Interaction between Chicken Cystatin and the Cysteine Proteinases Actinidin, Chymopapain A, and Ficin†

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Received June 28, 1989; Revised Manuscript Received September 26, 1989

ABSTRACT: The cysteine proteinase inhibitor cystatin, from chicken egg white, bound with equimolar stoichiometry to the cysteine proteinases actinidin, chymopapain A, and ficin. The changes of near-ultraviolet absorption and fluorescence induced by the binding differed appreciably for the three enzymes, indicating that these spectral changes arise predominantly from aromatic residues in the proteinases. In contrast, the near-ultraviolet circular dichroism changes were similar for all three enzymes, supporting previous evidence that these changes originate mainly from the single tryptophan residue in cystatin, Trp-104. The pseudo-first-order rate constant for the binding increased linearly with the inhibitor concentration up to as high concentrations as could be measured for the three proteinases. This behavior is consistent with the complexes being formed by simple, bimolecular reactions, as was concluded previously for the reaction of cystatin with active and inactivated forms of papain. The second-order association rate constant varied only about 4-fold, from 2.2×10^6 to 9.6×10^6 $M^{-1} \cdot s^{-1}$, for the three enzymes, the higher of these values being similar to that measured previously for the reaction with papain. These observations are consistent with the association rate being governed mainly by the frequency of collision between the binding areas of enzyme and inhibitor. All three cystatin–proteinase complexes dissociated to intact inhibitor, demonstrating reversibility. The dissociation rate constants varied about 20000-fold, from 4.6×10^{-7} s^{-1} for ficin to 1.1×10^{-2} s^{-1} for actinidin, reflecting substantial differences between the enzymes in the nature of the interactions with the inhibitor. The resulting variation in affinity of the inhibitor for the active enzymes, about 100000-fold, was reflected in a corresponding variation in affinity for inactivated enzyme forms. Deductions from published X-ray structures suggest that the considerably weaker binding of cystatin to actinidin ($K_d \sim 5$ nM) than to papain ($K_d \sim 60$ fM) may be due to different interactions between the N-terminal region of cystatin around Leu-8 and the S_2 subsite of the proteinase. Together, the results reinforce previous conclusions that the mechanism of interaction of cystatin with cysteine proteinases is appreciably different from that of reactions between serine proteinases and their protein inhibitors.

The reaction between chicken cystatin, a cysteine proteinase inhibitor from egg white (Fossum & Whitaker, 1968; Keilová & Tomasek, 1974; Anastasi et al., 1983), and papain has been studied as a model for the general mechanism of action of protein inhibitors of cysteine proteinases. The inhibitor forms a tight ($K_d \sim 60$ fM), equimolar complex with papain, in which the active site of the enzyme is inaccessible to substrates (Anastasi et al., 1983; Nicklin & Barrett, 1984; Lindahl et al., 1988; Björk et al., 1989). The X-ray crystal structure of chicken cystatin (Bode et al., 1988) suggests that the binding site of the inhibitor comprises the region around Gly-9, the Gln-Leu-Val-Ser-Gly sequence at residues 53–57, and the region around Trp-104, in agreement with earlier proposals (Ohkubo et al., 1984; Barrett et al., 1986; Abrahamson et al., 1987; Lindahl et al., 1988). These regions form a contiguous, hydrophobic, wedge-shaped edge of the molecule that is complementary to the active-site cleft of papain (Bode et al., 1988). In computer docking experiments, major contacts can be established between this edge and residues of the enzyme remote from the reactive cysteine, mainly Gln-19, Gln-23, Ala-136, and Trp-177, consistent with findings that interactions involving the active-site cysteine are of minor importance for the binding (Anastasi et al., 1983; Björk & Ylinenjärvi, 1989). The kinetics of the interaction are compatible with the complex being formed by a simple, reversible, bimolecular reaction with an association rate constant approaching that expected for a diffusion-controlled rate (Björk et al., 1989). The complex thus apparently is assembled with negligible conformational adaptation of either inhibitor or proteinase. Forms of papain inactivated by covalent attachment of different-size substituents to the active-site cysteine bind to cystatin with similar association rate constants as the active enzyme. However, the dissociation rate constant increases with increasing size of the inactivating group, leading to progressively lower binding affinities (Björk & Ylinenjärvi, 1989).

These findings show that the mechanism of the interaction between chicken cystatin and papain is appreciably different from that of reactions between serine proteinases and their protein inhibitors (Laskowski & Kato, 1980; Bode et al., 1988; Björk et al., 1989; Björk & Ylinenjärvi, 1989). However, the reactions between cystatin and other cysteine proteinases, or between such enzymes and other inhibitors, have not been studied in sufficient detail to allow conclusions whether this is the general mechanism of action of cysteine proteinase inhibitors. To elucidate this problem, we have studied the reaction of chicken cystatin with three other cysteine proteinases, actinidin, chymopapain A, and ficin. Characterization of the reaction with actinidin is of particular interest, since this is the only cysteine proteinase, besides papain, for which a high-resolution three-dimensional structure has been de-

† Supported by research grants from the Swedish Medical Research Council (Project 4212), the Swedish Council for Forestry and Agricultural Research (Project 546/88), the Knut and Alice Wallenberg's Foundation, and the King Gustaf V:s 80th Birthday Fund.

