Characterization by spectroscopic, kinetic and equilibrium methods of the interaction between recombinant human cystatin A (stefin A) and cysteine proteinases

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The near-UV spectroscopic changes induced by the binding of recombinant human cystatin A to papain were appreciably different from those induced by cystatin C, reflecting mainly interactions involving the single tryptophan of cystatin C, Trp-106. Cystatin A bound tightly and rapidly to papain and cathepsin L, with dissociation equilibrium constants of $\sim 10^{-11}$ – 10^{-13} M and association rate constants of 3×10^6 – 5×10^6 M⁻¹·s⁻¹. These affinities are at least 50–100-fold higher than previously reported values. The kinetics of binding to papain were consistent with a simple reversible bimolecular reaction mechanism, indicating that cystatin A, like chicken cystatin and cystatin C, binds to papain with no appreciable conformational adaptation of either reacting protein. Cystatin A bound more weakly to actinidin and cathepsins B, C and H, with dissociation equilibrium constants of 10^{-8} – 10^{-9} M. The weaker

binding to cathepsin B was largely due to a considerably reduced association rate constant ($\sim 4 \times 10^4$ M⁻¹·s⁻¹), consistent with the ‘occluding loop’ of cathepsin B markedly restricting the access of cystatin A to the active site. The lower affinities for actinidin and cathepsins C and H were due partly to lower association rate constants (2×10^5 – 6×10^5 M⁻¹·s⁻¹) but primarily to higher dissociation rate constants. The mode of binding of cystatin A to inactivated papains indicated that there is appreciably less space around the active-site cysteine of papain in the complex with cystatin A than in the complexes with chicken cystatin and cystatin C. An N-terminally truncated form of cystatin A, lacking the first six residues, had considerably lower affinity for papain than the full-length inhibitor, consistent with an intact N-terminal region being of importance for proteinase binding.

INTRODUCTION

Protein inhibitors of cysteine proteinases are ubiquitous in mammalian organisms. Most such inhibitors belong to the cystatin superfamily of related proteins, which comprises three individual families, I, II and III. The inhibitors in family I are small proteins, having about 100 amino acids and lacking disulphide bonds. Cystatins A and B (also called stefins A and B) and bovine stefin C are members of this family [1–3]. The cystatins in family II, to which cystatins C, D and S (and variants of the latter) as well as chicken cystatin belong, are somewhat longer, about 120 residues, and have two disulphide bonds [1,2,4]. The cystatins in family III are the kininogens, which are larger multifunctional glycoproteins of blood plasma, containing two inhibitory domains homologous with cystatins from family II [1]. All these inhibitors inactivate lysosomal cysteine proteinases, e.g. cathepsins B, H, L and S, and also some structurally related plant proteinases, such as papain and actinidin.

The X-ray structures of chicken cystatin and of a complex between cystatin B (stefin B) and papain have given considerable insight into the mechanism of inactivation of cysteine proteinases by cystatins [5,6]. These structures show that three regions of the inhibitors, namely the N-terminal region with a conserved glycine residue, a hairpin loop containing a similarly conserved Gln-Val-Val-Ala-Gly (QVVAG) sequence and a second hairpin loop close to the C-terminus, form a wedge-shaped binding surface of the inhibitor. This wedge is highly complementary to the active-site

cleft of the proteinase and presumably can interact with the latter with minimal conformational changes in either protein. These structural features are in agreement with the high stability of the enzyme–inhibitor complexes, with dissociation equilibrium constants as low as $\sim 10^{-14}$ M. They are also consistent with kinetic data indicating that the complexes form by simple reversible bimolecular reactions with rate constants close to that expected for a diffusion-controlled rate [7–9].

Compared with chicken cystatin and cystatins B and C, cystatin A has been relatively little characterized. The human inhibitor has been isolated from polymorphonuclear granulocytes and liver [10,11] and expressed in *Escherichia coli* [12–15]. Homologous inhibitors have also been purified from rat and bovine skin [16,17]. The X-ray structure of cystatin A has not been determined, but a recent analysis by NMR shows a similar overall structure to cystatin B [18]. Published equilibrium constants for the binding of the human inhibitor to cysteine proteinases are markedly discrepant for several proteinases, and rate constants have been reported only for the binding to two enzymes [11–13,19,20]. Results from site-directed mutagenesis of residues in the QVVAG region have been taken to indicate an insignificant role for this region in proteinase binding [13], in contrast with similar studies with chicken cystatin [21].

In this paper, we present a characterization of the interaction between human cystatin A and cysteine proteinases by spectroscopic, kinetic and equilibrium methods. The amounts of protein necessary for these studies demanded the use of a

Abbreviations used: ATVAQA-cystatin A, an N-terminally elongated form of cystatin A with six additional residues, Ala-Thr-Val-Ala-Gln-Ala-, from the OmpA signal peptide; DTT, dithiothreitol; E64, [N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]amido-4-guanidobutane; OmpA, outer membrane protein A of *Escherichia coli*; Ser⁷-cystatin A, an N-terminally truncated form of cystatin A lacking the first six residues.

