Kinetics of Binding of Chicken Cystatin to Papain†

Ingemar Björk,* Eva Alriksson, and Karin Ylinenjärvi

Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden

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ABSTRACT: The kinetics of binding of chicken cystatin to papain were studied by stopped-flow fluorometry under pseudo-first-order conditions, i.e., with an excess of inhibitor. All reactions showed first-order behavior, and the observed pseudo-first-order rate constant increased linearly with the cystatin concentration up to the highest concentration that could be studied, 35 μM. The analyses thus provided no evidence for a limiting rate resulting from a conformational change stabilizing an initial encounter complex, in contrast with previous studies of reactions between serine proteinases and their protein inhibitors. The second-order association rate constant for complex formation was 9.9×10^6 M^{-1} s^{-1} at 25 °C, pH 7.4, $I = 0.15$, for both forms of cystatin, 1 and 2. This value approaches that expected for a diffusion-controlled rate. The temperature dependence of the association rate constant gave an enthalpy of activation at 25 °C of 31.5 kJ mol^{-1} and an entropy of activation at 25 °C of -7 J K^{-1} mol^{-1}, compatible with no appreciable conformational change during the reaction. The association rate constant was independent of pH between pH 6 and 8 but decreased at lower and higher pH in a manner consistent with involvement of an unprotonated acid group with a pK_a of 4–4.5 and a protonated basic group with a pK_a of 9–9.5 in the interaction. The association rate constant was unaffected by ionic strengths between 0.15 and 1.0 but decreased somewhat at lower ionic strengths. Incubation of the complex between cystatin 2 and papain with an excess of cystatin 1 resulted in slow displacement of cystatin 2 from the complex. The displaced inhibitor was shown to be intact by several criteria. Analyses of the rate of displacement gave a first-order dissociation rate constant for the complex of $\sim 5.7 \times 10^{-7}$ s^{-1} ($t_{1/2} \sim 14$ days) at 25 °C, pH 7.4, $I = 0.15$, independent of the concentration of the displacing cystatin 1. These findings show that the inhibition of papain by chicken cystatin is best described as a simple, reversible bimolecular reaction, leading to formation of an inhibitor–proteinase complex with a dissociation equilibrium constant of ~ 60 fM.

Mammalian tissues contain a number of inhibitors of cysteine proteinases. To date, four such inhibitors, cystatins A, B, C, and S, which all have relative molecular masses of 12 000–14 000, have been identified in human tissues and secretions, and analogues to some of these have been shown in other mammals (Barrett et al., 1986). An analogue of human cystatin C has also been demonstrated in chicken egg white (Fossum & Whitaker, 1968; Keilová & Tomásek, 1974; Anastasi et al., 1983). This inhibitor is the best characterized tissue-type inhibitor, since it can be purified in reasonably large amounts. It is isolated as two apparently functionally identical forms, which differ in isoelectric point but have only a minimal, although still unidentified, difference in structure (Anastasi et al., 1983; Nicklin & Barrett, 1984; Lindahl et al., 1988). The reaction of chicken cystatin with papain has been studied in some detail as a model for other reactions between cystatins and cysteine proteinases. The inhibitor forms a tight, equimolar complex with the enzyme, in which the reactive cysteine of the latter is inaccessible to substrates and to thiol group reagents (Anastasi et al., 1983; Nicklin & Barrett, 1984; Lindahl et al., 1988). Such a complex can also be formed with papain in which the reactive cysteine is blocked (Anastasi et al., 1983). Residue Gly-9 and the Gln-Leu-Val-Ser-Gly-sequence at residues 53–57 have been suggested to contribute to the proteinase binding site of the inhibitor (Ohkubo et al., 1984; Barrett et al., 1986; Abrahamson et al., 1987). The binding of chicken cystatin to active papain is accompanied by pronounced changes of near-ultraviolet circular dichroism, ultraviolet absorption, and fluorescence emission (Lindahl et al., 1988). These changes, which originate partly from tryptophan residues around the active site of papain and from the single tryptophan residue of chicken cystatin, are compatible either with a conformational change of one or both proteins or with only local perturbations of the environment of aromatic residues (Lindahl et al., 1988). In this work, we have studied the association and dissociation kinetics of the reaction between chicken cystatin and papain with the aim of further characterizing the mechanism of the interaction.

