Interaction of chicken cystatin with inactivated papains

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Papain which was inactivated by covalent attachment of small substituents to the active-site cysteine, up to the size of a carbamoylmethyl group, bound with high affinity to chicken cystatin ($K_d < \sim 15 \mu\text{M}$), although less tightly than did active papain ($K_d \sim 60 \text{ fM}$). However, as the size of the substituent was increased further, the affinity decreased appreciably, generally in proportion to the size of the inactivating group. For instance the dissociation constants for papain inactivated with *N*-ethylmaleimide and [*N*-(1-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-(4-guanido)butane were 0.17 and $\sim 10 \mu\text{M}$ respectively. The spectroscopic changes accompanying the reaction of all but the most weakly binding ($K_d \geq 2 \mu\text{M}$) inactivated papains with cystatin were similar to those induced by the active enzyme. Interactions involving the reactive cysteine residue of papain are thus not crucial for high-affinity binding of the enzyme to cystatin, in accordance with a recently proposed model for the enzyme–inhibitor complex, based on computer docking experiments. In this model there is sufficient space around the reactive cysteine in the complex for a small inactivating group, explaining the tight binding of papains with such substituents. However, larger inactivating groups cannot be accommodated in this space and therefore must displace the inhibitor out of the tight fit with the enzyme, in agreement with the observed decrease in binding affinity with increasing size of bulkier substituents. The kinetics of binding of cystatin to inactivated papains were compatible with simple, reversible, bimolecular reactions, having association rate constants of $(7\text{--}9) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4, 25 °C, similar to what was shown previously for the binding of cystatin to active papain. The rate of association of the inhibitor with either active or inactivated papain thus appears to be primarily diffusion-controlled. The decreasing affinity of cystatin for papains inactivated with groups of increasing size was shown to be due to progressively higher dissociation rate constants, consistent with the greater impairment of fit between the binding regions of the two molecules.

INTRODUCTION

A number of protein inhibitors of cysteine proteinases have been identified in mammalian tissues. These inhibitors, called cystatins, all have relative molecular masses of 12000–14000 (Barrett *et al.*, 1986). An analogous inhibitor has also been demonstrated in chicken egg white and can be purified in reasonably large amounts (Fossum & Whitaker, 1968; Keilová & Tomasek, 1974; Anastasi *et al.*, 1983). The reaction between this inhibitor and papain has been studied as a convenient model for the general mechanism of action of cysteine proteinase inhibitors. Chicken cystatin forms a tight ($K_d \sim 60 \text{ fM}$) equimolar complex with papain, blocking the active site of the enzyme (Anastasi *et al.*, 1983; Nicklin & Barrett, 1984; Lindahl *et al.*, 1988; Björk *et al.*, 1989). Complex formation is accompanied by pronounced spectroscopic changes, most likely reflecting local perturbations of the environment of aromatic residues in both enzyme and inhibitor (Lindahl *et al.*, 1988; Björk *et al.*, 1989). The kinetics of the interaction are compatible with the complex being formed in a simple, reversible bimolecular reaction with an association rate constant approaching the limit expected for a rate controlled by macromolecular diffusion (Nicklin & Barrett, 1984; Björk *et al.*, 1989). The inhibitory reactive site has been suggested to include the peptide bond between Gly-9 and Ala-10, the

Gln-Leu-Val-Ser-Gly sequence at residues 53–57 and the region around Trp-104 of the inhibitor (Ohkubo *et al.*, 1984; Barrett *et al.*, 1986; Abrahamson *et al.*, 1987; Lindahl *et al.*, 1988). The recently published X-ray crystal structure of a variant of chicken cystatin shows that these regions form a contiguous hydrophobic, wedge-shaped edge of the molecule (Bode *et al.*, 1988). Moreover, computer docking experiments have shown that this edge is complementary to the active-site cleft of papain and thus presumably constitutes the proteinase binding region of the inhibitor.

Previous qualitative binding studies have indicated that cystatin can also form complexes with papain which has been inactivated by reaction of the active-site cysteine with irreversible sulphydryl-group reagents (Anastasi *et al.*, 1983). However, the nature of this interaction has not been elucidated. In particular, it is not known whether the inactivated papains bind to the inhibitor in the same manner as the active enzyme, nor to what extent the inactivating group affects the rate and affinity of the interaction. In this work we have used equilibrium and kinetic methods to study the binding of chicken cystatin to papain which has been inactivated with a series of reagents of different size to provide further information on the mode of interaction of cysteine proteinase inhibitors with their target enzymes.

