Bovine Proenteropeptidase Is Activated by Trypsin, and the Specificity of Enteropeptidase Depends on the Heavy Chain

(Received for publication, August 18, 1997, and in revised form, October 2, 1997)

Deshun Lu†, Xin Yuan‡, Xinglong Zheng, and J. Evan Sadler¶

From the Howard Hughes Medical Institute and the Departments of Medicine and of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Enteropeptidase, also known as enterokinase, initiates the activation of pancreatic hydrolases by cleaving and activating trypsinogen. Enteropeptidase is synthesized as a single-chain protein, whereas purified enteropeptidase contains a ~47-kDa serine protease domain (light chain) and a disulfide-linked ~120-kDa heavy chain. The heavy chain contains an amino-terminal membrane-spanning segment and several repeated structural motifs of unknown function. To study the role of heavy chain motifs in substrate recognition, secreted variants of recombinant bovine proenteropeptidase were constructed by replacing the transmembrane domain with a signal peptide. Secreted variants containing both the heavy chain (minus the transmembrane domain) and the catalytic light chain (pro-HL-BEK (where BEK is bovine enterokinase)) or only the catalytic domain (pro-L-BEK) were expressed in baby hamster kidney cells and purified. Single-chain pro-HL-BEK and pro-L-BEK were zymogens with extremely low catalytic activity, and both were activated readily by trypsin cleavage. Trypsinogen was activated efficiently by purified enteropeptidase from bovine intestine ($K_m = 5.6 \mu M$ and $k_{cat} = 4.0 s^{-1}$) and by HL-BEK ($K_m = 5.6 \mu M$ and $k_{cat} = 2.2 s^{-1}$), but not by L-BEK ($K_m = 133 \mu M$ and $k_{cat} = 0.1 s^{-1}$); HL-BEK cleaved trypsinogen at pH 5.6 with 520-fold greater catalytic efficiency than did L-BEK. Qualitatively similar results were obtained at pH 8.4. In contrast to this striking difference in trypsinogen recognition, the small synthetic substrate Gly-Asp-Asp-Asp-Asp-Lys-$b$-$b$-$b$-$b$-$b$ was cleaved with similar kinetic parameters by both HL-BEK ($K_m = 0.27 mM$ and $k_{cat} = 0.07 s^{-1}$) and L-BEK ($K_m = 0.60 mM$ and $k_{cat} = 0.06 s^{-1}$). The presence of the heavy chain also influenced the rate of reaction with protease inhibitors. Bovine pancreatic trypsin inhibitor preferred HL-BEK (initial $K_i = 99 nM$ and final $K_i = 1.8 nM$) over L-BEK ($K_i = 698 nM$ and $K_i = 6.2 nM$). Soybean trypsin inhibitor exhibited a reciprocal pattern, inhibiting L-BEK ($K_i = 1.6 nM$), but not HL-BEK. These kinetic data indicate that the enteropeptidase heavy chain has little influence on the recognition of small peptides, but strongly influences macromolecular substrate recognition and inhibitor specificity.

Enteropeptidase, originally named enterokinase when it was discovered by Pavlov (1), is a membrane-bound serine protease of the duodenal mucosa that cleaves trypsinogen to generate active trypsin. In almost all vertebrate species, a short trypsinogen activation peptide is released that terminates with the sequence Asp-Asp-Asp-Asp-Lys (2). Following activation, trypsin cleaves and activates other zymogens in pancreatic secretions, including chymotrypsinogen, proelastase, procarboxypeptidases, and some prolipases. Thus, enteropeptidase initiates a simple two-step enzymatic cascade that activates digestive hydrolases within the lumen of the gut. The biological importance of this pathway is demonstrated by the severe intestinal malabsorption and diarrhea that is caused by congenital enteropeptidase deficiency (3, 4).

