Kinetics of the pH-Induced Inactivation of Human Cathepsin L†

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ABSTRACT: Cathepsin L is known as the most unstable lysosomal cysteine proteinase at neutral or alkaline pH. The kinetics of inactivation of human cathepsin L was studied by mixing the enzyme with a substrate and recording the release of product. The inactivation was found to be a first-order process, and the rate of the process decreased with the substrate concentration. The substrate-independent inactivation rate constant k_{inact} was found to be 0.15 s^{-1} at pH 7.4 and 37 °C and increased 85-fold between pH 7.0 and 8.0. At pH 7.4, k_{inact} increased 3200-fold between 5 and 37 °C with an energy of activation 174.7 kJ/mol. Inactive cathepsin L did not reactivate at pH 5.5. The rate of inhibition of cathepsin L by stefin B or chicken cystatin at pH 7.4 was much faster than the rate of spontaneous inactivation of the enzyme. The stefin B–cathepsin L complex incubated at pH 7.4 released active enzyme at pH 5.5, suggesting that the cysteine proteinase inhibitors might act as extracellular carriers of the cysteine proteinases.

The lysosomal cysteine proteinases are generally thought to play an important role in the degradation of intracellular proteins (Barrett & Kirschke, 1981). Cathepsin L has a special place among them since it was found to be the most active lysosomal proteinase in the degradation of various protein substrates such as azocasein, elastin, or collagen (Barrett & Kirschke, 1981; Kirschke et al., 1982; Maciewicz et al., 1987; Mason et al., 1989). Like other lysosomal cysteine proteinases, this enzyme is optimally active in slightly acidic media and unstable under neutral or slightly alkaline conditions (Barrett & Kirschke, 1981). Human cathepsin L exhibits its maximum activity toward synthetic substrates at pH 5.5 and was found to be quite stable between pH 4.5 and 5.5. Above pH 6.0 and especially above pH 7.0 the activity is rapidly lost (Mason et al., 1985). Moreover, cathepsin L was found to be the most unstable lysosomal cysteine proteinase at neutral pH (Machleidt et al., 1986). Its rapid inactivation is accompanied by a loss of secondary structure: nearly all α-helices are disrupted (Dufour et al., 1988). Cathepsin L variants from other species have similar properties (Dufour et al., 1987; Kotnik et al., 1986; Mason, 1986; Mason et al., 1984, 1985; Towatari & Katunuma, 1988; Wada et al., 1987).

The most potent physiological regulators of the cathepsin L activity are the inhibitors from the cystatin superfamily (Abrahamson et al., 1991; Barrett et al., 1986; Machleidt et al., 1986; Turk & Bode, 1991). Their interactions were reported to be among the tightest known (Abrahamson et al., 1991; Barrett et al., 1984, 1986). Due to their concentrations in some biological fluids (Abrahamson et al., 1986; Löfberg & Grubb, 1979), cystatin C and the kininogens are thought to be the most important extracellular inhibitors of the cysteine proteinases (Abrahamson et al., 1991; Barrett et al., 1984, 1986; Machleidt et al., 1986). However, no data are available as yet concerning their interactions with the cysteine proteinases at neutral pH.

Although the inactivation of lysosomal cysteine proteinases at neutral pH has been known for a long time (Barrett & Kirschke, 1981), no detailed kinetic and thermodynamic investigations of this process are documented in the literature. The pH-dependent inactivation of cathepsin L was either not expressed in terms of inactivation rate constants (Dufour et al., 1988; Mason et al., 1985) or measured at only one pH value (Machleidt et al., 1986). Other investigators have also studied the inactivation of cathepsins B (Baici & Knöpfel, 1986; Baici et al., 1988; Willenbrock & Brocklehurst, 1985) or H (Willenbrock & Brocklehurst, 1985) but not in detail. Here we present for the first time a detailed kinetic study of the pH-induced inactivation of any lysosomal cysteine proteinase. Substrate was used to monitor this process by human kidney cathepsin L. The inactivation rate constant is studied as a function of pH and temperature and compared with the rate constant of cystatin-induced enzyme inactivation.