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0006-2960/90/0429-1770$02.50/0

termined (Baker, 1980; Kamphuis et al., 1985).

Materials and Methods

Chicken cystatin, forms 1 and 2, and papain (containing 0.90–1.0 mol of sulfhydryl groups/mol of protein) were purified and stored as described previously (Burke et al., 1974; Anastasi et al., 1983; Roberts et al., 1986; Lindahl et al., 1988). All studies were done with cystatin 1, unless otherwise indicated.

Actinidin was purified from kiwi fruit by the procedure developed by Brocklehurst et al. (1981). The purified enzyme gave one band in polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate (Davis, 1964; Weber & Osborn, 1969) and had a thiol content of 0.73 ± 0.16 (SD, n = 3) mol/mol of protein, as determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[1] (Ellman, 1959; Blumberg et al., 1970). The thiol content of the preparation probably was somewhat underestimated by the latter analysis, due to the slow reaction of actinidin with DTNB (McDowall, 1970). The specific activity, measured at 25 °C, pH 6.0, with 0.1 mM N^{α}-benzyloxycarbonyl-L-lysine p-nitrophenyl ester as substrate, was 67 μmol·min^{-1}·mg^{-1}, identical with that reported by Boland and Hardman (1972) and Brocklehurst et al. (1981). The enzyme was inactivated with methyl methanethiolsulfonate (Smith et al., 1975), stored, and reactivated before use in the same way as papain (Roberts et al., 1986; Lindahl et al., 1988).

Chymopapain A was isolated from a commercial, partially purified chymopapain preparation (Sigma, St. Louis, MO) by ion-exchange chromatography at pH 5.0 on S-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) with the use of the conditions developed by Baines and Brocklehurst (1982) for chromatography on SP-Sephadex (Pharmacia). The major peak of enzyme activity against N^{α}-benzoyl-DL-arginine p-nitroanilinide, shown to elute as chymopapain A in a separate chromatography on SP-Sephadex (Baines & Brocklehurst, 1982), was inactivated with methyl methanethiolsulfonate in a molar ratio of reagent to protein of 2.0. The inactivated enzyme was further purified by chromatography on S-Sepharose Fast Flow at pH 9.0 in the presence of 10 μM methyl methanethiolsulfonate under essentially the conditions used by Buttle and Barrett (1984) for separation on a Mono-S (Pharmacia) column. The purified protein gave one band in polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate (Reisfeld et al., 1962; Weber & Osborn, 1969). The inactivated enzyme was stored and reactivated before use in the same manner as papain (Roberts et al., 1986). The reactivated enzyme had a thiol content of 1.10 ± 0.15 (SD, n = 3) mol/mol of protein, as determined with DTNB. Its specific activity, measured at 40 °C, pH 6.8, with 2.5 mM N^{α}-benzoyl-DL-arginine p-nitroanilinide as substrate, was 0.17 μmol·min^{-1}·mg^{-1}, comparable to the value of 0.15 μmol·min^{-1}·mg^{-1} obtained by Buttle and Barrett (1984).

Ficin was purified from a commercial preparation of fig tree latex (United States Biochemical Corp., Cleveland, OH) by covalent chromatography on thiopropyl-Sepharose 6B (Pharmacia), essentially as described by Malthouse and Brocklehurst (1976). The enzyme eluted from the gel was reversibly inactivated with methyl methanethiolsulfonate in a molar ratio of reagent to protein of 2.0. The different ficin forms were separated by chromatography of the inactivated enzyme on (carboxymethyl)cellulose in the presence of 20 μM methyl methanethiolsulfonate by the procedure described for active ficin (Englund et al., 1968). Form III of the enzyme, the form characterized in most detail (Englund et al., 1968; Malthouse & Brocklehurst, 1976), was used for further analyses. The preparation gave one band in polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate (Reisfeld et al., 1962; Weber & Osborn, 1969). The inactivated enzyme was stored and reactivated before use as described for papain (Roberts et al., 1986). Reaction of the reactivated enzyme with DTNB gave 0.92 ± 0.07 (range, n = 2) mol of thiol groups/mol of protein. The specific activity, measured at 37 °C, pH 7.0, with 0.8 mM N^{α}-benzoyl-DL-arginine p-nitroanilinide as substrate, was 0.04 μmol·min^{-1}·mg^{-1}, somewhat lower than the value of 0.064 μmol·min^{-1}·mg^{-1} calculated from the data given by Englund et al. (1968).

The cysteine proteinases were inactivated with iodoacetamide, iodoacetic acid, and N-ethylmaleimide by the procedures used previously for the inactivation of papain (Björk & Ylinenjärvi, 1989).

Near-ultraviolet absorption difference, circular dichroism, and fluorescence emission spectra of cystatin, proteinase, and the cystatin–proteinase complex were measured as reported previously for the interaction between the inhibitor and papain (Lindahl et al., 1988). Difference spectra were calculated from the circular dichroism and fluorescence emission spectra as described in the same paper. Titrations of active or inactivated enzymes with cystatin for the determination of binding stoichiometries and affinities were monitored by the changes of fluorescence emission intensity accompanying the interactions and were evaluated by nonlinear least-squares regression (Lindahl et al., 1988; Björk & Ylinenjärvi, 1989).

