluted in a linear gradient of 0-80% acetonitrile in 0.1% TFA. Peaks were collected and identified by N-terminal sequencing with an Applied Biosystem 471 A pulsed liquid-phase sequencer.

RESULTS

Catalytic Properties of Mutant Trypsins Are Conserved

Replacement of Lys61, Arg117 or both with Asn does not significantly alter the kinetic parameters $k_{cat}$ and $K_M$ of trypsin (Table 1). All three mutant forms of trypsin display a ratio of $k_{cat}/K_M$ close to that of the wild-type value, thus these mutations did not substantially alter the catalytic efficiency of the enzyme.

Mutations Increase Stability of Trypsin against Autolysis

Measuring the residual activity as a function of incubation time, we found that all mutations resulted in an increase of stability of the enzymes against autolysis (Figure 1). This stabilisation was moderate when only Lys61 but considerable when Arg117 was replaced. The stabilizing effect of Ca$^{2+}$ on variants of trypsin depends on their respective stability in the absence of Ca$^{2+}$. The more stable the enzyme without Ca$^{2+}$, the less is its autolysis decelerated by that divalent cation. The double mutant the most stable enzyme tested, is almost completely insensitive to the presence of Ca$^{2+}$.
TABLE 2
Determination of Autolysis Cleavage Sites by N-Terminal Sequencing

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>N-Terminal sequence</th>
<th>Cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Ile-Val-Gly-Gly-Tyr-</td>
<td>(original N-terminus)</td>
</tr>
<tr>
<td></td>
<td>Leu-Gly-His-Asn-</td>
<td>Arg67-Leu68</td>
</tr>
<tr>
<td></td>
<td>His-Pro-Asn-Phe-Asp-</td>
<td>Lys90-His91</td>
</tr>
<tr>
<td></td>
<td>Lys-Thr-Leu-Asn-</td>
<td>Arg96-Lys97</td>
</tr>
<tr>
<td></td>
<td>Leu-Ser-Ser-Pro-Val-</td>
<td>Lys107-Leu108</td>
</tr>
<tr>
<td></td>
<td>Val-Ala-Thr-Val-Ala-</td>
<td>Arg117-Val118</td>
</tr>
<tr>
<td>K61N</td>
<td>Ile-Val-Gly-Gly-Tyr-</td>
<td>(original N-terminus)</td>
</tr>
<tr>
<td></td>
<td>Leu-Gly-His-Asn-</td>
<td>Arg67-Leu68</td>
</tr>
<tr>
<td></td>
<td>His-Pro-Asn-Phe-Asp-</td>
<td>Lys90-His91</td>
</tr>
<tr>
<td></td>
<td>Val-Ala-Thr-Val-Ala-</td>
<td>Arg117-Val118</td>
</tr>
<tr>
<td>R117N</td>
<td>Ile-Val-Gly-Gly-Tyr-</td>
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<td></td>
<td>Leu-Gly-His-Asn-</td>
<td>Arg67-Leu68</td>
</tr>
<tr>
<td></td>
<td>Leu-Ser-Ser-Pro-Val-</td>
<td>Lys107-Leu108</td>
</tr>
<tr>
<td>K61N/R117N</td>
<td>Ile-Val-Gly-Gly-Tyr-</td>
<td>(original N-terminus)</td>
</tr>
</tbody>
</table>

Wild type and mutant trypsins were incubated in 0.1M ammonium bicarbonate buffer pH 8.0 containing 1mM EDTA. The enzyme concentration was 5 x 10^{-6} M. After six hours incubation aliquots were taken out and the autolysis was stopped by adding 1M HCl. The samples were loaded onto an AQUAPORR OD-300 column (Applied Biosystem) and eluted in a linear gradient of 0-80% acetonitrile in 0.1% TFA. Peaks were collected and identified by N-terminal sequencing with an Applied Biosystem 471A pulsed liquid-phase sequencer.