Materials and Methods

The two cystatin forms, 1 and 2, were purified from chicken egg white as described previously (Anastasi et al., 1983; Lindahl et al., 1988). Active papain was prepared from crude papaya latex (type I, Sigma, St. Louis, MO) by affinity chromatography on a matrix-linked papain inhibitor (Blumberg et al., 1970; Burke et al., 1974). The preparations had a thiol content of 0.95–1.0 mol/mol of enzyme, as determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959; Blumberg et al., 1970). The enzyme was stored as the *S*-methylthio derivative of Cys-25 and activated before use as described previously (Roberts et al., 1986; Lindahl et al., 1988).

The kinetics of association between cystatin and papain were studied under pseudo-first-order conditions by fluorescence measurements in a Hi-Tech SF-4 stopped-flow spectrophotometer (Hi-Tech, Salisbury, Wilts, England) with a dead time

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* Address correspondence to this author.

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of about 2 ms. Excitation was at 280 nm with a 150-W xenon lamp, and emission was observed through a cutoff filter with 50% transmission at ~310 nm. Data were transferred to an Apple IIe computer by an analogue-to-digital converter with a sampling time of 40 μs. Reaction traces were analyzed by nonlinear least-squares regression or conventional first-order plots. All pseudo-first-order rate constants presented are averages of five to six individual measurements. At low signal levels (≤~20 nM papain), each such measurement was obtained by averaging of two reaction traces. All values are given together with their 95% confidence limits.

The kinetics of dissociation of the cystatin–papain complex were monitored by measurements of the displacement of cystatin 2 from its complex with papain by an excess of cystatin 1. Cystatin 2 was mixed with papain in a molar ratio of enzyme to inhibitor of 1.05 to form 10 μM complex. The low molecular weight cysteine proteinase inhibitor 1-[*N*-[(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane (E-64)[1] (Barrett et al., 1982) was then added to a concentration of 20 μM, followed after 1 min by cystatin 1 to a concentration of 100–300 μM. All concentrations are those in the final reaction mixture. The appearance of free cystatin 2 at 25 °C was measured at different time intervals by high-performance liquid chromatography of 100-μL portions of the reaction mixture on a Mono-Q ion-exchange column (Pharmacia, Uppsala, Sweden), eluted at a rate of 1–2 mL/min with a linear gradient (20 mL) from 20 mM Tris-HCl, pH 7.4, to the same buffer containing 0.2 M NaCl. The amount of free cystatin 2 was determined by comparison with a standard of 10 μM cystatin 2, run under the same conditions.

Cystatin 2 liberated from its complex with papain and isolated by ion-exchange high-performance liquid chromatography was labeled with ^{125}I by the chloramine-T procedure (Hunter, 1973) with the use of Iodobeads (Pierce, Oud-Beijerland, The Netherlands). The labeled protein was mixed with unlabeled, authentic cystatin 2, and the mixture was analyzed by rate electrophoresis on a 7–15% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate under reducing conditions (Blobel & Dobberstein, 1975). The gel was stained with Coomassie Brilliant Blue R-250, and the labeled protein was visualized by autoradiography on Fuji X-ray film.

Liberated cystatin 2 for amino acid sequence analyses was separated from buffer ions by reversed-phase high-performance liquid chromatography on an Aquapore C4 column (30 × 2.1 mm, pore size 30 nm; Brownlee, Santa Clara, CA). The bound protein was eluted with a 0–70% (v/v) gradient of acetonitrile in 0.05% (v/v) trifluoroacetic acid. Its amino-terminal sequence was then determined by degradation in an Applied Biosystems (Foster City, CA) 470A gas-phase sequencer, connected on-line to a 120A PTH analyzer, with the use of the 03CPTH program.

Titrations of papain with cystatin for estimation of binding constants were monitored by measurements of tryptophan fluorescence as described previously (Lindahl et al., 1988).

Protein concentrations were obtained by absorption measurements at 280 nm. Absorption coefficients of 0.87 (Anastasi et al., 1983) and 2.39 (Brocklehurst et al., 1973) $L \cdot g^{-1} \cdot cm^{-1}$ and relative molecular masses of 13 100 (Schwabe et al., 1984) and 23 400 (Husain & Lowe, 1969) were used for both forms of cystatin (Lindahl et al., 1988) and papain, respectively.

