Human cationic trypsinogen is activated by human enteropeptidase much more readily than bovine trypsinogen, the ratios \( k_{\text{cat}}/K_{\text{m}} \) being 330 and 11 \( \text{mM}^{-1} \text{s}^{-1} \), respectively. Conversely, porcine enteropeptidase activates bovine trypsinogen much more readily than bovine trypsinogen (\( k_{\text{cat}}/K_{\text{m}} = 2.4 \text{ mM}^{-1} \text{s}^{-1} \)). The primary structure of the activation region of human cationic trypsinogen has been investigated in an attempt to elucidate the basis for these findings. The sequence of the first 12 residues at the \( \text{NH}_2 \)-terminus of human cationic trypsinogen has been shown to be Asp-Lys-Ile-Val-Gly-Gly-Tyr-Asn-Cys-Glu-Glu-Asn. Furthermore, the activation peptide derived from human cationic trypsinogen has been isolated and shown to be the dipeptide Asp-Lys. This result is in contrast to the Val-(Asp),-Lys activation peptide from bovine trypsinogen and demonstrates that human cationic trypsinogen does not contain the (Asp), sequence present in many other mammalian trypsinogens. It is proposed that the high degree of specificity for activation of human cationic trypsinogen by human enteropeptidase is due to the preferential recognition of the novel activation peptide sequence in the human zymogen. Thus, these two functionally related proteins, cationic trypsinogen and enteropeptidase, may have evolved in a parallel manner in the human lineage.

PANCREATIC TRYPSIN PLAYS A KEY ROLE IN THE PROCESS OF PROTEIN DIGESTION, SINCE IT ACTIVATES TRYPsinogen AS WELL AS SEVERAL OTHER ZYMogens PRESENT IN PANCREATIC SECRETION, INCLUDING chymotrypsinogen, procarboxypeptidases A and B, and PROLASTase (1). Activation of the pancreatic zymogens is initiated by the activation of trypsinogen by intestinal enteropeptidase (2). Activation of bovine trypsinogen is accomplished by cleavage of an \( \text{NH}_2 \)-terminal hexapeptide with the unusual sequence Val-(Asp),-Lys from the zymogen molecule (3). While this cleavage can be catalyzed by trypsin (4), the rate constant for activation of bovine trypsinogen by porcine enteropeptidase is approximately 3 orders of magnitude greater than that for autoactivation (5, 6). Maroux et al. (6) have suggested that this unique specificity is due to the interaction of porcine enteropeptidase with the (Asp), sequence in the activation peptide. Thus, although enteropeptidase is a serine protease with apparent specificity toward arginine or lysine residues with respect to hydrolysis of ester or amide substrates (6, 7), trypsinogen appears to be the only known protein substrate for this enzyme (6).

Amino acid sequences have been determined for the activation peptides derived from several other trypsinogens (8–12). In each case, the (Asp), sequence is present. The only known exceptions are the activation peptides from African lungfish trypsinogens A and B (13). It has been suggested that this series of acidic residues constitutes a specific recognition site for activation of trypsinogen by enteropeptidase (6), and that the sequence has, therefore, been highly conserved during evolution (13).

The evidence presented in the accompanying communication (14) suggests that the activation peptide of human cationic trypsinogen does not contain an (Asp), sequence. We have demonstrated that both the rate of autoactivation and the nature of the dependence on \( \text{Ca}^{2+} \) for autoactivation of human cationic trypsinogen differ markedly from results obtained by other workers on autoactivation of the bovine zymogen. (4, 15). In this communication, we report the amino acid sequence for the activation peptide of human cationic trypsinogen and the sequence of the \( \text{NH}_2 \)-terminal region of cationic trypsin. The kinetic parameters for activation of human cationic trypsinogen by human and porcine enteropeptidases are also presented and are interpreted with respect to the sequence of the human activation peptide.