Bovine enteropeptidase is synthesized as a single-chain precursor of 1035 amino acid residues (5) that appears to require proteolytic activation, suggesting that enteropeptidase may not be the "first" protease of the digestive hydrolase cascade. Active enteropeptidase has been cleaved after Arg-800 to produce a disulfide-linked heterodimer with an amino-terminal ~120-kDa heavy chain and a ~47-kDa light chain; ~40% of the apparent mass of these polypeptides is due to glycosylation (6, 7). The enteropeptidase heavy chain consists of an amino-terminal membrane-spanning domain, a mucin-like domain, two repeats found in complement serine proteases C1r and C1s, a MAM domain (so named for similar motifs first identified in the metalloprotease meprin, the Xenopus laevis neuronal protein A5, and protein-tyrosine phosphatase Mu), and a macrophase scavenger receptor cysteine-rich repeat (reviewed in Ref. 8). The light chain is a typical chymotrypsin-like serine protease. The activation cleavage site between the heavy and light chains has the sequence Val-Ser-Pro-Lys ↓ Ile, which might be recognized by trypsin or other trypsin-like proteases. The identity of the endogenous proenteropeptidase activator is unknown. If trypsin were responsible in vivo, this would raise the logical question of how such a closed trypsin-enteropeptidase cycle could be initiated (reviewed in Ref. 8).

The determinants of enteropeptidase substrate specificity are not understood fully and may not be confined to the catalytic serine protease domain. The enteropeptidase light chain has been isolated in active form by partial reduction and alkylation of purified bovine intestinal enteropeptidase (9), with an average of ~3 alkylated cysteine residues/molecule. A similar protein has been made by expression of recombinant enteropeptidase light chain (10), which is predicted to have at least 1 unpaired cysteine. These light chain preparations had markedly reduced ability to activate trypsinogen, but normal activity toward Gly-Asp-Asp-Asp-Lys-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$-$b$. Similar selective defects in activity toward trypsinogen have been achieved by heating (6, 11) or acetylation (12) of two-chain enteropeptidase. Although the structural cause of the impaired trypsinogen activation is uncertain, these observations suggest...
Recombinant Bovine Enteropeptidase

that the catalytic center and at least one secondary binding site on enteropeptidase cooperate to recognize trypsinogen.

To determine the influence of the enteropeptidase heavy chain on substrate recognition, secreted variants of recombinant proenteropeptidase were prepared, with or without the heavy chain. Proenteropeptidase was shown to be a zymogen with extremely low intrinsic protease activity, and it was activated efficiently by trypsin. The presence of the heavy chain slightly inhibited proenteropeptidase activation and had almost no effect on the recognition of the small peptide substrate Gly-Asp-Asp-Asp-Lys-b-naphthylamide. However, the heavy chain had profound effects on the recognition of macromolecular substrates (trypsinogen) and inhibitors (pancreatic trypsin inhibitor and soybean trypsin inhibitor).

EXPERIMENTAL PROCEDURES

Materials—Bovine enteropeptidase (BEK)1 purified from intestine was purchased from Biozyme (San Diego, CA). Bovine trypsinsogen, bovine trypsin inhibitor (STI), and trypsin-Sepharose 4B were purchased from Worthington. The enteropeptidase substrate Gly-Asp-Asp-Asp-Lys-b-naphthylamide was purchased from Bachem (Philadelphia, PA). Chromogenic substrates S-2366 (Gluc-Arg-p-nitroanilide, where Gluc is pyroglycamin) and S-2765 (z-Arg-Arg-p-nitroanilide) were purchased from Chromogenix (Molndal, Sweden).

