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High-affinity binding of two molecules of cysteine proteinases to low-molecular-weight kininogen

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Abstract

Human low-molecular-weight kininogen (LK) was shown by fluorescence titration to bind two molecules of cathepsins L and S and papain with high affinity. By contrast, binding of a second molecule of cathepsin H was much weaker. The 2:1 binding stoichiometry was confirmed by titration monitored by loss of enzyme activity and by sedimentation velocity experiments. The kinetics of binding of cathepsins L and S and papain showed the two proteinase binding sites to have association rate constants $k_{ass,1} = 10.7\text{–}24.5 \times 10^6\ M^{-1}\ s^{-1}$ and $k_{ass,2} = 0.83\text{–}1.4 \times 10^6\ M^{-1}\ s^{-1}$. Comparison of these kinetic constants with previous data for intact LK and its separated domains indicate that the faster-binding site is also the tighter-binding site and is present on domain 3, whereas the slower-binding, lower-affinity site is on domain 2. These results also indicate that there is no appreciable steric hindrance for the binding of proteinases between the two binding sites or from the kininogen light chain.

Keywords: cathepsin; cystatin; cysteine; kinetics; kininogen; proteinase

Kininogens are large multifunctional glycoproteins in mammalian plasma that serve as parent molecules for vasoactive peptides, the kinins (DeLa Cadena & Colman, 1991). Three different types of kininogens have been identified, high-molecular-weight kininogen, low-molecular-weight kininogen, and T-kininogen. The latter variant has been found only in rat, and was shown to be the major acute-phase reactant in this species (Okamoto & Greenbaum, 1983; Kageyama et al., 1985). HK and LK bind to platelets and have the ability to prevent thrombin binding to, and activation of, human platelets (Jiang et al., 1992). Specific regions of HK are also involved in binding to neutrophils (Wachtvogel et al., 1994). Both human HK and LK are products of the same gene as a result of alternative mRNA splicing (Kitamura et al., 1985). The mature molecules are single-chain proteins but are converted to two-chain forms, consisting of a heavy and a light chain, by limited proteolysis by kallikreins, with release of the kinin segment (Müller-Esterl et al., 1986). The heavy chains of HK and LK are identical, whereas the light chain of HK is considerably larger than that of LK (Takagaki et al., 1985; Kellerman et al., 1986). The two chains are connected by a single disulfide bond (Müller-Esterl et al., 1986). The cleavage of HK by plasma kallikrein to yield two-chain HK results in a striking change in the conformation of the kininogen molecule (Weisel et al., 1994).

It is well established that kininogens inhibit the activity of cysteine proteinases (Ohkubo et al., 1984; Müller-Esterl et al., 1985; Sueyoshi et al., 1985). Sequence data have shown that the heavy chains are composed of three tandemly repeated cystatin-like domains, designated D1–D3 (Salvesen et al., 1986; Müller-Esterl et al., 1986). Domain D1 contains a Ca^{2+}-binding site but does not inhibit cysteine proteinases (Higashiyama et al., 1987). Domains D2 and D3, however, contain the QVVAG sequence that is common to the cysteine proteinase inhibitors, the cystatins, and inhibit cysteine proteinases, including papain and cathepsins B, H, and L (Salvesen et al., 1986; Vogel et al., 1988). On this basis, the kininogens are classified as family 3 of the cystatin superfamily (Barrett et al., 1986a). Domain D2 is unique among the cystatins in that it also inhibits calpain (Salvesen et al., 1986; Ylinenjärvi et al., 1995). This ability has been pro-

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Abbreviations: -AMC, aminomethyl coumaryl amide; HK, high-molecular-weight kininogen; LK, low-molecular-weight kininogen; -pNA, *p*-nitroanilide; Z-, benzoyloxycarbonyl.

posed to be due to a specific inhibitory region, distinct from the QVVAG sequence, forming a contiguous surface for interacting with calpain (Bradford et al., 1993).