The kinetics of binding of cystatin to the cysteine proteinases were studied under pseudo-first-order conditions (i.e., with an excess of inhibitor) by fluorescence measurements in a Hi-Tech SF-4 stopped-flow spectrophotometer (Hi-Tech, Salisbury, Wilts, England), as detailed previously (Björk et al., 1989).

The kinetics of dissociation of the complexes between cystatin and chymopapain A or ficin were monitored by measurements of the rate of displacement of cystatin 2 from its complexes with the enzymes by an excess of cystatin 1 in the same manner as in the corresponding studies with papain (Björk et al., 1989).

The rate of dissociation of the cystatin–actinidin complex was measured by a displacement method, in which reassociation of proteinase and inhibitor was prevented by addition of an excess of S-(carbamoylmethyl)papain. This inactivated form of papain reacts with cystatin with about a 4-fold higher association rate constant than actinidin (Björk & Ylinenjärvi, 1989; see also Results). Moreover, the dissociation rate constant of the cystatin–actinidin complex was estimated to be at least 25-fold higher than that of the complex between the inhibitor and S-(carbamoylmethyl)papain (which is ~1.1 × 10^{-4} s^{-1}; Björk & Ylinenjärvi, 1989) in preliminary experiments with the method used for chymopapain and ficin. Together, these data indicated at least a 100-fold lower affinity of actinidin than of the inactivated papain for cystatin. An excess of S-(carbamoylmethyl)papain therefore would be expected to efficiently trap the cystatin liberated from the cystatin–actinidin complex, leading to essentially irreversible dissociation of this complex. The rate of appearance of free actinidin was monitored by allowing the reaction to proceed in the presence of substrate and continuously recording the amount of product formed. These experimental conditions, together with the assumptions that dissociation of the complex is a first-order reaction and that substrate cleavage by the enzyme is fast compared to the rate of complex dissociation,

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, (ethylenedinitrilo)tetraacetate; Tris, tris(hydroxymethyl)aminomethane.

lead to the differential equation:

$$dP_t/dt = v_\infty(1 - e^{-k_{-1}t}) + v_0 \quad (1)$$

where P_t is the amount of product formed by substrate cleavage after time t, v_0 is the rate of substrate cleavage at $t = 0$ (i.e., at addition of displacer), v_∞ is this rate at $t = \infty$ (i.e., when all complex has dissociated), and k_{-1} is the first-order dissociation rate constant of the enzyme–inhibitor complex. Integration of this equation, together with the boundary condition that $P_t = 0$ at $t = 0$, gives

$$P_t = (v_\infty/k_{-1})(e^{-k_{-1}t} - 1) + v_\infty t + v_0 t \quad (2)$$

from which k_{-1} can be obtained. The experiments were carried out at 25 °C in the standard buffer (see below), containing 0.1% (w/v) poly(ethylene glycol). Complex was formed by mixing actinidin and cystatin to final concentrations of 200 and 300 nM, respectively, in a total volume of 2 mL. The enzyme concentration was chosen to give optimal experimental precision but less than 5% substrate consumption at the end of the experiment. After 10 min, the fluorogenic proteinase substrate carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) was added to a concentration of 10 μM, and the base-line fluorescence was measured in an SLM 4800S spectrofluorometer (SLM-Aminco, Urbana, IL) with excitation and emission wavelengths of 370 and 440 nm, respectively. The displacement was started by addition of *S*-(carbamoylmethyl)papain in an amount giving an initial molar ratio of free inactivated papain to cystatin–actinidin complex of 2–10, and the resulting fluorescence increase was recorded continuously. The amount of product formed by substrate hydrolysis was calculated from measurements of the fluorescence of a standard of 0.5 μM 7-amino-4-methylcoumarin (Peptide Institute). The dissociation rate constant was obtained by a three-parameter (k_{-1}, v_0, and v_∞) nonlinear least-squares regression of eq 2 to the data.

Cystatin 2 liberated from its complex with actinidin, chymopapain A, or ficin was prepared by displacement with an excess of cystatin 1, followed by isolation of the displaced inhibitor by ion-exchange high-performance liquid chromatography on a Mono-Q column (Pharmacia; Björk et al., 1989). The amino-terminal sequence of the liberated cystatin 2 and the behavior of the radiolabeled protein in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under reducing conditions were analyzed essentially by the procedures described previously (Björk et al., 1989). The only modification was that electrophoresis was done on a homogeneous 15% polyacrylamide gel.

All analyses were made in 50 mM Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 7.4, unless otherwise indicated. Protein concentrations were obtained by absorption measurements at 280 nm. The following absorption coefficients (in liters per gram per centimeter) and molecular weights were used: 0.87 and 13 100 for cystatin (Anastasi et al., 1983; Schwabe et al., 1984), 2.39 and 23 400 for papain (Brocklehurst et al., 1973; Husain & Lowe, 1969), 2.12 and 23 500 for actinidin (McDowall, 1970; Carne & Moore, 1978), 1.79 and 26 000 for chymopapain A (Robinson, 1975; Baines & Brocklehurst, 1982), and 2.1 and 25 000 for ficin (Englund et al., 1968). The absorption coefficients for the active enzymes were used also for inactivated enzymes (Björk & Ylinenjärvi, 1989).

