Isolation and Characterization of a Stable Activation Intermediate of the Lysosomal Aspartyl Protease Cathepsin D†

Gregory E. Conner* and Gary Richo
Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33101
Received June 12, 1991; Revised Manuscript Received September 19, 1991

ABSTRACT: Procathepsin D is the intracellular aspartyl protease precursor of cathepsin D, a major lysosomal enzyme. Procathepsin D is rapidly processed inside the cell, and, thus, examination of its proteolytic activation and structure has been difficult. To study this proenzyme, a nonglycosylated form of the human fibroblast procathepsin D was expressed in *Escherichia coli*, refolded in vitro, and purified by affinity chromatography on pepstatinyl agarose. Sequence analysis of the refolded, autoactivated enzyme allowed determination of the autoproteolytic cleavage site. The sequence surrounding this cleavage site between residues LeuP26 and IleP27 (in the "pro" region) resembled the first cleavage site found during activation of other aspartyl proteases. Thus, the autoactivated procathepsin D is analogous to the pepsin activation intermediate, which has been termed pseudopepsin. The enzymatic activity, thermal and pH stability, and fluorescence spectra of pseudocathepsin D were compared to mature, predominantly two-chain, cathepsin D isolated from human placenta. The results indicated that pseudocathepsin D and mature enzyme have a similar K_m toward a peptide substrate and cleave a protein substrate at identical sites. Temperature stability of the recombinant enzyme was similar to that of the tissue-derived enzyme. However, the recombinant enzyme had increased stability at low pH when compared to the glycosylated tissue-derived two-chain cathepsin D. Fluorescence spectra of the recombinant and tissue-derived enzymes were identical. Thus, the absence of asparagine-linked oligosaccharides and the presence of the remaining segment of propeptide did not significantly alter the structural and enzymatic properties of the enzyme.

Cathepsin D (EC 3.4.23.5) is the major intracellular aspartyl protease found in most tissues of higher eucaryotes (Barrett, 1977, Shewale et al., 1985, Tang & Wong, 1987). As a member of the aspartyl class of proteases, the amino acid sequence of cathepsin D is highly homologous to others of this group such as pepsin and renin (Shewale et al., 1985; Tang & Wong, 1987). Cathepsin D is located in lysosomes and endosomes, and is believed to play an important role in the normal degradation of proteins in these organelles.

Like other aspartyl proteases, cathepsin D is first synthesized as a larger inactive proenzyme which is subsequently proteolytically converted to mature, active forms. The conversion of procathepsin D to an active form of the enzyme (a process referred to as "activation") involves the removal of the amino-terminal region of the protein known as the "propeptide". Unlike most other aspartyl proteases which are secreted as proenzymes, procathepsin D is rapidly processed inside the cell to the mature, catalytically active forms of the enzyme (Hasilik & Neufeld, 1980; Erickson et al., 1981; Conner et al., 1987). This rapid intracellular proteolytic processing has made isolation of procathepsin D difficult and, thus, has prevented analysis of procathepsin D activation and structure. Small quantities of radiolabeled procathepsin D, isolated from media of cultured cells, can be partially processed to generate a form of cathepsin D which migrates on SDS gels faster than procathepsin D but slower than authentic single-chain cathepsin D (Hasilik et al., 1982; Samarel et al., 1986; Briozzo et al., 1988; Conner, 1989). This partially processed form is catalytically active. Formation of this partially processed cathepsin D is acid pH dependent and inhibited by pepstatin and, thus, is due to autocatalytic (Hasilik et al., 1982, Samarel et al., 1986; Briozzo et al., 1988) and, probably, intramolecular proteolysis (Conner, 1989). It is not known where in the molecule the autoproteolytic cleavage occurs, nor has it been possible to explore conditions which would allow further processing to the mature amino terminus of cathepsin D as has been done for other aspartyl proteases.

To gain a better understanding of procathepsin D activation, the molecule has been expressed in *Escherichia coli*. The enzyme was insoluble and inactive. After an extensive survey of variables in the refolding reaction, the bacterially expressed procathepsin D was renatured and rendered active. The formation of activity in this renaturation reaction was unusually slow (Conner & Udey, 1990). This paper examines the rate of activity formation in the refolding mixtures, describes the purification of renatured recombinant human enzyme, and defines the autocatalytic cleavage site. In addition, the enzymatic activity and structure of the partially processed enzyme are compared with mature, tissue-derived cathepsin D.