MATERIALS AND METHODS

Iodoacetic acid, dimethyl sulfoxide, and dimethylformamide were purchased from Merck (Darmstadt, Germany). Z-Phe-Arg-MCA[1] and dithiothreitol were purchased from Serva (Heidelberg, Germany), Z-Phe-Arg-pNA was from Bachem (Bubendorf, Switzerland). Stock solutions of the substrates were prepared in dimethylformamide (Z-Phe-Arg-pNA) or dimethyl sulfoxide (Z-Phe-Arg-MCA). Ep-475 and E-64 were from Peptide Research Institute (Osaka, Japan); EDTA and papain (2× crystallized) were from Sigma (St. Louis, USA). Papain (EC 3.4.22.2) was further purified as described previously (Blumberg et al., 1970) and active-site titrated with E-64 (Barrett et al., 1982). Bovine stefin B and chicken cystatin were isolated as described (Turk et al., 1992; Turk et al., 1983) and titrated with active site titrated papain. α_2-Macroglobulin was isolated as described previously (Kurecki et al., 1979). All other chemicals were of analytical grade. Unless otherwise stated, the kinetic experiments were done in phosphate buffer (100 mM Na_2HPO_4/KH_2PO_4, 1 mM EDTA, 2 mM dithiothreitol) of the desired pH and at 37 °C.

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[1] Abbreviations: CPI, cysteine proteinase inhibitor; E-64, L-3-carboxy-((*trans*-2,3-epoxypropyl)leucyl)amido(4-guanidino)butane; Ep-475, L-3-carboxy((*trans*-2,3-epoxypropyl)leucyl)amido(3-guanidino)butane; MCA, 4-methyl-7-coumarylamide; pNA, *para*-nitroanilide; Z, benzyloxycarbonyl.

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The activating buffer for all of the experiments was 400 mM sodium acetate buffer containing 1 mM EDTA and 8 mM dithiothreitol, pH 5.5. Protein concentrations of cathepsin L, stefin B, and chicken cystatin were determined by the method of Lowry et al. (1951). The concentration of papain was determined using a M_r of 23 400 (Husain & Lowe, 1969) and an absorption coefficient 2.39 L g^{-1} cm^{-1} (Brocklehurst et al., 1973).

Purification Procedure. Cathepsin L (EC 3.4.22.15) was purified from human kidneys using a method modified from that previously described for the purification of cathepsin L from human liver (Mason et al., 1985). All purification steps were carried out at 4 °C unless otherwise stated.

The two methods are identical up to the precipitation with solid ammonium sulfate and completely different after that step. The precipitate was resuspended in 0.1 M sodium acetate buffer containing 0.3 M NaCl and 1 mM EDTA, pH 5.0, and concentrated by ultrafiltration (Amicon YM-10). The concentrate was applied to a Sephacryl S-200 column (3 × 140 cm), equilibrated with the same buffer. Fractions (6.4 mL) were collected and assayed for catalytic activity using Z-Phe-Arg-MCA as a substrate as previously described (Barrett & Kirschke, 1981). Active fractions in the 20–35-kDa range were pooled, concentrated (Amicon YM-10), and dialyzed against 20 mM piperazine buffer containing 1 mM EDTA, pH 5.5. The dialyzed sample was then applied to a DEAE-Sephacel column (1.7 × 25 cm), equilibrated with the same buffer. The column was washed extensively, and bound proteins were eluted with a 0–0.4 M NaCl linear gradient in the starting buffer. Fractions (6.4 mL) were collected and assayed as described above. Cathepsin L eluted at 0.25 M NaCl. Cathepsin L-containing fractions were concentrated (Amicon YM-10), dialyzed against the above piperazine buffer and applied to a Mono Q column equilibrated with the same buffer. After washing, the bound proteins were eluted at room temperature with a 0–0.5 M NaCl gradient in the starting buffer (flow rate, 1 mL/min). Cathepsin L eluted at 0.25 M NaCl as two peaks. Cathepsin L was active-site titrated with Ep-475 as previously described (Barrett et al., 1982). From 1.5 kg of human kidneys we obtained 1.45 mg of pure cathepsin L.

