Interaction between Human Cathepsins K, L, and S and Elastins

MECHANISM OF ELASTINOLYSIS AND INHIBITION BY MACROMOLECULAR INHIBITORS*

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Proteolytic degradation of elastic fibers is associated with a broad spectrum of pathological conditions such as atherosclerosis and pulmonary emphysema. We have studied the interaction between elastins and human cysteine cathepsins K, L, and S, which are known to participate in elastinolytic activity in vivo. The enzymes showed distinctive preferences in degrading elastins from bovine neck ligament, aorta, and lung. Different susceptibility of these elastins to proteolysis was attributed to morphological differences observed by scanning electron microscopy. Kinetics of cathepsin binding to the insoluble substrate showed that the process occurs in two steps. The enzyme is initially adsorbed on the elastin surface in a nonproductive manner and then rearranges to form a catalytically competent complex. In contrast, soluble elastin is bound directly in a catalytically productive manner. Studies of enzyme partitioning between the phases showed that cathepsin K favors adsorption on elastin; cathepsin L prefers the aqueous environment, and cathepsin S is equally distributed among both phases. Our results suggest that elastinolysis by cysteine cathepsins proceeds in cycles of enzyme adsorption, binding of a susceptible peptide moiety, hydrolysis, and desorption. Alternatively, the enzyme may also form a new catalytic complex without prior desorption and re-adsorption. In both cases the active center of the enzymes remains at least partly accessible to inhibitors. Elastinolytic activity was readily abolished by cystatins, indicating that, unlike enzymes such as leukocyte elastase, pathological elastinolytic cysteine cathepsins might represent less problematic drug targets. In contrast, thyropins were relatively inefficient in preventing elastinolysis by cysteine cathepsins.

Elastic fibers are the key extracellular matrix component conferring elasticity to tissues such as blood vessels, lungs, and skin. The fibers are composed of a rubber-like network of highly stable and hydrophobic polymers of the protein elastin, associated with peripheral microfibrils (1, 2). Proteolytic degradation of elastic fibers leads to loss of tissue elasticity, which is associated with the development of different pathological conditions. A large repertoire of elastinolytic peptidases has been identified in human cells and tissues, including enzymes from four of the five catalytic classes of peptidases. The molecular mechanism underlying the process of elastinolysis has been thoroughly studied for human leukocyte elastase (HLE) (3–9), a serine peptidase produced by neutrophils, whose activity has been associated with the development of atherosclerosis and pulmonary diseases (10, 11). In addition, two other elastinolytic serine peptidases are present in neutrophils, cathepsin G and myeloblastin (leukocyte proteinase 3) (12).

Extracellular matrix degradation by monocyte-derived macrophages is because of the action of serine peptidases, matrix metalloproteases, and papain-like cysteine peptidases (cysteine cathepsins) (13–16). In inflammation, macrophages were found to secrete cathepsins B, K, L, and S, which were also identified as major contributors to tissue damage in chronic inflammatory conditions (13, 17). Six of the 11 cysteine cathepsins known in man (cathepsins B, F, K, L, S, and V) have been reported to exhibit elastinolytic activity in vitro (18–21). Numerous studies have implicated cathepsins K, L, and S, as well as some others, in extensive degradation of elastic fibers, which accompanies the development of pathological conditions of the cardiovascular system. In mice, cathepsin S was found to co-localize with regions of increased elastin breakdown in atherosclerotic plaques (22), and reduced atherosclerosis was observed in cathepsin S- and K-deficient mice (23, 24). In rats, up-regulation of cathepsin S and K activity was also observed following carotid artery injury (25). In human atheroma, cathepsins S and K were up-regulated in macrophages as well as smooth muscle cells (26), and increased cathepsin S levels were detected in sera of patients suffering from atherosclerosis and diabetes (27). Human macrophages in atherosclerotic plaques were shown to degrade elastin extracellularly as well as intracellularly. Cathepsins K and S were attributed major roles in extracellular degradation, whereas cathepsin V was identified as the major peptidase involved in lysosomal digestion (20).
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Increased levels of cathepsins K, S, and V were also observed in stenotic aortic valves (28), whereas cathepsin S has been connected to elastic lamina fragmentation in hypertensive heart failure (29). Similarly, increased cathepsin L levels were detected in abdominal aortic aneurysm, atherosclerosis, and stenosis (30).

Pathological elastin degradation by cysteine cathepsins has also been implicated in diseases of the pulmonary system. Alveolar macrophages are known to express cathepsins L and S (21, 31), whereas cathepsin K was shown to be expressed by lung epithelial cells (32). Increased cathepsin L activity was observed in bronchoalveolar lavage fluid of smokers (33), and increased levels of cathepsins B, H, K, L, and S were observed in bronchopulmonary dysplasia (34).