Results

Stoichiometry of Binding. Titrations of actinidin, chymopapain A, or ficin with chicken cystatin at protein concentrations much higher than the dissociation equilibrium constants of the complexes formed were monitored by the changes in intrinsic fluorescence accompanying the binding (see below). These analyses showed that the inhibitor bound to the three proteinases with stoichiometries of 0.93–1.04 (Figure 1), values experimentally indistinguishable from 1.0. Together with the thiol group and electrophoretic analyses (see Materials and Methods), these results indicate that the enzyme preparations were homogeneous and fully active in binding the inhibitor in an equimolar complex.

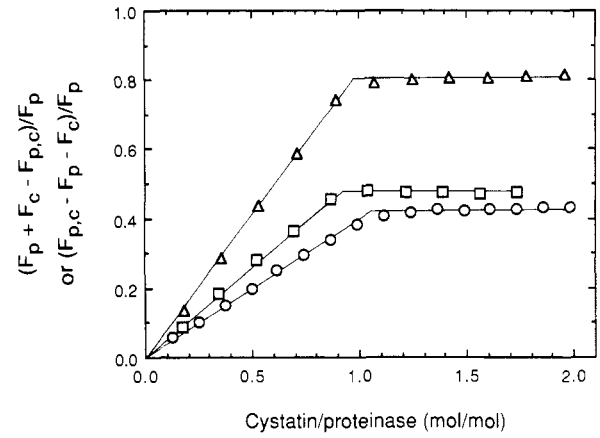

FIGURE 1: Titrations of actinidin, chymopapain A, and ficin with cystatin, monitored by measurements of tryptophan fluorescence. (○) Actinidin; (△) chymopapain A; (□) ficin. The initial proteinase concentration was 1 μM. Excitation was at 280 nm; fluorescence emission was measured at 350 nm in the titrations of actinidin and chymopapain A and at 333 nm in the titration of ficin. The data for actinidin and chymopapain A, the interaction of which with cystatin results in a fluorescence decrease, were plotted as $(F_p + F_c - F_{p,c})/F_p$, while the data for ficin, which binds to the inhibitor accompanied by a fluorescence increase, were plotted as $(F_{p,c} - F_p - F_c)/F_p$. F_p, fluorescence of proteinase; F_c, fluorescence of added cystatin; $F_{p,c}$, fluorescence of the proteinase–cystatin mixture.

Spectroscopic Changes. The changes of near-ultraviolet absorption, circular dichroism, and fluorescence induced by the interaction of cystatin with actinidin, chymopapain A, or ficin were analyzed at a molar ratio of inhibitor to enzyme of 1.2. This ratio was sufficient to give ≥98% saturation of the enzymes under the conditions used, as calculated from the equilibrium constants presented below.

Absorption difference spectra between the cystatin–proteinase complexes and the free proteins differed appreciably for the three enzymes (Figure 2). Although the spectra also were different from that measured for the cystatin–papain interaction (Lindahl et al., 1988), certain features of the spectra for chymopapain A and ficin, particularly the minima at ~300 and ~289 nm and the maxima at ~292 and ~285 nm, were reminiscent of the papain spectrum. The spectrum for actinidin, however, markedly deviated from the other three spectra, particularly in having a broad maximum at ~300 nm and peaks of low magnitude at lower wavelengths.

In contrast with this behavior, the near-ultraviolet circular dichroism difference spectra resulting from the cystatin–proteinase interactions were reasonably similar for the three proteinases, although certain minor differences were apparent (Figure 3). The spectra thus all had major minima at ~280 nm and shoulders or minor minima at ~287 nm. The spectra were also similar to that measured for the interaction of cystatin with papain (Lindahl et al., 1988).

Fluorescence studies showed that the interaction of cystatin with actinidin and chymopapain A was accompanied by a blue shift and a quenching of tryptophan fluorescence, together giving a maximal decrease in intensity at ~350 nm (Figure

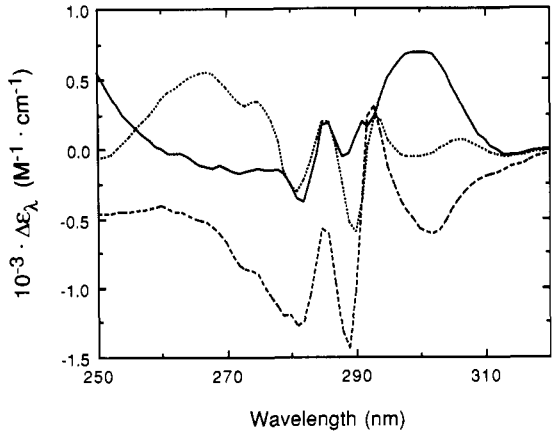

FIGURE 2: Near-ultraviolet absorption difference spectra measured between complexes of cystatin with actinidin, chymopapain A, or ficin and the free proteins. (—) Actinidin; (···) chymopapain A; (---) ficin. The proteinase concentration was 15 μM, and the molar ratio of cystatin to proteinase was 1.2.