Abbreviation used: E-64, [*N*-(1-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-4-guanido)butane.

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MATERIALS AND METHODS

Iodoacetic acid and iodoacetamide were obtained from Merck, Darmstadt, Germany, and Sigma, St. Louis, MO, U.S.A. respectively, and were recrystallized from water. *N*-Ethylmaleimide was from Sigma, *N*-*n*-butylmaleimide and *N*-benzylmaleimide from K & K Laboratories, Plainview, N.Y., U.S.A., and *N*-(3-carboxypropyl)maleimide from Fluka, Buchs, Switzerland. Methylmethanethiol sulphonate was purchased from Aldrich, Steinheim, Germany, and [N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido(4-guanido)-butane (E-64) from Peptide Institute, Osaka, Japan. All other chemicals were of reagent grade.

The purification and storage of cystatin forms 1 and 2 and papain (containing 0.95–1.0 mol of sulphhydryl groups/mol of protein) have been described previously (Burke *et al.*, 1974; Anastasi *et al.*, 1983; Roberts *et al.*, 1986; Lindahl *et al.*, 1988).

Papain (70–100 μ M) was inactivated at 25 °C with a series of sulphhydryl group reagents of varying size (see Table 1). The inactivation was monitored by measurements of papain activity and was continued until this activity was < 1% of that measured before the reaction. Reactions with iodoacetic acid and iodoacetamide were done for 5 min at a reagent concentration of 1 mM in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μ M-EDTA, pH 8.0. *N*-Ethylmaleimide, *N*-*n*-butylmaleimide and *N*-benzylmaleimide were allowed to react with the enzyme at a concentration of 1 mM for 45 min, 15 min and 5 min respectively in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μ M-EDTA, pH 7.4, while the inactivation with *N*-(3-carboxypropyl)maleimide required 4 mM reagent and a reaction time of 30 min in the pH 8.0 Tris buffer for completion. The reaction with the epoxysuccinyl peptide derivative E-64 (Barrett *et al.*, 1982) was for 2 min at pH 7.4 with a molar ratio of reagent to enzyme of 3.0. The inactivation of papain with methylmethanethiol sulphonate (Smith *et al.*, 1975) has been described previously (Roberts *et al.*, 1986; Lindahl *et al.*, 1988). Excess reagents were removed by chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden).

Papain activity was measured by addition of 100 μ l of enzyme (70–100 μ M) to 3 ml of 0.052 M-sodium phosphate buffer/1.03 mM-EDTA, pH 6.5, containing *N*- α -benzoyl-L-arginine *p*-nitroanilide, to give a final concentration of 316 μ M. The hydrolysis of the substrate at 25 °C was monitored by continuous recording of the absorbance at 410 nm for 1–3 min.

Near-ultraviolet absorption difference, circular dichroism and fluorescence emission spectra were measured essentially as described previously for the interaction between cystatin and active papain (Lindahl *et al.*, 1988). However, the molar ratios of cystatin to inactivated papain were increased up to 3.0 in the analyses of *S*-(*N*-ethylsuccinimidyl)papain and weaker-binding inactivated forms of the enzyme (see Table 1) to increase the degree of saturation with the inhibitor. Moreover, the concentrations of these inactivated papains were increased to 2 μ M in the measurements of fluorescence spectra, which were done in 3 mm cells to minimize inner-filter effects. Circular dichroism and fluorescence difference spectra between the complexes of the inactivated papains with cystatin and an equimolar mixture of the two proteins were calculated from the measured spectra as detailed earlier (Lindahl *et al.*, 1988).

Dissociation equilibrium constants for the interaction between cystatin and inactivated papains were measured by titrations of inactivated enzyme with inhibitor. These titrations were monitored by the decrease in tryptophan fluorescence accompanying the interaction, as detailed in a previous publication (Lindahl *et al.*, 1988), or, in the case of papain inactivated with E-64, by circular dichroism in a similar manner. The ratio between the concentration of inactivated papain and the dissociation constant of the complex was between 0.6 and 2.3 in all analyses except those with *S*-(carboxymethyl)-papain at pH 10.5 (see Fig. 4), in which the ratio was about 0.2. The titrations were continued until the molar ratio of cystatin to inactivated papain was 3–4, which gave a degree of saturation of inactivated enzyme with inhibitor of > 80% in most cases. However, in the fluorescence titrations of the weakly binding *S*-[*N*-(3-carboxypropyl)-succinimidyl]-papain the final saturation was only 70%, and in the titrations with *S*-(carboxymethyl)-papain at pH 10.5 only ~ 40% saturation was reached. Higher molar ratios of cystatin to papain were avoided, since high concentrations of the inhibitor contributed markedly to the observed fluorescence or circular dichroism and thus decreased the accuracy of the measurements. Dissociation constants were obtained by non-linear least-squares regression of the titration data to the equation derived for formation of an equimolar complex between the two reactants (Nordenman & Björk, 1978; Lindahl *et al.*, 1988).

