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Interaction of the Cysteine Proteinase Inhibitor Chicken Cystatin with Papain†

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ABSTRACT: The two forms of chicken cystatin, with different isoelectric points, that have been described previously were indistinguishable in analyses of amino- and carboxy-terminal residues, amino acid composition, and peptide maps. The two forms thus are highly similar and most likely differ only in an amide group or in a small charged substituent. The binding of either cystatin form to highly purified, active papain was accompanied by the same pronounced changes in near-ultraviolet circular dichroism, ultraviolet absorption, and fluorescence emission. These changes were compatible with perturbations of the environment of aromatic residues in one or both proteins of the complex, arising from local interactions or from a conformational change. Modification of the single tryptophan residue of cystatin, at position 104, with *N*-bromosuccinimide resulted in considerably smaller spectroscopic changes on binding of the inhibitor to papain, indicating that the environment of this residue is affected by the binding. Analogous modification of Trp-69 and Trp-177 of papain markedly affected the fluorescence changes observed on binding of cystatin to the enzyme, similarly suggesting that these two residues of papain are involved in the interaction. The fluorescence increase of papain at alkaline pH, arising from Trp-177 and due to deprotonization of the adjacent His-159, was abolished on binding of cystatin to the enzyme, further supporting the proposal that this region of papain participates in the interaction with the inhibitor. A stoichiometry of binding of either cystatin form to papain of 1:1 and a lower limit for the binding constant of 10^9 M^{-1} were determined by titrations monitored by either the ultraviolet absorption or fluorescence changes induced by the interaction.

Protein inhibitors of cysteine proteinases are widely distributed in mammalian tissues and are present also in plasma. Their function presumably is to protect the organism against uncontrolled action of endogenous or exogenous cysteine proteinases (Barrett et al., 1986). Those inhibitors of this group that are of tissue origin, cystatins, are small proteins with M_r 12000-14000. To date, four such cystatins, A, B, C, and S, have been demonstrated in human tissues and secretions, and analogues to some of these have been shown to occur also in other mammals (Barrett et al., 1986). The cysteine proteinase inhibitors of plasma are identical with the kininogens (Okhubo et al., 1984; Müller-Esterl et al., 1985; Sueyoshi et al., 1985), which exist in two partially identical forms, H-kininogen and L-kininogen with M_r ~100000 and 60000, respectively (Kato et al., 1981; Lottspeich et al., 1985; Kellerman et al., 1986). The kininogens also have other biological activities. Both forms are thus precursor molecules

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for vasoactive peptides, kinins, and H-kininogen also acts as a cofactor in the contact activation phase of blood clotting (Kato et al., 1981).

An analogue of human cystatin C has been identified in chicken egg white (Fossum & Whitaker, 1968; Keilová & Tomásek, 1974). In contrast with mammalian cystatins, this inhibitor can easily be purified in reasonably large amounts (Anastasi et al., 1983) and is thus the best characterized tissue-type inhibitor. Two forms of chicken cystatin with different isoelectric points can be isolated (Anastasi et al., 1983; Turk et al., 1983). Both forms rapidly inactivate a number of cysteine proteinases by forming tight complexes with the enzymes (Nicklin & Barrett, 1984). In these complexes, the active-site cysteine residue of the proteinase is inaccessible to substrates and to reaction with thiol group reagents (Anastasi et al., 1983; Nicklin & Barrett, 1984). Complex formation has been suggested to involve a specific reactive bond of the inhibitor, between Gly-9 and Ala-10 (Abrahamson et al., 1987). The inhibitor can also bind cysteine proteinases in which the reactive cysteine has been blocked, even with bulky substituents (Anastasi et al., 1983). However, the nature of the interaction with active or inactivated enzymes has not been further elucidated. In this report we present a characterization by several spectroscopic methods of the interaction of the two forms of chicken cystatin with active, highly purified papain as a model for other reactions between cysteine proteinase inhibitors and their target enzymes.

Materials and Methods

Proteins. Cystatin was purified from chicken egg white essentially as described by Anastasi et al. (1983). However, the chromatofocusing step was replaced by chromatography on a DEAE-Sephacel column (1.6 × 30 cm; Pharmacia, Uppsala, Sweden), equilibrated with 0.02 M Tris-HCl[1] and 0.02 M NaCl, pH 7.4, and eluted with a linear gradient to 0.2 M NaCl. Forms 1 and 2 of cystatin (Anastasi et al., 1983) appeared well separated at ~0.05 and ~0.11 M NaCl, respectively. Both forms gave one band in polyacrylamide gel electrophoresis under native conditions (Ornstein, 1964; Davis, 1964) or in the presence of sodium dodecyl sulfate with or without reducing agent (Weber & Osborn, 1969; Laemmli, 1970). The purified cystatin was stored at −70 °C in 0.05 M Tris-HCl, 0.1 M NaCl, and 100 μM EDTA, pH 7.4, at a concentration of 5–10 mg/mL. No loss of activity occurred during storage for several months.

Papain was isolated from 2× crystallized papain (type III; Sigma, St. Louis, MO) or from crude papaya latex (type I; Sigma) by affinity chromatography on a matrix-linked papain inhibitor, glycylglycyl-*O*-benzyl-L-tyrosyl-L-arginine (Peptide Institute, Osaka, Japan; Blumberg et al., 1970; Burke et al., 1974). The preparations had a thiol content of 0.94 ± 0.03 (SD, $n = 6$) mol/mol of enzyme, as determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959; Blumberg et al., 1970). Attempts to use an immobilized inhibitor without the benzyl group (Funk et al., 1979) resulted in much lower yields. The purified papain was stored for up to 6 months at 4 °C as a form with the reactive cysteine residue protected by an *S*-methylthio group. This derivative was obtained by addition of methyl methanethiolsulfonate (Aldrich, Milwaukee, WI) in a molar ratio of reagent to enzyme of 1.25 at pH 6.0 (Smith et al., 1975; Lewis et al., 1976; Roberts et al., 1986). Immediately before use, papain was reactivated as described by Roberts et al. (1986). The reactivated enzyme had a thiol content indistinguishable from that of freshly prepared papain.