Rate Constant k_{inact} for the Inactivation of Cathepsin L. Z-Phe-Arg-pNA of appropriate concentration was dissolved in 1.99 mL of the phosphate buffer. Cathepsin L was preactivated in the acetate buffer for 5 min, and the reaction was started by adding 10 μL of preactivated enzyme into the cuvette (2 nM final concentration). The progress of the reaction was then followed spectrophotometrically by monitoring the release of pNA at 410 nm. Cary 1 or Cary 2200 (Varian, Palo Alto, CA) spectrophotometers with thermostated cell holders were used for reactions lasting more than 5 min, and the absorbances were recorded continuously. At least 4–5 experiments were done at every substrate concentration.

For faster reactions we used a rapid kinetics accessory (stopped-flow) SFA-11 (Hi-Tech, Salisbury, U.K.) mounted on the Cary 2200 spectrophotometer or SF/PQ 53 (Hi-Tech) stopped-flow apparatus. One syringe was filled with buffers composed either of 200 mM Na_2HPO_4/KH_2PO_4 and 1 mM EDTA and 2 mM dithiothreitol or of 100 mM Tris, 0.2 M NaCl, 1 mM EDTA, and 2 mM dithiothreitol of desired pH in which Z-Phe-Arg-pNA of appropriate concentration was dissolved. The other syringe was filled with preactivated cathepsin L diluted in 1 mM EDTA, pH 5.5. Two to three hundred microliters of the solutions were used for each measurement, and 5–6 measurements were made at each substrate concentration. All inactivation rate constants were determined using at least five different substrate concentrations.

At temperatures below 20 °C the inactivation reaction was too slow to be measured as described above. A different method was thus used: 10 μL of preactivated enzyme (final concentration 2 nM) was added to the thermostated buffer solution (980 μL); after appropriate intervals, 10 μL of 1 mM Z-Phe-Arg-MCA was added to the mixture, followed 30 s (at 15 °C) or 2 min (at 5 °C) later by 1.5 mL of 5 mM iodoacetic acid, which stops the enzymatic reaction. The fluorescence of the released product was then measured at excitation and emission wavelengths of 370 and 460 nm, respectively. Residual activities, usually 10–15 data pairs (time, fluorescence) were obtained for each curve in this way.

Reversibility of Cathepsin L Inactivation. Ten microliters of preactivated cathepsin L (final concentration 0.3 μM) was added to 90 μL of prewarmed phosphate buffer. After a given interval of time, 900 μL of sodium acetate buffer (400 mM, 1 mM EDTA), pH 5.5, was added to this solution. Twenty-microliter aliquots of this diluted solution were then taken after appropriate intervals and added to 980 μL of 5 μM Z-Phe-Arg-MCA to test the residual activity (Barrett & Kirschke, 1981). After 2 min at 37 °C, the reactions were stopped by adding 2 mL of 2 mM iodoacetic acid. Control experiments were done as above except that phosphate buffer was replaced with activating buffer.

Inhibition of Cathepsin L by Cystatins under Neutral Conditions. The inhibition of cathepsin L by bovine stefin B or chicken cystatin was studied under pseudo-first-order conditions with $[I_0]$ = 0.15 μM, $[E_0]$ = 6 nM, and 30 μM Z-Phe-Arg-pNA. The fast release of *p*-nitroaniline was recorded using a DX 17MV sequential stopped-flow apparatus (Applied Photophysics, Leatherhead, U.K.). The experiments were done as described in the *Rate Constant k_{inact} for the Inactivation of Cathepsin L* section, except that the inhibitor was added to the syringe filled with phosphate buffer and substrate. In the control experiment the inhibitor was omitted.

