Maturation of Human Procathepsin B

PROENZYME ACTIVATION AND PROTEOLYTIC PROCESSING OF THE PRECURSOR TO THE MATURE PROTEINASE, IN VITRO, ARE PRIMARILY UNIMOLECULAR PROCESSES*

Lukas Mach, John S. Mort‡§, and Josef Glössel†

From the Zentrum für Angewandte Genetik, Universität für Bodenkultur, A-1180 Vienna, Austria, the ‡Joint Diseases Laboratory, Shriners Hospital for Crippled Children, Montreal, Quebec H3G 1A6, and the §Department of Surgery, McGill University, Montreal, Quebec H3A 2T6, Canada

Recombinant latent human procathepsin B produced in yeast was purified to near homogeneity. The purified recombinant proenzyme is activated in vitro under acidic conditions resulting in rapid conversion into the mature form of the proteinase. Activation as well as proteolytic maturation of the recombinant cathepsin B precursor were shown to be primarily concentration-independent processes indicating a unimolecular (i.e., intramolecular) mechanism. Only one cleavage site was identified, yielding a mature polypeptide with the same amino-terminal sequence as that found in recombinant active human cathepsin B obtained from yeast culture media. The same peptide bond is cleaved during processing of a nonactivatable mutant of procathepsin B by the purified mature enzyme (i.e., intermolecular processing). Thus, the complete proregion is liberated during proteolytic maturation of the recombinant cathepsin B precursor. This peptide may then act as a reversible inhibitor and stabilizer of the mature proteinase, and it appears likely that cathepsin B-propeptide complexes occur transiently during proteolytic maturation.

The role of the lysosomal cysteine proteinases, cathepsins B, H, L, and S, in general intracellular protein turnover is well documented (1). Individual enzymes may also fulfill more specialized functions such as bone resorption (2), cartilage breakdown (3), thyroglobulin fragmentation (4), and antigen processing (5). All of these proteinases are synthesized as latent precursors that are subsequently converted by limited proteolysis into their respective mature forms (6–11). The intracellular transport of the newly synthesized proenzymes relies mainly on the mannose 6-phosphate receptor system, although alternative pathways may exist for targeting of individual proteinases in certain cell types (12, 13). Originally, it was believed that the active forms of these enzymes reside solely in lysosomes, leading to the proposal that activation occurs in the same compartment (14). Recent reports demonstrate that mature lysosomal cysteine proteinases are also present in endosomal compartments (15), suggesting that activation may occur earlier in the biosynthetic pathway as already demonstrated for the maturation of the precursor of the aspartic proteinase cathepsin D (16, 17). However, the activation mechanisms, in vivo, for the latent proenzymes have not yet been elucidated.

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‡ To whom correspondence should be addressed: Zentrum für Angewandte Genetik, Universität für Bodenkultur, Gregor-Mendelstrasse 33, A-1180 Wien, Austria. Tel.: 43-1-47654 (ext. 2251); Fax: 43-1-3105175.

In vivo, metalloproteinase inhibitors have been reported to block the maturation of procathepsins B, H, and L in rat macrophages (18). However, it was not feasible to inhibit maturation of newly synthesized procathepsin B in human skin fibroblasts and human hepatoma cells (7, 10). In other studies, latent rat liver microsomal procathepsins B and L were activated in vitro at acid pH, and it was suggested that the maturation of microsomal procathepsin B relies on the action of endogenous cathepsin D (14). Recently, it has been reported that recombinant rat and human cathepsin B as well as recombinant human cathepsin S, produced in the yeast Saccharomyces cerevisiae, can be activated by an autocatalytic mechanism (19–21). Furthermore, it was demonstrated that a recombinant nonactivatable rat cathepsin B precursor could be processed by cathepsins B and L to yield a form resembling the mature enzyme (19).

It still remains to be determined whether autocatalytic maturation of procathepsin B may occur by an intramolecular reaction, as proposed for aspartic proteinases (22, 23) as well as for the serine proteinases of the subtilisin family (24–26). Recent evidence has been provided that a recombinant latent precursor of the plant cysteine proteinase papain, produced in Spodoptera frugiperda cells, may be activated by such a mechanism (27).