Construction of Expression Vectors—BEK cDNA clone A8 (5) was obtained from Genbank (Madison, WI) to generate plasmid pET-28bHL. Human prothrombin heavy chain, was constructed similarly except that an SmaI site was introduced at nucleotide 2453 in the first step by PCR with primers (complement of nucleotides 2998–3017 of BEK). The PCR product was digested with SfiI and BstXI. The resulting DNA was ligated with SpeI and partially digested with BstXI and then ligated to the PCR fragment to generate pBlue-HE. The desired fragment of pBlue-HE was isolated by digestion with XhoI and HindIII and then ligated to SalI/HindIII-digested His6 vector pET-28b(II+) (Novagen, Madison, WI) to generate plasmid pET-28bHL. Human protrombin signal peptide sequence was amplified by PCR using plasmid pMAD20 (31294

Formulation of L-BEK; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

1 The abbreviations used are: BEK, bovine enteropeptidase; BPTI, bovine pancreatic trypsin inhibitor; STI, soybean trypsin inhibitor; GD-K,NA, enteropeptidase-specific substrate Gly-Asp-Asp-Asp-Asp-Lys-b-naphthylamide; HL-BEK, recombinant two-chain enteropeptidase containing the light chain and 17 carboxyl-terminal residues of the heavy chain; pro-HL-BEK, single-chain zymogen form of HL-BEK; pro-L-BEK, single-chain zymogen form of L-BEK; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

2 The extent of the reaction was monitored by SDS-PAGE. Activated recombinant enteropeptidase was filtered and concentrated by ultrafiltration (Centronics-30, Amicon, Inc.). The concentration of activated enteropeptidase was determined by active-site titration with fluorescein monoo-p-guanidinobenzoate (Sigma) using trypsin tritiated with p-nitrophenyl-p-guanidinobenzoate as a reference standard (19, 20).
Recombinant Bovine Enteropeptidase

31295

Mechanism B:

\[ k \]

Mechanism C:

enteropeptidase (0.2 nM), and absorbance at 405 nm was recorded (two

Equation 1 (22),

squares regression (KaleidaGraph, Synergy Software, Reading, PA) to

and either 0–500 nM BPTI or STI. The reaction was initiated by adding

expression included the following (Mechanisms A–C).

at room temperature (21 °C) and contained 20 mM Tris-HCl, pH 8.0, 150

mM NaCl, 20 mM CaCl\(_2\), 0.1% polyethylene glycol 8000, 500 \(\mu\)M S-2366,

and either 0–500 nM BPTI or STI. The reaction was initiated by adding

enteropeptidase (0.2 nM), and absorbance at 405 nm was recorded (two

points/min) over 30 min. The data were fitted by nonlinear least-

squares regression (KaleidaGraph, Synergy Software, Reading, PA) to

Equation 1 (22),

\[ [P] = v_0 + (v_s - v_0)(1 - \exp(-k_{diss}t))/k_{diss} \]  

(Eq. 1)

where \([P]\) is the concentration of \(p\)-nitroanilide product at time \(t\), \(v_s\) is

reaction velocity at steady state, \(v_0\) is the initial reaction velocity,

and \(k_{diss}\) is the apparent rate constant for the transition from

\(v_s\) to \(v_0\).

Reaction mechanisms associated with relatively slow enzyme inhibition

include the following (Mechanisms A–C).

slow

Mechanism A:

\[ E + I \rightleftharpoons EI \]

\[ k_2 \]

\[ k_1 \]

Mechanism B:

\[ E + I \rightleftharpoons EI^+ \]

\[ k_2 \]

\[ k_3 \]

\[ k_4 \]

Mechanism C:

\[ E \rightleftharpoons E^+ \rightleftharpoons EI \]

\[ k_1 \]

\[ k_2 \]

\[ k_3 \]

\[ k_4 \]

In Mechanism A, enzyme and inhibitor associate slowly and therefore

approach equilibrium slowly. In Mechanism B, enzyme and inhibitor

rapidly and reversibly form an initial “loose” complex (EI), which

isomerizes slowly to the final complex (EI\(^+\)). Mechanism C involves the

slow isomerization of enzyme to a form (E\(^+\)) that binds inhibitor. These

mechanisms can be distinguished by the relationship of \(k_{diss}\) to inhibitor

concentration ([I]) (Equations 2–4).