MATERIALS AND METHODS

Human cationic trypsinogen was prepared as described in the accompanying report (14). Crude porcine enteropeptidase was obtained as a lyophilized powder from Miles Laboratories. Bovine trypsinogen was purchased from Sigma, Concanavalin A-Sepharose and DEAE-Sephadex A-50 were supplied by Pharmacia. Ultrogel ACA-22 was obtained from LKB. DEAE-cellulose (Whatman, DE52 microgranular) was obtained from Recce Angel. L-Asparyl-L-lysine was purchased from Bachem.

Preparation of Porcine Enteropeptidase—Porcine enteropeptidase was purified by a procedure based on that of Liopolnich and Light (16). Two grams of crude enteropeptidase powder were suspended in

**Structural Basis for the Specific Activation of Human Cationic Trypsinogen by Human Enteropeptidase**

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200 ml of 10 mM Mes buffer (pH 6.0) containing 20 mM NaCl, stirred for 2 h, clarified by centrifugation, dialyzed versus two changes of the same buffer, and applied to a column (4 x 20 cm) of DEAE-cellulose equilibrated with the same buffer. After washing nonadsorbed material through the column, the column was eluted with a linear gradient of 0.02 to 0.5 M NaCl in the same buffer. Enteropeptidase activity, which was eluted in the first part of the salt gradient, was pooled, concentrated by ultrafiltration, and applied to a column (2 x 90 cm) of Ultrogel ACA-22 (an agarose-polyacrylamide gel filtration resin) equilibrated in 10 mM sodium acetate (pH 5.5). Column fractions containing enteropeptidase activity were pooled, concentrated by ultrafiltration, and reapplied to the same column, whereupon a further increase in specific activity was obtained. The material was then passed through a column (1 x 2 cm) of concanavalin A-Sepharose in order to remove contaminating aminopeptidase activity, as described by Barns and Elmslie (17). The pass-through fractions containing enteropeptidase activity were pooled, concentrated by ultrafiltration, and stored at 4°C. An overall 300-fold increase in specific activity was obtained with respect to the initial extract.

Preparation of Human Enteropeptidase - Scrapings of duodenal mucosa, obtained from autopsy specimens at this hospital, were used as the source of human enteropeptidase, which was purified by a procedure based on that of Baratti et al. (18). The material was extracted by homogenization in 1% (w/v) sodium deoxycholate (pH 8.0). The extract was centrifuged and the pellet re-extracted with the same buffer. The remaining residual pellets were centrifuged and dialyzed extensively versus 10 mM Mes buffer (pH 6.0) containing 20 mM NaCl. The dialyzed material was centrifuged and loaded on a column (4 x 18 cm) of DEAE-cellulose equilibrated with the same buffer. After washing nonadsorbed material through the column, the column was eluted with a linear gradient of 0.02 to 0.5 M NaCl in the same buffer. Enteropeptidase activity eluted as a single peak early in the salt gradient.

The material from DEAE-cellulose chromatography was pooled, concentrated by ultratrituration, and applied to a column (2 x 20 cm) of Ultrogel ACA-50 equilibrated with 10 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. Column fractions containing enteropeptidase activity (partially separated from aminopeptidase activity) were pooled and passed through a column of concanavalin A-Sepharose as described above. While essentially all of the enteropeptidase activity passed through the column, approximately 40% of the aminopeptidase activity was retained. The remaining aminopeptidase activity was separated from enteropeptidase activity by chromatography on a column (2 x 20 cm) of DEAE-Sephadex A-50 equilibrated with 10 mM Tris-HCl (pH 8.0) containing 50 mM NaCl and eluted with 50 mM NaCl. This column was eluted with a linear gradient of 0.02 to 0.5 M NaCl in the same buffer. Enteropeptidase activity eluted as a single peak early in the salt gradient.

The preparation of human enteropeptidase was homogeneous by the criterion of discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19), and purification was determined by gel electrophoresis as described elsewhere in this section. The preparation was not homogeneous by the criterion of discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19), and purification was determined by gel electrophoresis as described elsewhere in this section.