In vivo, proteolytic activity of cysteine cathepsins is regulated by endogenous inhibitors, including the general cysteine cathepsin inhibitors cystatins (35), the more specific thyropins (36), and several others. The importance of the peptidase/inhibitor balance has been well investigated in tumor progression (reviewed in Ref. 37). Reduced cystatin C levels have also been observed in atherosclerosis and aortic aneurysm in human and mouse (38–40), and imbalance between cysteine cathepsins and their inhibitors was also reported in bronchopulmonary dysplasia (34).

In existing reports on the elastinolytic activity of cysteine cathepsins, the mechanism utilized by these peptidases to degrade elastin has not yet been investigated. In this work, we have studied the interaction between elastin and the human cathepsins K, L, and S, which have been most frequently implicated in extracellular elastolysis. Because the structural organization of elastic fibers differs between tissues, we used bovine elastins from three different sources as follows: aorta, lung, and neck ligament. We investigated the mode of cathepsin binding to elastin, the overall elastinolytic activity, and the effect of macromolecular inhibitors from the cystatin and thyropin families on the elastinolytic activity of cysteine cathepsins.

EXPERIMENTAL PROCEDURES

Elastins, Enzymes, and Inhibitors—All elastin types were purchased from Elastin Products Co., Inc. The elastins used in this study were all of bovine origin as follows: neck ligament elastin (number E70; particle size, 100–400 mesh), aortic elastin (number SB87; particle size, pass 100 mesh), lung elastin (number SB77; particle size, pass 100 mesh), and ETNA-elastin (number E60; prepared from neck ligament, salt–free, water-soluble).

Recombinant human cathepsin K (EC 3.4.22.38), cathepsin L (EC 3.4.22.15), and cathepsin S (EC 3.4.22.27) were produced in Escherichia coli according to published procedures (41–43). Active concentrations of all enzymes were determined by active site titrations with the irreversible inhibitor E-64 (Bachem, Switzerland).

Recombinant human stefin A was produced according to Ref. 44, and the cystatin-like domain 3 of human kininogen (kininogen domain 3) was isolated as described previously (45) and recombinant thyroglobulin type 1 (Tg1) domain of human testican-1 as described previously (46). The recombinant human p41 fragment of the major histocompatibility complex class II-associated invariant chain, recombinant Tg1 domain of human nidogen-1, and recombinant Tg1 domain 1 of human nidogen-2 were produced in-house. In brief, cDNA sequences coding for individual Tg1 domains were amplified by PCR and cloned into the pET-32b(+) expression vector (Novagen, Germany) using the NcoI and XhoI restriction sites. Soluble thioredoxin fusion proteins were then expressed in E. coli strain BL21 (DE3) pLysS (Novagen, Germany) at 37 °C. Fusion proteins were purified from cell lysates by immobilized nickel ion-affinity chromatography. The Tg1 domains were liberated from the fusion proteins by enterokinase cleavage and purified by ion-exchange chromatography. Samples were over 95% pure as visualized by SDS-PAGE. Active p41 fragment concentration was determined by active site titration of cathepsin L, and the concentrations of the nidogen Tg1 domains were determined spectrophotometrically at 280 nm.

Elastin Surface Area Determination and Scanning Electron Microscopy—The specific surface area (SSA) of solid and powdered elastins was measured by nitrogen adsorption at −196 °C using the Brunauer et al. method (47) (BET-method, Tristar, Micrometrics Inc, Gemini, The Netherlands). An eight point isotherm, 0.02 < p/p0 < 0.2, was used after degassing the samples at room temperature for 1 h. The morphology of the elastin samples was analyzed by scanning electron microscopy using a LEO 1530 Gemini (Oberkochen, Germany).

Kinetic Measurements—Binding of cathepsins to insoluble elastin was studied using an approach originally developed for describing the interaction of HLE with macromolecular substrates (9). With this method the hydrolysis of a low molecular mass fluorogenic substrate (called the reporter substrate) is measured in the presence of various amounts of a macromolecular substrate. In the assay, the enzyme is partitioned between the macromolecular substrate and the reporter substrate, and the perturbation caused by the macromolecular substrate to the kinetics of reporter substrate hydrolysis can be formally treated as that of an inhibitor. Kinetic measurements were performed in a C-61 fluorimeter (Photon Technology International) at 25 °C. Assays with cathepsins K and L were performed in 50 mM sodium phosphate buffer, pH 6.50, containing 2.5 mM EDTA and using the fluorogenic substrate benzoxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin (Bachem, Switzerland). In cathepsin K assays, the final concentration of the reporter substrate was 5 μM (Km = 7.5 μM), and in cathepsin L assays the concentration was 10 μM (Km = 0.93 μM). Assays with cathepsin S were performed in 50 mM sodium phosphate buffer, pH 6.50, containing 2.5 mM EDTA and 0.1% PEG-6000, with benzoxycarbonyl-Phe-Val-Arg-7-amino-4-methylcoumarin as the substrate (Bachem, Switzerland) at a final concentration of 10 μM (Km = 12.5 μM). Active enzyme concentrations in the assays were 60 pm for cathepsin K, 200 pm for cathepsin L, and 2 nm for cathepsin S.