Mechanism A:

\[ k_{diss} = k_2 + k_1[I](1 + [S]/K_s) \]  

(Eq. 2)

Mechanism B:

\[ k_{diss} = k_2 + k_1[I][1 + K(I) + K(I)/[S]/K_s]\]  

(Eq. 3)

Mechanism C:

\[ k_{diss} = k_2(1 + [S]/K_s) + k_1K_s[I] + K_s[I] + K_s[I]/[S]/K_s\]  

(Eq. 4)

where \([S]\) is the concentration of the competing substrate (S-2366) for

which \(K_s\) is the Michaelis constant and \(K_s\) (the dissociation constant

of the inhibitor-enzyme encounter complex) is \(k_2/k_1\), for Mechanism B and

\(k_2/k_1\) for Mechanism C (22). For Mechanism A, \(k_{diss}\) increases linearly

with [BPTI]. For Mechanism B, \(k_{diss}\) increases hyperbolically with

[BPTI]. For Mechanism C, \(k_{diss}\) decreases with increasing [BPTI].

Graphical distinction between Mechanisms A and B can be made by

transformation of Equation 2 or 3 to a double-reciprocal form to allow

plotting 1/(\(k_{diss} - k_i\)) or 1/(\(k_{diss} - k_i\)) versus 1/[I], where \(k_i\) (Mechanism A) or \(k_i\) (Mechanism B) = \(k_{diss}/v_0\) (22). For both mechanisms, the plot

is linear. For Mechanism A, the line passes through the origin. For

Mechanism B the y intercept is \(1/v_0\), the x intercept is \(-1/K(I)/1 +

(S/[K_s]), and the final \(K^*_i = K_s/K_s + K_s\). \(K_s\) and \(K_s\) were calculated from the values of \(k_{diss}\) and \(v_0\) that were determined by fitting to Equation 1. As indicated, values for

\(k_i\) (Mechanism A) or \(k_i\) (Mechanism B) were calculated by fitting appropriate parameters to Equation 2 or 3 by nonlinear least-squares regression. For Mechanism A, \(K_i = K_i + K_f\). For Mechanism B, the

final \(K^*_i = K_f/k_f + K_f\).

RESULTS

Expression and Purification of Recombinant Bovine En-teropeptidase—To facilitate the production of recombinant en-
teropeptidase, the membrane-spanning domain near the amino

terminus was replaced with the human prothrombin signal peptide,

allowing secretion into conditioned medium. A His\(_6\) tag was included after the signal peptidase cleavage site, ena-

bling purification by chromatography on Ni\(^{2+}\)-nitrilotriacetic acid resin. Two forms of recombinant enteropeptidase were

constructed (Fig. 1) to test the contribution of the heavy chain to

macromolecular recognition. HL-BEK was truncated just before the first low density lipoprotein receptor-like domain and contains the remainder of the heavy chain and the light chain. L-BEK was truncated after the last cysteine residue of the macrophage scavenger receptor-like domain and contains mainly the light chain attached to a short carboxyl-terminal segment of the heavy chain. This preserves the disulfide bond predicted to link Cys-788 of the heavy chain to Cys-912 of the light chain, based on the organization of chymotrypsinogen and other homologous serine proteases that have disulfide linkages between the protease domain and the activation peptide (Fig. 1). The masses calculated from amino acid composition are 101 kDa for pro-HL-BEK and 36 kDa for pro-L-BEK.

