Different Roles of the Two Disulfide Bonds of the Cysteine Proteinase Inhibitor, Chicken Cystatin, for the Conformation of the Active Protein†

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ABSTRACT: The Cys-71–Cys-81 disulfide bond of the cysteine proteinase inhibitor, chicken cystatin, was specifically reduced by thioredoxin or low concentrations of dithiothreitol. This cleavage, followed by S-carbamoylmethylation, induced a conformational change of the protein, as evidenced by changes in isoelectric point and circular dichroism spectra and by an increased susceptibility to digestion by nontarget proteinases. The proteinase binding ability and the immunological properties of the inhibitor, however, were not detectably altered, indicating that the conformational change was limited to the region around the disrupted bond. In contrast, reduction of both disulfide bonds of cystatin by higher concentrations of dithiothreitol and subsequent alkylation led to the slow conversion of the inhibitor into two forms lacking proteinase binding ability, indicative of more extensive conformational changes. Together, these results suggest that the less accessible Cys-95–Cys-115 disulfide bond of chicken cystatin, but not the more accessible Cys-71–Cys-81 bond, is of importance for maintaining the conformation of the inhibitor required for binding of target proteinases.

Most protein inhibitors of cysteine proteinases in mammalian organisms belong to the cystatin superfamily, which comprises three individual families, designated I, II, and III. The members of families I and II, the cystatins, are small, nonglycosylated proteins ($M_r \sim 11\,000$–$14\,000$) found mainly in tissues and secretions, whereas inhibitors of family III, the kininogens, are larger glycoproteins ($M_r \sim 60\,000$–$120\,000$) in blood plasma (Barrett et al., 1986). The cystatins of family I and II are structurally related but differ in certain aspects. Family I cystatins (also called stefins) are polypeptides of about 100 residues without disulfide bridges, whereas family II cystatins are somewhat longer, about 120 residues, and have two disulfide bridges (Barrett et al., 1986). The best characterized members of the latter family are chicken cystatin and human cystatin C. The two inhibitors inactivate mammalian cysteine proteinases, such as cathepsins B, H, and L, and several structurally similar plant proteinases, e.g., papain and actinidin, by forming tight ($K_d \sim 20$ nM–10 fM) equimolar complexes, blocking the active site of the enzymes (Anastasi et al., 1983; Nicklin & Barrett, 1984; Abrahamson et al., 1986, 1987, 1991; Lindahl et al., 1988, 1992a; Björk & Ylinenjärvi, 1990). The kinetics of these interactions are consistent with the complexes being formed by simple, reversible, bimolecular reactions with association rate constants approaching those of a diffusion-controlled rate (Björk et al., 1989; Björk & Ylinenjärvi, 1990; Lindahl et al., 1992a). This finding is in agreement with the crystal structures of chicken cystatin and of a complex of a family I cystatin with papain (Bode et al., 1988; Stubbs et al., 1990). These structures thus show that the proteinase binding site comprises three regions of the polypeptide chain, together forming a wedge-shaped edge of the inhibitor that fits into the active-site cleft of papain with minimal conformational changes of either protein.

The two disulfide bonds of chicken cystatin are both located in the carboxy-terminal half of the protein sequence (Grubb et al., 1984). The Cys-71–Cys-81 bond links a small segment of α-helical structure to the main β-sheet of the protein, and the Cys-95–Cys-115 bond joins the two carboxy-terminal strands of this sheet (Bode et al., 1988). In this work, we have assessed the importance of the two bonds for the structural integrity of the protein. We show that specific reduction of the more accessible Cys-71–Cys-81 bond by thioredoxin or dithiothreitol, followed by S-carbamoylmethylation, leads to a limited conformational change of the inhibitor which, however, does not detectably alter the proteinase binding ability. In contrast, following reduction of both disulfide bonds by dithiothreitol and subsequent alkylation, the inhibitor is slowly converted to species lacking proteinase inhibitory activity. These findings indicate that the Cys-95–Cys-115 disulfide bond, but not the Cys-71–Cys-81 bond, of cystatin is of importance for maintaining the active conformation of the inhibitor.