Materials and Methods

Isolation of Inclusion Bodies. Six liters of *E. coli* BL21-(DE3) (Rosenberg et al., 1987) carrying the procathepsin D plasmid pTCPSD2 was grown in LB broth, and expression from the T7 promoter was induced as described previously (Conner & Udey, 1990). Bacteria were harvested by centrifugation, resuspended in 20% sucrose/50 mM Tris-HCl, pH 8.0, and treated with 1 mg/mL egg white lysozyme for 10 min at 4 °C. After addition of 0.5 M EDTA, pH 8, to 5 mM final concentration, the incubation was continued for 20 min at 4 °C and for 1 h at 37 °C. Bacteria were then lysed by the addition of 3 volumes of water. The lysate was adjusted to 5 mM $MgCl_2$ and digested with 3 μg/mL DNase I for 30 min at room temperature. Inclusions were collected by centrifugation for 10 min at 15000*g* and washed 3 times by resus-

† This study was supported by NIH Grant GM35812 to G.E.C. and by the University of Miami BRSG.
* Author to whom correspondence should be addressed.

0006-2960/92/0431-1142$03.00/0

pension in 50 mM Tris, pH 8.0, and recentrifugation. Washed inclusions were resuspended in 10 mM Tris, pH 8.0, and 0.1 mM EDTA and stored in aliquots at −70 °C.

Cell Growth and Transfections. Mouse L cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 37 °C in 5% CO_2. Transfections were done using a modification (Conner et al., 1989) of the DEAE-dextran procedure of Sompayrac and Danna (1981). After transfection, cells were washed and allowed to recover for 24 h before addition of 20 mM sodium butyrate, pH 7.5. After 24 h in sodium butyrate, the cells were washed, and fresh medium with 17 μM chloroquine was added to the dishes. Media were collected 24 h later.

Renaturation and Pepstatinyl Agarose Chromatography. Procathepsin D was renatured from solubilized inclusion bodies by a slight modification of the previously published procedure (Conner & Udey, 1990). Renaturation and purification were carried out at room temperature. Briefly, inclusions were solubilized in 8 M urea, 10 mM Na-CAPS, pH 10.7, and 50 mM 2-mercaptoethanol at 2 mg/mL protein. After a 30-min incubation, the mixture was clarified by centrifugation and diluted 100-fold into water. The diluted material was adjusted to pH 8.7 with HCl and after 2 h was titrated to pH 3.7 with formic acid. After 24 h, oxidized glutathione was added to 5 mM and the incubation continued for various periods of time.

Immediately prior to pepstatinyl agarose chromatography, 0.1 volume of 1 M sodium formate, pH 3.5, was added to the refolding mixture. The mixture was applied to a pepstatinyl agarose column (Huang et al., 1979). The column was washed with 0.1 M sodium formate, pH 3.5, and eluted with 0.1 M sodium phosphate, pH 8.0 Protein was prepared for sequencing by two precipitations from 90% acetone.

For affinity purification of native glycosylated procathepsin D, the media from 18 100-mm dishes of mouse L cells transfected with the preprocathepsin D plasmid pCPSD1 were made 0.1 M sodium formate, pH 3.5, 0.1% Brij 35, and 0.4 M NaCl and applied to a pepstatinyl agarose column at 4 °C. The column was washed with 10 mM sodium formate, pH 3.5, 0.1% Brij 35, and 0.4 M NaCl and eluted with 20 mM Tris-HCl, pH 8.3, 0.02% Brij 35, and 0.4 M NaCl. For activation, the pH of the eluate was adjusted by addition of 0.1 volume of 1 M sodium formate, pH 3.5, and incubated at 37 °C for 1 h. The activated protein was lyophilized and resuspended in Laemmli sample buffer and separated on a 12% polyacrylamide gel, and the gel was electroblotted to an Immobilon-P membrane (Matsudaira, 1987).

Protein Sequence Determination. Amino acid sequences of proteins expressed in bacteria were determined using an Applied Biosystems Model 470A sequencer using pulsed liquid chemistry (courtesy of Dr. Keith Brew, Department of Biochemistry, University of Miami). Amino acid sequences of protein isolated from culture media were determined using an Applied Biosystems Model 470A protein sequencer using gas-phase chemistry (courtesy of Benne Parten, Protein Chemistry Core Laboratory at the University of Florida College of Medicine).

Enzyme Assays. Enzyme activity was measured spectrophotometrically using a p-nitro-derivatized synthetic peptide substrate. Standard assays were performed in 0.75 mL of 0.1 M sodium formate, pH 3.5, and 80 μM RS6 (Lys-Pro-Ile-Glu-Phe-4-nitro-Phe-Arg-Leu) at 30 °C (Dunn et al., 1986). The decrease in absorbance at 300 nm was recorded, and activity was determined by measurement of initial reaction rates. A unit of activity was defined as the amount of enzyme which caused a decrease of $A_{300nm} \times 10^{-6}$/s. Michaelis constants were calculated from activity determinations at five different substrate concentrations using the nonlinear regression analysis programs of Leatherbarrow (Leatherbarrow et al., 1985).