The kinetics of the reaction between cystatin and inactivated papains were studied under pseudo-first-order conditions by fluorescence measurements in a Hi-Tech SF4 stopped-flow spectrophotometer (Hi-Tech, Salisbury, Wilts., U.K.), as detailed previously (Björk *et al.*, 1988). The kinetics of dissociation of complexes between cystatin and *S*-(methylthio)- or *S*-(carbamoylmethyl)-papain were monitored by measurements of the displacement of cystatin 2 from its complexes with the inactivated papains by an excess of cystatin 1, as described for complexes with active papain (Björk *et al.*, 1989). The amount of cystatin 2 displaced after different times and the corresponding decrease in the amount of complex between cystatin 2 and inactivated enzyme were analysed by h.p.l.c. on a Mono-Q (Pharmacia) ion-exchange column (Björk *et al.*, 1989).

All analyses were carried out in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μ M-EDTA, pH 7.4, unless otherwise indicated. Protein concentrations were obtained by absorption measurements at 280 nm with the use of absorption coefficients and molecular masses reported previously (Lindahl *et al.*, 1988). The absorption coefficient of active papain was used also for all inactivated papains; spectra of reagents were run to ensure that no inactivating group contributed detectably to the observed absorbance at 280 nm.

RESULTS

Fully active papain, containing about 1 mol of sulphhydryl groups/mol of protein, was inactivated by a series of sulphhydryl-specific reagents, resulting in covalent attachment of groups of increasing size to the active-site cysteine of the enzyme (for nomenclature and structures of inactivating groups, see Table 1). The binding of chicken cystatin to these inactivated papains at pH 7.4, ionic strength 0.15, was characterized by spectroscopic

Table 1. Equilibrium and kinetic data for the interaction between cystatin and inactivated papains

The analyses were done with cystatin 1 (except where indicated otherwise) at 25 °C, pH 7.4, ionic strength 0.15. Most values are given with their 95% confidence limits and the number of measurements in parentheses. A dash (—) indicates that a value could not be determined. Most dissociation equilibrium constants (K_d) were obtained by titration, monitored by measurements of fluorescence emission at 358 nm (see Fig. 3). However, the value for E-64-inactivated papain was obtained by a titration monitored by circular dichroism measurements at 286 nm, and the value for S-(carboxymethyl)-papain was estimated by extrapolation to pH 7.4 of values measured by fluorescence titrations at higher pH (see Fig. 4). The relative decrease of fluorescence emission, $(F_p + F_c - F_{p,c})/F_p$, at 358 nm at saturation of inactivated papain with cystatin was about 30% saturation was reached, as calculated from the dissociation constant estimated by the titration monitored by circular dichroism. Association rate constants (k_{+1}), as well as dissociation rate constants (k_{-1}) for S-(N-ethylsuccinimide)-papain and weaker-binding inactivated papains, were obtained with cystatin 1 from the slopes and intercepts, respectively, of the plots in Fig. 5 and similar plots for the other inactivated papains. Dissociation rate constants for S-(methylthio)- and S-(carbamoylmethyl)-papain were obtained with cystatin 2 by displacement of this form from its complex with papain by an excess of cystatin 1. These data are averages of values obtained by analysis of both the appearance of free cystatin 2 and of the disappearance of the cystatin 2-papain complex. The dissociation rate constant for S-(carboxymethyl)-papain was calculated from k_{+1} and the estimated K_d .