Increased Stability Is due to Increased Resistance against Tryptic Attack

To follow the process of the autolysis of both the wild-type and mutant proteinases, sites where cleavages occurred were identified by N-terminal sequencing (Table 2 and Figure 2). We found that autolysis of the wild-type enzyme started with the cleavage of peptide bond Arg117-Val118. This cleavage was followed by fast and extensive digestion of the polypeptide chain at several sites between Lys61 and Arg117, leading to autoinactivation of the enzyme. In the case of the wild-type enzyme we detected peptide bond cleavages between the following pairs of residues: Arg67-Leu68, Lys90-His91, Arg96-Lys97, Lys107-Leu108 and Arg117-Val118. Additional cleavages after Lys61, Arg63, Lys105 and Lys113 would escape detection, as they would generate peptides too small to be resolved by the reverse-phase column (Figure 2). In K61N trypsin we could only detect cleavages at sites formed by Arg67, Lys90 and Arg 117. Mutation of Arg 117 to Asn protects nearly perfectly these sites against proteolytic attack. Only negligible cleavages occurred at Arg67 and Lys107. Further mutation of the enzyme by replacing Lys61 with Asn resulted in a trypsin mutant that is resistant almost completely to autolysis.

DISCUSSION

Decrease of the specific activity of a trypsin solution incubated at the pH optimum of the enzyme is due to autoproteolysis: trypsin molecules can cleave each other at lysyl and arginyl bonds. Rat trypsin has thirteen potential trypsin sensitive sites. The distribution of these residues in the trypsin sequence is uneven: only three sites are in the C-terminal domain, while ten are in the N-terminal domain. The latter ten residues are accumulated within a short segment of the trypsin structure bordered by Lys61 and Arg117. A model to explain the role of these sites in the process of autolysis is proposed as follows.

A peptide segment of 57 residues between Lys61 and Arg117 is part of the longest peptide chain not stabilized by disulfide bridges and may function as a built-in target for a self-destruct mechanism. As long as both ends of this segment are intact, cleavage sites in the middle appear to be protected from being hydrolysed by trypsin. Cleavage at Lys61 moderately while at Arg117 more effectively eliminates this protection. The first cleavage at any ends of this peptide segment is followed by cleavages at several trypsin sensitive sites between the two ends. This process can not be described as series of consecutive steps since cleavages appear to occur parallel. Thus, the decrease of activity can not be attributed to one or two particular cleavages at certain positions. The extensive process of autolysis does not seem to spread to the C-terminal domain. Here the rate of cleavages is much slower and independent on the autolysis of segment 61-117.

The above scenario explains the stabilizing effect of Ca^{2+} ions on dissolved trypsin. The Ca^{2+}-binding loop extends from Glu70 to Glu80, placing it within the "self-destruction" segment (Fig. 3). We suppose that Ca^{2+} can stabilize an autolysis-resistant conformation of the two ends of the polypeptide segment Lys61 to Arg117. If these sites are rendered resistant to proteolysis by site-directed mutagenesis, Ca^{2+} is no longer needed to protect against autolysis. Indeed, the stabilizing effect of Ca^{2+} is smaller with Lys61Asn and Arg117Asn then with the wild type enzyme; it is negligible in the double mutant.

Loss of activity during autolysis might simply be due to the extensive degradation of peptide segment 61-117 and the consequent disruption of protein structure. However, a more specific mechanism is also possible, as two members of the catalytic triad, His57 and Asp102 are located in this portion of trypsin. His57 is close to one of the ends of the above mentioned peptide segment while Asp102 is a part of it, and both should be displaced upon its degradation.

Our results on the Arg117Asn mutant of rat trypsin supports the view that natural Arg117His mutation of human trypsin may be responsible for human hereditary pancreatitis (HP). They found that a single G to A mutation in the 3rd exon of the cationic trypsinogen gene resulting in the substitution of...
Arg117 by His, is associated with the HP phenotype. Although the mutant form of the enzyme has not been available for kinetic studies in vitro, they proposed a reasonable model of how this mutation could lead to the observed clinical symptoms of pancreatitis. They assume that the Arg117His mutation does not alter the tertiary structure and activity of trypsin but abolishes an important autolytic site. Thus, the mutant enzyme would be protected from proteolysis by trypsin and other trypsin-like proteinases. This proteolysis is a supposed safety mechanism against prematurely activated trypsin in the pancreas.

Human cationic trypsin and rat anionic trypsin exhibit an extensive sequence homology within the region we found to be the major target for autolysis (78.5% sequence identity). Furthermore, Arg117 has been independently identified as an autolytic site in human trypsin (14). Therefore, our findings concerning autolysis of rat trypsin and its Arg117Asn mutant should be relevant to human trypsin and its Arg117His form. Presumably, Arg117His human trypsin, like the corresponding mutant of the rat enzyme, retains its catalytic activity and resists degradation by trypsin or other trypsin-like proteinases. Ours is the first in vitro evidence supporting the model for hereditary pancreatitis (13).

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REFERENCES