Stably transfected baby hamster kidney cells secreted recombinant pro-HL-BEK or pro-L-BEK to a final concentration of \(\sim 2\) mg/liter in conditioned medium, and both proteins were readily purified to homogeneity. Upon gel electrophoresis under reducing conditions (Fig. 2), pro-HL-BEK (second lane) and pro-L-BEK (seventh lane) migrated as single-chain species of

FIG. 1. Diagram of bovine proenteropeptidase constructs. The scheme at the top indicates the domain structure of full-length bovine enteropeptidase predicted from the CDNA sequence (5). HL-BEK represents a secreted recombinant form in which the signal anchor (SA) and alternatively spliced (AE) domains were replaced by the prothrombin signal peptide and a His\(_6\) tag. L-BEK represents a form in which all but the extreme carboxyl terminus of the heavy chain was replaced by the signal peptide and a His\(_6\) tag. Other repeated motifs are labeled: LDLR, low density lipoprotein receptor-like domain; C1r/C1s-like domain, LDLR-like domain; MAM, a domain named for similarity to meprin, X. laevis A5 protein, and protein tyrosine phosphatase Mu; MSCR, macrophage scavenger receptor-like domain. The arrows indicate the proteolytic activation cleavage site between the heavy and light chains of two-chain enteropeptidase. The disulfide bonding the heavy and light chains (−SS−) and the active-site residues His (H), Asp (D), and Ser (S) are indicated (5).

Expression and Purification of Recombinant Bovine En-
teropeptidase—To facilitate the production of recombinant en-
teropeptidase, the membrane-spanning domain near the amino

terminus was replaced with the human prothrombin signal peptide,

allowing secretion into conditioned medium. A His\(_6\) tag was included after the signal peptidase cleavage site, ena-

bling purification by chromatography on Ni\(^{2+}\)-nitrilotriacetic acid resin. Two forms of recombinant enteropeptidase were

constructed (Fig. 1) to test the contribution of the heavy chain to

macromolecular recognition. HL-BEK was truncated just before the first low density lipoprotein receptor-like domain and contains the remainder of the heavy chain and the light chain. L-BEK was truncated after the last cysteine residue of the macrophage scavenger receptor-like domain and contains mainly the light chain attached to a short carboxyl-terminal segment of the heavy chain. This preserves the disulfide bond predicted to link Cys-788 of the heavy chain to Cys-912 of the light chain, based on the organization of chymotrypsinogen and other homologous serine proteases that have disulfide linkages between the protease domain and the activation peptide (Fig. 1). The masses calculated from amino acid composition are 101 kDa for pro-HL-BEK and 36 kDa for pro-L-BEK.

Stably transfected baby hamster kidney cells secreted recombinant pro-HL-BEK or pro-L-BEK to a final concentration of \(\sim 2\) mg/liter in conditioned medium, and both proteins were readily purified to homogeneity. Upon gel electrophoresis under reducing conditions (Fig. 2), pro-HL-BEK (second lane) and pro-L-BEK (seventh lane) migrated as single-chain species of
The zymogens pro-HL-BEK and pro-L-BEK were activated readily by trypsin. The time course of pro-HL-BEK activation by 2 nM trypsin (Fig. 3) indicates that generation of enteropeptidase activity correlated with the appearance of a ~43-kDa fragment; no autocatalytic cleavage or activation was observed in the absence of trypsin. Trypsin (1 nM) cleaved and activated pro-L-BEK ~3-fold more rapidly than pro-HL-BEK (Fig. 4). The rate of pro-HL-BEK cleavage by trypsin was slightly faster in the presence of 5 mM calcium chloride than in 5 mM EDTA (data not shown), indicating that single-chain proenteropeptidase is a zymogen.

Trypsin-activated HL-BEK had an apparent mass of 140 kDa under nonreducing conditions (Fig. 2, fifth lane), similar to that of uncleaved pro-HL-BEK (fourth lane), and contained two disulfide-linked fragments of 133 and 43 kDa (second and third lanes). Compared with the unreduced trypsin-digested sample (Fig. 2, fifth lane), the 133-kDa heavy chain (third lane) stained relatively faintly, suggesting that the heavy chain may be sensitive to degradation by trypsin. The apparent mass of activated L-BEK was also similar to that of uncleaved pro-L-BEK (Fig. 2, eighth and ninth lanes), and upon reduction, L-BEK contained a 43-kDa fragment similar to that of HL-BEK (third lane). The predicted amino-terminal activation fragment of L-BEK therefore appears to be disulfide-linked to the 43-kDa fragment under nonreducing conditions. Amino acid sequencing of the 43-kDa fragment from either HL-BEK or L-BEK gave the sequence Ile-Val-Gly-Gly-Ser-Asp-Ser-Arg-Glu-Gly, indicating that cleavage occurred at the site predicted from the cDNA sequence, after Lys-800 of full-length BEK (5). No amino-terminal sequence could be obtained for pro-L-BEK, pro-HL-BEK, or the 133-kDa chain of HL-BEK, suggesting that the amino terminus of the heavy chain is blocked in these preparations.