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetate; E-64, 1-[*N*-[(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane; Tris, tris(hydroxymethyl)aminomethane.

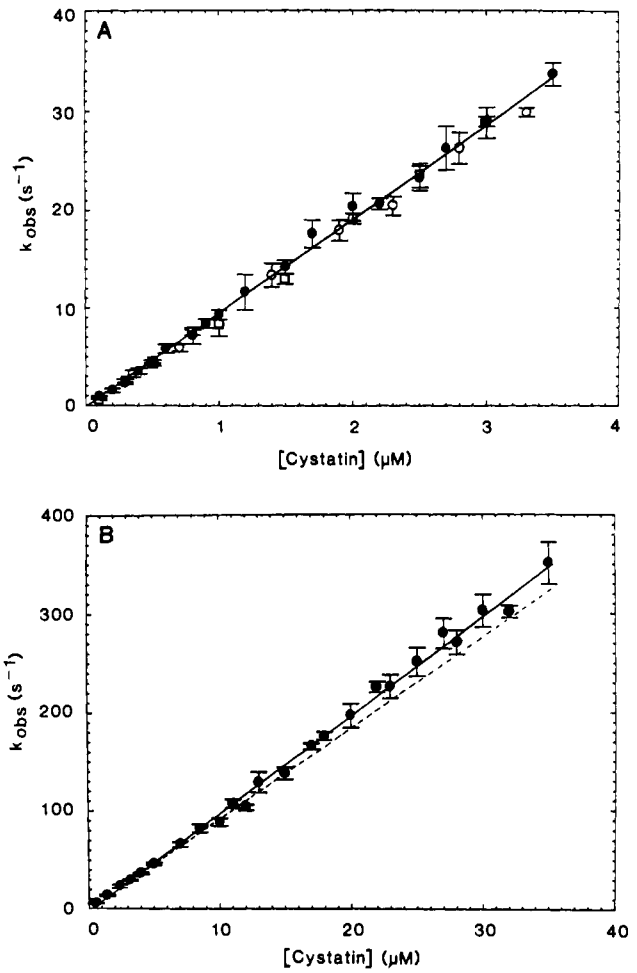

FIGURE 1: Observed pseudo-first-order rate constants (k_{obs}) for the binding of cystatin to papain at 25 °C, pH 7.4, and ionic strength 0.15, as a function of cystatin concentration. (A) Cystatin concentrations 0.1–3.5 μM; (B) cystatin concentrations 0.5–35 μM. (●) Cystatin 1; (○) cystatin 2. The buffer was 0.05 M Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 7.4; 0.1% (w/v) poly(ethylene glycol) 6000 was included in some experiments with cystatin 1 (□). The cystatin:papain ratio was maintained at 10:1 in all analyses. The dashed line in (B) represents the nonlinear least-squares fit of the lower 95% confidence limits to the equation resulting from the "standard mechanism" (eq 2) with the first step in rapid equilibrium, giving $K_1 \sim 500$ μM, $k_{+2} \sim 5000$ s^{-1}, and $k_{-2} \sim 0$.

Results

Association Kinetics. The kinetics of the binding of cystatin to papain were studied under pseudo-first-order conditions, i.e., at a molar ratio of cystatin to papain of 10:1, and were monitored by measurements of the decrease in tryptophan fluorescence accompanying the interaction (Lindahl et al., 1988). Initial studies were made with varying concentrations of cystatin at 25 °C, pH 7.4, and ionic strength 0.15 (Figure 1). First-order reactions were observed throughout the range of cystatin concentrations covered, 0.1–35 μM. Amplitudes were approximately proportional to papain concentrations (i.e., to concentrations of cystatin–papain complex) at cystatin concentrations <~10 μM, but decreased at higher concentrations as expected from loss of the initial part of the reaction in the dead time of the instrument. Studies at low cystatin concentrations (Figure 1A) showed an identical linear dependence of the observed pseudo-first-order rate constant on the concentration of inhibitor for the two forms of cystatin, 1 and 2. The slope of the regression line gave a second-order rate constant of 9.6 (95% confidence interval, 9.4–9.8) × 10^6 M^{-1} s^{-1}; the intercept of the line on the k_{obs} axis was indis-