Inactivating reagent	Inactivated papain formed	Structure of inactivating group	$10^6 \times K_d^*$ (M)	$(F_p + F_c - F_{p,c})/F_p$ at saturation	$10^{-6} \times k_{+1}$ ($M^{-1} s^{-1}$)	k_{-1} (s^{-1})	$K_d^\dagger$ (M)
Methyl methanesulphonate	S-(Methylthio)-	-S-CH ₃	—	0.72 ± 0.04 (3)	8.0 ± 0.3 (10)	$(5.7 \pm 1.2) \times 10^{-6}$ (6)	7.1×10^{-13}
Iodoacetamide	S-(Carbamoylmethyl)-	-CH ₂ -CONH ₂	—	0.69 ± 0.04 (3)	7.8 ± 0.4 (10)	$(1.1 \pm 0.3) \times 10^{-4}$ (4)	1.4×10^{-11}
Iodoacetic acid	S-(Carboxymethyl)-	-CH ₂ -COO ⁻	~ 0.0005	0.71 ± 0.06 (3)	6.7 ± 0.2 (10)	$\sim 3 \times 10^{-3}$	—
N-Ethylmaleimide	S-(N-Ethylsuccinimide)-		0.17 ± 0.06 (4) 0.19 ± 0.02 (6)†	0.69 ± 0.03 (4) 0.74 ± 0.02 (6)‡	8.1 ± 0.3 (13)	1.7 ± 0.7 (13)	0.21×10^{-6}
N-n-Butylmaleimide	S-(N-n-Butylsuccinimide)-		0.80 ± 0.10 (10) 0.90 ± 0.08 (4)‡	0.71 ± 0.02 (10) 0.76 ± 0.02 (4)‡	7.8 ± 0.3 (11)	7.0 ± 0.7 (11)	0.89×10^{-6}
N-(3-Carboxypropyl)maleimide	S-[N-(3-Carboxypropyl)succinimide]-		1.7 ± 0.4 (5)	0.39 ± 0.01 (5)	8.8 ± 0.5 (15)	13.6 ± 3.2 (15)	1.5×10^{-6}
N-Benzylmaleimide	S-(N-Benzylsuccinimide)-		0.56 ± 0.04 (9)	0.70 ± 0.08 (9)	6.8 ± 0.5 (9)	3.7 ± 1.0 (9)	0.54×10^{-6}
E-64	E-64-inactivated		R ~ 10	$< \sim 0.25$	—	—	—

* Measured values.

† Values calculated from k_{+1} and k_{-1} .

‡ Obtained with cystatin 2.

Fig. 1. Near-ultraviolet absorption difference spectra between complexes of cystatin 1 with active or inactivated forms of papain and the free proteins

—, Active papain; ·····, *S*-(methylthio)-papain; ----, *S*-(carboxymethyl)-papain; - · - · - ·, *S*-(*N*-ethylsuccinimidyl)-papain; - - - - -, *S*-(*N*-*n*-butylsuccinimidyl)-papain; · - · - ·, *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain; · - · - · - ·, E-64-inactivated papain. Protein concentrations were 13–22 μ M, and molar ratios of cystatin to papain were 1.2 for active papain, *S*-(methylthio)-papain and *S*-(carboxymethyl)-papain, 1.5 for *S*-(*N*-ethylsuccinimidyl)-papain, 2.0 for *S*-(*N*-*n*-butylsuccinimidyl)-papain and 3.0 for *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain and E-64-inactivated papain. The degree of saturation of papain with cystatin, calculated from the dissociation constants reported in Table 1, was > 95% in all analyses except that with E-64-inactivated papain, in which only ~ 75% saturation was achieved.

methods and by measurements of equilibrium and kinetic constants for the interaction. Most studies were done with cystatin 1, although cystatin 2 was also used in some experiments. The two forms of the inhibitor, which have different isoelectric points but differ only minimally in structure (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988), have previously been shown to interact with active papain in an identical or highly similar manner (Nicklin & Barrett, 1984; Lindahl *et al.*, 1988, Björk *et al.*, 1989).

Near-ultraviolet absorption and circular dichroism difference spectra showed that the interaction between cystatin 1 and most inactivated papains studied produced similar spectral changes to those observed with the active enzyme (Figs. 1 and 2), although certain minor differences were apparent. However, the binding of cystatin 1 to *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain and E-64-inactivated papain, to which the binding is weakest (see below), resulted in appreciably different absorption and circular dichroism difference spectra throughout the wavelength range compared with the corresponding spectra measured for either active papain or the other inactivated forms of the enzyme (Figs. 1 and 2). This conclusion is apparent also after allowance for the lower degree of saturation of E-64-inactivated papain with the inhibitor.