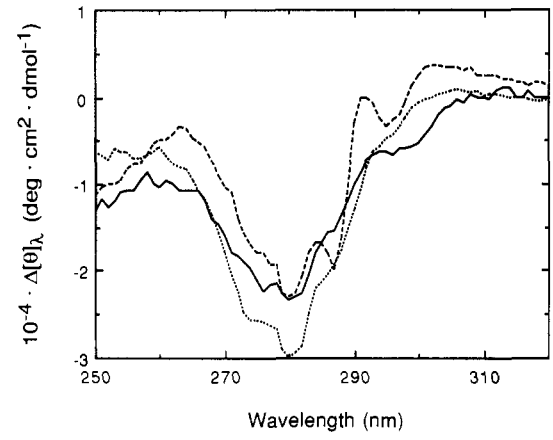

FIGURE 3: Near-ultraviolet circular dichroism difference spectra between complexes of cystatin with actinidin, chymopapain A, or ficin and the free proteins. (—) Actinidin; (···) chymopapain A; (---) ficin. The difference spectra were calculated from separately measured circular dichroism spectra of the complexes and the free proteins (Lindahl et al., 1988). The proteinase concentration was 15 μM, and the molar ratio of cystatin to proteinase was 1.2.

4). These changes were similar to those observed for the cystatin–papain interaction, although the relative decreases in fluorescence intensity were different from that given by papain (Figure 1; Lindahl et al., 1988). In contrast, the binding of cystatin to ficin produced a fluorescence increase with a minimal shift of the emission maximum, resulting in a maximal fluorescence enhancement at ~333 nm (Figure 4).

Kinetics of Association and Dissociation. The kinetics of binding of cystatin to actinidin, chymopapain A, and ficin were studied under pseudo-first-order conditions, i.e., at a molar ratio of cystatin to proteinase of 10:1, and were monitored by the fluorescence changes accompanying the interactions. The cystatin concentration was increased as high as possible, the limit being set by the dead time of the stopped-flow instrument (~2 ms). The approach of the fluorescence signal to its final value was a first-order process at all cystatin concentrations. Moreover, for all three proteinases, the observed pseudo-first-order rate constant increased linearly with cystatin concentration throughout the concentration range covered (Figure 5). The second-order association rate constant at 25 °C, pH 7.4, ionic strength 0.15, for the cystatin–ficin interaction, calculated from these data, was similar to that of the binding of the inhibitor to papain (which is 9.9×10^6 $M^{-1} \cdot s^{-1}$; Björk et al., 1989), while the corresponding rate constants for the reaction of cystatin with actinidin and chymopapain A were about 4-fold lower (Table I). The intercepts of the regression lines on the k_{obsd} axis were experimentally indistinguishable from zero, consistent with very low dissociation rate constants.

The rates of dissociation of the complexes between cystatin and chymopapain A or ficin were monitored by the method used previously for the determination of the dissociation rate

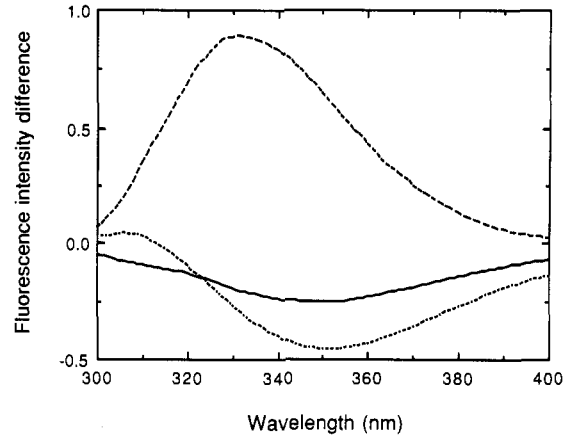

FIGURE 4: Fluorescence emission difference spectra between complexes of cystatin with actinidin, chymopapain A, or ficin and the free proteins. (—) Actinidin; (···) chymopapain A; (---) ficin. The difference spectra were calculated from separately measured corrected emission spectra of the complexes and the free proteins (Lindahl et al., 1988). Before these calculations, all spectra were normalized to a fluorescence intensity for actinidin of 1.0 at the wavelength of the emission maximum. The proteinase concentration was 1 μM, and the molar ratio of cystatin to proteinase was 1.2.

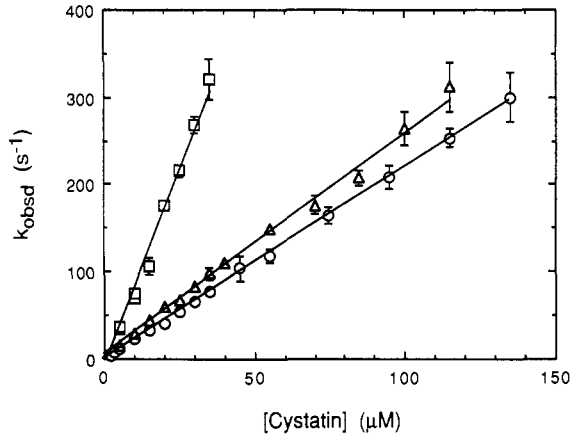

FIGURE 5: Observed pseudo-first-order rate constants (k_{obsd}) for the binding of cystatin to actinidin, chymopapain A, and ficin as a function of cystatin concentration. (○) Actinidin; (△) chymopapain A; (□) ficin. The analyses were done at 25 °C, pH 7.4, ionic strength 0.15. The molar ratio of cystatin to proteinase was maintained at 10:1 in all reactions. The vertical bars represent the 95% confidence intervals computed from four to eight individual experiments.