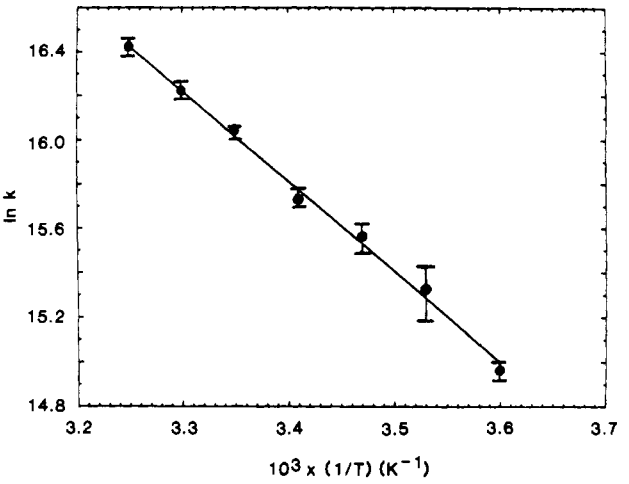

FIGURE 2: Logarithm of the second-order rate constants (k) for the binding of cystatin 1 to papain at pH 7.4, ionic strength 0.15, as a function of the inverse of the absolute temperature (T). The cystatin and papain concentrations were 3 and 0.3 μM, respectively, and the buffer was 0.05 M Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 7.4.

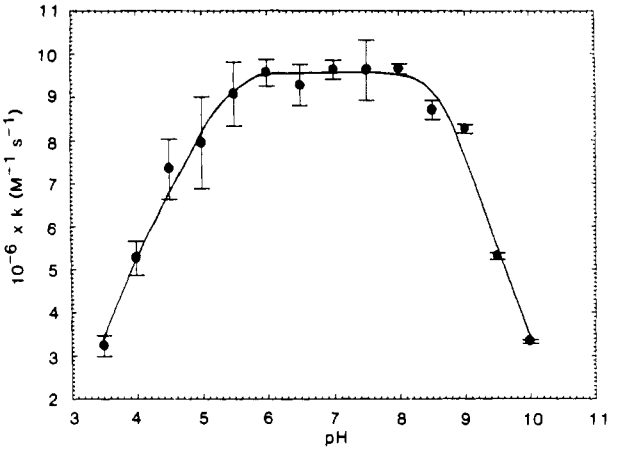

FIGURE 3: Second-order rate constants (k) for the binding of cystatin 1 to papain at 25 °C, ionic strength 0.15, as a function of pH. The cystatin and papain concentrations were 1.5 and 0.15 μM, respectively. The buffers were sodium formate at pH 3.5, sodium acetate at pH 4.0–5.5, sodium phosphate at pH 6.0–7.5, Tris-HCl at pH 8.0–9.0, and sodium carbonate at pH 9.5–10. All buffers were 0.02 M and contained NaCl to an ionic strength of 0.15 and 100 μM EDTA. The proteins were diluted extensively into the desired buffer (without any measurable effect on the pH of this buffer), and the experiments were carried out as soon as possible after temperature equilibration.

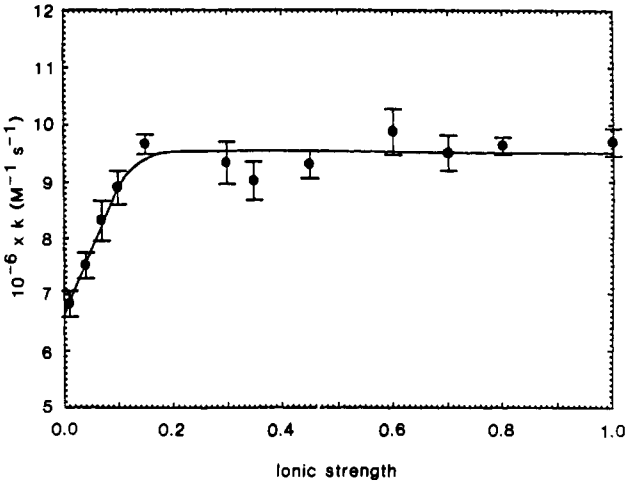

FIGURE 4: Second-order rate constants (k) for the binding of cystatin 1 to papain at 25 °C, pH 7.4, as a function of ionic strength. The cystatin and papain concentrations were 3 and 0.3 μM, respectively. The buffers were 0.0125 M Tris-HCl and 100 μM EDTA, pH 7.4, at an ionic strength of 0.01 and 0.05 M Tris-HCl and 100 μM EDTA, pH 7.4, containing the appropriate concentration of NaCl, at ionic strengths $\geq$0.04.