Variable amounts of elastin powder (ranging from 0.2 to 15 mg) were introduced into disposable 10 × 10-mm acryl cuvettes. 2 ml of the appropriate assay buffer were added to the powder, and the cuvette was then hermetically sealed and incubated overnight at room temperature. Immediately prior to the experiment, the mixture was supplemented with the appropriate substrate and DTT (final concentration 2.5 mM). Reaction
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Model 1

\[ \begin{align*}
M + S + E & \xrightarrow{K^*} ES \\
& \xrightarrow{\kappa} E + P
\end{align*} \]

Model 2

\[ \begin{align*}
M + S + E & \xrightarrow{K^*} ES \\
& \xrightarrow{\kappa} E + P
\end{align*} \]

Model 3

\[ \begin{align*}
M + S + E & \xrightarrow{K^*} ES \\
& \xrightarrow{\kappa} E + P
\end{align*} \]

was started by addition of the enzyme, and the progress was monitored continuously for 3 min at \( \lambda_{\text{ex}} = 383 \) nm and \( \lambda_{\text{em}} = 455 \) nm. To minimize light scattering, narrow excitation and emission bandwidths of 1 nm were used. During the assay the reaction mixture was continuously stirred using a built-in magnetic stirrer and a Teflon-coated stirring rod to avoid sedimentation of the insoluble material. Assays with soluble ETNA-elastin were performed under the conditions described above. A stock elastin solution (10 mg/ml) was prepared by dissolving ETNA-elastin in the appropriate assay buffer and diluted immediately prior to the experiment.

Kinetic Models for Cathepsin Binding to Elastins—To describe the binding of cysteine cathepsins to the surface of insoluble elastin, we propose three models of interaction, which are shown in Fig. 1. The three models and their corresponding equations are described with terminology and dimensions typical of interfacial enzyme catalysis, with the asterisk denoting processes occurring at the surface of insoluble elastin (48). \( A^*_M \) and \( K^* \) represents the elastin surface area per unit volume and the interfacial dissociation constant, respectively, both with dimensions \( \text{m}^2 \cdot \text{liter}^{-1} \); \([S]\) is the molar concentration of the reporter substrate, and \( K_m \) is its Michaelis constant. The models consist of two parts as follows: the upper parts represent the Michaelis-Menten path of reporter substrate hydrolysis, and the lower parts represent the adsorption of the enzyme to the matrix, followed by a catalytic step, which is shown in Fig. 1 as dashed boxes in each model. This part is not visible in the assays, because the elastin cleavage products (Q) are deprived of a spectroscopically measurable signal. Therefore, the macromolecular substrate can be formally regarded as a competitive inhibitor, and the measured “inhibition constant” of the macromolecular substrate (M) actually yields an analogous of the Michaelis constant. However, by using low enzyme concentrations and short reaction times, we can safely assume that the perturbation on the formation of the fluorescent product (P) caused by M yields a good estimate of the dissociation constant \( K^* \). In Model 1 the behavior of the macromolecular substrate is analogous to that of a linear competitive inhibitor and corresponds to enzyme adsorption to the matrix in a nonproductive manner followed by isomerization to a catalytically productive complex. The treatment of Models 2 and 3 is analogous to that of a competitive inhibitor, two molecules of which bind the enzyme in an ordered sequence. Model 3 differs from Model 2 by a dimensionless coefficient \( a \) that multiplies \( K^* \) and describes a change of the dissociation constant for the vacant site following the adsorption step, thus taking into account the cooperativity of the binding process.

In Fig. 1, Model 1 is described by Equation 1, which is equivalent to the equation for linear competitive inhibition; Model 2 is described by Equation 2, and for Model 3 Equation 3 applies. The interaction of cathepsins with soluble ETNA-elastin was analyzed using the same models, except that elastin surface area per unit volume (\( A^*_M \)) was replaced by mass concentration [M] in the corresponding equations, and the interface equilibrium dissociation constant \( K^* \), used to describe the adsorption of cathepsins to the surface of insoluble elastin, was replaced by the equilibrium dissociation constant in solution K (given in gliter \(^{-1} \)).

Discrimination between the models was performed by fitting the corresponding Equations 1–3 in Fig. 1 to experimental data using GraphPad Prism version 4.00 for Windows, GraphPad
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Software, San Diego. First, Equation 1 was fitted to the data, and the runs test was performed to check any systematic deviation of the fitted curve from the data. If there was no systematic deviation, a discrimination test was performed by analysis of variance of the difference between the sum of squares of Models 1 and 2 (extra sum-of-squares test). An F ratio and a p value were calculated as follows: for a p value less than 0.05, the more complicated model (Model 2) was chosen as appropriate. If fitting of Equation 1 did not pass the runs test, discrimination was performed with the extra-sum-of-squares test applied to Model 2 and Model 3.