Although it has been shown that two of the three isolated kininogen domains have inhibitory activity when studied separately (Salvesen et al., 1986; Vogel et al., 1988; Auerswald et al., 1993; Ylinenjärvi et al., 1995), and that the kininogens thus have the potential for binding two molecules of cysteine proteinases, the evidence regarding the binding stoichiometry of the intact molecules is conflicting (Gounaris et al., 1984; Ohkubo et al., 1984; Anderson & Heath, 1985; Higashiyama et al., 1986; Sueyoshi et al., 1988). Moreover, all studies were done with papain and ficin, and no data are available for the lysosomal cysteine proteinases, which are the physiological target enzymes for the kininogens (Barrett et al., 1986b).

In this work we demonstrate that human LK can bind two molecules of cathepsins H, L, S, and papain independently, whereas no inhibition of cathepsin B is apparent. Moreover, the rate constants for binding of the proteinases to the individual binding sites of LK are shown to differ significantly.

Results

Binding stoichiometry by fluorescence titration

Titration of active papain, having an active-site concentration of >0.95 mol/mol protein, with chicken cystatin gave an apparent inhibitor to enzyme binding stoichiometry of 1.1. As in previous studies, the chicken cystatin thus was fully active in forming an equimolar complex with papain (Bode et al., 1988; Lindahl et al., 1988). Similar titration of the other proteinases studied with chicken cystatin showed that these proteinases were 65–90% (depending on the enzyme) active in binding to the inhibitor. All proteinases were then titrated with LK and the data evaluated on the basis of concentrations of cystatin-binding enzyme. These experiments showed that 0.53–0.63 mol of LK were needed to saturate 1 mol of papain or of cathepsins L or S (Fig. 1A), giving apparent proteinase to LK binding stoichiometries of 1.9, 1.6, and 1.7 for the three enzymes, respectively. These values were independent of proteinase concentration in the range 50 nM to 2 μM for papain, 100–250 nM for cathepsin L, and 100–500 nM for cathepsin S. The results thus indicate two binding sites for papain and cathepsins L and S on each kininogen molecule, the observed apparent stoichiometries most likely reflecting the presence of some inactive material in the LK preparation. The shapes of the titration curves were consistent with dissociation equilibrium constants for both binding sites substantially lower than the proteinase concentrations.

Titration of cathepsin H with LK supported the presence of two proteinase binding sites on LK. At low concentration (200 nM), a plateau was reached at near-equimolar concentrations, indicating 1:1 binding stoichiometry (Fig. 1B). However, with increasing concentrations of cathepsin H, both the fluorescence change accompanying the interaction and the apparent cathepsin H to LK binding stoichiometry increased (Fig. 1B). This suggests the presence of a second binding site having substantially lower affinity, with a K_d in the micromolar range. Although cathepsin B was highly active in binding chicken cystatin, no detectable fluorescence change was observed in titration with LK, even at an enzyme concentration of 1 μM.

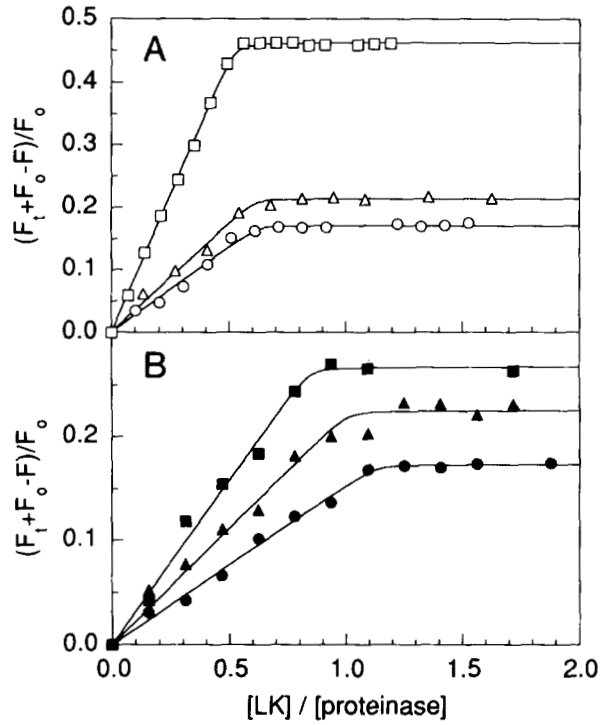

Fig. 1. Fluorescence titration of papain and cathepsins L, S, and H with LK. **A:** □, 2 μM papain; ○, 200 nM cathepsin L; △, 500 nM cathepsin S. **B:** ■, 1 μM cathepsin H; ▲, 500 nM cathepsin H; ●, 200 nM cathepsin H. Experimental conditions are described in the Materials and methods. F_t, fluorescence of added LK; F_o, fluorescence of proteinase; F, fluorescence of LK–proteinase mixture. Fitted curves were generated by nonlinear regression analysis (Olson et al., 1991).