tinguishable from zero. Addition of 0.1% (w/v) poly(ethylene glycol) to the buffer to decrease possible adsorption of the enzyme did not affect the observed rate constants (Figure 1A) and thus was not used in further experiments. The analyses were extended to higher protein concentrations only for cystatin 1 (Figure 1B). A linear dependence of k_{obs} on cystatin concentration was obtained also in this range, up to the highest concentration of the inhibitor that could be analyzed, i.e., one giving a reaction half-life of ~2 ms. A second-order rate constant of 1.00 (0.97–1.03) × 10^7 M^{-1} s^{-1} was calculated from the slope of this plot. The data in the high and low concentration range together gave a second-order rate constant of 9.9 (9.7–10.1) × 10^6 M^{-1} s^{-1}.

All further analyses of association kinetics were done with cystatin 1. The dependence of the reaction rate on temperature at pH 7.4, ionic strength 0.15 was studied in the interval 5–35 °C under pseudo-first-order conditions at a constant concentration of cystatin. Second-order rate constants were computed from the observed pseudo-first-order rate constants and were plotted against the inverse of the absolute temperature in an Arrhenius plot (Figure 2). An activation energy of 34 (95% confidence interval, 31.5–36.5) kJ mol^{-1}, corresponding to an enthalpy of activation at 25 °C of 31.5 (29–34) kJ mol^{-1}, was calculated from the slope of this plot. Similarly, an entropy of activation at 25 °C of −7 (−15 to +2) J K^{-1} mol^{-1} was computed from the intercept at $1/T = 0$. The larger uncertainty of the latter value is due to the long extrapolation involved.

The pH dependence of the rate of association of cystatin 1 with papain at 25 °C, ionic strength 0.15 was also studied under pseudo-first-order conditions at a constant cystatin concentration (Figure 3). The second-order rate constant calculated from the data was independent of pH between about pH 6 and 8 but decreased steeply at lower and higher pH values. An upper limit of ~10 nM for the dissociation equilibrium constant of the complex at both extreme pH values studied, 3.5 and 10, was estimated by titrations, monitored by tryptophan fluorescence, of 100 nM enzyme with inhibitor. The interaction is thus sufficiently tight throughout the pH range covered that the observed pseudo-first-order rate constant contains no appreciable contribution from the dissociation rate constant and therefore gives the correct second-order rate constant at all pH values studied. The experiments were not extended to lower or higher pH due to the instability of papain under these conditions (Stockell & Smith, 1957).

The variation of the rate of binding of cystatin 1 to papain with ionic strength was analyzed in a similar manner as the temperature and pH dependences of the reaction (Figure 4). Essentially identical second-order rate constants were measured between ionic strengths of ~0.15 and 1.0 at pH 7.4, 25 °C, while a small decrease was observed at lower ionic strengths.

Dissociation Kinetics. The rate of dissociation of the cystatin–papain complex was studied by displacement of cystatin 2 from its complex with the enzyme by an excess of cystatin 1 at 25 °C, pH 7.4, ionic strength 0.15. The basis for this experiment is the fact that the two forms of the inhibitor are functionally highly similar or identical, in particular in their association rate constants, but differ in charge. Displaced cystatin 2 thus can be rapidly separated from the other components of the reaction mixture and quantified by ion-exchange