An additional tool for interpreting the kinetics of cathepsin binding to elastin was the 90:10 ratio \( \frac{\text{kM,0.9}}{\text{kM,0.1}} \), i.e. the ratio of elastin surface areas necessary to reduce enzyme activity by 90 and 10%. For Model 1, the 90:10 ratio is always 81 (Equation 4), a characteristic of linear competitive inhibitors (49). For Model 2, the 90:10 ratio depends on the \([S]/K_m\) ratio (Equation 5), and for Model 3 it depends on the values of \([S]/K_m\) and \(a\) (see Equation 6).

Elastinolytic Assays—The elastinolytic activities of cathepsins K, L, and S were measured using a modified procedure of Schwabe (50). In brief, reaction mixtures containing 1.0 mg of insoluble elastin in a total volume of 200 \( \mu l \) of the appropriate assay buffer supplemented with 2.5 mM DTT (see under “Elastinolytic Assays”) were prepared. Reactions were started by addition of the enzyme and incubated in an Eppendorf Thermomixer Compact at 37 °C with shaking (1200 rpm) to avoid sedimentation. Enzyme concentrations in the assays were 285 nM (a, b, and c), 100 nM (d), and 100 nM (e). The reaction mixtures were incubated for 10–120 min, and the reactions then stopped by addition of trichloroacetic acid to a final concentration of 5% (w/v). The samples were centrifuged for 15 min at 14,000 × g, and 100 \( \mu l \) of clear supernatant were added to 3.0 ml of 0.2 mM sodium borate buffer, pH 8.5, and then combined with 1.0 ml of a fluorescamine solution (15 mg/100 ml in acetone) under vigorous stirring. The fluorescence of the labeled peptides was measured at \( \lambda_{ex} = 390 \) nm and \( \lambda_{em} = 480 \) nm. Appropriate blanks were run to take into account the fluorescence developed by elastin and enzyme alone. The measured fluorescence was compared with a standard curve, which was produced using glycine solutions of known concentrations. The rate of peptide release from elastin was determined as the slope of the curve obtained from multiple samples incubated for increasing periods of time and was then normalized to 1 pmol of enzyme.

Adsorption of Cathepsins on Elastin—The adsorption of cathepsins K, L, and S on elastin was studied by a modified procedure of Ying and Simon (12). The partitioning of the enzyme between the insoluble phase (elastin) and soluble phase (aqueous buffer) was studied in the presence of excess amounts of elastin to allow for concentration-independent adsorption of the enzyme. In brief, 100 \( \mu l \) of an elastin suspension (10 mg/ml) in 50 mM sodium phosphate buffer, pH 6.50, containing 2.5 mM EDTA and 2.5 mM DTT, was incubated with each cathepsin (concentrations ranging from 0.1 to 1 \( \mu l \)) for 10 min at 25 °C and with shaking to avoid sedimentation. The samples were then centrifuged for 3 min at 14,000 × g, and the enzymatic activity of the supernatant was measured fluorimetrically as described under “Kinetic Measurements.” In parallel, control samples without elastin were run and were used to calculate the percentage of enzyme adsorbed to the elastin surface.

Inhibition of Elastinolysis—The proteinaceous inhibitors used in this study were recombinant stefin A, kininogen domain 3, Tg1 domain of testican-1, the p41 fragment, Tg1 domain of nidogen-1, and Tg1 domain 1 of nidogen-2. Experiments were performed as described under “Elastinolytic Assays,” except that inhibitors were added to the mixture either prior to addition of the enzyme or following a 30-min preincubation of the enzyme with elastin. All samples were incubated for a total of 60 min, and released peptides were detected by reacting them with fluorescamine as described under “Elastinolytic Assays.” Appropriate blanks were run to account for fluorescence of elastin, enzyme, and inhibitor alone.

RESULTS

SSA and Microscopic Analysis of Elastin Particles—Bovine elastins from three different anatomical regions were analyzed by scanning electron microscopy to verify their morphological features as a support to the interpretation of our kinetic measurements (Fig. 2). Neck ligament elastin appeared predominantly as extended patches, or splinters thereof, with a rough surface, which was spotted by small fragments plausibly generated during extraction from the tissue and sample preparation (Fig. 2C). These small fragments are likely to substantially contribute to the SSA of neck ligament elastin (2.3 m\(^2\)/g). In contrast, lung elastin showed a distinct three-dimensional network composed of bundles of fibers (Fig. 2B, top). Not surprisingly, lung elastin had the largest SSA among the three samples investigated (3.3 m\(^2\)/g). The appearance of aortic elastin was intermediate between the others, being composed of mostly larger particles with highly structured surface (Fig. 2A, top) and an...
SSA of 1.9 m²/g. At high magnifications, neck ligament elastin appeared to be composed of multiple layers characteristic of predominantly two-dimensional structures, whereas lung elastin had a more uniform, fibrous structure distinctive of a three-dimensional connective tissue component. These properties reflect the physiological functions of the three elastin types. Neck ligament elastin, with an extended structure, acts as an elastic band allowing grazing animals to counterbalance the weight of their head and neck. Lung elastin forms a well-developed three-dimensional scaffold of fibers to support elasticity and to increase the gas-exchange area of the alveoli. Elastin in the media of the aorta is responsible for the viscoelastic mechanical properties of the blood vessel in close association with collagen and smooth muscle.