Tryptophan Modification. Tryptophan residues in cystatin and papain were modified with NBS. Cystatin was modified by successive additions of 10 mM NBS (Fluka, Buchs SG, Switzerland; recrystallized from concentrated acetic acid) in 0.05 M sodium acetate, pH 4.0, to 75 μM protein in the same buffer. The absorbance at 280 nm was measured after each addition, and the number of tryptophan residues modified was calculated from the absorbance decrease, corrected for dilution (Spande & Witkop, 1967). The inhibitory activity of the modified cystatin was measured after addition of skatole to a concentration of 500 μM to destroy excess reagent. Modified inhibitor to be used in other experiments was immediately separated from excess reagent by gel chromatography on Sephadex G-25 (Pharmacia). Inactivated papain was modified with NBS essentially as described by Lowe and Whitworth (1974). However, the papain concentration was 60–80 μM, and the active-site cysteine residue was protected by reaction with methyl methanethiolsulfonate instead of 2-hydroxyethyl disulfide. The number of tryptophan residues modified was calculated from the absorbance decrease. After removal of excess NBS by gel chromatography on Sephadex G-25, the modified papain was reactivated before use (Roberts et al., 1986).

Amino acid analyses were carried out on an LKB 4151 Alpha-Plus (LKB, Bromma, Sweden) or a Beckman 121M (Beckman Instruments, Fullerton, CA) amino acid analyzer after hydrolysis of the samples in evacuated tubes with 6 M HCl (containing 2–5 mg/mL phenol) at 110 °C for 24 h.

Peptide Mapping. Cystatin was reduced, carboxymethylated, and digested with trypsin as described previously (Björk & Jörnvall, 1986). The tryptic peptides were separated by reverse-phase high-performance liquid chromatography on a TSK ODS-120T UltroPac column (0.46 × 25 cm; LKB), eluted at 1 mL/min with a combination of linear gradients of acetonitrile (0–50%) in 0.1% trifluoroacetic acid.

Structural Analyses. Amino acid sequences were determined by degradations in a Beckman 890D liquid-phase sequencer. Phenylthiohydantoin derivatives were identified by reverse-phase high-performance liquid chromatography with a sodium acetate/acetonitrile gradient system, as described earlier (Kaiser et al., 1988). Hydrazinolysis was carried out at 110 °C for 6 h in evacuated tubes. Carboxypeptidase Y (Boehringer, Mannheim, FRG) was used in 1–4-h digestions at a molar ratio of 1:100 in 0.1 M pyridine acetate, pH 6.0.

Cystatin inhibitory activity was determined by a procedure based on that developed by Anastasi et al. (1983). A volume of 100 μL of sample (100–150 nM) in 0.125 M potassium phosphate and 0.1% Brij 35, pH 6.8, was incubated for 1 min at 40 °C with 20 μL of papain (1 μM; type III; Sigma) in the same buffer, containing 1.25 mM dithiothreitol. A 100-μL portion of this solution was then added to a mixture of 650 μL of phosphate buffer with the reducing agent and 250 μL of 20 μM papain substrate, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute), in H_2O. After an incubation time of 1 min at 40 °C, the hydrolysis of the substrate was stopped by addition of 1 mL of 0.1 M sodium monochloroacetate in 0.1 M sodium acetate, pH 4.3. The fluorescence of the liberated 7-amino-4-methylcoumarin was then measured in an Aminco-Bowman spectrofluorometer (SLM-Aminco, Urbana, IL) with excitation and emission wavelengths of 370 and 440 nm, respectively.

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetate; NBS, *N*-bromosuccinimide; Tris, tris(hydroxymethyl)aminomethane.

Spectroscopic Methods. All spectroscopic analyses were made in 0.05 M Tris-HCl, 0.1 NaCl, and 100 μM EDTA, pH 7.4, unless otherwise indicated. The buffer was treated with Chelex 100 (Bio-Rad, Richmond, CA) before addition of EDTA.

Ultraviolet absorption difference spectra were measured at 25.0 ± 0.1 °C with a bandwidth of 1 nm on the 0.05 absorbance scale of a Cary Model 219 spectrophotometer (Varian Instruments, Palo Alto, CA). The measurements were done with tandem cells, having 1-cm path length per compartment, and with papain concentrations of 16–20 μM. The two compartments of each tandem cell initially contained 2.00 mL of either papain solution or buffer. Identical volumes (5–10 μL in titrations of papain with cystatin; 100–150 μL in measurements of spectra at saturation) of cystatin (280–500 μM) were added to the protein compartment of the sample cell and to the buffer compartment of the reference cell. The same volume of buffer was also added to the protein compartment of the reference cell, and the absorption difference spectrum between sample and reference was then measured. In titrations, repeated additions of cystatin were made, and a spectrum was measured after each addition. The results were expressed as differences in molar absorption (based on the papain concentration) between sample and reference. Titration data were corrected for dilution (<10%).

Circular dichroism was measured at room temperature (22 ± 2 °C) with a Jasco J-41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Measurements in the far-ultraviolet (200–250-nm) region were carried out in cells with 0.1-cm path lengths and with papain and cystatin concentrations of 3.6 and 4.3 μM, respectively. Cells with 1-cm path lengths, papain concentrations of 13–16 μM, and cystatin concentrations of 16–24 μM were used in the near-ultraviolet (250–320-nm) region. The bandwidth was 2 nm in both wavelength regions. The results were expressed as mean residue ellipticities in the far-ultraviolet region and as molar ellipticities in the near-ultraviolet region. Mean residue weights of 110 for papain (Husain & Lowe, 1969) and of 113 for cystatin (Schwabe et al., 1984) were used in the calculations.

Fluorescence measurements were performed at 25.0 ± 0.1 °C in an SLM 4800S spectrofluorometer (SLM-Aminco). The excitation wavelength was 280 nm, except where otherwise stated. Cells with 1-cm path lengths were used, and samples were continuously stirred during measurements. Emission spectra were measured with a papain concentration of 1 μM, a cystatin concentration of 1.2–1.5 μM, and with excitation and emission bandwidths of 4 and 8 nm, respectively. The spectra were corrected for inner-filter effects and for the wavelength dependence of the instrumental response. Titrations of papain with cystatin were monitored by measurements at the wavelength at which the maximal fluorescence change was observed (350–355 nm) with excitation and emission bandwidths of 4–8 and 16 nm, respectively. Successive volumes (5 or 10 μL) of cystatin (600 nM–75 μM) were added to 2 mL of papain (10 nM–1 μM). Poly(ethylene glycol) (1 g/L) or dithiothreitol (10 μM) were included in the buffer in some titrations at a papain concentration of 10 nM. The data were corrected for inner-filter effects and dilution (<10%) and were plotted as $(F_p + F_c - F_{p,c})/F_p$. In this expression, which defines the observed fluorescence decrease as a positive quantity, F_p is the fluorescence of papain alone, F_c that of the added cystatin (measured in a parallel titration with buffer instead of papain), and $F_{p,c}$ that of the papain–cystatin mixture. Stoichiometries and dissociation constants were evaluated by nonlinear regression (Nordenman & Björk, 1978) with a Hewlett-Packard 9816 computer (Hewlett-Packard, Ft. Collins, CO) and the HP Statistical Library.