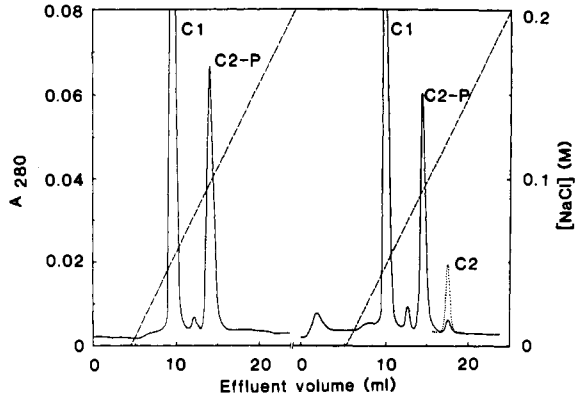

FIGURE 5: Analysis by ion-exchange high-performance liquid chromatography of the displacement of cystatin 2 from its complex with papain by an excess of cystatin 1. The complex between cystatin 2 and papain was formed at a concentration of 10 μM as described under Materials and Methods, and cystatin 1 was added to a concentration of 100 μM. The buffer was 0.05 M Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 7.4. The reaction mixture was analyzed immediately after addition of cystatin 1 (left) and after 95 h at 25 °C (right). (—) A_{280} of reaction mixture; (···) A_{280} of a 10 μM standard of cystatin 2; (- - -) NaCl concentration of eluting buffer. C1, cystatin 1; C2, cystatin 2; C2–P, complex between cystatin 2 and papain. The peak eluting between C1 and C2–P is an artifact due to the presence of EDTA in the sample.

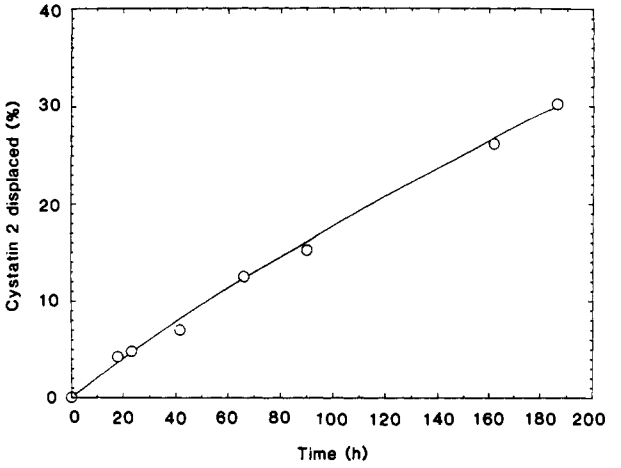

FIGURE 6: Amount of cystatin 2 displaced from its complex with papain by an excess of cystatin 1 at 25 °C, pH 7.4, and ionic strength 0.15, as a function of time. The conditions of the experiment were as described in the legend to Figure 5. The amount of cystatin 2 displaced from the complex was obtained from chromatograms such as that shown in Figure 5 by comparison with a standard and is given in percent of the maximal amount that would be expected to be displaced under the conditions used.

high-performance liquid chromatography (Figure 5). Provided that the binding is a simple, reversible equilibrium, such a displacement experiment will give the rate constant for the dissociation of cystatin 2 from papain at sufficiently high concentrations of the displacing cystatin 1 (Miller et al., 1980). The analogous experiment, i.e., displacement of cystatin 1 from its complex with papain by excess cystatin 2, could not be performed, as we found it impossible to quantitatively separate small amounts of cystatin 1 from the cystatin 1–papain complex at near-neutral pH. The low molecular weight cysteine proteinase inhibitor E-64 was added after the complex between cystatin 2 and papain had been formed to exclude possible slow proteolysis of the excess cystatin 1 by a small amount of proteinase not inactivated by cystatin, as has been described previously (Abrahamson et al., 1987). With increasing time after the addition of cystatin 1, an increasing amount of free cystatin 2, eluting from the ion-exchange column in the same position as authentic cystatin 2, slowly appeared (Figures 5 and 6). Only the initial part of the dissociation reaction could be monitored, as formation of a small amount of precipitate and the danger of bacterial growth precluded extension of the experiment to longer times. A dissociation rate constant for the complex between cystatin 2 and papain of 5.7 (95% confidence interval, 4.8–6.6) $\times 10^{-7}$ s^{-1} was calculated from first-order plots of the data in Figure 6 and similar experiments. This value is an average of five experiments at cystatin 1 concentrations of 100–300 μM (i.e., at molar ratios of cystatin 1 to complex between cystatin 2 and papain of 10–30), with no dependence on the concentration of cystatin 1. This independence shows that the concentration of the displacing cystatin 1 was sufficiently high to ensure that the observed rate constant well approximates the true dissociation rate constant of the complex (Miller et al., 1980). Evaluation from the initial rates of appearance of cystatin 2 gave a similar value for the rate constant, $\sim 5 \times 10^{-7}$ s^{-1}.