In the present study, we demonstrate that recombinant procathepsin B can be activated by an unimolecular autocatalytic mechanism and that the autocatalytic conversion of the proenzyme into the mature form of the proteinase under physiological relevant conditions is primarily the result of intramolecular processing.

EXPERIMENTAL PROCEDURES

Materials—Porcine pepsin, E-64,1 heparin from porcine intestinal mucosa (Grade II), and chondroitin sulfate from whale cartilage (grade A) were obtained from Sigma. Endoglucoamidase H and peptide-N-glycosidase F were supplied by Boehringer Mannheim. Z-Phe-Ala-CHN2 and Z-Phe-Phe-CHN2 were kindly supplied by Dr. E. Shaw (Friedrich-Miescher-Institut, Basel, Switzerland), and the cathepsin B-selective E-64 derivative CA-074 was a gift from Dr. N. Katsumata (Tokushima, Japan). Human liver cathepsin B, recombinant mature human cathepsin B (S115A),2 and a nonactivatable recombinant human

1 The abbreviations used are: E-64, N-([3-carboxy-2,3-trans-carboxyoxirane-2-carboxyl]-γ-leucyl-(4-quinuclidino)-butane; CA-074, N-([3-trans-propylcarbamoyloxirane-2-carboxyl]-γ-isoleucyl)-γ-proline-CHN2-βziazomethane; Z- benzoyloxycarbonyl; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

2 The single-letter code is used when referring to mutations within the procathepsin B sequence. In the numbering system used, the NH2 terminus (Leu) of the mature cathepsin B enzyme is designated as residue 1, and the proregion is assigned negative numbers decreasing toward the NH2 terminus. Because of a cloning artifact, all human procathepsin B variants have an NH2-terminal substitution [R-62H,S-61D,R-60K]. The NH2-terminal sequence of recombinant
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Cathepsin B startes with Glu-Ala-Glu-Ala-His-..., where the first 4 amino acids are the result of incomplete processing of the α-factor fusion construct.

cathpsin B [S115A,S298]) precursor were purified as described previously (20). A synthetic 56-residue peptide corresponding to the prreone of recombinant rat cathepsin B was synthesized and purified as reported (28). Affinity-purified rabbit antibodies to human liver cathepsin B were prepared as described previously (10). Affinity-purified antibdies to a synthetic peptide corresponding to residues 5-19 of human procathepsin B were used to control the activity of the proenzyme (20).

Production and Purification of Secreted Latent Human Procathepsin B Variants—For production of human procathepsin B [S115A], transformed BJ3501 cells were incubated in synthetic medium containing 2% (w/v) dextrose and 2% (w/v) casamino acids (Difco) to an optical density at 600 nm of 3.0 and incubated for 24 h at 39 °C. It was empirically found that the secreted recombinant enzyme was mainly in a latent state under these culture conditions (i.e., high initial cell density and short incubation periods). Latent recombinant human procathepsin B [S115A] was partially purified from the culture medium by anion exchange and gel filtration chromatography. Purification of the proenzyme was performed with affinity-purified recombinant human cathepsin B [S115A,S298] precursor (20). 2,2′-Dipyridyl disulfide was added after each purification step to a final concentration of 1 mM to prevent auto-processing. The partially purified enzyme sample (36 ml) was applied to a concanavalin A-Sepharose 4B column (Pharmacia Biotechology Inc.; 1 x 10 cm), equilibrated with 20 mM Tris/HCl buffer, pH 7.5, 0.5 mM NaCl, 1 mM CaCl2, and 1 mM MnCl2. After washing the column with equilibration buffer, bound glycoproteins were eluted stepwise with 0.1 M and subsequently 0.5 M α-methylmannoside in equilibration buffer. The procathepsin B-containing fractions (5 ml each) eluted at the higher haptan sugar concentration were of greater purity and were therefore used for further purification. This procathepsin B-containing sample (15 ml) was dialyzed against 20 mM Tris/HC1 buffer, pH 7.5, and then concentrated by ultrafiltration and fractionated by fast protein liquid chromatography on a Mono Q HR 5/5 column (Pharmacia) using a linear gradient up to 0.3 M NaCl in 20 mM Tris/HCl buffer, pH 7.5. Latent procathepsin B eluted at 200 mM NaCl. The peak fractions (1 ml each) from two consecutive runs were combined and stored at −20 °C. The final yield of purified human procathepsin B was 300 µg from 4 liters of yeast culture medium.