Binding stoichiometry by titration of enzyme activity

To confirm the results presented above, we monitored titrations of papain and cathepsins L and S with LK by the loss of enzyme activity against synthetic substrates. These and subsequent experiments were evaluated on the basis of concentration of active LK. The titration curves were linear and gave a proteinase/LK binding stoichiometry of 2:1 for all three proteinases (Fig. 2).

Binding stoichiometry by ultracentrifugation

An M_r of 51,100 was determined for LK by sedimentation equilibrium measurements. This value is in good agreement with those calculated from the amino acid sequence (Takagaki et al., 1985) and two different carbohydrate analyses (Ryley, 1979; Müller-Esterl et al., 1982) but appreciably lower than the value of 68,000 estimated by SDS-PAGE (Müller-Esterl et al., 1982). This difference indicates anomalous behavior of LK in SDS-PAGE, probably due to the glycosylation of the molecule. The sedimentation coefficients of LK and its complexes with papain, formed at a proteinase/LK molar ratios 1:1 or 2:1, were 3.0, 4.3, and 4.8 S, respectively. The latter value is consistent with that expected for a 2:1 papain/LK complex (Tanford, 1961), whereas the sedimentation coefficient for the equimolar complex is somewhat higher than the expected value of ~3.9 S. This discrepancy may be due to the asymmetrical schlieren peak observed in the latter experiment.

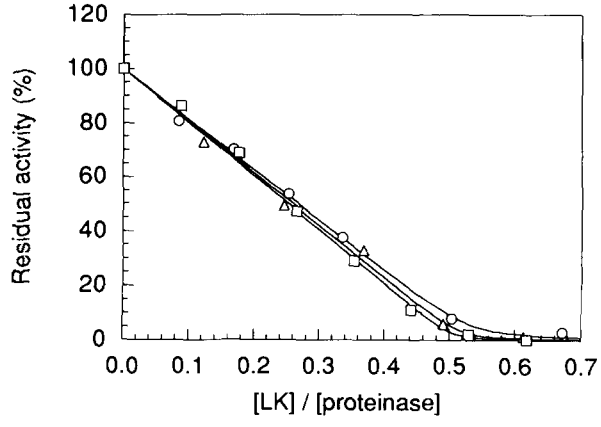

Fig. 2. Inhibition of papain and cathepsins L and S by increasing amounts of LK. □, 1 μM papain; ○, 500 nM cathepsin L; △, 250 nM cathepsin S. Fitted curves were generated by the nonlinear regression analysis according to Bieth (1984).

Kinetics of binding

The kinetics of binding of LK to target proteinases were studied by two different methods. In the first of these, the reactions were monitored by the decrease in tryptophan fluorescence accompanying the interaction. Most experiments were done under pseudo-first-order conditions with a 20-fold molar ratio of proteinase to LK, making detection of proteinase binding to both putative inhibitory sites possible. The fluorescence change was increased (Björk et al., 1989) and nonspecific hydrolysis of LK by excess proteinase was prevented by the use of inactive *S*-methylthio derivatives of the enzymes (Roberts et al., 1986). This derivatization has been shown only negligibly to affect the association rate constants for various cystatin-cysteine proteinase interactions (Björk et al., 1989; Lindahl et al., 1992). The kinetics for all three enzymes studied in this manner, i.e., papain and cathepsins L and S, showed two phases and were best fitted to a double-exponential function, as illustrated for papain and cathepsin S in Figure 3. The dependence of the observed