Kinetics of Substrate Cleavage by Enteropeptidase Variants—Pro-HL-BEK and pro-L-BEK were cleaved with immobilized trypsin to avoid trypsin contamination of the active forms, and the concentrations of HL-BEK, L-BEK, and purified intestinal BEK were determined by active-site titration. All three preparations had similar kinetic constants for cleavage of GD$_4$K-NA (Table I), and the values for $K_m$ and $k_{cat}$ were comparable to those reported for human enteropeptidase: $K_m = \ldots$
Kinetic constants were determined as described under “Experimental Procedures.” The data are listed as the mean ± S.E. for at least three independent determinations.

<table>
<thead>
<tr>
<th>Protease</th>
<th>GD, K-NA, pH 8.4</th>
<th>Trypsinogen, pH 5.6</th>
<th>Trypsinogen, pH 8.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ $\mu M$</td>
<td>$k_{cat}$ $s^{-1}$</td>
<td>$K_m$ $\mu M$</td>
</tr>
<tr>
<td>HL-BEK</td>
<td>0.27 ± 0.01</td>
<td>29.7 ± 1.5</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>L-BEK</td>
<td>0.60 ± 0.12</td>
<td>24.9 ± 2.5</td>
<td>133 ± 23</td>
</tr>
<tr>
<td>BEK</td>
<td>0.22 ± 0.03</td>
<td>24.1 ± 1.2</td>
<td>5.6 ± 0.9</td>
</tr>
</tbody>
</table>

$^{a}$ Reactions were performed at pH 5.6 and room temperature (21 °C) to minimize trypsinogen autoactivation (6).

$^{b}$ Reactions were performed at pH 8.4 and 37 °C in the presence of ovomucoid to prevent trypsinogen autoactivation (21).

These data are consistent with a slow binding inhibitor of Mechanism A.

Kinetic parameters determined for the reactions of enteropeptidase variants with BPTI and STI are summarized in Table II. The data with BPTI were consistent with a slow binding inhibitor of Mechanism B. For the reaction with BPTI with L-BEK, the final $K_s$ was 3-fold higher and the initial $K_s$ was 7-fold higher than for the corresponding reaction with HL-BEK. A reciprocal pattern was observed for reactions with STI: BEK and HL-BEK were completely resistant to STI, whereas L-BEK was inhibited more rapidly and more effectively by STI than by BPTI. Ovomucoid did not inhibit either HL-BEK or L-BEK (data not shown), confirming the suitability of ovomucoid to inactivate the trypsin product during kinetic studies of trypsinogen activation by these recombinant enteropeptidase variants.

**DISCUSSION**

The activation of trypsinogen is a key step in the activation of other digestive hydrolases within the lumen of the gut, and efficient catalysis of this reaction depends on enteropeptidase. Almost all vertebrate trypsinogens are activated by proteolytic cleavage of a Lys–Ile bond in an amino-terminal peptide that contains the sequence Asp-Asp-Asp-Asp-Lys-Ile (2). Molecular modeling of enteropeptidase suggests that specific basic residues on the surface of the catalytic subunit (light chain) interact directly with the acidic residues of trypsinogen activation peptides (25). Such interactions may account for the recognition of small peptide substrates, but probably are not sufficient to explain the recognition of trypsinogen. The isolated light
chain has been prepared by partial reduction of purified bovine enteropeptidase (9) or by expression of recombinant light chain (10). Both preparations had normal activity toward small synthetic peptides, but had dramatically reduced activity toward trypsinogen. Therefore, the recognition of small substrates requires only the light chain, whereas efficient cleavage of trypsinogen may also depend on the heavy chain. Similar selective defects in trypsinogen recognition were produced in two-chain enteropeptidase by heating (6, 11) or by acetylation (12). This behavior suggests that the catalytic center and at least one secondary substrate-binding site (exosite) cooperate to recognize trypsinogen, and exosites sensitive to these treatments could be located on the heavy chain or the light chain.