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recombinant inhibitor, expressed in *E. coli*. The results further elucidate the nature of the interaction between human cystatin A and its target proteinases and also form a basis for future studies of structure–function relationships of the inhibitor by site-directed mutagenesis.

MATERIALS AND METHODS

Enzymes

The purification, properties, storage and activation of papain (EC 3.4.22.2), actinidin (EC 3.4.22.14) and recombinant rat cathepsin B (EC 3.4.22.1) have been reported elsewhere [22,23].

Figure 1 Construction of the vector for expression of cystatin A

(a) Nucleotide and corresponding amino acid sequences of the engineered region of the cystatin A cDNA. The PCR-amplified cDNA fragment was digested with *DdeI*. The cleaved cDNA was ligated to a linker reconstituting the sequence encoding intact cystatin A upstream of the *DdeI* site and containing in frame the coding sequence for the OmpA signal peptide and a *Clal* cleavage site. This linker was assembled from four synthetic oligonucleotides; the boundary between the two oligonucleotides forming each strand is marked with a vertical line. The bold arrow marks the primary site of signal peptidase cleavage producing authentic cystatin A. The two thin arrows mark the secondary cleavage sites resulting in elongated forms of the inhibitor. (b) The expression vector carrying the sequence for the fusion protein between the OmpA signal peptide and cystatin A, cloned in via the *Clal* and *BamHI* sites. The latter site was introduced by the downstream PCR primer. The location of sequences for the λP_R promoter, the λ cl 857 temperature-sensitive repressor gene (allowing induction of expression by increasing the temperature [28]) and the Shine–Dalgarno (SD) initiator site, as well as relevant restriction sites, are shown.

Cathepsin L (EC 3.4.22.15) was from sheep liver [24]. Human cathepsins B and H (EC 3.4.22.16) from liver were purchased from Calbiochem (San Diego, CA, U.S.A.). Bovine spleen cathepsin C (EC 3.4.14.1) was from Unizyme, Hørsholm, Denmark. The inactivated papain forms, *S*-(methylthio)papain, *S*-(carbamoylmethyl)papain and *S*-(carboxymethyl)papain, were obtained as in previous work [25,26]. Forms 1 and 2 of chicken cystatin were isolated from egg white [22].

Expression and proteolytic cleavage of cystatin A

A region from nucleotide 49 in the codon for Ile-2 to nucleotide 373 in the 3' untranslated region of the cystatin A cDNA sequence [27] was amplified from human liver total RNA by PCR (GeneAmp RNA PCR kit; Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). This cDNA fragment was cleaved with *DdeI* (Figure 1) and ligated to a synthetic linker reconstituting the sequence coding for intact cystatin A upstream of the *DdeI* site and containing in frame a sequence encoding the signal peptide for outer membrane protein A (OmpA) of *E. coli* (Figure 1). The resulting cDNA segment was ligated into the expression vector pHD 389 (Figure 1), essentially identical with that used for cystatin C expression [28]. After transformation of *E. coli* with the construct, the correct sequence [27,29] of isolated clones was verified.

For production of recombinant cystatin A, transformed *E. coli*, strain MC 1061, was grown at 30 °C in Terrific Broth, containing 50 µg/ml ampicillin, to an absorbance at 550 nm of ~1, i.e. for 12–16 h. Expression was induced by raising the temperature to 42 °C, and the bacteria were grown for another 4–5 h. The cells were collected and the proteins in the periplasm were released by cold osmotic shock [28]. Cystatin A was isolated from the periplasmic extract by affinity chromatography on a column of matrix-linked *S*-(carboxymethyl)papain [22], eluted with 0.05 M NaHCO₃/NaOH/0.5 M NaCl/100 µM EDTA, pH 10.5. After neutralization of the eluate, batches containing 6–8 mg of protein were applied to a Bio-Gel Phenyl-5-PW column (0.75 cm × 7.5 cm; Bio-Rad), equilibrated with 0.1 M potassium phosphate/1.2 M (NH₄)₂SO₄, pH 7.0. The column was eluted at a flow rate of 1 ml/min with a linear gradient (total volume 80 ml) of 1.2–0 M (NH₄)₂SO₄ in the phosphate buffer. Eluted protein fractions were dialysed against 0.05 M Tris/HCl/0.1 M NaCl/100 µM EDTA, pH 7.4.

A truncated form of cystatin A having Ser-7 as its N-terminal amino acid (Ser⁷-cystatin A) was produced by digestion of the inhibitor (1 mg/ml) with subtilisin (EC 3.4.21.14; Boehringer, Mannheim, Germany) at an amount of 10 µg/mg cystatin for 115 h at 25 °C in 0.05 M Tris/HCl/0.1 M NaCl/2 mM CaCl₂, pH 7.4. The reaction was stopped by addition of PMSF to 100 µM. The truncated inhibitor was isolated by chromatography on a Bio-Gel Phenyl-5-PW column essentially as described above, except that the initial concentration of (NH₄)₂SO₄ was 1.5 M.