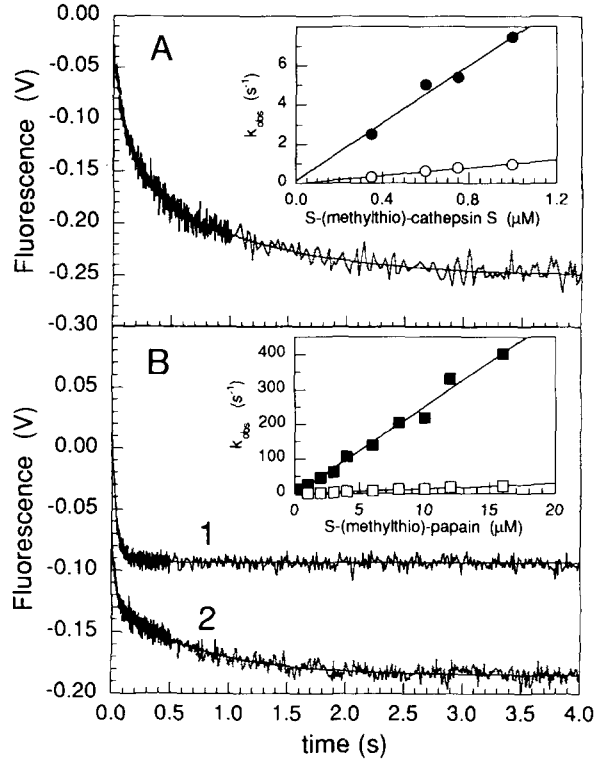

Fig. 3. Kinetics of binding of *S*-(methylthio)-cathepsin S and *S*-(methylthio)-papain to LK. The decrease of fluorescence accompanying the interaction was monitored as described in the Materials and methods. **A:** Double-exponential progress curve for the reaction between 1 μM *S*-(methylthio)-cathepsin S and 50 nM LK, with the best fit. Inset: Dependence of observed pseudo-first-order rate constants (k_{obs}) on *S*-(methylthio)-cathepsin S concentration. ●, $k_{obs,1}$; ○, $k_{obs,2}$. Solid lines were obtained by linear regression analysis. **B:** 1, Single-exponential progress curve for the reaction between 1 μM LK and 100 nM *S*-(methylthio)-papain, with the best fit. 2, Double-exponential progress curve for the reaction between 1 μM *S*-(methylthio)-papain and 50 nM LK, with the best fit. Inset: Dependence of observed pseudo-first-order rate constants (k_{obs}) on *S*-(methylthio)-papain concentration. ■, $k_{obs,1}$; □, $k_{obs,2}$. Solid lines were obtained by linear regression analysis.

Table 1. *Association rate constants for LK-cysteine proteinase interactions at 25 °C*[a]

Enzyme	$10^{-6} \times k_{ass,1}$ (M^{-1} s^{-1})	$10^{-6} \times k_{ass,2}$ (M^{-1} s^{-1})
S-(methylthio)-papain[b]	24.5 ± 0.6	1.4 ± 0.08
Papain[c]	21.4 ± 0.3	N.D.
S-(methylthio)-cathepsin L[b,d]	11.6 ± 1.5	0.83 ± 0.03
Cathepsin L[c]	10.7 ± 0.3	N.D.
S-(methylthio)-cathepsin S[b]	8.4 ± 0.9	1.2 ± 0.1
Cathepsin S[c]	18.1 ± 0.6	N.D.
Cathepsin H[c]	4.1 ± 0.2	N.D.
Cathepsin B[c]	<0.001	N.D.

[a] Experimental conditions were as described in the Materials and methods. N.D., not determinable.
[b] Determined by monitoring tryptophan fluorescence changes.
[c] Determined in the presence of substrate.
[d] Determined at enzyme concentrations of 0.5 and 1 μM.

pseudo-first-order rate constants for the two phases on proteinase concentration was analyzed for papain and cathepsin S and was found to be linear for both phases up to the highest concentration used (16 and 1 μM, respectively), as shown in Figure 3. Second-order association rate constants for the two phases were calculated from the slopes of these lines or from experiments at two enzyme concentrations for cathepsin L (Table 1). The ratios between the amplitudes of the fast and slow phases were 0.80 ± 0.03, 1.09 ± 0.07, and 0.33 ± 0.04 for papain, cathepsin S, and cathepsin L, respectively. These results are consistent with the proteinases binding by a simple, bimolecular reaction mechanism to two sites on LK, with the rate constants for the two sites differing 7–18-fold.