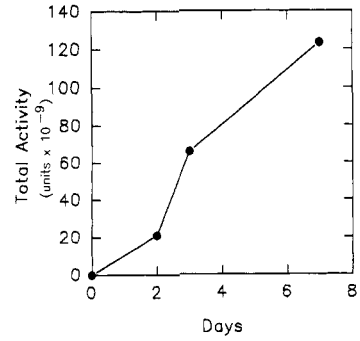

FIGURE 1: Formation of proteolytic activity during renaturation is a slow process. Bacterial inclusions containing procathepsin D were solubilized, refolded, and assayed with the synthetic peptide RS6 on the indicated days as described under Materials and Methods. A unit of activity is defined as a change of $A_{300} \times 10^{-6}$/s. Proteolytic activity increased slowly, suggesting that either refolding or activation was a slow process.

Results and Discussion

Formation of Proteolytic Activity during Refolding of Procathepsin D. After solubilization and refolding of human procathepsin D which was expressed in bacteria, pepstatin-inhibitable proteolytic activity appeared 24–48 h after acidification (Conner & Udey, 1990). Longer incubations (Figure 1) show that cathepsin D activity continued to increase for several days after acidification of the refolding mixture. Since acid-dependent autoproteolytic activation of glycosylated procathepsin D is known to occur within 10–30 min (Hasilik et al., 1982; Samarel et al., 1986; Briozzo et al., 1988; Conner, 1989), it was important to establish the reasons for the slow formation of activity in the nonglycosylated recombinant enzyme. Two possible explanations for the slow appearance of activity are (1) a slow, acid-dependent step in refolding or (2) slow autoproteolytic processing due to an abnormal feature of the recombinant.

These hypotheses can be differentiated by examining the forms of the enzyme found within the refolding mixture while activity continues to increase. If autocatalytic processing is the rate-limiting process, then a significant quantity of refolded but unprocessed procathepsin D will exist in the refolding mixture compared to refolded, processed forms of the enzyme. If refolding is the rate-limiting process, and proteolytic activation occurs rapidly after complete renaturation, very little procathepsin D would accumulate in the refolding mixture compared to refolded, processed enzyme. The amount and processing of refolded forms of the enzyme were measured using pepstatinyl affinity chromatography and SDS gel electrophoresis. Pepstatin binds to the active site of a variety of aspartyl proteases, and both correctly folded procathepsin D and processed forms of cathepsin D bind to pepstatinyl agarose at pH 3.5 (Conner, 1989), while denatured forms of the proenzyme and enzyme flow through the gel matrix.

At day 7, when activity was still increasing (Figure 1), the acidified refolding mixture was applied to a pepstatinyl agarose column to allow binding of refolded proenzyme and processed enzyme (Conner, 1989). After the column was washed with binding buffer, the column was eluted by raising the pH, and fractions were assayed for enzymatic activity. Table I shows that approximately 35% of the activity in the refolding mixture could be recovered in the bound and eluted fraction while almost no activity remained in the flowthrough fraction. This

Table I: Formation and Purification of Proteolytic Activity from the Refolding Mixture[a]

	total protein (mg)	total act. (units)[b]	sp act. (units/mg)	act. yield (%)	fold enrichment
refold mixture	52	139300	2680		
unbound	56	1200	22	1	0.008
pH eluate	0.65	50800	78000	36	29

[a] Inclusion bodies were solubilized and subjected to refolding conditions as described under Materials and Methods. Samples were taken from the refolding mixture after 7 days for protein and enzymatic assay. The remainder of the mixture was fractionated on pepstatinyl agarose and then assayed. [b] Units of activity are measured as the decrease of A_{300} absorbance units $\times 10^{-6}$ per second in the standard assay described under Materials and Methods.

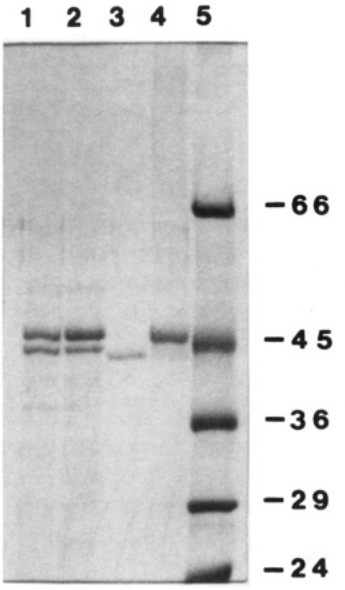

FIGURE 2: Slow appearance of proteolytic activity is due to slow folding. Refolded enzyme in the refolding mixture at day 7 was purified from denatured enzyme by pepstatin affinity chromatography under conditions described under Materials and Methods which allow both procathepsin D and also processed active enzyme to bind to the column (Conner, 1989). Samples from the fractions obtained from pepstatin affinity column were pooled, electrophoresed on a 12% SDS gel using a Laemmli gel system (Fisher et al., 1982), and visualized by Coomassie blue staining. The enzyme which bound and eluted from the column in a pH-dependent fashion had a smaller apparent molecular mass than procathepsin D, indicating that activation is not the slow step in the formation of proteolytic activity. The refolding mixture before application to the pepstatin column is shown in lane 1. The unbound fraction is shown in lane 2. The fraction bound and eluted in a pH-dependent fashion is shown in lane 3. The protein contained in the inclusions prior to refolding is shown in lane 4. Molecular mass standards seen in lane 5 are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsinogen (24 kDa).

yield is in agreement with that reported by others for isolation of cathepsin D from tissue by pepstatinyl agarose [e.g., see Huang et al. (1979)]. The loss of activity is thought to result from denaturation of the enzyme during elution from the affinity support. The data indicated that approximately 2.8% of the procathepsin D was refolded during the 7-day course of the experiment.