Dissociation of the Cathepsin L–Stefin B Complex. Cathepsin L–Stefin B complexes were made in phosphate buffer, pH 7.4, with $[E_0]$ = $[I_0]$ = 0.15 μM as follows: preactivated cathepsin L (30 μL) was added to 370 μL of prewarmed buffer + inhibitor solution. After 15 min of incubation at 37 °C, the solution was poured into one syringe of the DX 17MV sequential stopped-flow apparatus (Applied Photophysics, Leatherhead, U.K.). The second syringe was filled with a mixture of acetate buffer (0.4 M, 1 mM EDTA), pH 5.5, Z-Phe-Arg-pNA (250 μM), and α_2-macroglobulin (0.6 μM). A 10:1 volume ratio of mixing (acetate buffer mixture/sample) was used to achieve an unchanged final pH of 5.5. The release of the product was then monitored continuously at 410 nm. In the control experiment, cathepsin L was exposed to pH 7.4 in the absence of inhibitor. The stopped-flow apparatus was used to achieve efficient mixing of the reagents and early observation of the reaction.

Electrophoresis. Cathepsin L–CPI complexes were made as indicated above in the pH 7.4 phosphate buffer with $[E_0]$ = 0.6 μM and $[I_0]$ = 1 μM. After 15 min of incubation at 37 °C, the samples were electrophoresed under nondenaturing conditions. The electrophoresis was performed as described by Laemmli (1970) in 8 × 8 cm slabs of 10% homogeneous polyacrylamide gel in 0.1 M Tris buffer, pH 7.4, in the absence of SDS. After electrophoresis the gel was stained with Coomasie Brilliant Blue.

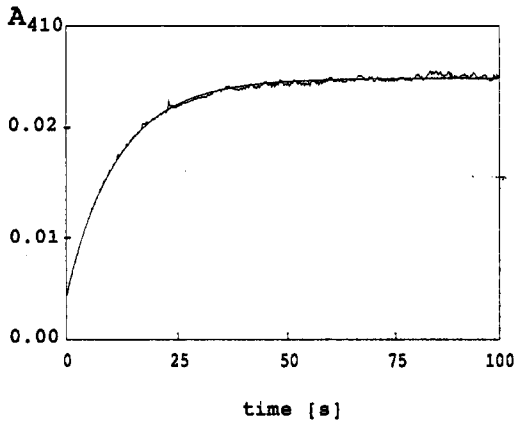


FIGURE 1: Progress curve for the inactivation of the cathepsin L at pH 7.4 and 37 °C. The reaction medium contained 2 nM cathepsin L and 30 μM Z-Phe-Arg-pNA. Other experimental details are described under Materials and Methods. The solid line crossing the stopped-flow trace is the theoretical first-order curve calculated using eq 1 and the best estimate of k_{obs} by nonlinear regression analysis.

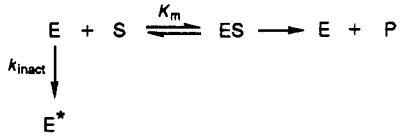
RESULTS

Kinetics of Cathepsin L Inactivation. Reaction of cathepsin L with Z-Phe-Arg-pNA at pH ≥7 resulted in an exponential release of pNA (absorbance at 410 nm) as shown in Figure 1. The substrate consumption was less than 5% in all the experiments, indicating that the shape of the progress curve is a consequence of enzyme inactivation. This progress curve could be fitted by nonlinear regression analysis to the following first-order relationship:

$$P = P_\infty(1 - e^{-k_{obs}t}) \quad (1)$$

where P represents the product concentration at a given time, P_∞ represents the product concentration at infinite time, and k_{obs} is the observed first-order rate constant. There is a good fit between the stopped-flow trace and the theoretical curve generated using eq 1 and the best estimate of k_{obs}, indicating that equation adequately describes the time-dependent inactivation of cathepsin L. The effect of enzyme concentration on the rate of inactivation was studied using 150 μM Z-Phe-Arg-pNA as the substrate in phosphate buffer at pH 7.4 and 37 °C. The enzyme concentration was varied from 0.6 to 5 nM. The observed rate constant was found to be independent of the enzyme concentration in the range studied. As shown in Figure 2, the observed first-order rate constant of inactivation decreases with the substrate concentration used in the experiments. The data were analyzed assuming the following:

Scheme I



where E represents active cathepsin L, S stands for substrate, P for product, and E* for the inactivated cathepsin L, and k_{inact} is the first-order rate constant of enzyme inactivation. This scheme assumes that k_{obs} is given by

$$k_{obs} = \frac{k_{inact}}{(1 + S_0/K_m)} \quad (2)$$

A nonlinear least squares fit of the data to eq 2 indicates that the dependency of k_{obs} upon $[S_0]$ may indeed be described by Scheme I. All kinetic data reported below were derived from k_{obs} vs $[S_0]$ measurements.