In another experiment, also monitored by tryptophan fluorescence, the relative concentrations were reversed, so that a 10-fold molar ratio of LK to *S*-(methylthio)-papain was used. In this case, monophasic kinetics were observed (Fig. 3), in contrast with the biphasic behavior evident at an excess of *S*-(methylthio)-papain. The observed pseudo-first-order rate constant

obtained in this manner at 1 μM LK, 24.1 s^{-1}, was in excellent agreement with the rate constant of 25.0 s^{-1} for the faster process determined at 1 μM *S*-(methylthio)-papain with an excess of the latter. The amplitudes of the two processes also agreed within 5% after correction for the difference in final complex concentration between the two experiments. These results are consistent with the proteinase binding predominantly to the faster-binding site at an excess of LK, which presumably has higher affinity for the enzyme, the slower-binding site remaining largely unoccupied.

In the second kinetic method, the reactions were monitored by continuous recording of the loss of enzyme activity in the presence of substrate. The experiments were done under pseudo-first-order conditions with a 10-fold molar ratio of LK to proteinase and thus allowed only determination of the association rate constant for the faster-binding site. All progress curves obtained with papain and cathepsins L and S had a final slope of zero, showing that the reactions were essentially irreversible at the inhibitor concentrations used and were thus fitted to a simple exponential function (Tian & Tsou, 1982; Turk et al., 1994a). In the case of cathepsin H, however, a small steady-state rate of product formation was observed in all experiments, and the progress curves were therefore analyzed with an equation derived for reversible inhibition (Morrison, 1982). The plots of the observed pseudo-first-order rate constant versus inhibitor concentration were linear for all LK-enzyme pairs investigated. Association rate constants were calculated from the slopes of these plots and were corrected for substrate competition (Table 1). These rate constants were similar to those determined for the binding of *S*-(methylthio)-enzymes to the faster-binding site, confirming that the inactivation of the enzymes indeed had a negligible effect on the inhibition rates by LK. The rate constants for binding of papain and cathepsins H and L were also similar to values determined previously for the binding of these enzymes to LK under comparable conditions (Abrahamson et al., 1986; Machleidt et al., 1986; Vogel et al., 1988; Auerswald et al., 1993). The steady-state velocities observed in the reactions between cathepsin H and LK indicated an upper limit of ~1 nM for the inhibition constant of this interaction. No inhibition of cathepsin B was detected, even when the enzyme activity of 0.8 nM cathepsin B with 1 μM LK was monitored for 2,000 s.

Discussion

Although two of the three isolated kininogen domains were shown to have inhibitory activity for cysteine proteinases (Salvesen et al., 1986; Vogel et al., 1988), the binding stoichiometry of the intact kininogens has remained unclear. The results reported were contradictory and suggested formation of either binary (Gounaris et al., 1984; Sueyoshi et al., 1988) or ternary complexes (Anderson & Heath, 1985; Higashiyama et al., 1986) between various forms of kininogens or their heavy chains and papain, which was always used as target proteinase. These discrepancies were presumably due largely to methodological problems. In some studies, an equimolar mixture of LK and papain was subjected to electrophoresis (Gounaris et al., 1984), or the binding was characterized with papain substituted with a large inactivating group (Sueyoshi et al., 1988), precluding detection of a ternary complex in both cases. In other work, commercial papain that had not been active-site titrated was used in combination with incomplete titration (Higashiyama et al., 1986) or insufficient reaction times for equilibrium to be reached (Anderson & Heath, 1985). Moreover, the possibility that the kininogen preparation may have contained a large proportion of inactive protein was not considered.

Here we demonstrate that human LK can simultaneously bind two molecules of cathepsins H, L, and S, which are its physiological target proteinases, as well as of the model enzyme, papain. This conclusion is based on several independent methods. Fluorescence titration of enzymes, having carefully standardized concentrations, with LK showed that papain and cathepsins L and S bound to LK with a 2:1 stoichiometry over a wide concentration range. Such titration also indicated that cathepsin H bound to LK with the same stoichiometry, although the inhibitor had a low affinity for the second enzyme molecule. Titration of the enzymes with LK, monitored by the loss of enzyme activity, fully corroborated these results. Further evidence for the formation of a ternary complex between LK and papain was obtained by sedimentation velocity experiments. Finally, stopped-flow analyses of reactions between papain or cathepsins L or S and LK kinetically confirmed the existence of two binding sites on LK, having different rates of association with target proteinases.