Recombinant human procathepsin B was also produced in S. cerevisiae strain YE409 as described above and partially purified by gel filtration (20). Conditioned media were obtained by culturing human hepatoma HepG2 cell monolayers for 24 h in serum-free medium in the presence of N2H4Cl to induce the secretion of newly synthesized lysosomal enzyme precursors (10). The conditioned media were centrifuged at 400 x g for 5 min to remove cell debris and then dialyzed against 25 mM Hepes/NaOH buffer, pH 7.5. The supernatant (43 ml) was concentrated, after the addition of 2 mM hydroxyl disulfide and 1 mM phenylmethylsulfonyl fluoride as proteinase inhibitors, by ultrafiltration and applied to an Affi-Gel Blue column (Bio-Rad; 1 x 10 cm), equilibrated in Hepes/NaOH buffer, pH 7.5. Human hepatoma procathepsin B did not bind to the matrix under these conditions. The breakthrough fractions (5 ml each) were combined, concentrated by ultrafiltration, and subjected to gel filtration (20).

In vitro Activation of Human Procathepsin B—All reactions were performed in polypropylene tubes (Eppendorf, Hamburg, Germany). For experiments with purified procathepsin B, the tubes were treated with Repel-Silane (Pharmacia) prior to use to prevent otherwise significant adsorption of the proenzyme to the tube walls. However, qualitatively similar results were obtained in untreated tubes.

The various procathepsin B-containing samples were incubated in 50 mM sodium acetate buffer, pH 4.5 or pH 5.5, for up to 30 min at 30 or 40 °C. The cathepsin B activity thus produced was determined spectrophotometrically at pH 6.0 as indicated below. Linear progress curves of substrate hydrolysis were observed under these conditions, since the activation process is effectively halted by the addition of Triton X-100. For the determination of in vitro procathepsin B activation as a function of pH, the reaction mixtures were incubated at 50 mM sodium formate (pH 3.0-4.0), 50 mM sodium acetate (pH 4.0-5.5), or 50 mM sodium phosphate (pH 5.5-7.0) at 37 °C. Quantitation of active cathepsin B thus released was carried out by continuous spectrophotometry. The effect of the proenzyme concentration on the activation rate of purified procathepsin B was determined as follows. Various concentrations of the recombinant human cathepsin B precursor, ranging from 0.75 to 7.5 µg/ml (i.e., 21-210 µM), were incubated in 50 mM sodium acetate buffer, pH 4.5, for 20 min at 37 °C. The reaction was then stopped by the addition of 0.1 M E-64. Bovine serum albumin (50 µg/ml) was added as a carrier, and the mixtures were heated for 5 min at 95 °C. The samples were then concentrated in vacuo and subjected to enzymatic glycosylation with either endoglucoamidase H or peptid N-glycosidase F followed by SDS-PAGE analysis and immunoblotting using 4-chloro-1-naphthol and N,N'-diethylphenylenediamine as peroxidase substrates (10, 20) unless stated otherwise. The determination of the concentration of recombinant procathepsin B was performed at procathepsin B concentrations ranging from 1.5 to 15 µg/ml in 50 mM sodium acetate buffer, pH 4.5, for 1, 2, and 5 min at 37 °C. Processing rates were determined by densitometric scanning of photographic films exposed to immunoblot developed with the chemiluminescence detection kit (Amersham Corp.) as outlined by the manufacturer. The linearity of the assay for the range of antigen used was established using known amounts of recombinant human cathepsin B. Inhibitors were added, as indicated, to the processing assays.