Acrylamide quenching (Eftink & Ghiron, 1976) of tryptophan fluorescence was analyzed by successive additions of 10 μL of ~8 M acrylamide (Eastman-Kodak, Rochester, NY) to 2 mL of protein (4 μM papain with or without 6 μM NBS-modified cystatin). The fluorescence intensity was measured with an excitation wavelength of 295 nm and an emission wavelength set at the maximum of the uncorrected emission spectrum for each sample. The corresponding bandwidths were 2 and 8 nm. The data were corrected for inner-filter effects and dilution (<7%) and were plotted according to a modified Stern–Volmer equation (Lehrer & Leavis, 1978).

The pH dependence of tryptophan fluorescence was studied by batchwise titrations (Johnson et al., 1981). All buffers (Johnson et al., 1981) were 0.02 M and contained NaCl to an ionic strength of 0.15 M and 100 μM EDTA. Volumes of 50 μL of concentrated protein solutions in 0.01 M Tris-HCl, 0.14 M NaCl, and 100 μL EDTA, pH 7.4, were pipetted into 2 mL of the appropriate buffer to final papain and cystatin concentrations of 2.0 and 2.4 μM, respectively. After thermal equilibration of the solutions, the fluorescence intensity was measured at an emission wavelength of 350 nm with excitation and emission bandwidths of 8 and 16 nm, respectively. The pH of the solutions was then determined. The observed intensities were corrected for inner-filter effects, and the intensities measured for papain above pH 8 were also corrected for the quenching caused by ionization of tyrosine side chains (Sluyterman & de Graaf, 1970; Johnson et al., 1981).

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 g^{-1} cm^{-1} and molecular weights of 13 100 (Schwabe et al., 1984) and 23 400 (Husain & Lowe, 1969) were used for both cystatin forms (see Results) and papain, respectively. Absorption coefficients for NBS-modified proteins were estimated from the decrease in absorbance measured during the modification reaction.

Results

Comparison of the Structures of Cystatins 1 and 2. There is disagreement in the literature on the structural differences between the two forms of chicken cystatin. Turk et al. (1983) have proposed that cystatin 1 is eight residues shorter than cystatin 2. However, others have determined an amino acid sequence for cystatin 1 identical with that reported for cystatin 2 by Turk et al. (1983) and have obtained the same amino-terminal sequence for the two forms (Anastasi et al., 1983; Schwabe et al., 1984). We found amino acid compositions for cystatins 1 and 2 that were identical within experimental error (±3%; results not shown). The two compositions also agreed as closely as can be expected for 24-h hydrolysates with the composition deduced from the published amino acid sequence of cystatin 1 (Schwabe et al., 1984). Moreover, the amino-terminal sequence was the same for the two forms, Ser-Glu-Asp-, in further agreement with the reported sequence of cystatin 1 (Schwabe et al., 1984). No carboxy-terminal amino acid could be demonstrated in either of the two forms by hydrazinolysis, consistent with Gln as the carboxy-terminal residue (Turk et al., 1983; Schwabe et al., 1984). Similarily, no amino acid was released by carboxypeptidase Y digestion, presumably because the adjacent disulfide bond (Turk et al., 1983; Schwabe et al., 1984) renders the terminal residue inaccessible to the enzyme. Peptide maps obtained by re-

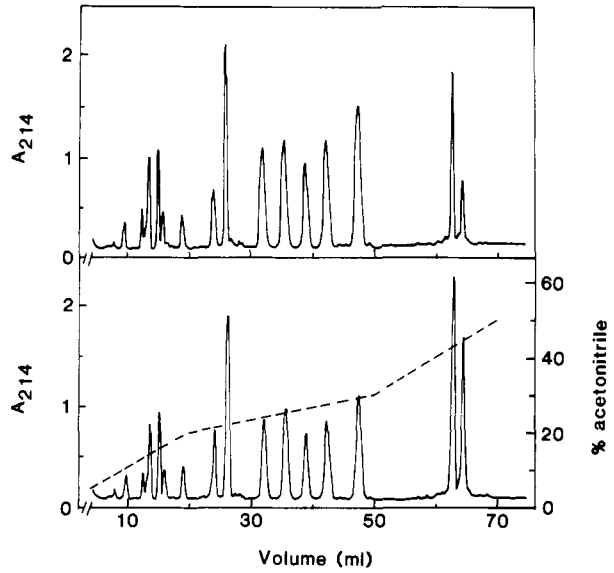

FIGURE 1: Separation by reverse-phase high-performance liquid chromatography of peptides produced by trypsin digestion of reduced and carboxymethylated cystatins 1 and 2. Lower panel, cystatin 1; upper panel, cystatin 2. (—) Absorbance at 214 nm; (---) percent acetonitrile. About 70 nmol of digested protein was applied to the column.

verse-phase high-performance liquid chromatography of tryptic peptides of the two forms showed peaks at identical positions, with the only detectable difference being in the relative height of the last peak (Figure 1). Finally, we were unable to demonstrate a difference in mobility between cystatins 1 and 2 in sodium dodecyl sulfate/polyacrylamide gel electrophoresis under a variety of conditions, and also when the two forms were mixed (not shown). Together, the data thus provide no evidence for any amino acid difference between the two cystatin forms. Although we have not been able to localize the structural difference, it must be small, most likely a difference in an amide group resulting from deamidation or in a small charged substituent on only one of the two forms. Consequently, we have used the molecular weight calculated from the amino acid sequence of cystatin 1 (Schwabe et al., 1984), i.e., 13 100, for both forms.

Spectroscopic Changes on Interaction of Cystatin with Papain. The interaction was studied by spectroscopic methods at pH 7.4 and an ionic strength of 0.15 with active, highly purified papain and with both cystatins 1 and 2. The molar ratio of inhibitor to enzyme in the analyses was 1.2, sufficient to achieve >98% saturation under the conditions used, as deduced from quantitative binding studies presented below.

Absorption difference spectra between the cystatin–papain complex and the free proteins demonstrated that a complicated pattern of changes in ultraviolet absorption in the aromatic wavelength region accompanies the interaction (Figure 2). The spectra thus showed considerable detail, with maxima at 292, 285, and 265 nm and minima at 300, 289, and 282 nm. Identical results were obtained for cystatins 1 and 2.