To investigate the determinants of enteropeptidase substrate specificity, variants were constructed that contain or lack most of the heavy chain. Proteolytic removal of the transmembrane segment appears to have little effect on trypsinogen activation by bovine (9) or porcine (25) enteropeptidase; therefore, the putative membrane-spanning domain was replaced with a signal peptide to enable purification of secreted recombinant proteins from conditioned medium (Fig. 1).

Special attention was paid to the structure of the construct that encodes pro-L-BEK. Several chymotrypsin-like serine proteases have an “extra” disulfide bond that covalently links the activation peptide to the protease domain, and alignment of enteropeptidase with a subfamily of serine proteases suggests that the last cysteine of the heavy chain (Cys-788) is linked to the light chain (Cys-912, or Cys-122 in chymotrypsin numbering) (5). To preserve this predicted disulfide bond and to avoid the generation of an unpaired or abnormally paired cysteine, the construct retained the carboxyl-terminal 17 amino acids of the heavy chain that correspond to the chymotrypsin activation peptide. Covalent association of the short activation peptide and the catalytic domain was confirmed by demonstrating that under nonreducing conditions, pro-L-BEK and trypsin-activated L-BEK have similar electrophoretic mobility, whereas after reduction, the apparent mass of L-BEK (45 kDa) is substantially smaller than that of pro-L-BEK (60 kDa) (Fig. 2).

Both pro-HL-BEK and pro-L-BEK were purified as single-chain proteins and were found to have little (if any) catalytic activity. In particular, pro-HL-BEK had no more than 0.002-fold the activity of two-chain HL-BEK toward GD4K-NA (Fig. 3). The predicted activation cleavage site of proenteropeptidase...
occurs in the sequence VSPKIVGG, which has the appearance of a good site for cleavage by trypsin (5). In fact, trypsin readily activated both pro-HL-BEK and pro-L-BEK (Fig. 4) by cleaving them after Lys-800. Pro-HL-BEK was activated ∼3-fold more slowly than L-BEK, indicating that the heavy chain has a modest inhibitory effect on the recognition of proenteropeptidase by trypsin.

The concentrations of proenteropeptidase tested (0 to ∼1 μM) were not sufficient to allow accurate determination of the $K_m$ for cleavage by trypsin, which must be >1 μM (data not shown). However, trypsin cleaved pro-HL-BEK at a rate of 1.0 s$^{-1}$ and pro-L-BEK at a rate of 2.0 s$^{-1}$ (each at 50 nM), and these turnover numbers are similar to those of other good trypsin substrates. Thus, proenteropeptidase is made as a single-chain zymogen, and it is activated efficiently by trypsin; enteropeptidase, in turn, activates trypsinogen, begging the question of how such a closed cycle could be initiated in vivo. Additional study is required to determine whether trypsinogen or proenteropeptidase is sufficiently “leaky” for this purpose, or whether another responsible protease may be present in duodenal mucosa or pancreatic secretions.