MATERIALS AND METHODS

Proteins. Forms 1 and 2 of chicken cystatin were purified and stored as described previously (Anastasi et al., 1983; Lindahl et al., 1988). The two forms have the same amino acid sequence and are functionally identical but differ in their isoelectric points, owing to Ser-80 being phophorylated in cystatin 2 (Lindahl et al., 1988; Björk et al., 1989; Laber et al., 1989). Most experiments were done with cystatin 1.

Papain (EC 3.4.22.2) was purified from papaya latex (Sigma, St. Louis, MO) (Burke et al., 1974; Lindahl et al., 1988). The enzyme (containing 0.9–1.0 mol of thiol groups/mol of protein) was stored as its inactive *S*-methylthio derivative and was activated for 15 min with 1 mM dithiothreitol before use (Roberts et al., 1986; Lindahl et al., 1988). *S*-(*N*-Ethylsuccinimidyl)papain was prepared by reaction with *N*-ethylmaleimide as described earlier (Björk & Ylinenjärvi, 1989).

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)

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and 23 400 (Husain & Lowe, 1969) were used for cystatin and papain, respectively.

Reduction with the Thioredoxin System. Chicken cystatin 1 or 2 (40 μM) was reduced with 5 μM thioredoxin, 0.1 μM thioredoxin reductase (both from IMCO Co., Stockholm, Sweden) and 0.4 mM NADPH at 25.0 °C in 0.05 M Tris[1]-HCL, 0.1 M NaCl, and 100 μM EDTA, pH 7.4. The extent of the reduction was monitored by continuous measurements of the decrease in absorbance at 340 nm due to the consumption of NADPH (Holmgren, 1984). At the end of the experiment, i.e., after 10 or 50 min in the reactions with cystatin 1 or 2, respectively, the liberated thiol groups were blocked by reaction with 1 mM iodoacetamide for 10 min in the dark. The reduced and S-carbamoylmethylated protein was isolated by an initial separation from low-molecular-mass reactants on Sephadex G-50 Superfine (Pharmacia LKB Biotechnology, Uppsala, Sweden). A subsequent ion-exchange chromatography on a column (0.5 × 5.5 cm) of DEAE-Sephacel (Pharmacia LKB), eluted with a linear gradient (total volume of 20 mL) from 0.02 M Tris-HCl and 0.02 M NaCl, pH 8.0, to the same buffer containing 0.2 M NaCl, further separated the reduced and alkylated cystatin 1 or 2 (which appeared in the breakthrough fraction and at ~0.07 M NaCl, respectively) from thioredoxin and thioredoxin reductase. In most preparations, the reduced and alkylated cystatin was finally separated from a small amount (<10%) of unreduced protein by ion-exchange chromatography on a 1-mL Mono-Q column (Pharmacia LKB), eluted with a linear gradient (total volume of 20 mL) from 0.02 M Tris-HCl, pH 7.4, to the same buffer containing 0.2 M NaCl. Reduced and alkylated cystatin 1 and 2 appeared at ~0.02 and ~0.1 M NaCl, respectively, slightly ahead of the corresponding form of the intact protein.

For determination of the number of disulfide bonds cleaved and identification of these bonds, cystatin 1 that had been reduced by the thioredoxin system for 10 min under the conditions described above was alkylated with 5 mM iodo-[2-^{14}C]acetic acid (sp act. 37 GBq/mol) for 30 min in the dark (Holmgren, 1984). The number of disulfide bonds reduced was calculated from liquid scintillation measurements of 100-μL portions of 35–49 μM protein, isolated by chromatography on Mono-Q as described above, and 10–130 μM iodo[2-^{14}C]acetic acid standards. The purified, labeled protein was denatured in guanidinium chloride, reduced with 10 mM dithiothreitol, carboxymethylated, and digested with trypsin as described previously (Björk & Jörnvall, 1986). The tryptic peptides were separated by reversed-phase HPLC on a TSK ODS-120T Ultropac column (0.46 × 25 cm; Pharmacia LKB), eluted at 1 mL/min with a combination of linear gradients of acetonitrile (0–20% in 20 min, 20–30% in 30 min, 30–50% in 20 min, and 50–70% in 10 min) in 0.1% trifluoroacetic acid. Labeled peptides were identified by liquid scintillation, and amino-terminal sequences of these peptides were determined by degradation in an Applied Biosystems (Foster City, CA) 470A gas-phase sequencer, connected on-line to a 120A PTH analyzer.