**Kinetics of Cathepsin Binding to Elastin**—Enzyme binding to the surface of elastin was studied kinetically using a method originally developed for HLE (9). The behavior of cysteine cathepsins was basically different from that observed for HLE. In fact, the binding of the latter produced progress curves similar to those observed for slow-binding inhibitors, whereas progress curves obtained with cysteine cathepsins were linear from the very beginning of the reaction, and an initial lag phase was not observed.

Plots of progress curve slopes versus amount of elastin present in the reaction mixture (surface area per unit volume, \(A_{M}^{*}\), for insoluble elastins, and mass concentration, \([M]\), for soluble ETNA-elastin) showed profiles of various shapes. The equations corresponding to the models described in Fig. 1 were fitted to each set of data points, and model discrimination was performed with strict statistical criteria, as detailed under “Experimental Procedures.” The best fit curves obtained for each cathepsin/elastin combination are shown in Fig. 3, which for the sake of clarity also reports the calculated kinetic parameters (equilibrium dissociation constant \(K^{*}\) or \(K\) for insoluble and soluble substrate, respectively, as well as the coefficient \(a\) when applicable) and the 90:10 ratios (both experimental and calculated).

Plots of residual velocity \(v_{i}/v_{0}\) versus mass concentration \([M]\) of soluble ETNA-elastin produced hyperbolic curves, which were optimally described by Model 1 (Fig. 1). The equilibrium dissociation constant \(K\) decreased in the order cathepsin K > cathepsin L > cathepsin S, and the value of the 90:10 ratio was close to 81. Thus, soluble elastin formally behaved as a classical linear competitive inhibitor of the reporter substrate.

The profiles of cathepsin binding to the surface of insoluble elastins (plotted as residual velocity \(v_{i}/v_{0}\) versus the surface area per unit volume of elastin, \(A_{M}^{*}\)) deviated from hyperbolic behavior. Instead, the profiles were of various sigmoid shapes and were best fitted by either Model 2 or Model 3 (Fig. 1), depending on the cathepsin/elastin combination (Fig. 3). These models take into account the sequential binding of the enzyme to two sites of elastin and can be interpreted as follows. Binding to the first macromolecular site (\(M\) in Fig. 1) corresponds to the adsorption of the enzyme onto the surface of the elastin particle, and binding to a second macromolecular site represents a rearrangement of the adsorbed enzyme resulting in the formation of a catalytically productive complex. Deviation from Model 1 was also confirmed by the experimentally determined 90:10 ratio, which was in the range of 15–40, as opposed to 81 for Model 1. As shown by the dissociation constants \(K^{*}\) and the coefficient \(a\), the second step in Models 2 and 3, i.e. the formation of a catalytically competent complex, was either favored by the adsorption step or was a process with lesser propensity to occur as the adsorption onto elastin.

**Adsorption of Cathepsins on Elastin**—In addition to the kinetics of cathepsin binding to elastin, we compared the overall adsorption of cathepsins K, L, and S on the surface of insoluble elastins by measuring the partitioning of the enzymes between solid and aqueous phase. Overall adsorption is a measure of the number of binding sites available to each enzyme and is different from the dissociation constant \(K^{*}\) (Fig. 3), which describes the affinity of cathepsins for elastin binding sites independently of their number.

As shown in Fig. 4, the enzymes differed markedly in their adsorption properties. 68% of cathepsin K was adsorbed on the surface of aorta and lung elastins and 80% on the surface of elastin from neck ligament. In contrast, cathepsin L showed a preference for the soluble phase. Less enzyme was adsorbed on aorta (15%) and neck ligament elastin (7%), and very little cathepsin L was retained in the solid phase with lung elastin as the substrate. The adsorption of cathepsin S was intermediate between cathepsins K and L, with 46% cathepsin S adsorbed on aorta elastin, 60% on lung elastin, and 28% on neck ligament elastin. Altogether these results show that the number of binding sites for cathepsin L on elastin is obviously smaller than that for cathepsins K and S.

**Elastinolysis by Cysteine Cathepsins**—Overall elastinolytic activity is a composite effect containing contributions of the relative affinities of the enzymes for elastins and of their mode of binding and catalysis. The elastinolytic activity of cathepsins K, L, and S was determined using an assay with relatively high enzyme concentrations and long incubation periods. The peptidases showed distinctive preferences in degrading elastins from different sources (Fig. 5). The rate of peptide release from soluble ETNA-elastin was 1 order of magnitude higher than peptide release from insoluble elastins. Cathepsin L was the most potent, cathepsin S activity was 90% of cathepsin L activity, and the rate of peptide release by cathepsin K was only 55% of that by cathepsin L.