Far-ultraviolet circular dichroism studies showed that the spectrum measured for the cystatin–papain complex reproducibly was slightly more negative than that calculated for an equimolar mixture of the two proteins (Figure 3). Thus, a small decrease in circular dichroism in the peptide bond region is induced by the interaction. Experiments with cystatin 2 gave similar results as those presented for cystatin 1. The spectra for both cystatin forms differed somewhat from that reported for cystatin 1 by Schwabe et al. (1984) in that the minima had higher magnitudes (~−7000 deg cm^2 dmol^{-1}) and

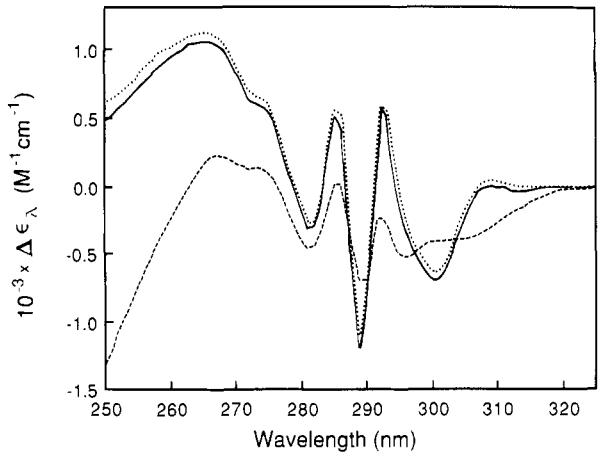

FIGURE 2: Ultraviolet absorption difference spectra measured between complexes of cystatin 1, cystatin 2, or NBS-modified cystatin 1 with papain and the free proteins. (—) Cystatin 1; (···) cystatin 2; (---) NBS-modified cystatin 1. The papain concentration was 15.8–16.4 μM, and the molar ratios of cystatin to papain were 1.2 and 1.5 in experiments with intact and modified cystatin, respectively.

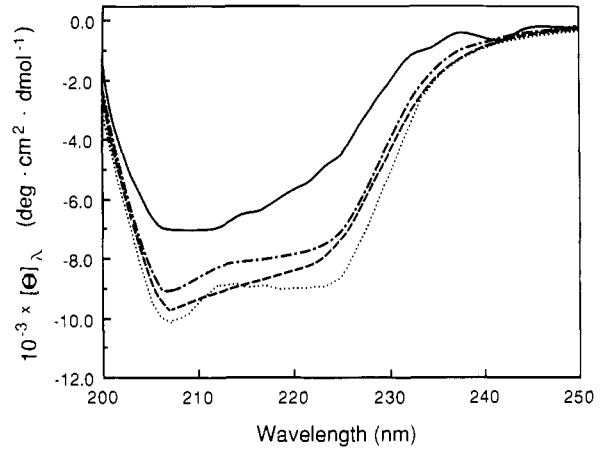

FIGURE 3: Far-ultraviolet circular dichroism spectra of cystatin 1, papain, and the complex between cystatin 1 and papain. (—) Cystatin 1; (···) papain; (---) complex between cystatin 1 and papain; (-·-) spectrum calculated for an equimolar mixture of cystatin 1 and papain. The papain and cystatin concentrations in the measurements were 3.6 and 4.3 μM, respectively. In the calculations of the spectrum for the cystatin–papain complex, the contribution due to excess, free cystatin was subtracted from the measured spectrum.

were shifted to a shorter wavelength (~208 nm). The papain spectrum had a similar shape as that measured by Barel and Glazer (1969) for a less active preparation, but the minimum had a somewhat lower ellipticity, ~−10 000 deg cm^2 dmol^{-1}.

A much larger spectral change on interaction of cystatin with papain was evident in measurements of near-ultraviolet circular dichroism. Thus, the experimental spectrum for the cystatin–papain complex was markedly lower throughout the wavelength range than the spectrum calculated for an equimolar mixture of the two proteins (Figure 4A). Difference spectra (Figure 4B) calculated from the spectra presented for cystatin 1 and from analogous data for cystatin 2 show that the interactions of the two cystatin forms with papain produce highly similar changes of near-ultraviolet circular dichroism. The difference spectra also reveal that the largest decrease occurs at 281 nm, with a local minimum of higher magnitude at 287 nm.

The binding of cystatin to papain also resulted in a decrease of the wavelength of the maximum of the corrected tryptophan fluorescence emission spectrum. This maximum shifted from 329 nm for the spectrum calculated for an equimolar mixture

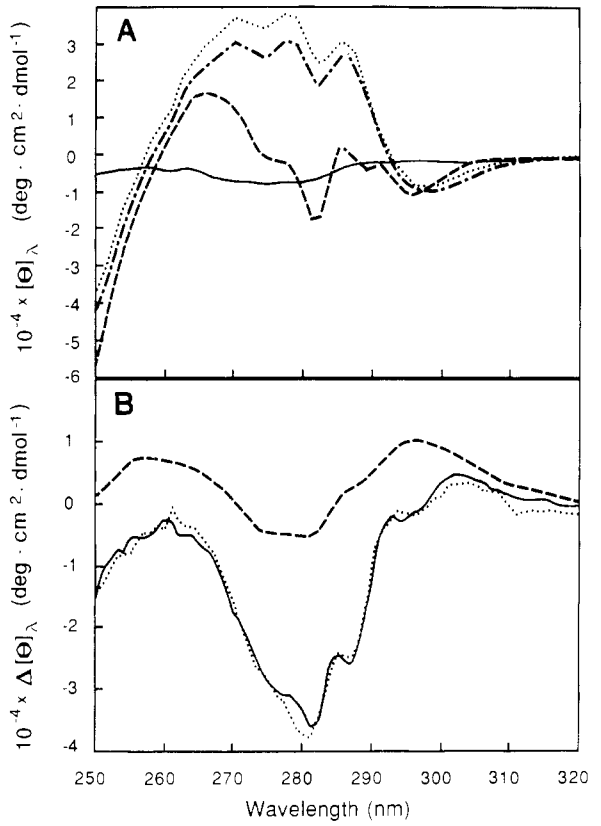

FIGURE 4: (A) Near-ultraviolet circular dichroism spectra of cystatin 1, papain, and the complex between cystatin 1 and papain. (—) Cystatin 1; (···) papain; (- - -) complex between cystatin 1 and papain; (-·-) spectrum calculated for an equimolar mixture of cystatin 1 and papain. The papain and cystatin concentrations in the measurements were 13.4 and 16.1 μM, respectively. The spectrum for the cystatin–papain complex was calculated as described in the legend to Figure 3. (B) Near-ultraviolet circular dichroism difference spectra between complexes of cystatin 1, cystatin 2, or NBS-modified cystatin 1 with papain and the free proteins. (—) Cystatin 1; (···) cystatin 2; (- - -) NBS-modified cystatin 1. The difference spectra were calculated from the spectra in (A) and corresponding spectra for the other two proteins. The molar ratios of cystatin to papain were 1.2 and 1.5 in experiments with intact and modified cystatin, respectively.