Table I: Association Rate Constants (k_{+1}), Dissociation Rate Constants (k_{-1}), and Calculated Dissociation Equilibrium Constants ($K_d = k_{-1}/k_{+1}$) for the Binding of Cystatin to Actinidin, Chymopapain A, and Ficin[a]

enzyme	$10^{-6} \times k_{+1}$ ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	K_d (M)
actinidin	2.2 ± 0.05 (16)	(1.1 ± 0.2) × 10^{-2} (9)	5 × 10^{-9}
chymopapain A	2.6 ± 0.1 (14)	(2.4 ± 0.2) × 10^{-6} (2)	9 × 10^{-13}
ficin	9.6 ± 0.8 (9)	(4.6 ± 0.1) × 10^{-7} (2)	5 × 10^{-14}

[a] The analyses were done at 25 °C, pH 7.4, ionic strength 0.15. The values are given with their 95% confidence limits, and the number of measurements in parentheses, or with the range if the value is an average of two measurements.

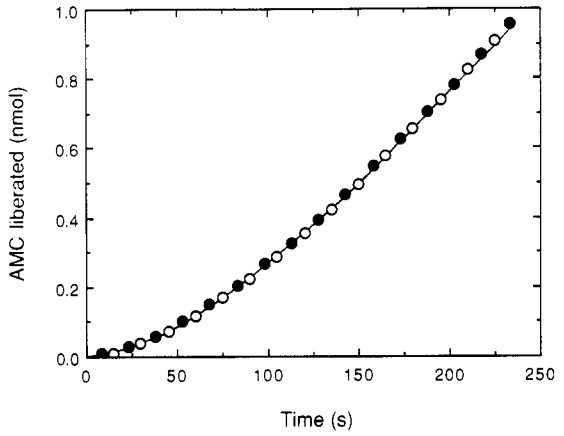

FIGURE 6: Kinetics of dissociation of the cystatin–actinidin complex, monitored by the appearance of actinidin activity. The analyses were done at 25 °C, pH 7.4, ionic strength 0.15. Essentially irreversible dissociation of the complex was induced by trapping the liberated inhibitor with an excess of *S*-(carbamoylmethyl)papain. The appearance of actinidin activity was continuously monitored in the presence of a fluorogenic substrate (see Materials and Methods). AMC, 7-amino-4-methylcoumarin produced by actinidin cleavage of the substrate. The initial molar ratio of free *S*-(carbamoylmethyl)papain to cystatin–actinidin complex was 2.0 (○) and 10.0 (●). The solid line represents the nonlinear least-squares fit of eq 2 to the data, giving $k_{-1} = 0.0122\ s^{-1}$, $v_0 \approx 0$, and $v_\infty = 5.4\ pmol \cdot s^{-1}$.

constants of complexes between the inhibitor and active or inactivated forms of papain (Björk et al., 1989; Björk & Ylinenjärvi, 1989). In this procedure, cystatin 2 was displaced from its complexes with the two proteinases by a 10-fold excess of cystatin 1, and the liberated inhibitor was quantified by high-performance liquid chromatography on an ion-exchange column. A time-dependent appearance of free cystatin 2 compatible with a first-order reaction was observed for the complexes with both proteinases. The dissociation rate constants obtained (Table I) correspond to half-lives of ~3.3 and ~17 days for the complexes of cystatin with chymopapain A and ficin, respectively.

The rate of dissociation of the cystatin–actinidin complex was too high to be monitored by the method used for the complexes with chymopapain A and ficin. Instead, the dissociation rate constant of this complex was obtained from studies of the displacement of active actinidin from the complex by an excess of an inactivated form of papain, *S*-(carbamoylmethyl)papain, which binds consideraby tighter to cystatin than actinidin (Björk & Ylinenjärvi, 1989). The release of actinidin was monitored by continuously recording the amount of product formed by hydrolysis of a fluorogenic substrate by the liberated enzyme. The rate of product formation, and hence the rate of appearance of free actinidin, was independent of the concentration of the displacing inactivated papain (Figure 6). This behavior indicates that the concentration of the latter was sufficiently high to effectively trap all the cystatin dissociating from the complex and thus to give a rate constant well approximating the true dissociation rate constant of the complex. The data could be well fitted to eq 2, which describes the time dependence of product formation resulting from a first-order dissociation reaction (Figure 6). This fit gave a dissociation rate constant of the cystatin–actinidin complex corresponding to a half-life of ~1 min (Table I). The final rate of product formation (Figure 6) was identical, within experimental error, with the rate given by the amount of free actinidin used to form the complex, demonstrating that all complex had dissociated at the end of the experiment.

Dissociation equilibrium constants for the three cystatin–proteinase complexes were calculated from the association and dissociation rate constants (Table I) and ranged from ~50 fM for the complex with ficin to ~5 nM for the actinidin complex.