Fig. 2. Near-ultraviolet circular dichroism difference spectra between complexes of cystatin 1 with active or inactivated forms of papain and the free proteins

—, Active papain; ·····, *S*-(methylthio)-papain; ----, *S*-(carboxymethyl)-papain; - · - · - ·, *S*-(*N*-ethylsuccinimidyl)-papain; - - - - -, *S*-(*N*-*n*-butylsuccinimidyl)-papain; · - · - ·, *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain; · - · - · - ·, E-64-inactivated papain. Protein concentrations were 13–15 μ M. Molar ratios of cystatin to papain and degrees of saturation were as given in the legend to Fig. 1.

Fluorescence spectra (results not shown) showed that a decrease of the wavelength of the emission maximum accompanies the interaction of cystatin 1 with all those inactivated papains for which data could be obtained, i.e. all except E-64-inactivated papain. This blue shift was from 340–344 nm for the spectrum calculated for an equimolar mixture of inactivated enzyme and inhibitor to 323–328 nm for the spectrum measured for the complex. The blue shift was accompanied by a decrease of the intensity at the emission maximum; together the two effects resulted in the largest decrease in fluorescence intensity occurring at 355–358 nm. These spectral changes were comparable to those observed for the binding of cystatin to active papain (Lindahl *et al.*, 1988). The relative intensity decrease at 358 nm at saturation of the inactivated enzyme with inhibitor, determined by fluorescence titrations (Fig. 3), was similar for all but the two weakest-binding inactivated papains (Table 1), and this value was also similar to that observed with active papain (Lindahl *et al.*, 1988). In contrast, the interaction of cystatin 1 with *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain produced an appreciably lower fluorescence decrease, and an even smaller decrease was estimated for E-64-inactivated papain (Table 1). Reliable data could not be obtained with the latter form of inactivated papain because of the small fluorescence change and the low affinity of the interaction with the inhibitor. The fluorescence decrease caused by cystatin 2 was determined for two of the inactivated papains and was found to be similar to that induced by cystatin 1 (Table 1).

The affinity of cystatin for the different inactivated papains was determined by titrations of inactivated

Fig. 3. Titrations of inactivated papains with cystatin 1, monitored by measurements of tryptophan fluorescence

□, *S*-(*N*-Ethylsuccinimidyl)-papain, 0.4 μM ; ■, *S*-(*N*-*n*-butylsuccinimidyl)-papain, 1 μM ; △, *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain, 1 μM . F_p , fluorescence of inactivated papain; F_c , fluorescence of added cystatin; $F_{p,c}$, fluorescence of the mixture of inactivated papain and cystatin. The measurements were done with excitation and emission wavelengths of 280 nm and 358 nm respectively. The expression on the ordinate defines the relative fluorescence decrease on binding of cystatin to inactivated papain as a positive quantity. The solid lines represent the computer fits of the data to the theoretical binding equation for the formation of an equimolar complex between the two reactants. The dissociation equilibrium constants and maximum fluorescence decreases giving these lines were 0.19 μM and 0.68 for *S*-(*N*-ethylsuccinimidyl)-papain, 0.85 μM and 0.73 for *S*-(*N*-*n*-butylsuccinimidyl)-papain and 1.7 μM and 0.39 for *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain.

enzyme with inhibitor. Most such titrations were monitored by the decrease in tryptophan fluorescence accompanying the binding; three representative titrations are shown in Fig. 3. However, due to the low-affinity and low-fluorescence decrease of the interaction of cystatin with E-64-inactivated papain, titrations of this inactivated enzyme were monitored by near-ultraviolet circular dichroism. The dissociation constants were evaluated on the basis of an equimolar binding stoichiometry, as has been demonstrated previously for the interaction of cystatin with active papain (Lindahl *et al.*, 1988) and as was also shown by fluorescence titrations for the tighter-binding inactivated papains in this work (results not shown). The three forms of inactivated enzyme with the smallest groups linked to the reactive cysteine, i.e. *S*-(methylthio)-, *S*-(carbamoylmethyl)- and *S*-(carboxymethyl)-papain, bound to cystatin 1 with affinities too tight to be measurable by titrations at pH 7.4 ($K_d \leq 1$ nM), as was also found previously for active papain (Lindahl *et al.*, 1988). However, a dissociation constant of ~ 0.5 nM could be estimated for the binding of cystatin 1 to *S*-(carboxymethyl)-papain at this pH by extrapolation of data obtained by titrations at higher pH (see below and Fig. 4). The forms of papain inactivated with the maleimide reagents showed appreciably weaker binding to cystatin 1, with dissociation constants ranging from 0.17 to 1.7 μM at pH 7.4, and generally increasing with the size of the inactivating group (Table 1). E-64-inactivated papain, with the largest