The enteropeptidase activity was measured during purification procedures by a method previously used in this laboratory (23). A solution of enteropeptidase DMZ with NPG (24) was incubated with Tris-HCl buffer at pH 6.0, 0.01 or 0.1 M CaCl₂, and enteropeptidase as indicated. Incubation was terminated after 15 min by boiling the enzyme, or 10 min for human cationic trypsinogen, by addition of 10 μl of 2 M HCl. The amount of trypsin formed was then determined spectrophotometrically with TosArgOMe (22).

In all kinetic measurements, duplicate determinations were made at each substrate concentration. The concentration range for human trypsinogen was 0.02 to 0.33 mg/ml, while the range of bovine trypsinogen employed was 0.03 to 0.40 mg/ml. The data obtained were first plotted according to the method of Linekar and Wetter (25) to check for linearity of the reciprocal plot and to test for the presence of inhibitory material in the sample. Each point was then plotted on a semilog plot, and the parameters Kₐ and Vₐₐₐ were obtained from a parallel experiment in order to determine the specific activity of the [HIDFP].

Kinetic Measurements - In order to determine the parameters Kₐ and Vₐₐₐ for trypsinogen activation by human and porcine enteropeptidases, the assay conditions of Baratti et al. (21) were employed. Varying concentrations of human cationic trypsinogen or bovine trypsinogen were incubated at 25°C in a total volume of 0.5 ml in polypropylene tubes containing 28 mM sodium succinate (pH 5.60), 1.0 or 10 mM CaCl₂, and enteropeptidase as indicated. Incubation was terminated after 1.0 or 1.5 min for bovine trypsinogen, or 10 min for human cationic trypsinogen, by addition of 10 μl of 2 M HCl. The amount of trypsin formed was then determined spectrophotometrically with TosArgOMe (22).

The specific activity of trypsin produced (micrograms of TosArgOMe hydrolyzed per mm per μmol of trypsin) was determined by activation of bovine and human trypsins with their respective enteropeptidases at pH 5.6, followed by assay with TosArgOMe. A single band of radioactivity was observed after autoradiography according to the method of Gray (29) as previously described (19). Autodigestion of trypsins was prevented by incubation for 2 min at 100°C in bicine buffer containing 1% sodium dodecyl sulfate prior to reaction with dianisyl chloride. Dns-amino acids were identified by thin layer chromatography after 4- or 18-h hydrolysis at 110°C in 6 N HCl.

NH₂-terminal Sequence Determination - A sample of human cationic trypsinogen (240 nmol) was reduced and alkylated with iodoacetamide by the method of Grady et al. (28) prior to automatic Edman degradation (31) with a Beckman DMQ 890 C Sequencer (28). The resulting sequence was sequenced by the method of Shaw (32) in order to determine the sequence range for human trypsinogen. Varying concentrations of trypsinogen were incubated with TosArgOMe (22) in order to determine Kₐ and Vₐₐₐ for trypsinogen activation by human and porcine enteropeptidases.

NH₂-terminal Amino Acid Residues - Determination of NH₂-terminal residues was performed according to the dianisyl method of Gray (28) prior to automatic Edman degradation (31) with a Beckman DMQ 890 C Sequencer (28). The sample of trypsinogen was subjected to digestion by trypsin for 15 min at 37°C in 0.01 M NaCl containing 0.05 M Tris-HCl (pH 7.5). The active fractions were pooled, adjusted to pH 3.5, and trypsin was added. The resulting trypsin was then reduced and alkylated with iodoacetamide as described above.

The PTH derivatives derived from each sequenator cycle were identified by at least two of the following methods: high pressure liquid chromatography and amino acid analysis, and thin layer chromatography. High pressure liquid chromatography was performed on a Waters Model 6000 A chromatograph using a Micro Bondapak C18 column (33). Gas-liquid chromatography was performed on a Varian 1400A chromatograph (34). Prior to amino acid analysis, PTH derivatives were hydrolyzed in 6 N HCl containing 0.1% SnCl₃ for 4 h at 150°C in order to recover the free amino acids as described by Mendez and Lai (35). Thin layer chromatography was performed by following the procedure described by Baratti et al. (36). Cysteine was identified as PTH-Cys(Cm) by high pressure liquid chromatography. Amino acid analysis of hydrolyzed material from this step yielded only alanine. A commercially obtained sample of PTH-Cys(Cm), when hydrolyzed under the conditions described above, also gave alanine after amino acid analysis. Asparagine was identified as PTH-asparagine by high pressure liquid chromatography and as aspartic acid by amino acid analysis.
Activation of Human Cationic Trypsinogen