Analysis by SDS gel electrophoresis of the refolding mixture and fractions from the pepstatin affinity chromatography (Figure 2) demonstrated that the slow appearance of activity after acidification of the refolding mixture is due to a slow step in refolding of the proenzyme. After incubation in the acidic refolding mixture, a portion of the procathepsin D (Figure 2, lane 4) was converted to a faster moving species (Figure 2, compare lane 1 and lane 4), but neither procathepsin D nor this faster moving species bound to pepstatinyl agarose (Figure 2, lane 2). Instead, another species of still lower apparent molecular mass bound to the column and was eluted by raising

```
Porcine pepsinogen          LVKVPLVRKKSLRQNLIKDG.KLKDFLKTHKHNPASKYFPEAAAL
Bovine prochymosin          AEITRIPLYKGKSLRKALKEHG.LLEDFLQKQQYGISSKYSGF
Human procathepsin D        LVRIPLHKFTSIRRTMSEVGGSVEDLIAKGPVSKYSQAVPAVTE
Pseudocathepsin D (Rec)                                 IAKGPVSKYSQAVPAVTE
Pseudocathepsin D (Nat)                                 IAKGPVSKYSQAVPAVTE
```

FIGURE 3: Comparison of human procathepsin D cleavage sites with those of other aspartyl proteases. Amino-terminal sequences of porcine pepsinogen, bovine prochymosin, human procathepsin D, and both recombinant (Rec) and native (Nat) pseudocathepsin D are shown. The left arrowheads indicate the N-terminal residues after autocatalytic cleavage to the pseudoforms of the enzymes. The right arrowheads indicate the cleavage site which generates the mature protein. The mature amino terminus of human cathepsin D is based on sequence homology with porcine cathepsin D (Huang et al., 1979). Conserved residues surrounding the first cleavage site of each molecule are underlined.

the pH of the buffer (Figure 2, lane 3). This species, which exhibited pH-dependent pepstatin binding, was visualized by SDS gel analysis of the unfractionated refolding mixture after application of larger amounts of sample (data not shown). Procathepsin D was not observed in the fraction bound to pepstatin. These data indicated that the slow acquisition of activity was not the result of slow activation of proenzyme but instead is most likely the result of a slow step in refolding of the denatured protein. Continued incubation of refolding mixtures, after depletion by pepstatinyl agarose chromatography, allowed activity formation to proceed (data not shown), providing further evidence in support of this conclusion.

Addition of pepstatin prevented formation of all proteolytic products in the acidified refolding mixture (data not shown). Since aspartic proteases are rarely found in bacteria, this pepstatin inhibition argues against the possibility that other contaminating proteases from bacteria are involved in activation and proteolysis in this system. Pepstatin inhibition of processing in the refolding mixture also confirmed that correctly folded, recombinant procathepsin D was capable of binding pepstatin similar to native glycosylated proenzyme.

The exact identity of the faster moving protein species in the refolding mixture, which does not bind to pepstatin, is not known. Since correctly folded enzyme, with or without amino-terminal processing, is capable of binding to pepstatin, this species must be a denatured form.

Active Cathepsin D Isolated after Refolding Is a Stable Pseudoform of the Enzyme. Since electrophoretic analysis of the enzyme which bound and eluted from the pepstatin column showed one species, sequential Edman degradation was performed on this fraction without further purification. Comparison of the first 10 residues of this protein to the amino-terminal sequence of the procathepsin D before renaturation (Conner & Udey, 1990; Faust et al., 1985; Conner et al., 1989) demonstrated that 26 amino acids had been removed from the amino terminus (Figure 3).