Table II: Dissociation Equilibrium Constants (in nM) for the Binding of Cystatin to Inactivated Forms of Actinidin, Chymopapain A, and Ficin[a]

	inactivating group			
enzyme	*S*-(methylthio)	*S*-(carbamoylmethyl)	*S*-(carboxymethyl)	*S*-(*N*-ethylsuccinimidyl)
actinidin	12 ± 7 (8)	35 ± 17 (3)	170 ± 50 (3)	>10000
chymopapain A	≤1	ND[b]	≤1	120 ± 30 (3)
ficin	≤1	ND	≤1	310 ± 70 (8)

[a] The analyses were done at 25 °C, pH 7.4, ionic strength 0.15. The values are given with their 95% confidence limits and the number of measurements in parentheses. [b] ND, not determined.

Analyses of Dissociated Inhibitor. Cystatin 2 was displaced from its complex with actinidin, chymopapain A, or ficin by incubation with a 10-fold excess of cystatin 1 for ~30 min, ~4 days, and ~8 days, respectively, and was isolated by ion-exchange high-performance liquid chromatography (Björk et al., 1989). The amino-terminal sequence of the inhibitor liberated from all three complexes was Ser-Glu-Asp-, identical with that of intact cystatin 2 (Schwabe et al., 1984; Lindahl et al., 1988). Moreover, in all three cases, the released inhibitor, labeled with ^{125}I, migrated identically with unlabeled, authentic cystatin 2 in sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions, when the labeled and unlabeled proteins were mixed.

Binding of Inactivated Enzymes. Actinidin, chymopapain A, and ficin were inactivated by reaction of the active-site thiol group with reagents of increasing size, as in previous studies with papain (Björk & Ylinenjärvi, 1989). The affinities between cystatin and the inactivated enzymes were measured by titrations, monitored by the fluorescence changes induced by the interactions, at protein concentrations of 50–500 nM, comparable to the dissociation equilibrium constants of the complexes. Cystatin bound considerably weaker to inactivated forms of actinidin than to the corresponding inactivated forms of the other two enzymes (Table II). The affinity of the inhibitor for these inactivated actinidins decreased with increasing size of the inactivating group in a manner similar to that observed for inactivated forms of papain (Björk & Ylinenjärvi, 1989). The affinity of cystatin for chymopapain A or ficin inactivated with the *S*-(*N*-ethylsuccinimidyl) group (Table II) was comparable to that for *S*-(*N*-ethylsuccinimidyl)papain (Björk & Ylinenjärvi, 1989). The affinities between the inhibitor and the other three inactivated forms of these two enzymes (Table II) were too tight to be determined by fluorescence titrations, as was also found previously for the corresponding inactivated forms of papain (Björk & Ylinenjärvi, 1989).

Discussion

These results show that the reaction of chicken cystatin with the cysteine proteinases actinidin, chymopapain A, and ficin, like the reaction with papain (Björk et al., 1989), is best described by the simple, one-step, reversible bimolecular mechanism:

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

where P is proteinase, I inhibitor, and PI their complex. As expected for this mechanism, the pseudo-first-order rate constant for complex formation, measured with excess inhib-

itor, increased linearly with the inhibitor concentration up to the highest concentration that could be studied with the stopped-flow method for the reactions with all three proteinases. Moreover, the dissociation of the three complexes was consistent with first-order reactions (even though this could be rigorously shown only for the complex with actinidin, due to the slow dissociation of the other two complexes) and produced intact inhibitor. A reversible, bimolecular mechanism is in agreement with the model proposed for the binding of cystatin to papain, based on computer docking experiments (Bode et al., 1988). This model thus suggests that a tight complex can be formed with negligible conformational adaptation of either protein. Moreover, in the model, cystatin is bound to the enzyme in a manner different from that of a substrate, making cleavage of the inhibitor unlikely.

The data for the interaction of cystatin with all cysteine proteinases studied so far thus agree with a simple, bimolecular mechanism, a reaction model different from the standard mechanism for serine proteinase inhibitors (Luthy et al., 1973; Quast et al., 1974; Laskowski & Kato, 1980; Björk et al., 1989). However, a two-step binding reaction similar to the latter mechanism cannot be completely excluded. This reaction scheme, in which a Michaelis-type complex is initially formed, followed by conversion of this intermediate to the stable complex, would result in a hyperbolic dependence of the observed pseudo-first-order rate constant on cystatin concentration (Fersht, 1985; Björk et al., 1989). Conceivably, only the initial, approximately linear portion of this curve may have been accessible in our studies. However, as discussed previously for the reaction with papain (Björk et al., 1989), in this case the rate constant for stabilization of the complex must be high ($>5000\ s^{-1}$) and the initial complex very weak ($K_d >$ 2 mM for actinidin and chymopapain A).