Other Methods—The enzymatic activity of cathepsin B was determined spectrophotometrically in 0.1 mM sodium phosphate buffer, pH 6.0, containing 2 mM EDTA, 1 mM Z-Arg-Arg-methylcoumarylamide (Bachem, Bubendorf, Switzerland) as substrate (34). The enzyme activity was assayed under conditions described (30). The effect of synthetic cyclic proteinase inhibitors on the activity of human procathepsin B was determined as reported previously (19).

Slab gel electrophoresis (10 or 12.5% polyacrylamide) in the presence of SDS under reducing conditions was performed according to Laemmli (31) or, when indicated, using the Tris/Tricine buffer system developed by Schagger and von Jagow (35) with 3% (w/v) Triton X-100 added to the sample buffer and subsequently on to nitrocellulose sheets (33) or polyvinylidene difluoride membranes (34). Subsequent immunoblotting analysis was carried out as described (10, 20). NH2-terminal amino acid sequence analysis was performed as reported (19). Total protein was determined according to Lowry et al. (35) using bovine serum albumin as a standard.

RESULTS

Purification of Latent Recombinant Human Procathepsin B—A latent form of human procathepsin B has been purified from the conditioned media of transformed variants of the vacular proteinase-deficient S. cerevisiae yeast strain BJ3501 to near homogeneity by a combination of anion exchange chromatography, gel filtration and affinity chromatography on concanavalin A-Sepharose 4B by minor modifications of the method described previously for the isolation of a nonactivatable recombinant human cathepsin B precursor (20). When the purified proenzyme was treated in vitro with pepsin, a specific activity of 11.9 units/mg was determined. On the basis of these results it was calculated that 66% of the theoretical maximum activity could be generated by in vitro activation of the cathepsin B precursor, since the fully active mature recombinant enzyme was found to exhibit a specific activity of 22.3 units/mg under the same assay conditions. Recombinant latent human procathepsin B produced in yeast strain BJ3501 was heterogene-
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**FIG. 1. Purification and characterization of recombinant human procathepsin B.** Purified human liver cathepsin B (two-chain form; lane 1), purified recombinant human cathepsin B (lane 2), purified recombinant human procathepsin B after treatment with endoglucosaminidase H (lane 3), and the purified recombinant human nonactivatable cathepsin B [C29S] precursor, produced in the mnn9 mutant yeast strain, before (lane 4) or after (lane 5) treatment with endoglucosaminidase H were subjected to SDS-PAGE reducing conditions. Polypeptides were detected by Coomassie Blue staining. The molecular mass standards used were bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and bovine $\beta$-lactoglobulin (18.4 kDa).

**FIG. 2. Effect of pH on in vitro activation of procathepsin B.** Procathepsin B (3 pg/ml) was activated in 50 mM sodium formate buffer (pH 3.0-4.0), 50 mM sodium acetate buffer (pH 4.0-6.0), or 50 mM sodium phosphate buffer (pH 6.0-7.5) for 10 min at 40°C. The generated cathepsin B activity is expressed as percentage of the maximum achieved.

**TABLE I**

Effect of various compounds on autocatalytic activation of recombinant human procathepsin B

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Relative activation</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>2 mM</td>
<td>104</td>
</tr>
<tr>
<td>PMSF*</td>
<td>2 mM</td>
<td>105</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>1 mM</td>
<td>104</td>
</tr>
<tr>
<td>Cysteine</td>
<td>20 mM</td>
<td>122</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>2 mM</td>
<td>6</td>
</tr>
<tr>
<td>Dipyridyl disulfide</td>
<td>2 mM</td>
<td>6</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.2 mg/ml</td>
<td>136</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>0.2 mg/ml</td>
<td>169</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>10.2 mg/ml</td>
<td>5</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.2 mg/ml</td>
<td>12</td>
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* PMSF, phenylmethylsulfonyl fluoride.

ous due to extensive modification of the carbohydrate moiety with outer chain oligosaccharides, but after enzymatic deglycosylation gave a single protein band of the expected size of 36 kDa upon SDS-PAGE analysis (Fig. 1). The low amount of latent recombinant human procathepsin B secreted by transformants of the mnn9 mutant yeast strain YE409, where modification of N-linked oligosaccharides is minimized, precluded purification of this more homogeneous form of the human cathepsin B precursor to homogeneity.