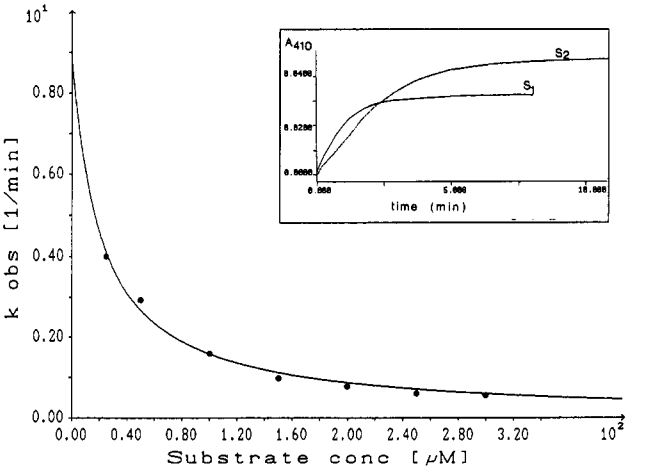


FIGURE 2: Effect of substrate concentration on the observed rate constant of inactivation, k_{obs}, at pH 7.4 and 37 °C. The k_{obs} was determined as described in the legend to Figure 1 using 2 nM cathepsin L and variable concentrations of Z-Phe-Arg-pNA. Every circle represents an average of at least five different runs at the same substrate concentration. The solid line is the theoretical curve, calcualted using eq 2 and the best estimates of k_{inac} and K_m calculated by nonlinear regression analysis. (Inset) Progress curves for the inactivation of cathepsin L at pH 7.4 and 37 °C with two different substrate concentrations (S_1 = 150 μM and S_2 = 300 μM).

Table I: Effect of pH on the Rate of Inactivation of Human Cathepsin L at 37 °C[a]

pH	k_{inact} (s^{-1})	$t_{1/2}$ (s)
7.0	0.00862 ± 0.0006	80.4
7.3	0.0813 ± 0.0084	8.52
7.35	0.0963 ± 0.0115	7.19
7.4	0.150 ± 0.013	4.63
7.5	0.254 ± 0.022	2.73
8.0	0.736 ± 0.051	0.94

[a] The best estimates for the inactivation rate constant k_{inact} are given together with their standard errors obtained by nonlinear regression analysis and with their corresponding half-lives of inactivation ($t_{1/2} = \ln 2/k_{inact}$).

pH and Temperature Dependence of k_{inact}. The effect of pH on k_{inact} was studied in the pH range 7.0–8.0. Phosphate buffers of desired pH were used for all experiments except at pH 8.0, where Tris buffer was used. There is an 85-fold increase of k_{inact} between pH 7.0 and 8.0 (Table I). This increase is particularly dramatic between pH 7.0 and 7.3. The very low half-lives of inactivation illustrate the very high rates of cathepsin L inactivation at neutral pH (Table I).

The effect of temperature on k_{inact} was studied in the temperature range of 5–37 °C and at pH 7.4. The rate constant increases 3200-fold between these two limits ($t_{1/2}$ = 244.6 min at 5 °C and 4.6 s at 37 °C). The Arrhenius plot shown in Figure 3 yielded an activation energy of 174.7 kJ mol^{-1} (41.8 kcal mol^{-1}).

Reversibility of the Inactivation of Cathepsin L. To determine whether the inactivation of cathepsin L is a reversible process, cathepsin L was inactivated at pH 7.4 and incubated at pH 5.5 and 37 °C. The reactivation was followed for 2 h. No increase in catalytic activity was observed, even when the enzyme was exposed to neutral pH for shorter periods, such as 5 min.