The rate constant for the binding of papain to the faster-binding site in intact LK identified in this work, $\sim 2.5 \times 10^7\ M^{-1} \cdot s^{-1}$ agrees well with the values of $2.4\text{-}3.0 \times 10^7\ M^{-1} \cdot s^{-1}$ reported for papain binding to domain 3 isolated from plasma LK or expressed in *E. coli* (Vogel et al., 1988; Auerswald et al., 1993). Similarly, the rate constant for cathepsin L binding to the faster-binding site, $\sim 1 \times 10^7\ M^{-1} \cdot s^{-1}$, is in agreement with the values of $0.5\text{-}3.0 \times 10^7\ M^{-1} \cdot s^{-1}$ measured for the binding of cathepsin L to intact LK (Machleidt et al., 1986; Auerswald et al., 1993) and somewhat lower than that reported for recombinant domain 3 (Auerswald et al., 1993). Moreover, the rate constants of $\sim 1.5 \times 10^6$ and $6 \times 10^5\ M^{-1} \cdot s^{-1}$ for binding of papain and cathepsin L, respectively, to the slower-binding site in intact LK are comparable to previously reported values of $\sim 3\text{-}5 \times 10^6$ for the binding of these enzymes to proteolytically produced or recombinant domain 2 (Vogel et al., 1988; Ylinenjärvi et al., 1995). Unfortunately, no data are available for the other proteinases studied. Nevertheless, these comparisons lead to the conclusion that the faster-binding site in intact LK is the site located in domain 3, whereas the slower-binding site is that of domain 2.

The different binding kinetics of the two domains are reflected also in different affinities. Free domain 3 thus has been shown to have higher affinity for proteinases than free domain 2, with 5–500-fold lower inhibition constants reported for papain and cathepsin L (Salvesen et al., 1986; Vogel et al., 1988; Auerswald et al., 1993; Ylinenjärvi et al., 1995). Proteinase binding to intact kininogen will therefore predominantly occur to this domain, as reflected in the approximate agreement of rate and inhibition constants determined for intact LK and free domain 3 (Vogel et al., 1988; Auerswald et al., 1993). Our results demonstrate that there is no major difference in kinetic behavior between the domains in intact LK and the isolated domains. This observation indicates that there is no appreciable steric hindrance for the binding of proteinases to domain 2 by an enzyme molecule bound to the faster-binding, high-affinity domain 3 or by the presence of the light chain, as suggested earlier (Sueyoshi et al., 1988; Higashiyama et al., 1986).

The kininogens are the major physiological plasma inhibitors of cysteine proteinases, due to their high concentration (Abra-

hamson et al., 1986) and affinity. However, the functional significance of the tandem arrangement of inhibitory domains in the kininogen heavy chain remains unclear. This work shows that proteolytic release of the inhibitory domains from the parental molecule to enhance their inhibitory potential is unnecessary. Nevertheless, inhibitory kininogen fragments have been found in some body fluids under pathological conditions (Lenarčič et al., 1987; Vogel et al., 1988), although both the origin and function of these cleavages are unknown. The inhibitory potential of the high-affinity domain 3 against papain-like cysteine proteinases is sufficiently large (Bieth, 1984) that the low-affinity domain 2 presumably remains largely unoccupied by such proteinases under normal conditions. However, if necessary, domain 2 may serve to enhance the inhibitory capacity of the kininogens, providing more efficient regulation of cysteine proteinase activity. In addition, a major function of domain 2 appears to be the inhibition of calpain (Salvesen et al., 1986; Bradford et al., 1993; Ylinenjärvi et al., 1995).