In a separate experiment, cystatin 2 displaced from its complex with papain after ~10 days of incubation with a 10-fold excess of cystatin 1 was isolated by the ion-exchange chromatography procedure. The liberated, ^{125}I-labeled protein migrated identically with unlabeled, authentic cystatin 2 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, also when the labeled and unlabeled proteins were mixed. Moreover, the amino-terminal sequence of the protein was found to be Ser-Glu-Asp-Arg-Ser-Arg-Leu-, identical with that of native cystatin 2 (Schwabe et al., 1984; Lindahl et al., 1988). Amounts of 85–25 (for Arg) nmol of phenylthiohydantoin derivatives giving this sequence were recovered; maximally, 10–15% of other phenylthiohydantoin derivatives were found in each step. Since the sequence deduced from these additional derivatives could not be identified in the cystatin sequence (or in papain), the derivatives appear to have arisen from contaminations of unknown origin rather than from fragmentation of cystatin 2.

Discussion

The data obtained for the kinetics of binding of chicken cystatin to papain show that the reaction can be best described by the simple, one-step, reversible bimolecular mechanism:

$$P + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PI \qquad (1)$$

where P is proteinase, I inhibitor, and PI their complex. For this mechanism, plots of the observed pseudo-first-order rate constant for complex formation, measured in the presence of excess inhibitor, vs inhibitor concentration will be linear, the slope of the line giving k_{+1} and the intercept on the ordinate giving k_{-1} (Frost & Pearson, 1961; Fersht, 1985). Such linear plots were obtained for the cystatin–papain reaction up to the highest cystatin concentration that could be studied by the stopped-flow method and gave an association rate constant, k_{+1}, of 9.9×10^6 M^{-1} s^{-1} at 25 °C, pH 7.4, ionic strength 0.15. The dissociation rate constant, k_{-1}, in this mechanism could not be determined from the stopped-flow data, since the intercept of the plot on the ordinate was indistinguishable from zero. However, the displacement experiments, in which an inhibitor shown to be intact by several criteria was liberated, demonstrated that the reaction is reversible, although the reverse reaction is very slow. A dissociation rate constant of $\sim 5.7 \times 10^{-7}$ s^{-1} at 25 °C, pH 7.4, ionic strength 0.15, corresponding to a half-life of ~14 days, was obtained for the cystatin 2–papain complex from these experiments. Reversibility of cystatin–cysteine proteinase reactions is further

indicated by studies of the activity of the inhibitor against cathepsin B and dipeptidyl peptidase I (Nicklin & Barrett, 1984). The proposed simple, reversible bimolecular reaction mechanism (eq 1) is also supported by the similar results obtained for reactions between cystatin and several inactivated forms of papain (Björk and Ylinenjärvi, unpublished experiments).

Another mechanism should be considered in interpreting the results, namely, the "standard mechanism" shown for the association of several serine proteinases with their protein inhibitors (Laskowski & Kato, 1980). Somewhat simplified, this mechanism can be written as

$$P + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} PI^* \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} P + I_M \qquad (2)$$

Thus, a loose, Michaelis-type complex, PI, is initially formed, followed by conversion of this intermediate to the stable complex, PI*, by a structural rearrangement giving rise to the signal by which the reaction is monitored. This complex slowly decomposes to free proteinase and a proteolytically modified inhibitor, I_M, cleaved at its reactive bond. For many proteinase–proteinase inhibitor reactions, the latter step is essentially irreversible (i.e., $k_{-3} \sim 0$). The initial step has been shown to be in rapid equilibrium (i.e., $k_{-1} \gg k_{+2}$) for several reactions between inhibitors of serine proteinases and their target enzymes (Luthy et al., 1973; Quast et al., 1974; Olson & Shore, 1982), and under these conditions, this mechanism is consistent with the simple exponential behavior under conditions of inhibitor excess observed in this work. However, in this case, the observed pseudo-first-order rate constant varies with the concentration of inhibitor according to the equation:

$$k_{obs} = k_{-2} + k_{+2}[I]/([I] + K_1)$$

(Strickland et al., 1975; Olson et al., 1981; Fersht, 1985), where K_1 is the dissociation constant for the first, rapid-equilibrium step (i.e., $K_1 = k_{-1}/k_{+1}$). Thus, plots of k_{obs} vs [I] show hyperbolic behavior, with an intercept on the ordinate of k_{-2} and a limiting rate at high [I] of $k_{+2} + k_{-2}$, and with K_1 being given by the concentration of inhibitor at which $k_{obs} = k_{+2}/2 + k_{-2}$.