Inhibition of Cathepsin L by CPIs at pH 7.4. The cystatins are known as tight, reversibly binding inhibitors of cathepsin L under slightly acidic conditions (Abrahamson et al., 1991; Barrett et al., 1986; Machleidt et al., 1986). Enzymatic and electrophoretic methods were used to study their ability to

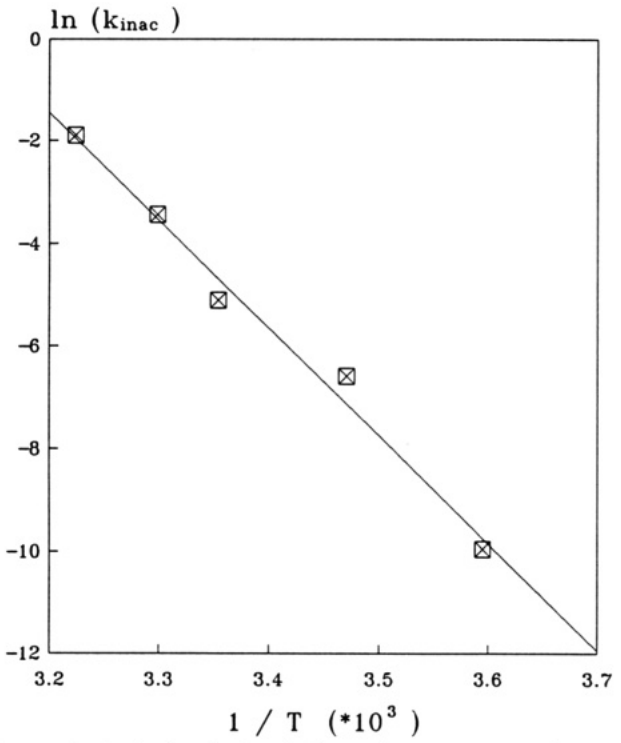


FIGURE 3: Arrhenius plot for the first-order rate constant, k_{inact}, at pH 7.4. The k_{inact} values were determined as shown in Figure 2. The solid line was calculated using the best estimate of energy of activation calculated by linear regression analysis.

bind cathepsin L under neutral conditions. The inhibitor concentrations used in the former experiments were comparable to or lower than the physiological concentrations of some CPIs (Abrahamson et al., 1986) in order to determine whether the CPIs are of physiological significance at neutral pH (Bieth, 1980).

The inhibition of cathepsin L by the CPIs was investigated by adding the enzyme to a mixture of CPI and substrate and recording the formation of product with a stopped-flow apparatus. As a control, cathepsin L was added to substrate in the absence of inhibitor. Virtually no substrate breakdown was observed when stefin B or chicken cystatin were present. In contrast, a significant amount of product was released during the spontaneous inactivation of cathepsin L (Figure 1).

The association could also be studied by nondenaturing electrophoresis. As shown in Figure 4, a stefin B–cathepsin L complex is clearly visible, since it migrates differently to the constituent proteins. Furthermore, practically all of the reacted stefin B formed a complex with cathepsin L. The multiple bands, visible for the free proteins, are not due to impurities in the preparations but to their ability to occur in isoforms. The volume of the stefin B–cathepsin L complex loaded on the gel (100 μL, lane 1) was larger than that of stefin B (20 μL, lane 2). Therefore lane 1 spread during the electrophoresis and apparently overlapped with lane 2 (Gallagher & Smith, 1991). Similar results were obtained also with chicken cystatin and cathepsin L (data not shown). This experiment shows in a nonenzymatic way that the complexes between cathepsin L and CPIs can be formed even under neutral conditions and thus confirms our stopped-flow experiments.