In degrading insoluble elastins, all three enzymes had a preference for degrading elastin from the aorta when compared with elastins from lung and neck ligament. Cathepsins L and S were similar in their action with an increasing preference for elastins in the order neck ligament < lung < aorta. With all three substrates, cathepsin L showed higher elastinolytic activity than cathepsin S. Cathepsin K was the most active enzyme in degrading neck ligament and aorta elastin, but it showed scarce elastinolytic activity toward lung elastin.

The results in Fig. 5 complement those shown in Figs. 3 and 4. As stated above, \(K^{*}\) (Fig. 3) gives information on the affinity of enzymes for elastins, and the adsorption results in Fig. 4 show how many binding sites are available on elastins for the three peptidases. Fig. 5 is a measure of the catalytic efficiency of the cathepsins and explains what could not be seen in the binding experiments, i.e. Fig. 1, boxed paths of Models 1–3. Thus, the
whole mechanism of elastinolysis is understood from the three concepts together.

Inhibition of Elastinolysis by Macromolecular Inhibitors—One of the most striking properties of HLE is its resistance against macromolecular inhibitors (3). In this study we have tested the ability of macromolecular inhibitors to prevent the elastinolytic activity of cysteine cathepsins. We tested the following inhibitors: stefin A, kininogen domain 3, the p41 fragment, the Tg1 domain of testican-1, and the Tg1 domains of nidogen-1 and nidogen-2. The first two belong to the cystatins and are nonspecific cysteine cathepsin inhibitors. The remaining molecules are members of the thyropin family and are specific inhibitors of individual cysteine cathepsins: the p41 fragment inhibits cathepsin L (51) and cathepsin K, the Tg1 domain of testican-1 inhibits cathepsin L (46), and the Tg1 domains of nidogens inhibit cathepsin K.

The effect of the inhibitors was tested with and without preincubation of the enzymes with elastin, and it was shown that the susceptibility of cysteine cathepsins to inhibition was independent of their preincubation with elastin. As shown in Fig. 6, stefin A and kininogen domain 3 were highly effective and com-

\[ K^* = 0.73 \pm 0.08 \]
\[ a = 2.8 \pm 1.1 \]
\[ 90/10 = 30.4 (30.0) \]

\[ K^* = 0.62 \pm 0.02 \]
\[ a = 2.3 \pm 1.1 \]
\[ 90/10 = 11.9 (13.1) \]

\[ K^* = 1.62 \pm 0.04 \]
\[ a = 2.3 \pm 1.1 \]
\[ 90/10 = 21.0 (20.8) \]

\[ K^* = 2.0 \pm 0.3 \]
\[ a = 5.8 \pm 3.8 \]
\[ 90/10 = 38.7 (38.2) \]

\[ K^* = 0.9 \pm 0.2 \]
\[ a = 3.2 \pm 2.4 \]
\[ 90/10 = 33.7 (33.6) \]

\[ K^* = 6.5 \pm 1.7 \]
\[ a = 0.27 \pm 0.17 \]
\[ 90/10 = 18.6 (14.7) \]

\[ K^* = 2.7 \pm 0.8 \]
\[ a = 0.32 \pm 0.24 \]
\[ 90/10 = 15.7 (15.5) \]

\[ K^* = 3.5 \pm 0.1 \]
\[ a = 1.5 \]
\[ 90/10 = 14.8 (14.8) \]

\[ K^* = 5.6 \pm 0.9 \]
\[ a = 0.36 \pm 0.14 \]
\[ 90/10 = 15.6 (15.7) \]

\[ K^* = 0.39 \pm 0.04 \]
\[ 90/10 = 81.4 (81.0) \]

\[ K^* = 0.29 \pm 0.02 \]
\[ 90/10 = 81.4 (81.0) \]

\[ K^* = 0.085 \pm 0.004 \]
\[ 90/10 = 81.1 (81.0) \]

FIGURE 3. Kinetics of cathepsin binding to elastin. Equations describing the proposed models of interaction (Fig. 1) were fitted to experimental data obtained for each cathepsin/elastin combination. Only best fit curves are shown. For each fit, the calculated values of the equilibrium dissociation constant, i.e. \( K^* \) for insoluble elastins (dimensions m^2/liter^{-1}) and \( K \) for soluble ETNA-elastin (dimensions g/liter^{-1}), and the dimensionless coefficient \( a \) (when applicable) are shown. Also given for each cathepsin/elastin combination are experimental as well as calculated (in parentheses) 90:10 ratios.