In Vitro Activation of Human Procathepsin B—Upon incubation at acid pH, purified recombinant procathepsin B is readily activated (Fig. 2). The pH optimum for activation was determined to be 4.5. The maximum specific activity thus generated was 1.42 units/mg protein, which represents 12% of the specific activity of the proenzyme when activated with pepsin. In this regard, it is important to note that the proregion of cathepsin B, once liberated, can act as a tight binding reversible inhibitor of the mature proteinase (28). Substantial activation was found at pH 5.5 (71% of the maximum), which is of physiological relevance with regard to the intracellular trafficking of newly
purified recombinant procathepsin B (7.5 µg/ml) was incubated in 50 mM sodium acetate buffer, pH 4.5, for 30 min at 40 °C in the presence of the indicated inhibitors at a final concentration of 100 µM or with the solvent dimethyl sulfoxide (Me2SO; 5% (v/v) final concentration) alone. A zero time point sample served as a control (Co). Panel B, cultures of yeast strain YE409 transformants producing human procathepsin B [S115A] were incubated for 2 days at 30 °C in the presence of various concentrations of the inhibitors as indicated and 1% (v/v) dimethyl sulfoxide or the solvent alone (control), and secreted proteins were collected. After enzymatic deglycosylation with endoglucoaminidase H, the samples were subjected to SDS-PAGE (10%) and subsequent immunoblotting using antibodies to human liver cathepsin B. For molecular mass standards, see the legend to Fig. 1.

It has been reported recently that acidic macromolecules such as dextran sulfate accelerate the in vitro activation of procathepsin L under physiologically relevant conditions (38). In a similar fashion, heparin and chondroitin sulfate exhibited activating effects on recombinant procathepsin B, albeit less dramatically. Interestingly, the addition of non-ionic detergents prevented procathepsin B activation, and a similar effect was also seen with exogenous proteins (Table I). Although the physiological relevance of these findings is not yet clear, it appears that the added proteins inhibit activation by exerting surface active properties rather than acting (solely) as competitive substrates. The inhibitory effect of non-ionic detergents on procathepsin B activation was an important finding since it provided a convenient method for halting the activation process.

In Vivo Processing of Human Procathepsin B—At pH 4.5, the proteolytic maturation of recombinant human procathepsin B to the mature single chain form of the enzyme with the apparently molecular mass of 30 kDa was demonstrated to be a rapid process at physiologically relevant temperatures, although it appears that the kinetics were somewhat slower than for the autocatalytic activation reaction, suggesting that activation and processing of procathepsin B are different processes (Fig. 3A). This conversion into the mature enzyme was not blocked by the addition of serine, aspartic, and metalloproteinase inhibitors. However, cysteine proteinase inhibitors drastically reduced precursor processing, although concentrations as high as 100 µM were required for complete inhibition (Fig. 4A). The complete prevention of proenzyme maturation in vivo by the cathepsin B-selective inhibitor CA-074 confirmed that the conversion of procathepsin B into the mature enzyme relies entirely on the action of cathepsin B itself. Similarly, the maturation of heterologous human procath-epsin B in yeast in vivo could be blocked by the addition of Z-Phe-Ala-CHN2 or CA-074 to the culture medium (Fig. 4B). Latent recombinant human procathepsin B was also autocatalytically processed at pH 5.5 at albeit slightly slower rates than observed at pH 4.5 (Fig. 3A). At pH 4.5 the partially purified recombinant human proenzyme produced in the mnn9 mutant yeast strain was subject to autocatalytic cleavage at rates comparable to that of the purified latent human cathepsin B precursor, demonstrating that the heterogeneous carbohydrate moiety present in the latter exerts no significant influence on procathepsin B processing. Again, proenzyme activation seemed to proceed faster than autoprocessing. Taken together, these results suggest that proteolytic maturation of the latent proenzyme probably involves two sequential processes, namely proenzyme activation and subsequent autocatalytic procathepsin B cleavage into the mature form of the proteinase.