Fig. 4. The logarithm of the dissociation equilibrium constant (K_d) for the binding of cystatin to *S*-(carboxymethyl)-papain as a function of pH

○, Cystatin 1; ●, cystatin 2. The buffers were 0.02 M-Tris/HCl in the pH range 8.2–9.0 and 0.02 M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ in the range 9.5–10.5; all buffers also contained NaCl to an ionic strength of 0.15, and 100 μM -EDTA. The vertical bars represent the 95% confidence intervals computed from three experiments. The broken line indicates extrapolation of the data for cystatin 1 to pH 7.4 to give an approximate dissociation constant of 0.5 nM at this pH.

inactivating group, bound weakly to the inhibitor with a dissociation constant of ~ 10 μM ; this value is somewhat uncertain due to the low circular dichroism change induced by the binding (Fig. 2). The affinity of the binding of cystatin 2 to inactivated papains was assessed with two such forms of the enzyme, *S*-(*N*-ethylsuccinimidyl)- and *S*-(*N*-*n*-butylsuccinimidyl)-papain, and was found to be indistinguishable from that of cystatin 1 (Table 1).

To further characterize the interactions between cystatin and the three tightly-binding inactivated papains, we investigated the pH dependence of these interactions. No such dependence could be observed for *S*-(methylthio)- or *S*-(carbamoylmethyl)-papain, which bound to cystatin 1 with dissociation constants of ≤ 1 nM at pH 9.5 as well as at pH 7.4. In contrast, the affinity between cystatin 1 and *S*-(carboxymethyl)-papain was markedly dependent on pH, the logarithm of the dissociation constant increasing linearly with a slope of ~ 1.3 from pH 8.2 to 10.5 (Fig. 4). Measurements could not be extended to higher pH because of low binding affinity and instability of the proteins. The affinity of cystatin 2 for *S*-(carboxymethyl)-papain at pH 9.0 was similar to that of cystatin 1 at this pH (Fig. 4).

The kinetics of the binding of cystatin 1 to the inactivated papains were investigated by stopped-flow fluorimetry under pseudo-first-order conditions, i.e. with excess inhibitor. All reactions showed simple exponential behaviour. Reactions of cystatin 1 with most inactivated papains were studied only up to inhibitor concentrations of 5–10 μM , as illustrated for four such reactions in Fig. 5(a). However, in studies with two of the inactivated forms of the enzyme, the inhibitor concentrations were increased as much as possible (Fig. 5b), the limit being set by the dead-time of the instrument. All resulting plots

Fig. 5. Observed pseudo-first-order rate constants (k_{obs}) for the binding of cystatin 1 to inactivated papains as a function of cystatin concentration

(a) Low cystatin concentrations; (b) high cystatin concentrations. ○, *S*-(Methylthio)-papain; ●, *S*-(carboxymethyl)-papain; □, *S*-(*N*-ethylsuccinimidyl)-papain; ■, *S*-(*N*-*n*-butylsuccinimidyl)-papain. The ratio of cystatin to inactivated papain was maintained at 10:1 in all analyses. The vertical bars represent the 95% confidence intervals computed from five to six individual experiments.

of the observed pseudo-first-order rate constant versus cystatin concentration were linear (Fig. 5), consistent with all reactions being second-order, as was found previously for the reaction with active papain (Björk *et al.*, 1988). Second-order association rate constants and first-order dissociation rate constants (for those interactions for which the latter were experimentally distinguishable from zero) were computed from the slopes and intercepts, respectively, of the plots in Fig. 5(a) and comparable plots for the other inactivated forms of the enzyme. The association rate constants only varied between $\sim 7 \times 10^6$ and $\sim 9 \times 10^6$ M⁻¹ s⁻¹ at 25 °C for the different inactivated papains (Table 1) and were thus comparable to the value of 9.9×10^6 M⁻¹ s⁻¹ measured for the interaction of cystatin with active papain (Björk *et al.*, 1989). In contrast, the dissociation rate constants increased appreciably with decreasing affinity of cystatin

for the inactivated papains (Table 1); the values obtained correspond to half-lives of the complexes from ~ 0.4 to ~ 0.05 s. Dissociation equilibrium constants calculated from the association and dissociation rate constants agreed well with those measured by fluorescence titrations (Table 1).