Reduction with Dithiothreitol. Cystatin 1 or 2 (100 μM) was reduced with 1 mM dithiothreitol at 25 °C in 0.02 M Tris-HCl and 1 mM EDTA, pH 9.0. The progress of the disulfide bond cleavage was monitored by the appearance of protein thiol groups. 5,5′-Dithiobis(2-nitrobenzoic acid) was added at a final concentration of 100 μM to 10-fold dilutions of portions of the reaction mixture, removed at different times, into 0.05 M Tris-HCl, pH 8.1, containing sodium arsenite at a final concentration of 2.5 mM to complex the excess dithiothreitol (Zahler & Cleland, 1968; Grubb et al., 1984). The absorption at 412 nm was measured continuously, and the number of protein thiol groups was determined from the absorption extrapolated to zero time (Ellman, 1959; Zahler & Cleland, 1968). The number of thiol groups produced in cystatin 1 after a 60-min reduction was also determined by removing excess dithiothreitol by gel chromatography on Sephadex G-25 in 0.02 M acetic acid and measuring the absorbance at 412 nm after addition of a solution of the reduced protein to an equal volume of 650 μM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.2 M Tris-HCl and 1 mM EDTA, pH 8.0.

For identification of the disulfide bonds cleaved by 1 mM dithiothreitol, cystatin 1 was reduced for 45 min under the conditions given above and was then alkylated with 7 mM iodo[2-^{14}C]acetic acid as described for the reduction with thioredoxin. After gel chromatography of the reduced and alkylated protein on Sephadex G-25, followed by dialysis overnight, labeled tryptic peptides were isolated and sequenced as in the corresponding analyses of the thioredoxin-reduced protein.

Cystatin 1 (100 μM) was also reduced with 10 mM dithiothreitol for 120 min at 25 °C in 0.02 M Tris-HCl and 1 mM EDTA, pH 9.0. The number of disulfide bonds cleaved was determined by removing excess reducing agent from a portion of the reaction mixture by gel chromatography in 0.02 M acetic acid and measuring the liberated thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid), as described for the reaction with 1 mM dithiothreitol. The remainder of the reduced protein was S-carbamoylmethylated by reaction with 25 mM iodoacetamide for 30 min in the dark in 0.12 M Tris-HCl and 1 mM EDTA, pH 8.2, followed by overnight dialysis at 4 °C against 0.02 M Tris-HCl, pH 7.4. The reduced and alkylated protein was subjected to ion-exchange chromatography on a 1-mL Mono-Q column, eluted with a linear gradient (total volume of 20 mL) from 0 to 0.2 M NaCl in 0.02 M Tris-HCl, pH 7.4.

Spectroscopic, Equilibrium Binding, and Kinetics Methods. Near-ultraviolet absorption difference spectra, near-ultraviolet and far-ultraviolet circular dichroism spectra, and fluorescence emission spectra of intact or reduced and alkylated cystatin, papain, and the inhibitor–papain complexes were measured as reported previously (Lindahl et al., 1988; Björk & Ylinenjärvi, 1989). Difference spectra were calculated from measured circular dichroism and fluorescence emission spectra as described by Lindahl et al. (1988).

Titrations of active or inactivated papain with reduced and alkylated cystatin for the determination of binding stoichiometries or affinities were monitored by the decrease of fluorescence emission intensity accompanying the interactions (Lindahl et al., 1988; Björk & Ylinenjärvi, 1989). Stoichiometries were determined for the interaction with active papain at proteinase concentrations of 1–2 μM. Affinities were measured for the binding to *S*-(*N*-ethylsuccinimidyl)papain at concentrations of inactivated enzyme comparable with the dissociation equilibrium constant of the complex formed. The fluorescence emission was measured at the wavelength of the maximum change (350 nm) with excitation at 280 nm. Stoichiometries and dissociation constants were obtained by non-linear least-squares regression of the titration curves (Nordenman & Björk, 1978; Lindahl et al., 1988).