Materials and methods

Materials

Z-Phe-Arg-AMC was purchased from Serva (Heidelberg, Germany), and Z-Phe-Arg-pNA, Z-Arg-Arg-pNA, and H-Arg-AMC from Bachem (Bubendorf, Switzerland). Stock solutions of the substrates were prepared in dimethylsulfoxide (Merck, Germany). Human LK, chicken cystatin, and bovine cathepsins B, H, S, and L were purified as described previously (Zvonar et al., 1979; Turk et al., 1983; Müller-Esterl et al., 1988; Dolenc et al., 1992; Popovič et al., 1993). Papain (2× crystallized; Sigma, St. Louis, Missouri, USA) was further purified by affinity chromatography (Blumberg et al., 1970); the purified enzyme had a thiol content of 0.96 ± 0.03 mol/mol protein. Proteinases were inactivated with methylmethanethiol sulfonate as described elsewhere (Roberts et al., 1986). The concentration of LK was determined from a specific absorbance coefficient of 0.78 L g^{-1} cm^{-1} (Müller-Esterl et al., 1982) and an M_r of 51,100, determined by analytical ultracentrifugation (see Results). Concentrations of proteinases and of chicken cystatin were determined as described previously (Lindahl et al., 1988; Turk et al., 1995).

Experimental conditions

Unless otherwise stated, all experiments were done at 25 °C. The buffers were 100 mM phosphate, pH 6.0, for cathepsins B, H, and S and papain, and 50 mM acetate, 100 mM NaCl, pH 5.5, for cathepsin L. All buffers contained 1.5 mM EDTA. The slightly acidic pH was used because of the instability of lysosomal cathepsins at neutral pH (Turk et al., 1993a, 1994b).

Fluorescence titration

Fluorescence titration of proteinases with chicken cystatin or LK were done in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, Illinois, USA) as described previously (Lindahl et al., 1988; Björk & Ylinenjärvi, 1989). The binding curves were analyzed by nonlinear least-squares computer fitting to the equilibrium binding equation (Olson et al., 1991).

Titration of enzymatic activity

Papain or cathepsins L or S were incubated with increasing amounts of LK from 5 to 90 min (at enzyme concentrations of ≥500 and 5 nM, respectively), and the residual activity of the proteinase was then measured as described earlier (Turk et al., 1993b). The substrates were Z-Arg-Arg-pNA (100 μM) at high enzyme concentrations (≥500 nM) and Z-Phe-Arg-pNA (100 μM) at lower concentrations. The data were analyzed by computer fitting to the theoretical binding equation (Bieth, 1984).

Analytical ultracentrifugation

Sedimentation equilibrium measurements were done at 20 °C in an MSE Centriscan analytical ultracentrifuge (MSE, Crawley, UK) and were monitored by photoelectric scanning (Yphantis, 1964). Sedimentation velocity was measured at 20 °C in the same instrument with the schlieren detection system (Schachmann, 1959). The concentrations of L-kininogen were 0.6 and 1.4–3.1 mg/mL in the two types of experiment, respectively. A partial specific volume of 0.717 was calculated for LK from the amino acid sequence and carbohydrate composition (Müller-Esterl et al., 1982; Takagaki et al., 1985).

Kinetic analyses

The kinetics of inhibition of cysteine proteinases by LK were analyzed in an SX-17MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK). The reactions were monitored either by the decrease of tryptophan fluorescence emission accompanying the interaction or by the formation of fluorescent product released by the proteinase in experiments done in the presence of substrate. The decrease of tryptophan fluorescence was monitored at an excitation wavelength of 280 nm and with an emission cutoff filter with ~50% transmission at 320 nm. A 20-fold molar ratio of proteinase to LK or a 10-fold ratio of LK to proteinase was used. Reactions in the presence of substrate were analyzed as described previously (Turk et al., 1994a), except that a 1:1 (v/v) mixing ratio was used throughout. Cathepsins L, B, S and papain were assayed with 5 μM Z-Phe-Arg-AMC, whereas 10 μM H-Arg-AMC was used for cathepsin H. The substrate consumption and the concentration of organic solvent were less than 5% and 1% (v/v), respectively, in all experiments. Association rate constants were corrected for substrate competition with the use of K_m values of 7 μM for cathepsin L, 11 μM for cathepsin S (both A. Čolič, unpubl. results), 65 μM for papain (Zucker et al., 1985), and 115 μM for cathepsin H (Turk et al., 1995).

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