Despite the similarity of the general mechanism of the reactions between cystatin and actinidin, chymopapain A, and ficin, several differences between the interactions are apparent. The near-ultraviolet absorption and fluorescence changes accompanying the binding of the inhibitor to the three proteinases thus were considerably different, consistent with these spectral changes originating largely from interactions involving aromatic amino acid residues in the proteinases. In contrast, similar changes of near-ultraviolet circular dichroism were induced by the interaction of cystatin with the three enzymes and with papain (Lindahl et al., 1988), supporting the conclusion from earlier studies by chemical modification that these changes arise primarily from perturbations around the only tryptophan of the inhibitor, Trp-104 (Lindahl et al., 1988). Moreover, the similarity of the spectra is consistent with this residue interacting with homologous structures in the enzymes. The rate constants for the reactions of cystatin with the three proteinases also differed appreciably. The magnitude and moderate 4-fold variation of the association rate constants is consistent with the association rate being governed predominantly by the frequency of collision between the binding areas of enzyme and inhibitor (Björk et al., 1989), the differences most likely mainly reflecting different sizes of these areas in the three proteinases. However, besides a limited target area, steric interference with complex formation may also be responsible for the variation in association rate constants and for these constants being lower than that theoretically calculated for a diffusion-controlled reaction, $\sim 10^9\ M^{-1}\cdot s^{-1}$ (Jencks, 1969; Gutfreund, 1972). The variation in dissociation rate constants was considerably larger, about 20000-fold, and must be due to substantial differences between the three enzymes in the nature of the interactions with the inhibitor. As a consequence of the different binding kinetics, the affinity of cystatin for the three enzymes differed about 100000-fold. A corresponding variation in affinity was apparent also for the binding of cystatin to inactivated forms of the three proteinases, consistent with previous conclusions from studies with papain that interactions of considerable strength occur between the inhibitor and regions of the enzymes outside the reactive cysteine (Anastasi et al., 1983; Björk & Ylinenjärvi, 1989). The ability of cystatin to bind with appreciable affinity to enzymes with large inactivating groups distinguishes it further from serine proteinase inhibitors, which generally do not bind to proteinases inactivated with bulky substituents (Foster & Ryan, 1965; Feinstein & Feeney, 1966; Travis & Salvesen, 1983). An exception is hirudin, which binds with appreciable affinity to active-site-blocked derivatives of thrombin (Markwardt, 1970; Stone & Hofsteenge, 1986).

A comparison of cystatin binding to papain (Lindahl et al., 1988; Björk et al., 1989; Björk & Ylinenjärvi, 1989) and actinidin is of particular interest, since the three-dimensional structures of these two cysteine proteinases are known from X-ray crystallography (Drenth et al., 1968, 1971; Baker, 1980; Kamphuis et al., 1985). These studies have shown that the active-site regions of the two enzymes are highly similar, the only significant differences being restricted to the hydrophobic specificity pocket, the S_2 subsite (Berger & Schechter, 1970). The major change is that the position of Ser-205 at the end of this subsite in papain is occupied by Met-211 in actinidin, making the pocket noticeably shorter (Baker, 1980). Other differences are that Trp-69 in papain is replaced by Tyr-69 in actinidin and that Pro-68, Phe-207, and Val-133 in papain become Ile-70, Ser-213, and Ala-136, respectively, in actinidin (Baker et al., 1980). Because the only structural changes of importance occur in the S_2 subsite, it is likely that any differences in the binding of cystatin to papain and actinidin arise mainly from interactions between the inhibitor and this region of the enzymes. The model for the cystatin–papain interaction, although proposed from X-ray crystallography studies of a form of the inhibitor lacking an amino-terminal octapeptide, suggests that Leu-8 in the amino-terminal segment of intact cystatin would interact with papain in the S_2 subsite (Bode et al., 1988). The importance of this segment of the inhibitor in the binding to papain is supported by the lower affinity of amino-terminally truncated cystatin forms (Abrahamson et al., 1987; Machleidt et al., 1989). Cystatin binds substantially stronger to papain ($K_d \sim 60$ fM; Björk et al., 1989) than to actinidin ($K_d \sim 5$ nM), the difference in affinity corresponding to a difference in the unitary free energy of binding (Gurney, 1953; Karush, 1962) from ~ -85 to $\sim -57\ kJ\cdot mol^{-1}$. This finding thus indicates that interactions between Leu-8 in cystatin and the S_2 subsite of papain confer appreciably stability to the cystatin–papain complex, whereas the corresponding interactions in the complex with actinidin are much weaker. However, the different electrostatic fields in the active-site clefts of papain and actinidin (Pickersgill et al., 1988) may also contribute to the different binding energies. A further inference from a comparison of cystatin binding to the two proteinases is that the replacement of Trp-69 in the S_2 subsite of papain by Tyr in actinidin most likely is reflected in the different near-ultraviolet absorption and fluorescence changes seen with the two enzymes. This deduction is in agreement with previous evidence that Trp-69 in papain is involved in the interaction with the inhibitor (Lindahl et al., 1988). Those features of the spectra for the cystatin–actinidin complex that are consistent with perturbations of the environment of tryptophan residues presumably arise from in-

teractions involving Trp-104 of cystatin and Trp-184 of actinidin. The latter residue is homologous to Trp-177 of papain, which has been shown previously to be affected by the binding (Lindahl et al., 1988).

Registry No. Cystatin, 81989-95-9; actinidin, 39279-27-1; chymopapain A, 9001-09-6; ficin, 9001-33-6; cysteine proteinase, 37353-41-6.

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