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

The kinetics of association of reduced and alkylated cystatin with papain were studied by stopped-flow fluorometry at cystatin concentrations from 0.2 to 4 μM under pseudo-first-order conditions (i.e., with an excess of inhibitor), as detailed previously (Björk et al., 1989). The kinetics of dissociation of the complex between reduced and alkylated cystatin and papain were monitored by measurements of the rate of displacement of the reduced and alkylated form 2 of the inhibitor from its complex with the enzyme by an excess of intact cystatin 1 in the same manner as described earlier (Björk et al., 1989).

All spectroscopic, equilibrium binding and kinetic studies were made at 25 °C in 0.05 M Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 7.4.

Miscellaneous Procedures. Immunodiffusion was done in a 1% agarose gel in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4, with an antiserum against intact chicken cystatin (generously donated by Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, England).

Analytical isoelectric focusing was performed with the PhastSystem (Pharmacia LKB) on precast gels covering the pH range 5–8. Isoelectric points were determined by comparisons of the focusing positions of the samples with those of standard proteins (Pharmacia LKB).

Intact or reduced and alkylated cystatin (75–90 μM) were digested with subtilisin or thermolysin at 25 °C in 0.05 M Tris-HCl, 0.1 M NaCl, and 2 mM $CaCl_2$, pH 7.4, at an enzyme to cystatin weight ratio of 1:100. At the end of the digestions, subtilisin and thermolysin were inactivated by 100 μM phenylmethanesulfonyl fluoride or 4 mM EDTA, respectively. After addition of SDS to 1% (w/v), the samples were heated to 100 °C and were then analyzed by SDS–PAGE on 15% slab gels under reducing conditions.

The proteinase inhibitory activity of intact or reduced and alkylated cystatin was determined by incubating appropriate concentrations of the inhibitor with 40 nM papain for 2 min in 0.05 M Tris-HCl, 0.1 M NaCl, 100 μM EDTA, 100 μM dithiothreitol, and 0.1% (w/v) poly(ethylene glycol), pH 7.4. The remaining papain activity was then measured by diluting a portion of the reaction mixture into a 5 μM solution of the papain substrate, carbobenzoxy-L-phenylalanyl-L-arginine (4-methylcoumarinyl-7-amide) (Peptide Institute, Osaka, Japan), in the Tris buffer and measuring the increase in fluorescence emission at 440 nm (with excitation at 370 nm) for 2–5 min.

RESULTS

Specific Reduction of the Cys-71–Cys-81 Disulfide Bond by Thioredoxin or Dithiothreitol. (a) Reduction with Thioredoxin. Reduction of cystatin 1 and 2 by 5 μM thioredoxin resulted in cleavage of 0.95–1.0 of the two disulfide bonds in both forms of the inhibitor, as monitored by the decrease in absorbance at 340 nm due to the consumption of NADPH (Figure 1A). However, cystatin 2 was reduced almost 10-fold more slowly by thioredoxin than cystatin 1. Prolonged reaction, up to several hours, did not increase the amount of disulfide bonds reduced in either form of the protein.

Reaction of cystatin 1, reduced for 10 min by the thioredoxin system, with iodo[2-^{14}C]acetic acid gave an incorporation of 1.95 ± 0.1 (SD; n = 3) mol of *S*-carboxymethyl groups/mol of protein, confirming that one disulfide bond had been reduced. More than 90% of the radioactivity was recovered in two tryptic peptides, which had the amino-terminal sequences Thr-Thr-Gln/CmCys-Pro-Lys and Ser-Ser-Gly-Asp-Leu-. Comparison with the known sequence and disulfide bond arrangement of chicken cystatin (Schwabe et al., 1984; Grubb et al., 1984) showed that the Cys-71–Cys-81 disulfide bond thus was cleaved by thioredoxin.