Dissociation of the Cathepsin L–Stefin B Complex by α_2-Macroglobulin and Substrate. To determine whether stefin-bound cathepsin L retains catalytic activity even if the complex is exposed to alkaline pH, we dissociated this complex at pH 5.5, a pH where human cathepsin L is stable and exhibits

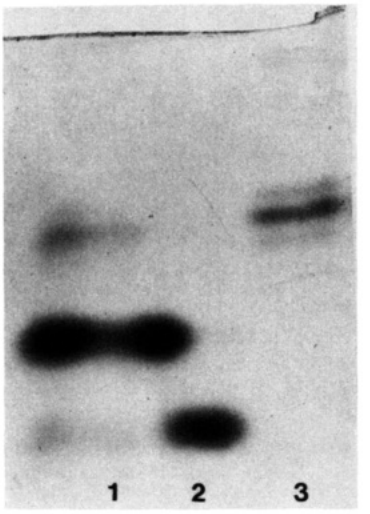


FIGURE 4: Association of cathepsin L and stefin B at pH 7.4 and 37 °C. Nondenaturing electrophoresis was performed as described under Materials and Methods. (Lane 1) Cathepsin L–stefin B complex; (lane 2) stefin B; (lane 3) cathepsin L.

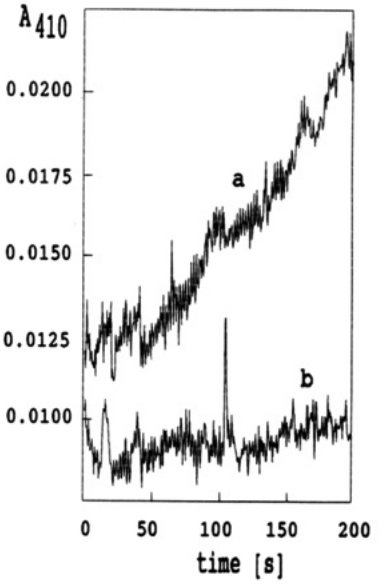


FIGURE 5: Dissociation of the cathepsin L–stefin B complex at pH 5.5 and 37 °C. The complex (0.15 μM) was prepared at pH 7.4 and 37 °C and diluted 10-fold into a pH 5.5 buffer containing 200 μM Z-Phe-Arg-pNA and 0.6 μM α_2-macroglobulin, after which the release of product was recorded (curve a). Curve b represents the control experiment, where cathepsin L was exposed to the denaturing conditions in the absence of stefin B.

maximal catalytical activity. We used stefin B since the stefins are generally known as weaker inhibitors of the cysteine proteinases than the cystatins (Abrahamson et al., 1986; Barrett et al., 1986; Turk & Bode, 1991). The dissociation of the enzyme–inhibitor complex was promoted by the combined action of dilution, reaction of free enzyme with α_2-macroglobulin, which forms an irreversible and enzymatically active complex with proteinases (Pagano et al., 1984; Starkey & Barrett, 1977), and reaction of free enzyme with the substrate in high concentration which also diagnoses the presence of active enzyme. The increase in absorbance shown in Figure 5, which corresponds to a continuous substrate degradation, indicates that cathepsin L has been released from its complex with stefin B. Identical results were obtained when the enzyme–inhibitor complex was incubated for as long as 1 h at neutral pH. The results from separate runs did not differ and were reproducible to a great extent. In the control

experiment, where cathepsin L was incubated at pH 7.4 in the absence of stefin B, no substrate was degraded (Figure 5).

DISCUSSION

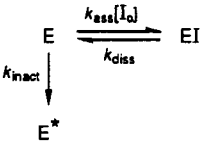
We have shown that the inactivation of cathepsin L at neutral pH in the presence of substrate is the first-order process where the rate decreases with the substrate concentration in a way that strongly suggests that the substrate protects the enzyme from inactivation. First-order inactivation of lysosomal cysteine proteinases in the presence of substrate has been observed previously (Baici & Knöpfel, 1986; Machleidt et al., 1986). However, one study did not investigate the effect of substrate concentration on the rate of inactivation (Machleidt et al., 1986) while the other used substrate concentrations below K_m at which the rate of inactivation did not vary with substrate concentration (Baici & Knöpfel, 1986) (see eq 2).