The standard mechanism cannot be completely excluded by our data, since the measurements, although extended to as high inhibitor concentrations as technically possible, may have been restricted to only the initial, approximately linear portion of the hyperbolic curve. However, in this case, the limiting rate, corresponding to k_{+2} since $k_{-2} \sim 0$, must be $> \sim 5000\ s^{-1}$, with K_1 being $>500\ \mu M$ (Figure 1). This value for k_{+2} is 15–500-fold higher than corresponding values measured for reactions between serine proteinase inhibitors and their target enzymes (Luthy et al., 1973; Quast et al., 1974; Olson et al., 1982) and would represent a high, although still possible, rate constant for a protein conformational change (Fersht, 1985). A further observation making the standard mechanism less likely is that only intact inhibitor has been shown to dissociate from the complex with papain, both under native conditions, as in this work, and on denaturation by sodium dodecyl sulfate (Anastasi et al., 1983; Nicklin & Barrett, 1984).

Nicklin and Barrett (1984) have reported an apparent bimolecular rate constant for the association of chicken cystatin with papain of $\sim 1 \times 10^7\ M^{-1}\ s^{-1}$ at 40 °C, pH 5.85. This value is remarkably similar to that obtained in this work, in view of the experimental difficulties involved in measurements of enzyme activity at the low papain concentrations used (40 pM). The association rate constant for the cystatin–papain interaction is of the same order of magnitude as the highest bimolecular rate constants measured for protein–protein interactions and approaches the limit anticipated for a rate controlled by macromolecular diffusion (Alberty & Hammes, 1958; Gutfreund, 1972; Means et al., 1974; Fersht, 1985). Identical association rate constants were observed for cystatins 1 and 2, demonstrating that the small structural difference between the two forms, so far unidentified (Lindahl et al., 1988), negligibly affects the rate of binding of the inhibitor to the enzyme. It is likely that the two forms of the inhibitor also dissociate from their complexes with papain with similar or identical rates, although the dissociation of the complex between cystatin 1 and papain could not be analyzed. Thus, the rate constants for the dissociation of cystatins 1 and 2 from complexes with several inactivated papains are indistinguishable, although higher than those for the dissociation from complexes with the active enzyme (Björk and Ylinenjärvi, unpublished experiments). Furthermore, the two forms have similar inhibition constants for cathepsin B and dipeptidyl peptidase I (Nicklin & Barrett, 1984). The cystatin–papain dissociation rate, although slow, is almost 10-fold faster than that reported for the tightest complex between a small protein inhibitor of serine proteinases and its target enzyme studied so far, that between the basic pancreatic trypsin inhibitor and trypsin (Vincent & Lazdunski, 1972). Together, the association and dissociation rate constants give a dissociation equilibrium constant for the cystatin–papain complex of $\sim 6 \times 10^{-14}$ M (i.e., ~60 fM), similar to that reported for the basic pancreatic trypsin inhibitor–trypsin complex (Vincent & Lazdunski, 1972). This value corresponds to a unitary free energy change (Gurney, 1953; Karush, 1962) for the binding reaction of $\sim -85\ kJ\ mol^{-1}$ and thus reflects a very tight interaction.

The absence of a demonstrable conversion of an initial cystatin–papain complex to a more stable form indicates that no appreciable conformational adaption of either inhibitor or proteinase occurs during complex formation. The moderate enthalpy of activation and the small negative entropy of activation are also compatible with no significant conformational change during the reaction. Thus, the spectral changes accompanying formation of the complex most likely reflect predominantly local interactions affecting aromatic amino acid residues in the manner discussed previously (Lindahl et al., 1988) rather than a change affecting the conformation of either protein.

The effects of pH and ionic strength on the cystatin–papain association rate are comparable with the corresponding effects on the rate of hydrolysis of small substrates by papain (Stockell & Smith, 1957). The decrease of the association rate constant at low and high pH is compatible with involvement of an unprotonated acid group with pK_a 4–4.5 and a protonated basic group with pK_a 9–9.5 in the interaction. However, an effect of low and high pH on the conformation of either cystatin or papain, resulting in a reduced association rate, is also possible. The small decrease of the association rate constant below an ionic strength of ~0.15 may reflect the presence of one or more specific charged groups in the reactive region of either or both proteins. However, it may also be due only to an increased general electrostatic repulsion between the two proteins in the absence of counterions.

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Registry No. Papain, 9001-73-4; cystatin, 81989-95-9.

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