3 P. Meh and B. Lenarčič, unpublished data.
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Proteolytic destruction of elastic fibers is associated with the development of various pathological conditions, such as atherosclerosis and pulmonary emphysema (10, 52). The molecular basis of elastinolysis has been thoroughly studied for HLE, a serine peptidase secreted by neutrophils. Binding of HLE involves a slow initial complex formation with elastin, followed by isomerization to a tightly bound complex. Once bound, HLE does not readily dissociate from its substrate but moves along the elastin fibril (3). Therefore, macromolecular inhibitors are hindered in gaining access to the active center of the enzyme and to reduce its elastinolytic activity (3–8). Besides HLE, neutrophils contain two other serine peptidases, cathepsin G and myeloblastin (53, 54). The behavior of cathepsin G is similar to HLE, whereas myeloblastin makes use of a different elastinolytic mechanism, which does not involve the formation of a tightly bound complex with elastin (12) and is similar to the mechanism we report here for cysteine cathepsins.

Several reports described the ability of cysteine cathepsins to degrade elastin and other insoluble extracellular substrates, such as collagens (18–21, 55–58). Details on the molecular mechanism of cysteine cathepsin action at the surface of insoluble substrates have, however, not been investigated before. In this study we have shown that the mechanism of elastinolysis of the cysteine cathepsins differs from that of HLE. Notably, a tightly bound complex between cathepsins and elastin was not observed. Based on our results, we propose that elastin degradation by cathepsins K, L, and S proceeds in cycles, as illustrated in Fig. 7. The initial step is a rapid adsorption of the enzyme on the elastin surface in a nonproductive manner. The adsorbed enzyme then rearranges to a catalytically productive complex in which the active center of the enzyme binds to an elastin peptide susceptible to proteolytic attack. This part of the mechanism is based on the kinetics of cathepsin binding to the surface of insoluble elastin, which operates according to either Model 2 or Model 3 (Figs. 1 and 3). In comparison, when acting on soluble elastin, adsorption of the enzyme on an insoluble surface does not apply, and the enzyme directly and rapidly binds the substrate in a catalytically productive manner, as proposed in Model 1 (Figs. 1 and 3). This is also the mechanism employed by HLE when acting on soluble macromolecular substrates (9). Once the cleaved peptides are released from the active site, the enzyme can either dissociate from the elastin surface and then re-adsorb at a distinct site, or can remain adsorbed and form a new catalytically competent complex. The results in Fig. 4 show that cathepsin K tends to remain adsorbed on elastin, whereas cathepsin L very likely acts in rapid cycles of adsorption and desorption. On the other hand, cathepsin S makes use of both pathways in an approximately equal extent, even though its adsorption properties strongly depend on the type of elastin.

The overall elastinolytic activity was a combined effect, containing contributions of enzyme binding to elastin and the catalytic properties of the enzyme, which were specific for each cathepsin/elastin combination. In our experimental approach, aimed at mimicking elastinolytic activity in vivo, the elastin sur-
face area available to the cysteine cathepsins was very large, so that the cycle shown in Fig. 7 can take place a number of times, as shown by the following calculation. Considering for the cysteine cathepsins an approximate molecular mass of 25,000 Da and dimensions of $5 \times 3 \times 2.5 \text{ nm}$, one molecule of enzyme will occupy an area of about $15 \times 10^{-18} \text{ m}^2$. The highest enzyme concentration used in our kinetic experiments was 2 nM for cathepsin S (and much lower for cathepsins K and L). If all of the enzyme molecules of cathepsin S present in 1 liter ($1.2 \times 10^{15}$ molecules) were adsorbed at the insoluble surface, the area covered would be 0.018 m$^2$. Lung elastin, for instance, had an SSA of 3.3 m$^2$/g, i.e. in 1 liter of a suspension containing 2 g/liter of it, as in a typical kinetic experiment, the available surface area was 6.6 m$^2$. Thus, a theoretical maximum of about 0.27% of the total elastin SSA available was covered by enzyme.

We investigated the action of cysteine cathepsins on neck ligament elastin as an experimental reference model for the action of elastinolytic enzymes on a substrate with a predominantly two-dimensional spatial organization. Yet because of the involvement in a variety of pathological conditions of the cardiovascular and pulmonary systems, degradation of aorta and lung elastins is of particular interest. All three cathepsins showed highest activity against aorta elastin when compared with elastins from other sources, and cathepsin K activity was more than 2-fold larger than that of cathepsins L and S. Multiple studies have described the involvement of these cathepsins in the degradation of elastic fibers of blood vessel walls in vivo, which contributes to the development of pathological conditions such as atherosclerosis, aortic aneurysms, stenosis, and heart failure (22–30). In contrast, cathepsin K showed very little activity in degrading lung elastin, but cathepsin L was the most efficient enzyme in degrading this substrate, with activity comparable with that observed with aorta elastin. In the lung, cathepsins L and S are secreted by alveolar macrophages, which are thought to contribute to the development of lung emphysema

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**FIGURE 6.** Effect of macromolecular inhibitors on the elastinolytic activity of cathepsins K, L, and S. The inhibitors used were stefin A (StfA), kininogen domain 3 (Kin.d3), the p41 invariant chain fragment (p41 Ii), the thyroglobulin type 1 (Tg1), domain of testican 1 (Tst1), Tg1 domain of nidogen-1 (Nid1), and Tg1 domain 1 of nidogen-2 (Nid2.d1). Enzymes were incubated with elastin (from neck ligament, 1 mg per 200 μl of reaction mixture) in the presence of equimolar (eq.) or 10-fold excess amounts of inhibitors at 37 °C for 60 min. Released peptides were reacted with fluorescamine, and detected fluorescence was compared with a control sample without added inhibitor.