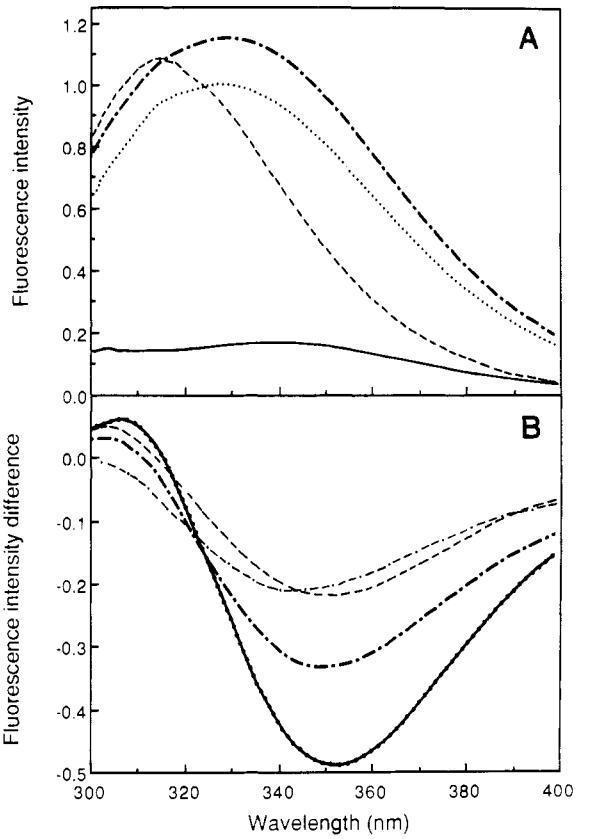

FIGURE 5: (A) Corrected fluorescence emission spectra of cystatin 1, papain, and the complex between cystatin 1 and papain. (—) Cystatin 1; (···) papain; (- - -) complex between cystatin 1 and papain; (-·-) spectrum calculated for an equimolar mixture of cystatin 1 and papain. The papain and cystatin concentrations in the measurements were 1.0 and 1.2 μM, respectively. The spectrum for papain has been normalized to 1.0. The spectra for cystatin, the cystatin–papain complex, and the equimolar mixture of the two proteins have been recalculated to reflect the same concentration as papain, i.e., 1 μM. (B) Fluorescence emission difference spectra between complexes of cystatin 1, cystatin 2, or NBS-modified cystatin 1 with papain and the free proteins, and between complexes of cystatin 1 with two forms of NBS-modified papain and the free proteins. (—) Cystatin 1/papain; (···) cystatin 2/papain; (- - -) NBS-modified cystatin 1/papain; (-·-) cystatin 1/NBS-modified papain with 1.1 residues modified; (-···-) cystatin 1/NBS-modified papain with 2.2 residues modified. The difference spectra were calculated from the spectra in (A) and corresponding spectra for the other proteins. The molar ratio of cystatin to papain was 1.2 in experiments with intact proteins and 1.5 in experiments with modified inhibitor or enzyme.

of the proteins to 314 nm for the spectrum actually measured for the complex, as shown for cystatin 1 in Figure 5A. This blue shift was accompanied by a small decrease of maximum emission intensity. Again, cystatins 1 and 2 produced identical results, as evidenced by difference spectra calculated from complete sets of spectra for the two forms (Figure 5B). These spectra furthermore show that the largest decrease in fluorescence intensity occurs at about 353 nm.

Stoichiometries and Binding Constants of the Cystatin–Papain Interaction. Stoichiometries of binding of cystatin 1 or 2 to papain were determined by titrations of the enzyme with the two inhibitor forms at high protein concentrations. Experiments monitored by measurements of absorbance difference spectra gave a cystatin/papain stoichiometry of 1.1 for both cystatins 1 and 2 (Figure 6). Similarly, a corresponding stoichiometry of 1.0 was obtained for the two cystatin forms from titrations monitored by measurements of tryptophan fluorescence (Figure 7A).

Attempts were also made in fluorescence titrations of papain with cystatin 1 or 2 to decrease the protein concentration sufficiently to allow determinations of binding constants. Unfortunately, even the lowest papain concentration compatible with acceptable experimental precision (10 nM, i.e., ~0.2 mg/L) was too high to give a good estimate of the binding constant. However, it was sufficiently low to cause

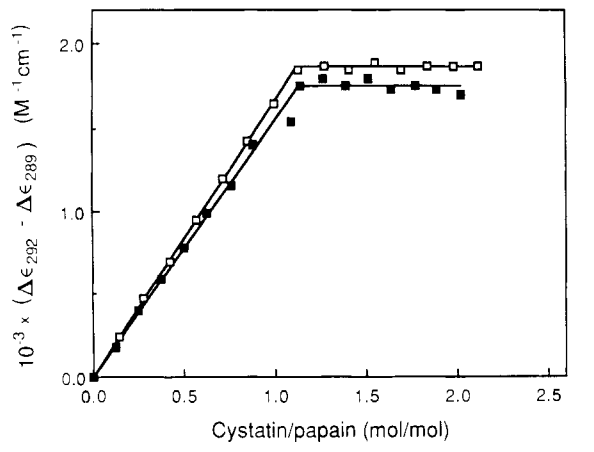

FIGURE 6: Titrations of papain with cystatins 1 and 2, monitored by measurements of ultraviolet absorption difference spectra. (□) Cystatin 1; (■) cystatin 2. The initial concentration of papain was 18–20 μM. The ordinate shows the difference between the molar absorbance differences measured at 292 and 289 nm (see Figure 2).