RESULTS

NH₂-terminal Amino Acid Residue of Human Cationic Trypsinogen—Three separate preparations of human cationic trypsinogen obtained during the course of this work were subjected to NH₂-terminal amino acid determination as described under "Materials and Methods." In each case, a single major Dns-Asp residue was identified after acid hydrolysis, indicating that the NH₂-terminus of human cationic trypsinogen is aspartic acid or asparagine.

Activation Peptide of Human Cationic Trypsinogen—The peptide fraction isolated from activated human trypsinogen was found to contain a single major component, detected with fluorescamine (37), after paper electrophoresis in pyridine acetate buffers at pH 3.5 and pH 6.5. The electrophoretic mobility at pH 6.5 was consistent with that of a neutral peptide. Amino acid analysis of a sample of the peptide fraction after hydrolysis in 6 M HCl for 24 h at 110°C yielded aspartic acid and lysine in a molar ratio of 1:1.3, with minor contamination from several other amino acids. NH₂-terminal analysis indicated a single major spot of Dns-Asp after thin layer chromatography. Amino acid analysis prior to acid hydrolysis yielded a single component which was eluted in the second buffer, between the elution times of isoleucine and leucine. A sample of commercially obtained L-aspartyl-n-lysine was eluted at the same position. From these results, it was concluded that the single major peptide isolated from autoactivated human cationic trypsinogen has the sequence Asp-Lys.

A separate sample of human cationic trypsinogen was activated with 0.1% by weight of human enteropeptidase for 10 min at 25°C in 20 mM sodium succinate (pH 5.2) containing 20 mM CaCl₂. Autoactivation by trypsin produced during the incubation was minimized, but not prevented, under these conditions. A single major peptide was isolated from Sephadex G-25 gel filtration of this incubation mixture. This activation peptide had properties identical with those described above.

NH₂-terminal Sequences of Human Cationic Trypsinogen and Trypsin—The results obtained from sequenator analyses of samples of human cationic trypsinogen, and of cationic trypsin prepared by activation of trypsinogen by enteropeptidase, are presented in Fig. 1. Unambiguous identification of PTH-derivatives released during sequential Edman degradation was obtained up to residue 19 in cationic trypsinogen and residue 10 in cationic trypsin. The results clearly show that the NH₂-terminal Asp-Lys constitutes the activation peptide of human cationic trypsinogen, confirming the results obtained by isolation of peptide material from autoactivated cationic trypsinogen. The first 16 residues in the sequence of bovine trypsinogen (39) are presented for comparison. Three differences in sequence were observed, in addition to the change in the activation peptide, at positions 6, 8, and 9 in the numbering system shown in Fig. 1.

Kinetic Parameters for Activation of Human and Bovine Trypsinogens by Human and Porcine Enteropeptidases—Initial attempts at demonstrating activation of human cationic trypsinogen by a partially purified preparation of porcine enteropeptidase suggested that bovine trypsinogen was activated much more readily than human cationic trypsinogen under the same conditions. Other experiments indicated that a preparation of human enteropeptidase, obtained by extraction of duodenal scrapings, activated human cationic trypsinogen much more rapidly than bovine trypsinogen.

The initial observations were confirmed with preparations of human and porcine enteropeptidase purified free of interfering enzyme activities (trypsin, aminopeptidase) following published procedures, as described under "Materials and Methods." Although both human and porcine enteropeptidases were highly purified when compared with the initial extracts, neither preparation was considered to be homogeneous. Thus, active site concentrations determined by titration with [¹²⁵I]DIFP were employed in order to estimate kcat values. Kinetic parameters were determined using the conditions described by Baratti et al. (21) except that the CaCl₂ concentration was increased to 10 mM in order to limit autoactivation of human trypsinogen, which was shown to occur readily at pH 5.6 in 1 mM CaCl₂ (14). In all instances, the rate of activation was linear for the times of incubation indicated (see "Materials and Methods"). The results obtained in two of the kinetic measurements, plotted according to Lineweaver and Burk (25), are shown in Fig. 2.