A similar intermediate in procathepsin D activation with an unknown amino terminus has been previously reported after in vitro activation of small quantities of radiolabeled glycosylated procathepsin D isolated from tissue culture cells

(Hasilik et al., 1982; Samarel et al., 1986; Briozzo et al., 1988; Conner, 1989). To confirm the identity of the activation intermediates obtained from cultured cells and from the refolding mixtures, a eucaryotic expression system was used to increase the amount of native glycosylated procathepsin D available for activation and sequencing. Mouse L cells were transfected with the plasmid pCPSD1 which encodes human preprocathepsin D (Conner et al., 1989). Sodium butyrate was used to enhance expression from the SV40 promoter (Gorman & Howard, 1983), and chloroquine was added to media to block intracellular processing and increase secretion of procathepsin D. Proenzyme was purified from 90 mL of culture media by pepstatin affinity chromatography, and the eluate was activated in vitro by incubation at pH 3.5. The sample was resolved by SDS gel electrophoresis and transferred to Immobilon-P for sequencing. The amino-terminal five amino acids of the activation intermediate, derived from secreted glycosylated human procathepsin D, were identical to those obtained from the refolding mixture (Figure 3). This demonstrated the direct conversion of procathepsin D to the intermediate. In addition, these data indicated that the lack of glycosylation of the proenzyme was not responsible for formation of the intermediate form.

The sequence surrounding the cleavage site was similar to the first cleavage site used during activation of several aspartyl proteases (Tang & Wong, 1987) which generates the pseudoform of these enzymes. The glutamic and aspartic acid residues in procathepsin D at positions P2 and P3 of the substrate are found in similar positions in prochymosin, while the corresponding pepsinogen residues are glutamine and asparagine. The conservation of sequence surrounding the cleavage site suggests that these residues may contribute important structural features of the propeptide which are recognized during autoproteolysis.

In contrast to the other aspartyl proteases, continued incubations between pH 2.0 and 6.5 did not increase the mobility of this form, indicating that further processing to the mature enzyme had not occurred (data not shown). Thus, the intermediate form was a stable and active pseudocathepsin D, which corresponded to previously detected intermediates in the activation of other aspartyl proteases (Dykes & Kay, 1976; Asbaek Christensen et al., 1977; Barkholt Pedersen et al., 1979; Kageyama & Takahashi, 1985).

The inability to generate, by autoproteolysis, smaller forms of the recombinant protein which correspond to mature cathepsin D could be explained by two possibilities: either full processing requires another enzyme or pseudocathepsin D is not an in vivo intermediate in autocatalytic processing. At this time, we cannot distinguish between these two possibilities. Although pepsinogen is capable of complete autocatalytic processing to pepsin (Kanegami & Takahashi, 1982), prorenin is incapable of autoproteolytic activation and must be processed by another activating enzyme (Inagami et al., 1985). It remains to be determined whether procathepsin D is activated by either or both of these mechanisms.

Pseudocathepsin D Is Enzymatically and Structurally Similar to Mature Cathepsin D. The activity of recombinant pseudocathepsin D was characterized using the synthetic peptide substrate RS-6 (Lys-Pro-Ile-Glu-Phe-4-nitro-Phe-Arg-Leu) (Dunn et al., 1986) and compared to cathepsin D isolated from human placenta. The pH optima of the recombinant and placental enzymes were found to be identical at pH 3.5. The K_m values of pseudocathepsin D and placental cathepsin D were 223 ± 88 and 230 ± 51 μM, respectively (average of nine determinations).

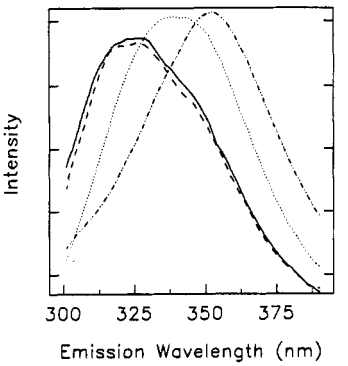

FIGURE 4: Comparison of pseudocathepsin D and mature cathepsin D fluorescence. Purified pseudocathepsin D (—) and placenta cathepsin D (– –) were diluted to 10 μg/mL in 0.1 M sodium formate, pH 3.5, and excited at 280 nm with a 4-nm bandwidth. Fluorescence spectra were recorded at 1-nm intervals with a 5-nm bandwidth between 300 and 390 nm. The spectrum of procathepsin D in 7 M urea and 0.1 M sodium formate, pH 3.5 (–··–), and after 100-fold dilution of the urea is shown for comparison (···). The data demonstrated that the spectra of the folded forms of the enzymes are nearly identical.