Dissociation rate constants for the complexes between the two tightly-binding inactivated papains, *S*-(methylthio)-papain and *S*-(carbamoylmethyl)-papain, and cystatin were measured by displacement of cystatin 2 from its complexes with the inactivated papains by an excess of cystatin 1. This method is similar to that used for the determination of the dissociation rate constant for the complex between cystatin and active papain (Björk *et al.*, 1989). However, since the complexes with the inactivated papains dissociated more rapidly than the complex with the active enzyme, the reactions could be followed to near-completion and could be monitored both by the liberation of cystatin 2 and by the disappearance of its complexes with the inactivated enzymes. Both evaluation procedures resulted in first-order plots that were linear to at least about 75% of the reaction and gave similar dissociation rate constants for each of the two inactivated enzymes, further attesting to the validity of the method (Björk *et al.*, 1989). The dissociation rate constants obtained (Table 1) correspond to half-lives of ~ 34 h and ~ 1.8 h for the complexes between cystatin and *S*-(methylthio)-papain or *S*-(carbamoylmethyl)-papain respectively. Dissociation equilibrium constants of ~ 0.7 pM and ~ 14 pM respectively for the two complexes were calculated from these data and the measured association rate constants. The dissociation rate constant for the complex between cystatin and *S*-(carboxymethyl)-papain could not be measured by this method, the reaction being too rapid. However, a value corresponding to a half-life of ~ 230 s was calculated from the association rate constant and the estimated equilibrium constant (Table 1).

DISCUSSION

These results show that chicken cystatin binds tightly to papain in which the active-site cysteine has been blocked with small inactivating groups. The dissociation equilibrium constant for the binding to *S*-(methylthio)-papain is thus as low as ~ 0.7 pM, only about 10-fold higher than the value of ~ 60 fM obtained for the binding to active papain (Björk *et al.*, 1989). The affinity of cystatin for papain inactivated with the somewhat larger *S*-(carbamoylmethyl) group is also appreciable ($K_d \sim 14$ pM). These values correspond to relatively small differences in unitary free energy change of the reaction (Gurney, 1953; Karush, 1962) from -85 kJ mol⁻¹ for active papain to -79 and -72 kJ mol⁻¹ for *S*-(methylthio)-papain and *S*-(carbamoylmethyl)-papain respectively. The similar spectroscopic changes accompanying complex formation further indicate that the inhibitor binds to these inactivated papains in a similar manner as to the active enzyme. It is thus apparent that interactions involving the reactive cysteine residue of papain are not crucial for high-affinity binding of the enzyme to cystatin, as was also indicated by previous qualitative binding studies (Anastasi *et al.*, 1983). This conclusion is in general agreement with results of analogous studies with some small serine proteinase inhibitors, which bind tightly to anhydrotrypsin or

anhydrochymotrypsin, in which the reactive serine has been converted to dehydroalanine (Ako *et al.*, 1974; Vincent *et al.*, 1974). However, the latter modification results in a decrease of the size of the catalytic residue, in contrast to the modification of the active-site cysteine of papain in this work.

As the size of the substituent on the reactive cysteine of papain increases, the affinity of cystatin for the modified enzyme decreases appreciably, as shown by the papains inactivated with the different maleimide reagents and E-64. The unitary free energy change for the binding of the inhibitor to these inactivated papains is thus ~ -38 to ~ -48 kJ mol⁻¹, i.e. about half that of the binding to the active enzyme. However, the similar spectroscopic changes induced on complex formation indicate that cystatin still interacts with papain inactivated with the smaller of these reagents in a qualitatively similar manner as with the active and tightly-binding inactivated forms of the enzyme. Only when the inactivating group becomes very large, and the affinity thus is considerably reduced (to $K_d \geq \sim 2$ μ M), is the mode of interaction of inactivated enzyme with inhibitor appreciably altered, as suggested by the different spectroscopic changes accompanying the binding of cystatin to *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain and E-64-inactivated papain than to the other inactivated papains. This general behaviour is drastically different from that observed for serine proteinase inhibitors, which do not bind with detectable affinity to enzymes inactivated with bulky substituents, such as a tosyl or a di-isopropylphosphoryl group (Foster & Ryan, 1965; Feinstein & Feeney, 1966; Rosenberg & Damus, 1973; Travis & Salvesen, 1983).