Although deletion of the heavy chain had only a modest effect on proenteropeptidase activation, it had a dramatic and selective effect on enteropeptidase substrate specificity. BEK, HL-BEK, and L-BEK had similar kinetic parameters for cleavage of GD$_2$K-NA, indicating that the heavy chain does not strongly influence the recognition of small substrates (Table I). With the physiological substrate trypsinogen, BEK and HL-BEK also had similar values for $K_m$ (5.6 μM), and the values for $k_{cat}$ differed <2-fold (Table I), indicating that the transmembrane and alternative exon domains have little effect on trypsinogen activation. This is consistent with the observation that proteolytic release of enteropeptidase from membranes did not impair trypsinogen cleavage (26). In contrast to these small effects, deletion of the entire heavy chain markedly inhibited the cleavage of trypsinogen (Table I). Compared with HL-BEK, the catalytic efficiency ($k_{cat}/K_m$) of L-BEK was decreased ∼520-fold at pH 5.6 and ∼47-fold at pH 8.4. Therefore, heavy chain motifs between the first low density lipoprotein receptor-like domain and the macrophage scavenger receptor-like domain (Fig. 1) strongly influence the recognition of trypsinogen. The defect in trypsinogen activation is not explained by changes in the S2–S5 subsites of L-BEK since GD$_2$K-NA cleavage is preserved. These results suggest that the enteropeptidase heavy chain contains an exosite that interacts directly with trypsinogen.

In addition to promoting trypsinogen activation, the heavy chain also affects the reactions of enteropeptidase with macromolecular protease inhibitors. BEK purified from intestine was reported to be inhibited by BPTI with a dissociation constant of ∼5 nM (7), and recombinant HL-BEK was also found to be inhibited by BPTI with a final $K_{i}$ of 1.8 nM (Table II). In addition, the kinetics were typical of slow binding inhibition (Fig. 5). Detailed analysis (Fig. 6) was consistent with a mechanism in which a low affinity complex was formed rapidly with $K_i$ of 99 nM, and this complex slowly isomerized to the final high affinity complex with a forward rate constant ($k_f$) of 0.88 min$^{-1}$. Deletion of the heavy chain had little effect on $k_f$, modestly increased the final $K_{i}$ to 6.2 nM, and increased the initial $K_{i}$ ∼7-fold to ∼700 nM (Table II). An opposite pattern was observed for STI. BEK is completely resistant to STI (7), and HL-BEK also was not inhibited by STI. However, deletion of the heavy chain rendered L-BEK susceptible to slow inhibition by STI with a $K_{i}$ of 1.6 nM (Table II). In addition, the kinetics of the L-BEK reaction with STI did not indicate the existence of an initial low affinity complex.

Isolated enteropeptidase light chain prepared by partial reduction and alkylation (9) and a recombinant form with an unpaired cysteine residue (10) also are inhibited by STI. Thus, deletion of the heavy chain impairs the inhibition of enteropeptidase by BPTI, but enables inhibition by STI. Unlike typical enteropeptidase substrates, BPTI and STI do not have acidic amino acids adjacent to the site that interacts with the catalytic center, and for this reason, the enteropeptidase heavy chain may be a particularly important determinant of reaction rates with these and other inhibitors.

The kinetic effects of deleting the enteropeptidase heavy chain clearly implicate it in the recognition of macromolecular substrates and inhibitors. The activation of trypsinogen is enhanced by the heavy chain, and its influence is especially significant under acid conditions (pH 4–6) that commonly occur in the duodenum. The resistance of enteropeptidase to most protease inhibitors also appears to depend, in part, on the heavy chain. The unusual properties of the enteropeptidase-trypsinogen interaction appear analogous to those of certain blood coagulation factors for which the noncatalytic domains of the serine proteases contribute to macromolecular recognition. These systems provide detailed insight into how serine proteases and their substrates are modified during evolution to perform specific, regulated functions by exploiting structural features of both catalytic and noncatalytic domains.

**Acknowledgments**—We thank Dr. Alex Kurosky and Steve Smith (University of Texas, Medical Branch at Galveston) for amino acid sequencing.

**REFERENCES**

10. LaVallie, E. R., Rhee, M., Racie, L. A., Diblasio, A. E., Ferenz, C., Grant,
Recombinant Bovine Enteropeptidase