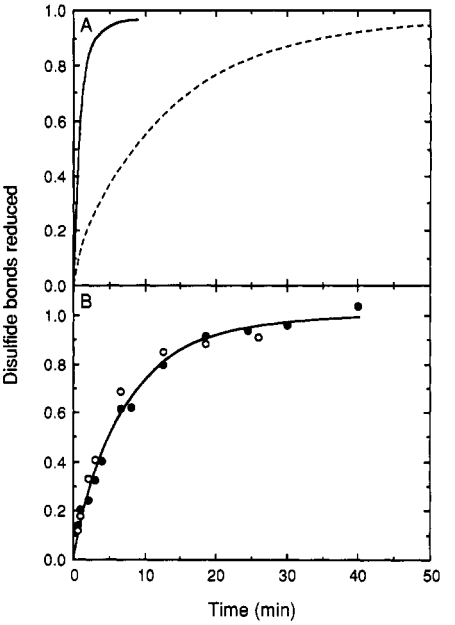

FIGURE 1: Kinetics of reduction of disulfide bonds in cystatin 1 and 2 by 5 μM thioredoxin (A) or 1 mM dithiothreitol (B): (—, ○) cystatin 1; (– –, ●) cystatin 2.

(*b*) *Reduction with Dithiothreitol.* Reaction of cystatin 1 and 2 by 1 mM dithiothreitol for ~45 min at pH 9.0 also resulted in cleavage of about one disulfide bond in both forms of the protein, as monitored by the liberation of protein thiol groups, measured with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of the reducing agent after trapping of this reagent with arsenite (Figure 1B). In this case, however, no difference in the rate of the reaction between cystatin 1 and 2 was apparent. Analyses of cystatin 1, reduced for 60 min by 1 mM dithiothreitol, with 5,5′-dithiobis(2-nitrobenzoic acid) after removal of excess dithiothreitol gave 2.00–2.05 (range, n = 2) mol of thiol groups/mol of protein, verifying that one disulfide bond was cleaved. Reaction of similarly reduced cystatin 1 with iodo[2-^{14}C]acetic acid resulted in >90% of the incorporated radioactivity being recovered in the same peptides as in the thioredoxin-reduced protein, demonstrating that the Cys-71–Cys-81 disulfide bond was cleaved also by 1 mM dithiothreitol.

(*c*) *Properties of Cystatin with the Cys-71–Cys-81 Disulfide Bond Cleaved.* Although thioredoxin and low concentrations of dithiothreitol thus can reduce the same disulfide bond of cystatin, all characterization of the reduced inhibitor was done with cystatin reduced by thioredoxin, because of the greater potentiality for specificity of this reaction (Holmgren, 1984; see also below). Most studies were done with cystatin 1. Before the analyses, the liberated thiol groups were blocked by *S*-carbamoylmethyl groups.

Thioredoxin-reduced and S-carbamoylmethylated cystatin 1 showed a reaction of complete identity with intact cystatin 1 in immunodiffusion analyses with antibodies against the intact inhibitor. In spite of this immunological similarity, the reduced and alkylated inhibitor had circular dichroism spectra both in the far- and near-ultraviolet wavelength regions that were appreciably different from the corresponding spectra of the intact inhibitor (Figure 2). Moreover, the reduced and alkylated cystatin had an isoelectric point, as determined by isoelectric focusing, of 7.4, distinctly higher than that of 6.8 shown by the intact protein. This finding is in agreement with the reduced and alkylated inhibitor eluting ahead of intact

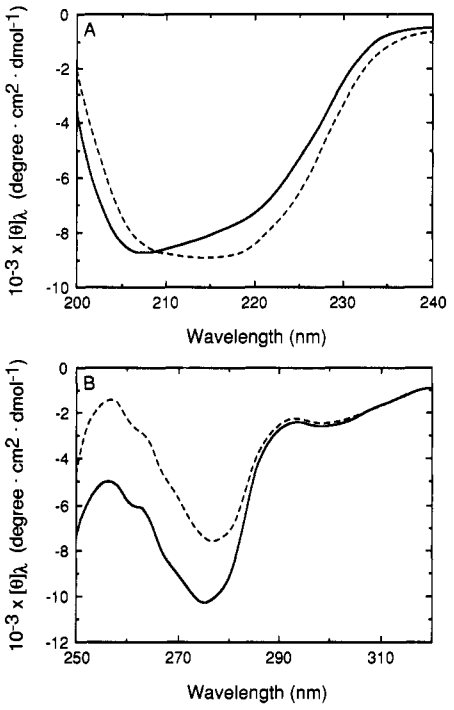

FIGURE 2: Circular dichroism spectra of intact and thioredoxin-reduced and S-carbamoylmethylated cystatin 1. (A) Far-ultraviolet-wavelength region; (B) near-ultraviolet-wavelength region. (—) Intact cystatin; (– –) reduced and alkylated cystatin. The unit on the ordinate is mean residue ellipticity in (A) and molar ellipticity in (B).