These binding data can be interpreted on the basis of the recently proposed model for the interaction between chicken cystatin and active papain, deduced from computer docking experiments (Bode *et al.*, 1988). The modelling studies suggest that cystatin, unlike inhibitors of serine proteinases, does not bind to the target enzyme in a substrate-like manner. Instead, major contacts are made between two hairpin loops of the inhibitor, around Val-55 and Trp-104, which have conformations quite different from those of a bound substrate, and residues of the enzyme remote from the reactive cysteine, mainly Gln-19, Gln-23, Ala-136 and Trp-177. These contacts, which can be established with negligible alterations of the conformation of either protein, produce a tight fit between inhibitor and enzyme, explaining the observed high affinity of the binding. The reactive site Cys-25 does not appear to be directly involved in the interaction, in accordance with the conclusion discussed above. The model further suggests that there is sufficient space around the reactive cysteine in the complex for an inactivating group of about the size of a carbamoylmethyl group, in agreement with the tight binding of papains having active-site substituents of this size or smaller. However, larger inactivating groups cannot be accommodated in this space and therefore must displace the inhibitor out of the tight fit with the enzyme, in a manner preserving the general mode of interaction but leading to the observed progressive decrease in binding affinity with increasing size of the substituent. Sufficiently large inactivating groups could also qualitatively alter the manner in which the inhibitor binds to the inactivated enzyme, explaining the different spectroscopic changes obtained for papain inactivated with such groups.

The affinity of cystatin for *S*-(carboxymethyl)-papain and *S*-[*N*-(3-carboxypropyl)succinimidyl]-papain at near-neutral pH was lower than the corresponding affinity measured for papain inactivated with an uncharged group of similar size, the carbamoylmethyl and *N*-*n*-butylsuccinimidyl groups respectively. A negatively-charged substituent at the active site of papain thus apparently reduces the strength of the interaction between cystatin and the inactivated enzyme. Although the proposed binding region of cystatin is primarily positively charged, this behaviour may be caused by repulsion between the negatively-charged group on the inactivated enzyme and a restricted region of negative potential in the contact edge of cystatin, formed by exposed carbonyl groups (Bode *et al.*, 1988). The affinity of cystatin for *S*-(carboxymethyl)-papain decreased markedly at high pH; the linear increase, with a slope of about 1, of the logarithm of the dissociation constant with pH up to pH 10.5 indicates that deprotonization of a group with $pK_a > 11$, presumably an arginine residue, is responsible for this effect (Fersht, 1985). Possible candidates for such a role are Arg-6 and Arg-52 in cystatin, which are close to the putative proteinase-binding region (Bode *et al.*, 1988).

The two cystatin forms, 1 and 2, were found to have similar affinities for the two inactivated papains to which the binding of the two forms were compared at pH 7.4, and also for *S*-(carboxymethyl)-papain at pH 9.0. It is therefore reasonable to conclude that cystatins 1 and 2 have similar affinities also for active papain and for the tight-binding inactivated forms of the enzyme, although only the affinity between cystatin 2 and these papain forms could be obtained (see Björk *et al.*, 1989). These findings thus reinforce previous conclusions from spectroscopic and kinetic studies that the minor structural difference between the two cystatin forms, so far unidentified, negligibly affects the inhibitory activity (Anastasi *et al.*, 1983; Nicklin & Barrett, 1984; Lindahl *et al.*, 1988; Björk *et al.*, 1989).

Previous kinetic studies have shown that the binding of chicken cystatin to active papain can be best described as a simple, one-step, reversible, bimolecular reaction (Björk *et al.*, 1989). The present work provides strong evidence that this mechanism is valid also for the interaction of cystatin with inactivated papains. The pseudo-first-order rate constant, measured under conditions of cystatin excess, thus varied linearly with the cystatin concentration, even up to as high concentrations of the inhibitor as were technically possible for the two inactivated papains studied under these conditions. Moreover, dissociation equilibrium constants calculated from association and dissociation rate constants agreed excellently with the corresponding dissociation equilibrium constants that could be measured by fluorescence titrations. The association rate constants were similar for the reactions of cystatin with all inactivated papains studied and also similar to that for the reaction with the active enzyme (Björk *et al.*, 1989). Like the rate of association between cystatin and active papain (Björk *et al.*, 1989), the rate of the reaction with inactivated enzymes thus appears to be primarily governed by the frequency of encounter between the two molecules, i.e. to be largely diffusion-controlled. This conclusion is consistent with no appreciable conformational change of either reacting protein accompanying complex formation, as is also indicated by

the computer docking experiments with active papain (Bode *et al.*, 1988). Instead, the lower affinity of cystatin for inactivated papains than for the active enzyme is due to a higher rate of dissociation of such forms of the enzyme from the inhibitor as a consequence of the impaired fit between the binding regions of the two molecules.

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