Table I shows the kinetic parameters obtained for activation of bovine and human cationic trypsinogens by porcine or human enteropeptidases. Anderson et al. (40) have recently demonstrated that the kinetic parameters for hydrolysis of bovine trypsinogen by bovine enteropeptidase are similar to those reported by Marouxi et al. (6) and by Baratti et al. (21) for activation of the same zymogen by porcine enteropeptidase. Thus, the term homologous will be applied to the combination of bovine trypsinogen and porcine enteropeptidase as well as to the corresponding human proteins. The values of K₅₀ and kcat shown for the homologous combinations were derived from statistical analysis of kinetic data obtained at two levels of enteropeptidase as described under "Materials and Methods." For the heterologous combinations, neither K₅₀ nor kcat could...
Activation of Human Cationic Trypsinogen

**DISCUSSION**

The activation peptide of human cationic trypsinogen has been shown to be the dipeptide Asp-Lys by two separate approaches. First, the activation peptide was isolated following autoactivation or enteropeptidase-catalyzed activation of thezymogen and was shown to be Asp-Lys. No other peptides were observed after Sephadex G-25 gel filtration of the activation mixtures followed by high voltage paper electrophoresis of the peptide material. In addition, the NH₂-terminal sequence of human cationic trypsinogen was shown to be identical with that of human cationic trypsin except for the addition of the Asp-Lys dipeptide.

The Asp-Lys sequence of the activation peptide of human cationic trypsinogen has been shown to be the dipeptide Asp-Lys by two separate approaches. First, the activation peptide was isolated following autoactivation or enteropeptidase-catalyzed activation of thezymogen and was shown to be Asp-Lys. No other peptides were observed after Sephadex G-25 gel filtration of the activation mixtures followed by high voltage paper electrophoresis of the peptide material. In addition, the NH₂-terminal sequence of human cationic trypsinogen was shown to be identical with that of human cationic trypsin except for the addition of the Asp-Lys dipeptide.

The Asp-Lys sequence of the activation peptide of human cationic trypsinogen is quite different from those reported for several other mammalian trypsinogens, all of which contain the sequence (Asp)₂-Lys. While no evidence of heterogeneity in the NH₂-terminus of cationic trypsinogen has been observed, it is possible that chemical hydrolysis or partial cleavage by acid proteases of a longer activation peptide occurred during isolation of thezymogen. Bovine trypsinogen was employed in order to assess the stability of the activation peptide sequence to the purification steps carried out at low pH (14) as follows. A sample of bovine trypsinogen was incubated at pH 2.6 for 24 h at 4°C with 5% by weight of a dialyzed extract of acetone powder of human pancreas (centrifuged at (NH₄)₂SO₄ precipitation). A second sample of bovine trypsinogen was incubated in pH 2.6 buffer alone for 24 h at 4°C. In both cases, a single NH₂-terminal valine was found, as was the case with an unincubated control sample of bovine trypsinogen. This result demonstrated that the (Asp)₂ sequence of the bovine zymogen is not hydrolyzed under these conditions. Although this does not exclude the possibility that a putative human cationic trypsinogen with an extended NH₂-terminus would be degraded under the same conditions, it is consistent with the contention that the human zymogen isolated in this laboratory has not been altered during purification. Finally, the possibility of autolytic partial degradation of trypsinogen by neutral proteases prior to preparation of acetone powders cannot be excluded.