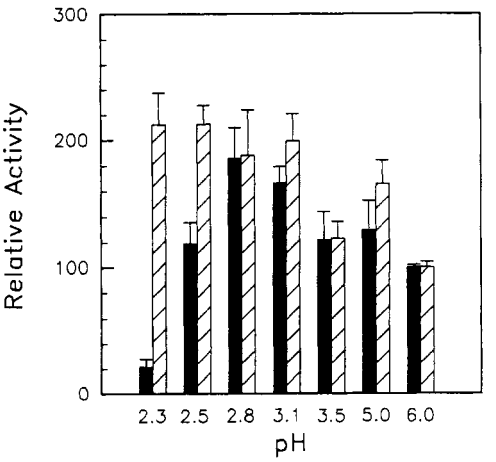

FIGURE 5: Stability of pseudocathepsin D and mature cathepsin D to pH preincubation. Purified pseudocathepsin D and placental cathepsin D were preincubated at the indicated pH, in either 0.01 M sodium formate, sodium acetate, or sodium phosphate at 20 μg/mL for 5 min at 30 °C. Samples were then diluted into 0.1 M sodium formate, pH 3.5, for assay using the peptide substrate RS6. The percent of activity obtained after incubation at pH 6.5 was arbitrarily set as 100%, and the relative activity of the sample after preincubation was plotted as a function of pH. Solid bars represent pseudocathepsin D, and open bars represent placental cathepsin D. Standard errors of the means were calculated from at least four separate determinations. The data show that the single-chain pseudocathepsin D is more stable at acidic pH than the mature two-chain enzyme obtained from placenta.

Pseudocathepsin D was compared to the mature glycosylated two-chain enzyme by spectrofluorometry and by evaluation of the stability of enzyme activity to preincubation at different pHs and temperature. Fluorescence spectra of the two enzymes were examined at 30 °C in 0.1 M sodium formate, pH 3.5 (Figure 4), and found to be identical to each other. When these proteins were compared to urea-denatured procathepsin D, before and after dilution of the urea, significant differences were seen. These data suggested that the overall structure of the pseudocathepsin D closely resembled that of fully processed cathepsin D. The stability of recombinant pseudocathepsin D and tissue-derived cathepsin D to pH and temperature was examined by preincubation of the enzymes at various pHs and temperatures followed by assay under standard conditions (Materials and Methods). The recombinant enzyme was significantly more stable below pH 3.5 when compared to the tissue-derived enzyme (Figure 5), despite the lack of glyco-

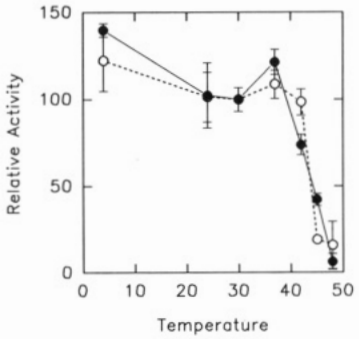

FIGURE 6: Stability of pseudocathepsin D and mature cathepsin D to temperature. Purified pseudocathepsin D and placental cathepsin D at 20 μg/mL were preincubated for 5 min in 0.1 M sodium phosphate, pH 8.0, at the indicated temperatures. Samples were then cooled on ice and diluted into standard enzyme assay conditions. Preincubation at the assay temperature, 30 °C, was arbitrarily set as 100%, and the relative activity remaining after preincubation was plotted as a function of temperature. Open circles represent pseudocathepsin D, and solid circles represent placental cathepsin D. Standard errors of the means were calculated from at least four separate determinations and in some cases were smaller than the symbols used. The data demonstrated that the two enzymes have similar temperature stability, with pseudocathepsin D demonstrating a sharper transition to the inactive state.

sylation. This increase in stability may be due to the structural difference between single-chain pseudocathepsin D and the predominant two-chain form seen in preparations from human tissue. A difference in stability between two-chain and single-chain forms was previously reported for cathepsin D from bovine uterus (Sapolsky & Woessner, 1972).

Temperature stabilities of the recombinant pseudocathepsin D and the tissue-derived two-chain cathepsin D were almost indistinguishable (Figure 6). The temperature-dependent transition of pseudocathepsin D from an active to an inactive state occurs over a narrower temperature range than the two-chain enzyme, perhaps reflecting that pseudocathepsin D is a pure form whereas the placental enzyme is a combination of forms present in the tissue. Finally, the specificity of the enzyme was compared using acid-denatured hemoglobin as a protein substrate (Figure 7). Examination of the proteolytic products generated by each enzyme using SDS–PAGE indicated no difference between the native and recombinant enzyme in terms of cleavage specificity toward this protein substrate.

These data suggested that the presence of the uncleaved N-terminal 18 amino acids of pseudocathepsin D did not alter the activity or overall structure of the enzyme when compared to completely processed enzyme. In addition, the absence of asparagine-linked oligosaccharide on the recombinant enzyme also did not destabilize the structure or affect the enzymatic activity of the recombinant protein.

Conclusions

Purification of refolded recombinant procathepsin D expressed in bacteria has allowed study of the activation and structure of this protein and the comparison of these characteristics with the human placental forms of the enzyme. Immediately following proper refolding, autocatalytic processing resulted in the conversion of procathepsin D to an active, stable form of the enzyme. Edman degradation of this activated recombinant enzyme demonstrated that a pseudocathepsin D was generated by proteolytic processing. The N-terminal sequence of this pseudocathepsin D was identical to the N-terminal sequence of the activation intermediate generated by autoproteolysis of glycosylated procathepsin D isolated from media of cultured cells.