cystatin in anion-exchange HPLC. Reduced and alkylated cystatin was also digested by proteolytic enzymes in a manner quite different from that of the intact inhibitor. Subtilisin and thermolysin, at a weight ratio of enzyme to substrate of 1:100, thus completely cleaved the reduced and alkylated inhibitor at 25 °C into two fragments with apparent molecular weights in SDS–PAGE of ~7000 and ~4000 in 10 min and 30 min, respectively. No corresponding cleavage was seen with intact cystatin, although both enzymes cleave off a small peptide from the amino-terminal end of the protein, with no discernible change of apparent molecular weight (Lindahl et al., 1992b).

The interaction of saturating amounts of thioredoxin-reduced and S-carbamoylmethylated cystatin 1 with papain resulted in near-ultraviolet absorption, circular dichroism, and fluorescence emission difference spectra that were experimentally indistinguishable from the corresponding spectra for intact cystatin (Lindahl et al., 1988). Titrations, monitored by the decrease in intrinsic fluorescence accompanying the interaction, of papain with the reduced and alkylated cystatin gave an apparent binding stoichiometry of 1.00 ± 0.05 (SD; $n = 4$) mol of inhibitor/mol of proteinase, indicating that all molecules of the reduced and alkylated inhibitor were fully active in binding to the enzyme. No change of this apparent stoichiometry was observed after storage of the protein at 4 °C for several days. The association rate constant for the binding of the reduced and alkylated inhibitor to papain, measured with cystatin 1, and the dissociation rate constant of the resulting complex, measured with cystatin 2 for methodological reasons (Björk et al., 1989), were highly similar to the corresponding rate constants for the interaction of intact cystatin with papain (Table I). As a consequence, the dissociation equilibrium constant for the binding of the reduced and alkylated cystatin to papain was indistinguishable from that of the intact inhibitor (Table I). Likewise, the dissociation equilibrium constant for the binding of the reduced and alkylated cystatin to an inactivated form of papain, *S*-(*N*-ethylsuccinimidyl)papain, was similar to the corresponding equilibrium constant for intact cystatin (Table I).

Reduction of Both Disulfide Bonds by Dithiothreitol. (*a*) *Reduction.* Reaction of cystatin 1 with a higher concentration of dithiothreitol than that used in the experiments described above, 10 mM, for 120 min at pH 9.0 led to the cleavage of 1.8 ± 0.1 (SD; $n = 5$) disulfide bonds, as determined after removal of excess reducing agent by analyses with 5,5′-dithiobis(2-nitrobenzoic acid) of the number of thiol groups liberated. Prolonged incubation or reaction of the protein with 100 mM dithiothreitol did not appreciably increase this value, consistent with both disulfide bonds being fully cleaved.

(*b*) *Properties of Fully Dithiothreitol-Reduced Cystatin.* The activity of the fully reduced and alkylated cystatin 1 was found to decrease with time. Analyses following dialysis at 4 °C for about 24 h after disulfide bond cleavage and blocking of the liberated thiol groups showed that the reduced and alkylated inhibitor had only about 60% of the proteinase inhibitory activity of intact cystatin. In agreement with this finding, the apparent stochiometry of binding of the inhibitor to papain, measured by a fluorescence titration, was about 1.5, reflecting the presence of only about 65% active protein. The amount of active protein further decreased with longer storage at 4 °C. Preparative anion-exchange HPLC of the reduced and alkylated cystatin after 24 h of dialysis gave three main peaks (Figure 3A) The protein in all three fractions had the same mobility as intact cystatin in SDS–PAGE and showed a reaction of complete identity with the intact inhibitor in immunodiffusion analyses. The major of the three fractions (fraction 1) had full proteinase binding ability when it was analyzed immediately after having been isolated (Figure 4), but the other two fractions (2 and 3) were essentially inactive. The proportion of the inactive fractions was consistent with the reduced inhibitory activity of the preparation. Analytical rechromatography of fraction 1 soon after its isolation gave virtually only one peak (Figure 3B), whereas material eluting at the positions of the inactive fractions 2 and 3 progressively appeared with increasing time of incubation at 25 °C (Figure 3B). This behavior was paralleled by a corresponding decrease in the proteinase binding ability of fraction 1, reflected in an increase of the apparent stoichiometry of binding to papain (Figure 4) and in a decrease of inhibitory activity.