Upon in vitro autocatalytic processing of procathepsin B, a 6-kDa polypeptide was liberated which cross-reacted with an anti-peptide antibody to the NH2-terminal region of human procathepsin B (Fig. 3B). Since following enzymatic deglycosylation this polypeptide comigrated with the synthetic rat cathepsin B propeptide, the cleaved fragment appears to represent the complete proregion of human procathepsin B. It seems likely that in vitro activation of procathepsin B proceeds via the production of a noncovalent complex between mature cathepsin B and its propeptide. The liberated propeptide probably acts as a reversible inhibitor of the mature form of the enzyme under the experimental conditions used. For the generation of fully active enzyme from this complex, it appears that an even more acidic milieu is required than for the initial processing reaction, as degradation of the synthetic rat cathepsin B propeptide by mature cathepsin B itself occurs only at pH values below 4.5 (28). The NH2-terminal sequence of the 30-kDa polypeptide obtained upon autocatalytic procathepsin B processing was de-
Panel B, Primarily Intramolecular Processes

An analysis was performed using linear regression. The standard deviations of the relative rates of activation mechanism for procathepsin B were found for mature recombinant human cathepsin B, analyzed by SDS-PAGE. The samples were treated with endoglucosaminidase H and then incubated in 50 mM sodium acetate buffer, pH 4.5, for 1, 2, and 5 min at 30 °C. The generated cathepsin B activity was then determined and processed. Processing rates were obtained by denaturing conditions mimicking the milieu in endosomal/lysosomal compartments. Secreted human hepatoma procathepsin B was also activated under similar conditions in a cysteine proteinase-dependent manner. These results confirm our previous findings that the processing of a recombinant nonactivatable variant of the latent proenzyme by organellar lysates of human hepatoma cells relies on the action of endogenous cathepsin B (20).

**DISCUSSION**

It was originally postulated that activation of mammalian lysosomal cysteine proteinase precursors in vitro relies on the action of endogenous aspartic proteinases (14). In contrast, purified procathepsin L isolated from conditioned media of cultured murine fibroblasts was shown to be autokatallytically activated at pH 3.0 (39), as was also demonstrated for the guinea pig sperm cathepsin L precursor (40). We now provide direct evidence that recombinant human procathepsin B can be autokatallytically activated and processed by unimolecular mechanisms under pH conditions mimicking the milieu in endosomal/lysosomal compartments. Secreted human hepatoma procathepsin B was also activated under similar conditions in a cysteine proteinase-dependent manner. These results confirm our previous findings that the processing of a recombinant nonactivatable variant of the latent proenzyme by organellar lysates of human hepatoma cells relies on the action of endogenous cathepsin B (20).

**In Vitro Activation and Processing of Procathepsin B Are Primarily Intramolecular Processes**

An intramolecular activation mechanism for procathepsin B would imply a zero order reaction where the rate of proenzyme processing would be independent of the precursor concentration. When a plot of the relative activation rate versus proenzyme concentration was extrapolated toward zero, the hypothetical activation rate was clearly different from null, demonstrating the occurrence of an unimolecular reaction (Fig. 5A). The decrease in the efficiency of proenzyme activation at the lower concentrations may indicate additional intermolecular cleavage as the proenzyme concentration increases. In vitro procathepsin B processing was also found to be primarily an intramolecular process, although additional intermolecular precursor cleavage may be involved (Fig. 5B). However, autokatallytically processing of latent procathepsin B into the mature form of the enzyme is complete after incubation for 30 min at pH 4.5, whereas the addition of mature cathepsin B in catalytic amounts to a nonactivatable variant of human procathepsin B at pH 4.5 for up to 30 min did not result in significant processing under the same conditions, although the enzymatic activity of the mature proteinase added was equivalent to the maximum activity generated in the course of autokatallytically activation of latent procathepsin B. Under the conditions used, procathepsin B processing thus appears to be primarily an intramolecular reaction.