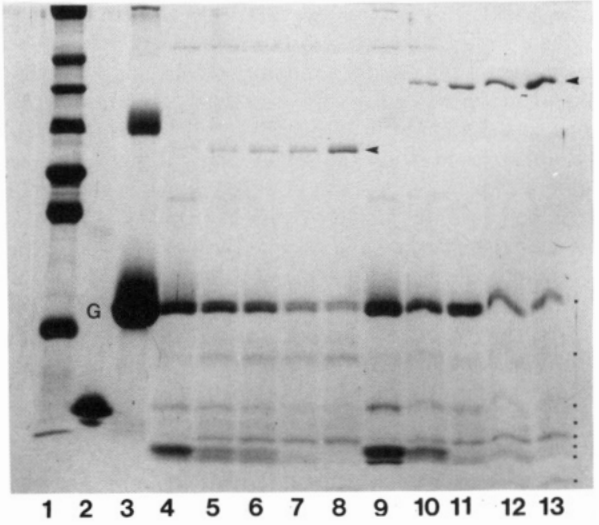

FIGURE 7: Digestion of hemoglobin by pseudocathepsin D and native cathepsin D. Acid-denatured hemoglobin (25 μg, lane 3) was incubated at 30 °C for 1 h with increasing amounts of recombinant native human cathepsin D (lanes 4–8) or pseudocathepsin D (lanes 9–13). Digested samples were TCA-precipitated and resuspended in Laemmli sample buffer and separated on an 18% polyacrylamide gel using an SDS–Tricine buffer system (Schagger & von Jagow, 1987). Following electrophoresis, the gel was stained with Coomassie blue. Molecular mass standards seen in lane 1 are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (21 kDa), and lactalbumin (14 kDa). Lane 2 is the molecular mass standard glucagon (8 kDa). Arrows indicate the position of the heavy chain from placental cathepsin D (lanes 4–8) and the single chain of pseudocathepsin D (lanes 9–13). TCA-precipitable peptides produced by degradation with the two enzymes were identical and are marked with a dot.

The enzymatic activity of recombinant pseudocathepsin D was compared to human placental cathepsin D using both synthetic peptide substrate and acid-denatured hemoglobin. The pH optima and temperature stability of the two forms of the enzyme were indistinguishable, while the recombinant pseudocathepsin D was more stable to acidic pH. The Michaelis constants of the two enzymes were nearly identical. The proteolytic products following digestion of hemoglobin were identical. Fluorescence spectra of the recombinant and tissue-derived forms of the enzyme were nearly identical, indicating that they are structurally similar. Thus, neither the presence of the N–terminal sequence nor the lack of N-linked oligosaccharide altered the structural and enzymatic characteristics of the pseudocathepsin D when compared to fully mature tissue-derived cathepsin D.

Acknowledgments

We thank Karen DeGalan and Marisol Holderby for excellent technical assistance. We also thank Paula Scarborough and Dr. Ben Dunn for supplying peptide substrates and for extensive advice and discussion.

References

Asbaek Christensen, K., Barkholt Pedersen, V., & Foltmann, B. (1977) *FEBS Lett. 76*, 214–218.

Barkholt Pedersen, V., Asbaek Christensen, K., & Foltmann, B. (1979) *Eur. J. Biochem. 94*, 573–580.

Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., Ed.) pp 209–248, North-Holland Publishing Co., New York.

Briozzo, P., Morisset, M., Capony, F., Rougeot, C., & Rochefort, H. (1988) *Cancer Res. 48*, 3688–3692.

Conner, G. E. (1989) *Biochem. J. 263*, 601–604.