DISCUSSION

Predictions from the X-ray structure of chicken cystatin, in which both disulfide bonds of the protein are largely buried in the molecule (Bode et al., 1988), suggest that the two bonds should be about equally accessible to reducing agents. Nevertheless, this work demonstrates that the Cys-71–Cys-81 bond is considerably more susceptible to reduction than the Cys-95–Cys-115 bond. The former bond is thus easily cleaved both by the reducing protein, thioredoxin, and by low concentrations of dithiothreitol, whereas the latter bond is refractory to reduction by thioredoxin and requires reaction with a 10-fold higher dithiothreitol concentration during a longer time to be cleaved. Interestingly, the Cys-71–Cys-81 bond is reduced considerably more slowly by thioredoxin in cystatin 2 than in cystatin 1, presumably due the phosphate group on Ser-80 in cystatin 2 (Laber et al., 1989) interfering with the access of thioredoxin to the adjacent disulfide bond. As no corresponding rate difference was observed with dithiothreitol, this finding attests to the high degree of specificity shown by thioredoxin in reducing protein disulfide bonds (Holmgren, 1984).

Table I: Kinetic and Equilibrium Data for the Interaction of Intact or Thioredoxin-Reduced and S-Carbamoylmethylated Chicken Cystatin with Active Papain or *S*-(*N*-Ethylsuccinimidyl)papain[a]

cystatin	active papain			*S*-(*N*-ethylsuccinimidyl)papain
	$10^{-6}k_{+1}$ (M^{-1} s^{-1})	$10^{7}k_{-1}$ (s^{-1})	$10^{14}K_d$ (M)	$10^{7}K_d$ (M)
intact[b]	9.6 ± 0.2 (18)	5.7 ● 0.9 (5)	5.9	1.7 ± 0.6 (4)
reduced, alkylated	8.7 ± 0.4 (10)	4.9 ± 1.2 (3)	5.6	1.3 ± 0.4 (3)

[a] The association rate constants (k_{+1}) for the interaction with active papain were measured with cystatin 1 by stopped-flow fluorometry, whereas the corresponding dissociation rate constants (k_{-1}) were measured with cystatin 2 by displacement experiments. The dissociation equilibrium constants (K_d) for the interactions with active papain were calculated from these association and dissociation rate constants. The dissociation equilibrium constants for the interactions with *S*-(*N*-ethylsuccinimidyl)papain were measured by fluorescence titrations. All measured values are given with their 95% confidence limits and with the number of measurements in parentheses. [b] The values for intact cystatin are taken from Björk et al. (1989) and Björk and Ylinenjärvi (1989).

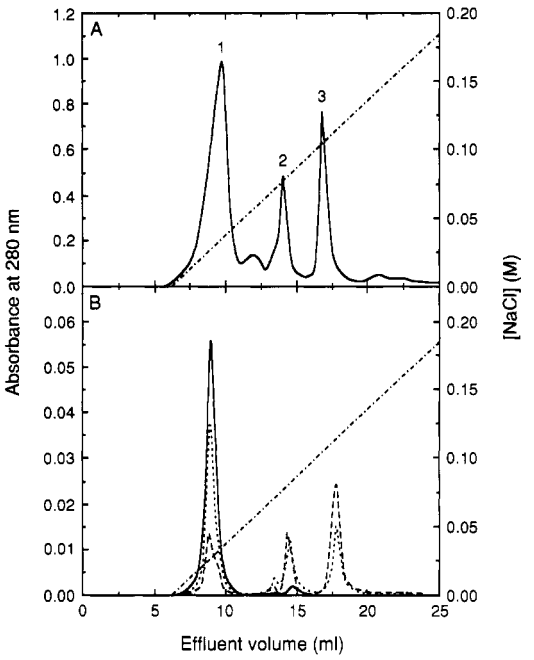

FIGURE 3: Anion-exchange HPLC of fully dithiothreitol-reduced and S-carbamoylmethylated cystatin 1. (A) Preparative separation of 4.5 mg of protein after dialysis at 4 °C for 24 h following reduction and alkylation. (B) Analytical rechromatography of fraction 1 (80 μg) from the preparative separation in (A) after 1 (—), 24 (···), and 96 (- - -) h at 25 °C; (– · –) NaCl concentration.