The difference in sequence between the activation peptides of human and bovine trypsinogens is reflected in the kinetics of activation of thesezymogens by human and porcine enteropeptidases. Thus, the kinetic data presented in Table I provide strong evidence that human cationic trypsinogen, with an Asp-Lys activation peptide, is a much better substrate for human enteropeptidase than is bovine trypsinogen. Indeed, the kinetic parameters obtained with the homologous combinations ofzymogen and enteropeptidase, as shown in Table I, are quite similar, suggesting that human enteropeptidase recognizes the Asp-Lys sequence in human cationic trypsinogen as effectively as porcine enteropeptidase recognizes the (Asp)₂ sequence in bovine trypsinogen. This kinetic evidence provides strong support for the contention that the human cationic trypsinogen isolated in this laboratory is identical with that secreted by the human pancreas. On the other hand, human trypsinogen appears to be a very poor substrate for porcine enteropeptidase. This finding is consistent with the data presented by Maroux et al. (6) indicating that the peptide Asp-Lys-Ile-Val-Gly, which corresponds to the NH₂-terminus of human trypsinogen, does not significantly inhibit the activation of bovine trypsinogen by porcine enteropeptidase.

The (Asp)₂ sequence of bovine trypsinogen has been proposed by Maroux et al. (6) to be a highly specific recognition site for activation by porcine enteropeptidase. The finding of an (Asp)₂ sequence in activation peptides from trypsinogens of several other species (8-12) supports this view. However, Folet et al. (41) recently presented evidence that procozymogen, which contains the sequence -Asp-Asp-Gly-Gly-Lys in its activation peptide, is activated by porcine enteropeptidase. In addition, Maroux et al. (6) showed that the peptide (Asp)₂-Lys-Ile-Val-Gly is hydrolyzed readily by porcine enteropeptidase. These results suggest that the (Asp)₂ sequence is not an absolute requirement for peptide hydrolysis by porcine enteropeptidase.

The NH₂-terminal amino acid sequence Ile-Val-Gly-Tyr is present in all trypsins analyzed to date, including human cationic trypsin. This NH₂-terminal sequence has, therefore,

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**TABLE I**

**Kinetic parameters for activation of human and bovine trypsinogens by human and porcine enteropeptidases**

<table>
<thead>
<tr>
<th>Enteropeptidase substrate</th>
<th>Trypsinogen substrate</th>
<th>Kₘ</th>
<th>kₐ</th>
<th>kₐ/Kₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Human</td>
<td>7.2 x 10⁻³</td>
<td>2.3</td>
<td>330</td>
</tr>
<tr>
<td>Human</td>
<td>Bovine</td>
<td>4.5 x 10⁻³</td>
<td>2.8</td>
<td>630</td>
</tr>
<tr>
<td>Porcine</td>
<td>Bovine</td>
<td>4.5 x 10⁻³</td>
<td>2.8</td>
<td>630</td>
</tr>
</tbody>
</table>

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2 C. Largman, J. H. Johnson, and M. C. Geokas, unpublished observations.
been more highly conserved than the (Asp) sequence in the activation peptide and may be involved in the binding of the zymogen to enteropeptidase. Furthermore, the kinetic data presented in this report strongly suggest that the binding site of human enteropeptidase differs from that of the porcine enzyme. This difference between the two enteropeptidases is currently under investigation in this laboratory.

The three internal sequence differences shown in Fig. 1 can be accounted for by point mutations resulting from single base changes. When compared to bovine trypsinogen, the loss of several amino acids in the human activation peptide could be due to a deletion mutation. An alternative mechanism is suggested by the work of Devillers-Thiery et al. (42) who have shown that in a cell-free protein synthesis system canine trypsinogen is synthesized via a precursor containing approximately 14 additional residues. If a similar mechanism is operative in vivo, then a mutation affecting the processing of trypsinogen is synthesized via a precursor containing approximately 14 additional residues. If a similar mechanism is operative in vivo, then a mutation affecting the processing of trypsinogen is presumably accompanied by a mutation affecting the kinetic properties of human enteropeptidase. One other case of parallel evolution has recently been described in a brief report by Figarella et al. (46) who have suggested by the work of Devillers-Thiery et al. (42) who have shown that in a cell-free protein synthesis system canine trypsinogen is synthesized via a precursor containing approximately 14 additional residues. If a similar mechanism is operative in vivo, then a mutation affecting the processing of trypsinogen is...
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Structural basis for the specific activation of human enteropeptidase.
J W Brodrick, C Largman, M W Hsiang, J H Johnson and M C Geokas


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