Conner, G. E., & Udey, J. A. (1990) *DNA Cell Biol. 9*, 1–9.
Conner, G. E., Blobel, G., & Erickson, A. H. (1987) in *Lysosomes: Their Role in Protein Breakdown* (Glaumann H., & Ballard, J., Eds.) pp 151–162, Academic Press, London.
Conner, G. E., Udey, J. A., Pinto, C., & Sola, J. (1989) *Biochemistry 28*, 3530–3533.
Dunn, B. M., Jimenez, M., Parten, B. F., Valler, M. J., Rolph, C. E., & Kay, J. (1986) *Biochem. J. 237*, 899–906.
Dykes, C. W., & Kay, J. (1976) *Biochem. J. 153*, 141–144.
Erickson, A. H., Conner, G. E., & Blobel, G. (1981) *J. Biol. Chem. 256*, 11224–11231.
Faust, P. L., Kornfeld, S., & Chirgwin, J. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4910–4914.
Fisher, P. A., Berrios, M., & Blobel, G. (1982) *J. Cell Biol. 92*, 674–686.
Gorman, C. M., Howard, B. W., & Reeves, R. (1983) *Nucleic Acids Res. 11*, 7631–7648.
Hasilik, A., & Neufeld, E. F. (1980) *J. Biol. Chem. 255*, 4937–4945.
Hasilik, A., von Figura, K., Conzelmann, E., Nehrkorn, H., & Sandhoff, K. (1982) *Eur. J. Biochem. 125*, 317–321.
Huang, J. S., Huag, S. S., & Tang, J. (1979) *J. Biol. Chem. 254*, 11405–11417.
Inagami, T., Misono, K., Chang, J.-J., Takii, Y., & Dykes, C. (1985) in *Aspartic Proteinases and Their Inhibitors* (Kostka, V., Ed.) pp 319–337, Walter de Gruyter, Berlin.
Kageyama, T., & Takahashi, K. (1982) *Biochem. Biophys. Res. Commun. 107*, 1117–1122.
Kageyama, T., & Takahashi, K. (1985) in *Aspartic Proteinases and Their Inhibitors* (Kostka, V., Ed.) pp 265–282, Walter de Gruyter, Berlin.
Leatherbarrow, R. J., Fersht, A. R., & Winter, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7840–7844.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Rochefort, H., Capony, F., Garcia, M., Cavailles, V., Freiss, G., Chambon, M., Morisset, M., & Vignon, F. (1987) *J. Cell. Biochem. 35*, 17–29.
Rosenberg, A. H., Lade, B. N., Chui, D., Linl, S. W., Dunn, J. J., & Studier, F. W. (1987) *Gene 56*, 125–135.
Samarel, A. M., Worobac, S. W., Ferguson, A. G., Decker, R. S., & Lesch, M. (1986) *Am. J. Physiol. 250* C589–C596.
Sapolsky, A. I., & Woessner, J. F. (1972) *J. Biol. Chem. 247*, 2069–2076.
Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Shewale, J. G., Takahashi, T., & Tang, J. (1985) in *Aspartic Proteinases and Their Inhibitors* (Kostka, V., Ed.) pp 101–116, Walter de Gruyter, Berlin.
Sompayrac, L. M., & Danna, K. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7575–7578.
Tang, J., & Wong, R. N. S. (1987) *J. Cell. Biochem. 33*, 53–63.

Linkage between Proton Binding and Amidase Activity in Human γ-Thrombin†

Raimondo De Cristofaro,‡ John W. Fenton II,§ and Enrico Di Cera*,‡

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, Missouri 63110, New York State Department of Health, Wadsworth Center for Laboratory and Research, Box 509, Albany, New York 12201, and Departments of Physiology and Biochemistry, Albany Medical College of Union University, Albany, New York, 12208

Received June 11, 1991; Revised Manuscript Received October 14, 1991

ABSTRACT: The amidase activity of human γ-thrombin has been studied in the pH range 6–10 as a function of NaCl concentration and temperature. As recently found for human α-thrombin [Di Cera, E., De Cristofaro, R., Albright, D. J., & Fenton, J. W., II (1991) *Biochemistry 30*, 7913–7924], the Michaelis–Menten constant, K_m, shows a bell-shaped dependence over this pH range with a minimum around pH 7.9 in the presence of 0.1 M NaCl at 25 °C. The catalytic constant, k_{cat}, has a bell-shaped pH dependence with a maximum around pH 8.6. A thermodynamic analysis of these parameters has enabled a characterization of the linkage between proton and substrate binding, its dependence on NaCl concentration, and the relevant entropic and enthalpic contributions to binding and catalytic events. Three groups seem to be responsible for the control of γ-thrombin amidase activity as a function of pH. One of these groups has pK values that are significantly different from those found for α-thrombin, and all groups show slightly perturbed enthalpies of ionization. The dependence of γ-thrombin amidase activity on NaCl concentration is different from that of α-thrombin. Increasing NaCl concentration always decreases the substrate affinity for the enzyme in the case of α-thrombin, regardless of pH. In the case of γ-thrombin, such an effect is observed only in the pH range 7.5–9, and a reversed linkage is observed at pH <7 and >9.5. It is proposed that the perturbed functional properties of γ-thrombin compared with the native enzyme are due to structural perturbations of the anion binding exosite for the recognition of fibrinogen.

Human thrombin, the enzyme that catalyzes the critical reaction leading to clot formation in the coagulation cascade, is a member of the large family of serine proteases. Although its catalytic mechanism does not differ from that of other serine proteases, the way thrombin amidase activity is affected by salts, pH, and temperature is unusual. The thermodynamic bases of this behavior have recently been explored in a quantitative way (De Cristofaro & Di Cera, 1990; Di Cera et al., 1991) and can now be related to the underlying structural features of the enzyme (Bode et al., 1989). A natural question that arises in this connection is how the insertion loops contribute to thrombin's unique regulatory properties and

† This work was supported in part by National Institutes of Health Research Grants RR05389 (E.D.C.) and HL13160 (J.W.F.).
* Address correspondence to this author.
‡ Washington University School of Medicine.
§ New York State Department of Health and Albany Medical College of Union University.

0006-2960/92/0431-1147$03.00/0