**Fig. 5. Effect of proenzyme concentration on in vitro autokatallyticity (panel A) and processing (panel B) of procathepsin B.**

Panel A, varying amounts of procathepsin B (0.75–7.5 μg/ml) were incubated in 50 mM sodium acetate buffer, pH 4.5, for 1, 2, and 5 min at 30 °C. The generated cathepsin B activity was then determined and expressed as percentage of the maximum activation rate observed. Panel B, varying amounts of procathepsin B (1.5–15 μg/ml) were incubated in 50 mM sodium acetate buffer, pH 4.5, for 1, 2, and 5 min at 30 °C. The samples were treated with endoglucosaminidase H and then analyzed by SDS-PAGE (10%) and immunoblotting using antibodies to human liver cathepsin B. The immunoblots were developed by the chemiluminescence method, and processing rates were obtained by densitometric analysis of the exposed films. Conversion of the proenzyme into mature cathepsin B was expressed as percentage of the maximum processing rate observed. The calculated data points have been fitted by linear regression analysis. The standard deviations of the relative rates of in vitro activation and processing, respectively, versus procathepsin B concentration are indicated.

terminated to be Phe-Thr-Glu-Asp-Leu-Leu-Pro-..., the same NH2 terminus as found for mature recombinant human cathepsin B. These results suggest that autokatallytically procathepsin B processing proceeds via cleavage of a single peptide bond, which is identical to the processing site found for the cleavage of a nonactivatable variant of procathepsin B by the mature enzyme (19).

In Vitro Activation and Processing of Procathepsin B Are Primarily Intramolecular Processes—An intramolecular activation mechanism for procathepsin B would imply a zero order reaction where the rate of proenzyme processing would be independent of the precursor concentration. When a plot of the relative activation rate versus proenzyme concentration was extrapolated toward zero, the hypothetical activation rate was clearly different from null, demonstrating the occurrence of an unimolecular reaction (Fig. 5A). The decrease in the efficiency of proenzyme activation at the lower concentrations may indicate additional intermolecular cleavage as the proenzyme concentration increases. In vitro procathepsin B processing was also found to be primarily an intramolecular process, although additional intermolecular precursor cleavage may be involved (Fig. 5B). However, autokatallytically processing of latent procathepsin B into the mature form of the enzyme is complete after incubation for 30 min at pH 4.5, whereas the addition of mature cathepsin B in catalytic amounts to a nonactivatable variant of human procathepsin B at pH 4.5 for up to 30 min did not result in significant processing under the same conditions, although the enzymatic activity of the mature proteinase added was equivalent to the maximum activity generated in the course of autokatallytically activation of latent procathepsin B. Under the conditions used, procathepsin B processing thus appears to be primarily an intramolecular reaction.
proteinases (44). However, the inhibitor E-64 was present throughout the purification procedure, hence the proenzyme would have been inactivated.

Recently, it has been proposed that the processing of recombinant propapain proceeds through generation of a processing intermediate (27), suggesting the occurrence of multiple autocatalytic cleavage steps. For recombinant cathepsin B, only one cleavage site could be identified in the course of autocatalytic maturation, and there was no evidence for the existence of a processing intermediate. Differences in the primary structures of the proregions may account for the observed lack of processing intermediates during cathepsin B maturation since this enzyme seems to belong to a different subgroup of the papain superfamily as compared with the other mammalian cysteine proteinases (45). However, it appears likely that a noncovalent complex between the mature enzyme and its propeptide is transiently formed.

It has now been demonstrated that at least two cysteine proteinases may be autocatalytically activated and converted to the mature enzyme by a unimolecular activation mechanism. This reaction mode may be complemented to some extent by intermolecular processing, as maturation of a nonactivatable mutant cathepsin B variant by the active proteinase occurs by cleavage at the same site as detected in the course of intramolecular proenzyme processing. By analogy, it has been reported for the activation of pepsinogen that the intramolecular activation mechanism dominates below pH 3.0, whereas at higher pH values pepsinogen activation relies on intermolecular cleavage (21).

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