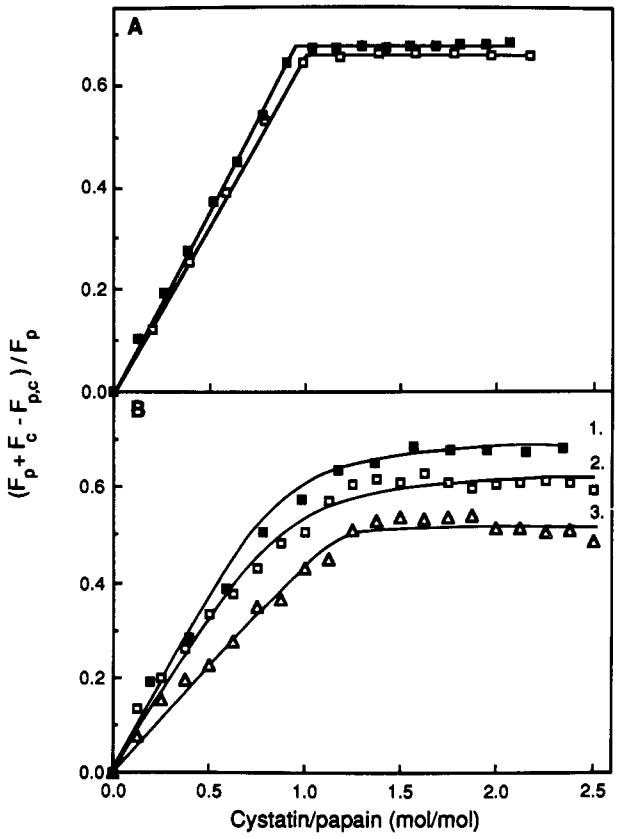

FIGURE 7: Titrations of papain with cystatins 1 and 2, monitored by measurements of tryptophan fluorescence. (A) Initial papain concentration 1 μM; (B) initial papain concentration 10 nM. (□ and △) Cystatin 1; (■) cystatin 2. F_p, fluorescence of papain; F_c, fluorescence of added cystatin; $F_{p,c}$, fluorescence of the papain–cystatin mixture. In (B) the solid lines represent the computer fits of the data to the theoretical binding equation. The stoichiometries and binding constants giving these lines were 0.9 and 1.8×10^9 M^{-1} (curve 1), 0.9 and 1.9×10^9 M^{-1} (curve 2), and 1.2 and 1.5×10^{10} M^{-1} (curve 3), respectively.

some irreproducibility in the analyses, reflected primarily in a variation of the maximal fluorescence decrease between titrations (see experiments in Figure 7B). This problem was not alleviated by the inclusion of poly(ethylene glycol) or dithiothreitol in the buffer, or by extended rinsing of the pipette and fluorescence cell with sample before measurements. Thus, the variable fluorescence decrease does not appear to be due to adsorption of protein to the cell or to oxidation of the essential cysteine residue of papain. In spite of this irreproducibility, the experiments allow the conclusion that the binding constant is $>10^9$ M^{-1} for both cystatins 1 and 2.

Modification of the Tryptophan Residue of Cystatin and Interaction of the Modified Inhibitor with Papain. Since both interacting proteins contain aromatic residues, the spectroscopic changes in the near-ultraviolet region accompanying their interaction can arise from such residues in one or both of the proteins. To elucidate this problem, we chemically modified the single tryptophan, at position 104 (Turk et al., 1983; Schwabe et al., 1984), in cystatin with NBS. These experiments were only done with cystatin 1, since the spectral changes induced by the two forms of the inhibitor are highly similar. About 0.9 tryptophan residue was modified at an NBS/cystatin ratio of 3–3.5 (Figure 8). Moreover, the fluorescence emission maximum of cystatin modified under these conditions was shifted to ~305 nm, with minimal fluorescence at 340 nm, indicating absence of tryptophan (results not shown). Amino acid analyses showed that no other residue, except maximally 0.3 of the 5 tyrosines of the protein,

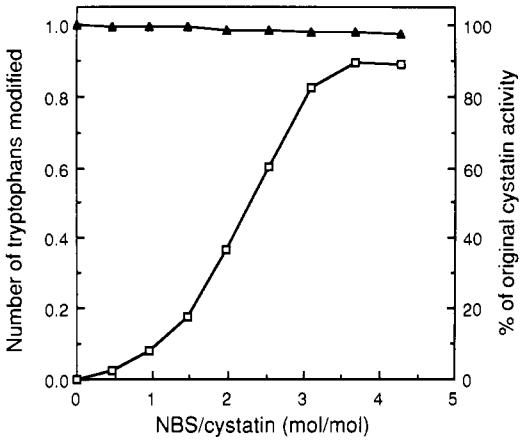

FIGURE 8: Modification of the single tryptophan residue in cystatin 1 with NBS. (□) Number of tryptophan residues modified, calculated from the decrease of absorbance at 280 nm; (▲) cystatin activity in percent of that measured before modification.

was affected. The modification had only minimal effect on cystatin activity under the conditions used in the assay (Figure 8). However, titrations of papain with the modified cystatin, monitored by measurements of the fluorescence decrease (see below), showed that the modified inhibitor bound more weakly to the enzyme than intact cystatin, i.e., with a binding constant of $\sim 8 \times 10^7$ M^{-1}. Therefore, the spectroscopic changes accompanying the interaction were analysed with a molar ratio of modified cystatin to papain of 1.5 to ensure >98% saturation of the enzyme. Measured or calculated difference spectra showed that binding of the NBS-modified cystatin to papain produced near-ultraviolet spectroscopic changes markedly different from those caused by binding of the unmodified inhibitor (Figures 2, 4, and 5). The largest effect of the modification was observed in the circular dichroism analyses, in which the binding of the modified cystatin to papain produced only small changes.

Studies of the quenching of tryptophan fluorescence by acrylamide (Eftink & Ghiron, 1976) showed that about 90% of the tryptophan fluorescence of papain was accessible to the quencher (results not shown). This value was reduced to about 70% on saturation of the enzyme with the NBS-modified cystatin. Only a minimal effect on the effective quenching constant (~4 M^{-1}) was observed.

Modification of Tryptophan Residues of Papain and Interaction of the Modified Enzyme with Cystatin. Further attempts to elucidate the origin of the spectroscopic changes accompanying the interaction were made by modification of tryptophan residues in papain with NBS. Previous studies have shown that Trp-69 of papain is specifically modified at a molar ratio of NBS to papain of 2, whereas Trp-177, but no other tryptophan, is also modified at a molar ratio of 4 (Lowe & Whitworth, 1974). In our work, the necessary protection of the reactive cysteine of the enzyme during the modification was achieved by reversible conversion of this cysteine to an *S*-methylthio derivative. The fluorescence decrease on addition of successive amounts of NBS to the inactivated papain was found to follow closely that reported previously (Lowe & Whitworth, 1974). Moreover, reaction of NBS with the inactivated papain in molar ratios of 2 and 4 resulted in the modification of 1.1 and 2.2 tryptophan residues, respectively. We thus conclude that the modification occurred in the manner described by Lowe and Whitworth (1974). Titrations, monitored by measurements of the fluorescence decrease (see below), showed that the reactivated papain forms with 1.1 and 2.2 tryptophans modified bound to cystatin 1 with binding