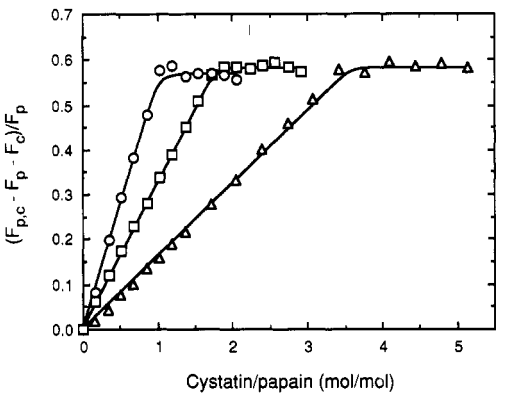

FIGURE 4: Titrations, monitored by measurements of tryptophan fluorescence, of papain with fully dithiothreitol-reduced and S-carbamoylmethylated cystatin 1 at different times after its isolation. The measurements were made with fraction 1 from the preparative anion-exchange HPLC of the reduced and alkylated inhibitor (Figure 3A) after 1 (○), 27 (□), and 97 (△) h at 25 °C following isolation of the fraction. The initial papain concentration was 1 μM. Variables: F_p, fluorescence of papain; F_c, fluorescence of added cystatin; $F_{p,c}$, fluorescence of the papain–cystatin mixture. The solid lines represent the fits of the data to the theoretical binding equation by nonlinear least-squares regression.

These studies also show that specific reduction and alkylation of the Cys-71–Cys-81 disulfide bond in cystatin leads to a conformational change of the protein, as evidenced by several criteria. However, this change does not detectably affect the proteinase-binding ability or the immunological properties of the inhibitor, indicating that the three-dimensional structure is only altered locally around the disrupted bond. This conclusion is supported by the observation that digestion of the reduced and alkylated protein, but not of the native protein, by nontarget proteinases produces fragments with sizes compatible with cleavage having occurred in this region. The negligible importance of the structure around the Cys-71–Cys-81 disulfide bond for inhibitory activity is in agreement with the localization of this bond in three-dimensional structure of the protein, i.e., joining the appending α-helix to the main body of the protein at an appreciable distance from the proteinase-binding region (Bode et al., 1988).

In contrast to cleavage of the Cys-71–Cys-81 disulfide bond of cystatin alone, reduction and alkylation of both disulfide bonds of the protein destabilizes the conformation required for inhibitory activity. The nascent, fully reduced, and alkylated inhibitor thus apparently retains full proteinase-binding ability but is slowly converted to two forms lacking activity, indicative of extensive structural alterations occurring as a result of the disulfide bond cleavage. The relative amounts of these two forms presumably are governed by the rate constants of the two conformational change pathways. These findings suggest that the less accessible disulfide bond of cystatin, the Cys-95–Cys-115 bond, is of importance for maintaining the active protein conformation. As this bond links the two carboxy-terminal strands of the major β-sheet of cystatin (Bode et al., 1988), this conclusion indicates that the structural integrity of this β-sheet, the stability of which apparently is decreased by cleavage and alkylation of the Cys-95–Cys-115 bond, is crucial for the proteinase-binding ability of the inhibitor.

Chicken cystatin and human cystatin C have highly similar structural and functional properties (Grubb & Löfberg, 1982; Turk et al., 1983; Schwabe et al., 1984; Abrahamson et al., 1987; Björk et al., 1989; Lindahl et al., 1992a) and, in particular, have disulfide bonds at homologous positions (Grubb et al., 1984). It is therefore likely that the different roles of the two disulfide bonds for the conformation of the active protein are the same in the two inhibitors and possibly also in all family II cystatins.

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