**FIGURE 7.** Schematic representation of the proposed elastinolytic mechanism of cathepsins K, L, and S. The enzyme is first adsorbed on elastin in a nonproductive manner. The adsorbed enzyme then interacts with another elastin site to form a catalytically productive complex. Following cleavage and release of the products, the enzyme either dissociates from elastin or remains adsorbed on the surface (dashed arrow).
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(21, 31, 33), whereas cathepsin K is expressed by bronchial and alveolar epithelial cells (32) and has been proposed to play a major role in lung tissue homeostasis (59).

Overall, cathepsins K, L, and S behave as powerful elastolytic enzymes in vitro using assays performed under optimal conditions. In vivo, their activity and stability are influenced by environmental parameters, such as pH and ionic composition. Cathepsin S is known to be stable at neutral pH (60), whereas cathepsins K and L are relatively short lived (18, 61). Macrophages circumvent this problem by utilizing a vacuolar-type H⁺-ATPase to acidify the pericellular milieu where cathepsins are secreted (13). Another likely factor that may influence the elastolytic activity of cysteine cathepsins in vivo is the presence of glycosaminoglycans, which were shown to inhibit the collagenolytic activity of cathepsins L and S and to stimulate cathepsin K activity (56).

Independently of the dynamic aspects of peptidase binding to elastins, the relative elastolytic activities of various enzymes can be deduced from previous studies using the molar concentration of the enzymes as a normalization parameter. This comparison is qualitative because some cysteine peptidases are unstable at neutral pH, where other elastolytic enzymes exert their maximum activity. However, a general trend can be safely deduced from published data as follows (enzymes are listed in decreasing order of elastinolytic potential): cathepsin V > cathepsin K > cathepsin S > pancreatic elastase > leukocyte elastase > cathepsin G > gelatinase A > macrophage elastase > myeloblastin > cathepsin F > cathepsin L (12, 18, 20).

In vivo, excessive connective tissue degradation is often because of failure of endogenous inhibitors to prevent ongoing proteolysis. In atherosclerosis and lung diseases, imbalance between cysteine peptidases and their inhibitors has been observed as a result of up-regulation of cysteine cathepsin activity and/or down-regulation of endogenous inhibitors, such as cystatin C (38–40). In our experiments, the elastinolytic activity of cathepsins K, L, and S was readily abolished by inhibitors of the cystatin family (stefin A and kininogen domain 3), suggesting that, in contrast to HLE, it is not the inefficiency but the unavailability of inhibitors that is responsible for proteolysis of extracellular matrix components by cysteine cathepsins. Furthermore, their susceptibility to inhibitors makes cysteine cathepsins appealing drug targets in diseases such as atherosclerosis and pulmonary emphysema.

In contrast to cystatins, thyropins were relatively inefficient in inhibiting the elastinolytic activity of cathepsins K and L, and none could drive enzyme activity to zero. Interestingly, the p41 fragment, which had been characterized as a strong inhibitor of cathepsin L (51) using a low molecular mass substrate, failed to inhibit this peptidase at equimolar inhibitor/enzyme concentrations in the presence of elastin. These results support the hypothesis that the p41 fragment acts as a chaperone for cathepsin L, maintaining a pool of catalytically active extracellular cathepsin L by shielding it from inactivation at neutral pH (62). The presence of elastin apparently lowers the affinity of the p41 fragment for cathepsin L, causing release of active enzyme from the complex and at the same time preventing its re-association with the p41 fragment. The most efficient inhibitor of all thyropins was the Tg1 domain of testican-1, despite the fact that it is also susceptible to cleavage by cathepsin L (46). However, the Tg1 domains of nidogens and the p41 fragment were relatively ineffective in inhibiting cathepsin K activity. It is worth noting that recombinant Tg1 domains performed as stand-alone proteins in our assays, whereas in vivo they are modular parts of larger proteins (with the exception of the p41 fragment), which may affect their activity. Nevertheless, our results show that thyropins are not efficient in preventing extensive proteolytic activity. Given their poor effectiveness on one hand and their high specificity for individual cysteine cathepsins on the other hand, the thyropins seem not to function as cysteine peptidase inhibitors per se, as is the case of cystatins. Rather, they seem to perform a different role in the regulation of cysteine cathepsin activity with mechanisms that remain to be identified.

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