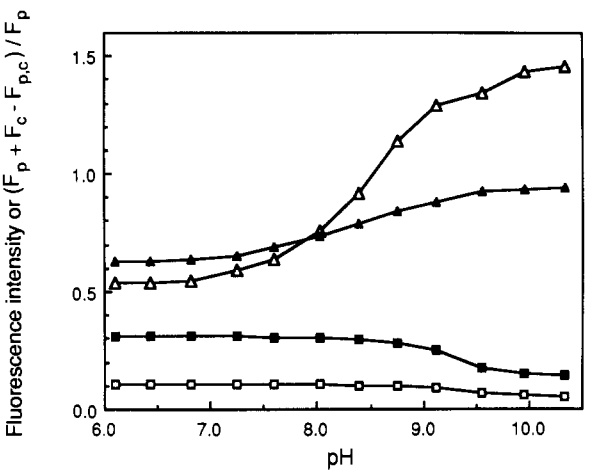

FIGURE 9: Dependence of the fluorescence of cystatin 1, papain, and the complex between cystatin 1 and papain on pH. (□) Cystatin 1; (△) papain; (■) complex between cystatin 1 and papain; (▲) fluorescence decrease on interaction of cystatin 1 with papain, calculated as $(F_p + F_c - F_{p,c})/F_p$ (see legend to Figure 7 and Materials and Methods). The concentrations of papain and cystatin 1 were 2.0 and 2.4 μM, respectively. The fluorescence intensity is plotted in arbitrary units.

constants $\geq 10^9$ and $\sim 4 \times 10^7$ M^{-1}, respectively. Fluorescence emission difference spectra were calculated from experiments with a molar ratio of cystatin 1 to modified papain of 1.5 to ensure >98% saturation. These spectra showed that modification of the two tryptophan residues resulted in a progressively smaller decrease in fluorescence accompanying the interaction with the inhibitor (Figure 5B). The maximum fluorescence decrease was also shifted to shorter wavelengths (Figure 5B).

Dependence of the Tryptophan Fluorescence of the Cystatin–Papain Complex on pH. The tryptophan fluorescence of active papain has been shown previously to increase markedly at alkaline pH, with a pK_a of about 8.5 (Sluyterman & de Graaf, 1970; Johnson et al., 1981). A similar behavior was found also in this work (Figure 9). In contrast, the fluorescence of both cystatin and its complex with papain remained constant up to pH ~9 and decreased slightly at higher pH with an apparent pK_a of ~9.5 (Figure 9), possibly reflecting tyrosyl ionization. These changes lead to a much larger fluorescence decrease accompanying the cystatin–papain interaction at pH ~10 than at neutral pH (Figure 9). No change of fluorescence was observed for either the free proteins or the complex in the acid pH range, down to about pH 3.6 (results not shown). Titrations of papain with cystatin at pH 3.9 and 9.6 showed that the complex was ≥95% saturated at these pH values under the conditions used.

Discussion

The complex spectroscopic changes in the near-ultraviolet region accompanying the binding of chicken cystatin to papain indicate that the environment of several aromatic amino acids in one or both proteins is perturbed by the interaction. Thus, the peaks in the absorption difference spectrum at 300 and 292 nm suggest that tryptophan residues are involved, whereas the peaks at 289 and 282 nm are indicative of changes around tyrosine residues (Donovan, 1969, 1973a,b). The broad peak around 260 nm may be due partly to phenylalanine or cystine residues and may also contain contributions from tryptophans (Donovan, 1973a). The peaks at 302, 293, and 287 nm in the near-ultraviolet circular dichroism difference spectrum most likely are due to perturbation of tryptophan residues, whereas the 281-nm peak is compatible with changes around either tryptophan or tyrosine residues (Strickland, 1974). Moreover, the magnitude of this difference spectrum, corresponding to an $\epsilon_L - \epsilon_R$ of ~11 M^{-1} cm^{-1}, suggests that at least 3–4 aromatic residues are affected (Strickland, 1974). The fluorescence changes induced by the binding further show that the environment of one or more tryptophan side chains are perturbed. These changes may arise from an altered conformation of either or both proteins induced by the binding. However, they may also, wholly or partly, originate from local interactions affecting chromophoric groups of the two proteins without involvement of a conformational change (Donovan, 1969, 1973a; Strickland, 1974). The small circular dichroism change in the far-ultraviolet region is not necessarily in conflict with the latter alternative, since such local interactions may also contribute to the far-ultraviolet circular dichroism (Holladay & Puett, 1976; Holladay et al., 1977).

Several conclusions can be drawn regarding which aromatic residues of the two proteins are affected by the interaction. The considerably smaller spectral changes observed with cystatin in which the only tryptophan residue, Trp-104, was modified strongly suggest that the environment of this residue is perturbed on binding to papain. Interactions involving this tryptophan appear to be predominantly responsible for the near-ultraviolet circular dichorism changes induced by the binding, although they also contribute appreciably to the absorbance and fluorescence changes. The importance of this residue for the interaction is further indicated by the decrease in the affinity of cystatin for papain accompanying its modification. Trp-104 of cystatin thus appears to be located in or close to the region of the inhibitor that binds to the cysteine proteinase, a region in which Gly-9 and the Gln-Leu-Val-Ser-Gly sequence at residues 53–57 also have been implicated (Ohkubo et al., 1984; Barrett et al., 1986; Abrahamson et al., 1987).

In papain, the three-dimensional structure of which is known (Drenth, 1968, 1971), four of the five tryptophan residues are located around the active site and might thus be affected by the interaction with cystatin. Two of these residues, Trp-69 and Trp-177, are at the surface of the protein, on each side of the reactive cysteine and close to the substrate binding pocket. Previous studies have shown that these two tryptophan residues participate in substrate binding and turnover (Lowe & Whitworth, 1974). The other two residues, Trp-26 and Trp-181, are at about the same distances from the active site as the surface tryptophans but are buried in the interior of the protein. The smaller fluorescence changes and the blue shift of the fluorescence difference spectrum on binding of cystatin to papain in which the two surface tryptophans were modified (Lowe & Whitworth, 1974) indicate that these residues are primarily affected by the binding. Several observations are consistent with the effect being partly due to shielding of the two residues from solvent by the inhibitor. Thus, the fluorescence blue shift, with a maximal fluorescence change at about 350 nm, and the absorbance difference maximum at 292 nm on binding of cystatin to unmodified papain both indicate of a more hydrophobic environment around tryptophans (Donovan 1969, 1973; Chen et al., 1969). Moreover, the acrylamide quenching studies show that tryptophan residues of papain are less exposed in the complex with cystatin than in the free enzyme. The conclusion that the interaction involves Trp-69 and Trp-177 of papain is further supported by the decreased affinity of papain for cystatin following modification of Trp-177 and is also consistent with the role of the two residues in papain catalysis (Lowe & Whitworth, 1974). However, the environment around other aromatic residues of papain, such as the two buried tryptophans and

three tyrosines, Tyr-61, Tyr-67, and Tyr-88, located reasonably close to the reactive cysteine residue, may also be affected by the binding.