At pH 7.4, k_{inact} increases more than 3000-fold between 5 and 37 °C. This enormous increase in rate corresponds to an activation energy of 41.3 kcal mol^{-1}, a value that is close to the activation energy (46.1 kcal mol^{-1}) for the alkaline pH induced unfolding of staphylococcal nuclease (Chen et al., 1991), suggesting that the unfolding of the cathepsin L might perhaps explain its in activation. This view is supported by the finding that the inactivation of chicken cathepsin L is accompanied with a loss of α-helix content (Dufour et al., 1988). The substrate and the CPIs might stabilize the activity of cathepsin L by preventing pH-induced unfolding of the protein. X-ray diffraction data clearly show that the cystatins form a molecular complex wich buries the active site of the enzyme and stabilizes its active conformation (Bode et al., 1988; Stubbs et al., 1990).

Renaturation studies were unsuccessful, indicating that the alkaline inactivation of cathepsin L is an irreversible process. The irreversibility of the alkaline pH inactivation was also shown for cathepsin B (Barrett, 1973). Our findings on fully processed cathepsin L differ from those reported for the precursor form of mouse cathepsin L (Mason et al., 1987), where pH inactivation was shown to be a reversible process. This is not surprising since, according to the thermodynamic hypothesis of protein folding, the information required for a protein to attain its native, active conformation is contained in the primary structure of the completely unfolded polypeptide chain. Anfinsen and Scheraga (1975) showed that proteins cleaved after biosynthesis and folding were unable to refold properly after denaturation. This is true for a wide range of globular proteins, especially those derived from a precursor, such as pepsin (Blumenfeld et al., 1960), pepsinogen (Ahmad & McPhie, 1978), and also cathepsin D (Turk et al., 1981). Comparison of the reversibility of the inactivation of the precursor of cathepsin L (Mason et al., 1987) with the irreversibility of the inactivation of fully processed cathepsin L suggests that the above hypothesis (Anfinsen & Scheraga, 1975) is also valid for cathepsin L.

Cathepsin L can come into contact with the inhibitors from the cystatin superfamily even under neutral pH conditions, when it is released from the lysosomal system into the extracellular fluid or the cytoplasm of some cells. We have therefore studied the inactivation of cathepsin L in the presence and absence of some representatives of the cystatins. Both stopped-flow and electrophoresis experiments indicate that the latter are able to form complexes with cathepsin L at neutral pH. We therefore provide experimental support to previous hypotheses (Abrahamson et al., 1986; Barrett et al., 1984; Machleidt et al., 1986). Moreover, at pH 7.4 the rate of inhibition of cathepsin L by the cystatins is significantly higher than the rate of spontaneous inactivation of the enzyme. The competition between the two inactivation reactions is illustrated by

Scheme II



where $[I_0]$ is the concentration of CPIs in the extracellular fluid, EI is the cathepsin L–CPI complex, and k_{ass} and k_{diss} are the rate constants of association and dissociation, respectively. The CPIs are tight binding cathepsin L inhibitors with K_i ($= k_{diss}/k_{ass}$) values in picomolar range (Abrahamson et al., 1991; Machleidt et al., 1986). In addition, the concentration of cystatin C and kininogens in biological fluids is at least 10-fold higher than that used in the current experiments. Hence, $k_{ass}[I_0] \gg k_{diss}$, which means that the CPI-induced inhibition of cathepsin L in vivo is pseudo-irreversible (Bieth, 1980). In addition, we have shown that $k_{ass}[I_0] \gg k_{inac}$. It may thus be concluded that most of the cathepsin L molecules released into the extracellular milieu form an EI complex, only a minor part being transformed into E*. The latter route, i.e., the enzyme inactivation at neutral pH, is commonly thought to be the major way used in vivo to prevent cysteine proteinase-mediated proteolysis in extracellular fluids.

We have shown that catalytically active cathepsin L may be released from its complex with CPI even if the latter is incubated under pH conditions where free cathepsin L is rapidly inactivated. This property leads us to suggest that cystatin C and/or kininogens might serve as cysteine proteinase "reservoirs" (Bieth, 1986) that rapidly take up the enzyme released from lysosomes, carry them, and release them at sites where they can play further biological functions.

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