That Trp-177 of papain is involved in the interaction with cystatin is further supported by the studies of the pH dependence of the fluorescence of the cystatin–papain complex. In the active enzyme, the fluorescence of Trp-177 is markedly increased by the deprotonization of His-159, which occurs with a pK_a of ~8.5. This increase is due to the loss of the charge on the histidine residue considerably reducing the quenching of the fluorescence of the adjacent Trp-177 (Lowe & Whitworth, 1974; Johnson et al., 1981). The absence of a corresponding fluorescence change in the cystatin–papain complex may be due to a shift of the pK_a of His-159, induced by cystatin binding, to a value outside the pH range investigated. Alternatively, the fluorescence of Trp-177 in the complex is no longer affected by the state of ionization of His-159. This may occur either because the orientation of the indole ring relative to the histidine side chain is different or because the environment around the tryptophan side chain is considerably perturbed by the interaction with cystatin. Nevertheless, it is apparent that the region of papain around His-159 and Trp-177, close to the reactive cysteine, is affected by the binding of the inhibitor.

The spectroscopic changes shown here on binding of chicken cystatin to papain are appreciably different from those reported for the interaction of rat cystatin α (also called thiol proteinase inhibitor or TPI) with the enzyme (Takeda et al., 1986). In particular, a positive absorption difference spectrum throughout the aromatic wavelength region, with a double maximum around 280 nm, and only a small change of near-ultraviolet circular dichroism, centered around 300 nm, were obtained in the latter study. Moreover, a larger decrease of the maximum of the fluorescence emission spectrum than that shown here and a small increase, instead of a decrease, of far-ultraviolet circular dichroism were observed by Takeda et al. (1986). Since rat cystatin α contains no tryptophan, the different spectroscopic changes shown by the two inhibitors are consistent with our conclusion that the single tryptophan of chicken cystatin is affected by the interaction with papain. Evidence for perturbation of the environment of Trp-177 of papain accompanying the binding of the inhibitor was also presented for rat-cystatin α, in agreement with our work. However, the interpretation of the studies with the rat inhibitor are complicated by the fact that only commercial, partially active papain was used.

The equimolar stoichiometry of binding of chicken cystatin to papain established by this study is in contrast with earlier work indicating that about two molecules of inhibitor were required for inhibition of one molecule of enzyme (Nicklin & Barrett, 1984). However, the equimolar binding ratio is in agreement with the apparent molecular weight of chemically cross-linked cystatin–papain complexes in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Nicklin & Barrett, 1984). The reason for these discrepant findings is unclear, although the use of only partially active papain may have influenced the results of the inhibition studies (Nicklin & Barrett, 1984). The lower limit of $\sim 10^9$ M^{-1} estimated for the association constant for the binding of cystatin to papain indicates a very tight interaction and is consistent with the low K_i for the inhibition suggested previously (Nicklin & Barrett, 1984). No difference in the binding of cystatin 1 or 2 to papain was apparent with any of the qualitative or quantitative methods used to characterize this binding. Thus, the small, illusive difference in structure between the two forms of cystatin negligibly affects the interaction of the inhibitor with the proteinase.

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Registry No. Trp, 73-22-3; cystatin, 81989-95-9; papain, 9001-73-4.

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Mechanism of Binding of Horse Liver Alcohol Dehydrogenase and Nicotinamide Adenine Dinucleotide†

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ABSTRACT: The binding of NAD^+ to liver alcohol dehydrogenase was studied by stopped-flow techniques in the pH range from 6.1 to 10.9 at 25 °C. Varying the concentrations of NAD^+ and a substrate analogue used to trap the enzyme–NAD^+ complex gave saturation kinetics. The same maximum rate constants were obtained with or without the trapping agent and by following the reaction with protein fluorescence or absorbance of a ternary complex. The data fit a mechanism with diffusion-controlled association of enzyme and NAD^+, followed by an isomerization with a forward rate constant of 500 s^{-1} at pH 8: $E \rightleftharpoons E\text{–}NAD^+ \rightleftharpoons {}^*E\text{–}NAD^+$. The isomerization may be related to the conformational change determined by X-ray crystallography of free enzyme and enzyme–coenzyme complexes. Overall bimolecular rate constants for NAD^+ binding show a bell-shaped pH dependence with apparent pK values at 6.9 and 9.0. Acetimidylation of ϵ-amino groups shifts the upper pK to a value of 11 or higher, suggesting that Lys-228 is responsible for the pK of 9.0. Formation of the enzyme–imidazole complex abolishes the pK value of 6.9, suggesting that a hydrogen-bonded system extending from the zinc-bound water to His-51 is responsible for this pK value. The rates of isomerization of $E\text{–}NAD^+$ and of pyrazole binding were maximal at pH below a pK of about 8, which is attributable to the hydrogen-bonded system. Acetimidylation of lysines or displacement of zinc–water with imidazole had little effect on the rate of isomerization of the $E\text{–}NAD^+$ complex. Rate constants from a computer simulation suggest that the isomerization partially controls the transient phase of 1-propanol oxidation.

The three-dimensional structures of horse liver alcohol dehydrogenase (EC 1.1.1.1) and its complexes show that the two domains of each subunit move closer together upon forming complexes with NAD^+ and pyrazole (Eklund et al., 1982a), NAD^+ and trifluoroethanol (Plapp et al., 1978), NAD^+ and *p*-bromobenzyl alcohol (Eklund et al., 1982b), and NADH and dimethyl sulfoxide (Eklund et al., 1981). This conformational change is thought to be important for catalysis because residues in the active site are repositioned and water is excluded from the active site (Eklund & Brändén, 1987; Colonna-Cesari et al., 1986; Eklund et al., 1984). Identifying the step, or steps, in the mechanism where the conformation changes and assessing the factors that control the change are the subject of this work.

Steady-state results suggest that the enzyme–NAD^+ complex isomerizes, as the rate constant for dissociation of NAD^+ calculated on the assumption of the ordered bi bi mechanism is 2-fold less than the turnover number for the reduction of acetaldehyde (Wratten & Cleland, 1963; Plapp et al., 1986). Pressure relaxation studies on the enzyme–NAD^+ complex have given preliminary estimates of the rates of isomerization

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