Cystatin A was digested with cytosolic (EC 3.4.11.1; Sigma) and microsomal (EC 3.4.11.2; Boehringer) leucine aminopeptidase in 0.05 M Tris/HCl/0.1 M NaCl, pH 8.5, and 0.06 M potassium phosphate, pH 7.3, respectively for up to 17 h at 37 °C with enzyme concentrations of 10–20 µg/mg of cystatin A.

Spectroscopy

Near-UV absorption difference spectra, near-UV and far-UV CD spectra and fluorescence emission spectra of cystatin A, papain and the complex between the inhibitor and the proteinase were measured as reported previously [8,22,26]. Near-UV CD

and fluorescence emission difference spectra between the complex and the free proteins were calculated from separately measured spectra [22]. Estimates of secondary structure content from far-UV CD spectra were obtained by the CONTIN program [30].

Titration of active or inactivated forms of papain with cystatin A for the determination of binding stoichiometries or affinities were monitored by the changes in fluorescence emission intensity accompanying the interactions [22,26] (see also below). Stoichiometries and dissociation constants were obtained by non-linear least-squares regression analysis of the titration curves [22].

Association kinetics

The kinetics of binding of cystatin A or an N-terminally elongated form of the inhibitor, ATVAQA-cystatin A, to active or inactivated papains were studied by stopped-flow fluorescence measurements under pseudo-first-order conditions (i.e. with an excess of inhibitor), as detailed previously [7]. The inhibitor concentration range was 0.2–100 μM and 0.4–5 μM for reactions of cystatin A with active papain and inactivated papains respectively and 0.2–2.5 μM for reactions of ATVAQA-cystatin A with active papain.

The rate of binding of cystatin A to actinidin or cathepsins B, C, H and L, and of ATVAQA-cystatin A to cathepsin H, was evaluated under pseudo-first-order conditions by continuous measurement of the loss of enzyme activity in the presence of a fluorogenic substrate, essentially as in earlier work [23,31]. The substrate was carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) for actinidin and cathepsin L, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute) for cathepsin B, glycyl-L-phenylalanine 4-methylcoumaryl-7-amide (Bachem, Bubendorf, Switzerland) for cathepsin C and L-arginine 4-methylcoumaryl-7-amide (Bachem) for cathepsin H. The substrate concentrations were 5–20 μM ; substrate hydrolysis never exceeded 5%. The inhibitor concentration was maintained at at least 10-fold higher than that of the enzyme and was varied over a 10–20-fold range for each enzyme. The K_m values used for correction of the apparent association rate constants [23,31] for substrate competition have been reported elsewhere [9,23,24,31–34].

Dissociation kinetics

The kinetics of dissociation of complexes between cystatin A or ATVAQA-cystatin A and papain or recombinant rat cathepsin B were analysed by virtually irreversibly trapping the enzyme dissociated from the complex by an excess of chicken cystatin, thus preventing reassociation of enzyme and inhibitor [7,26]. The complex concentrations were 10 and 6.5 μM for papain and cathepsin B respectively, and the concentrations of displacing chicken cystatin (form 2) were 50–300 μM for papain and 65–200 μM for cathepsin B. The dissociation rate was sufficiently low to be monitored by the appearance of the tight complex between the displacing chicken cystatin and the liberated enzyme by chromatography on a Mono Q (Pharmacia Biotech, Sollentuna, Sweden) ion-exchange column [26].

The rate of dissociation of the complex between cystatin A and S-(methylthio)papain was analysed by similarly trapping the inactivated enzyme liberated from 1 μM complex by 2–8 μM chicken cystatin (form 1). However, because of the rapid dissociation, the reaction rate was instead monitored by tryptophan fluorescence. This was feasible because the liberated S-(methylthio)papain binds essentially irreversibly to the displacing chicken cystatin and with a larger fluorescence decrease than that caused

by formation of the original complex with cystatin A [25] (see also the Results section). The dissociation was thus accompanied by an exponential decrease in fluorescence with time, from which the dissociation rate constant was obtained by non-linear least-squares regression analysis.

The dissociation kinetics of the complexes of cystatin A with cathepsins C or H were monitored by preventing reassociation of enzyme and inhibitor by trapping cystatin A dissociated from the complexes (30–170 nM for cathepsin C, 8–17 nM for cathepsin H) with a large excess (15–20 μM for cathepsin C, 1.2–5 μM for cathepsin H) of inactive S-(carbamoylmethyl)papain. The rate of concomitant release of cathepsin C or H activity was analysed with the substrates glycyl-L-phenylalanine 4-methylcoumaryl-7-amide (10 μM) and L-arginine 4-methylcoumaryl-7-amide (10 μM) respectively essentially as in previous work [8].

Inhibition constants

Inhibition constants for the interaction of cystatin A with actinidin or cathepsins B, C, H and L, and of ATVAQA-cystatin A with cathepsin H, were obtained from the equilibrium rates of cleavage of a fluorogenic substrate by the enzyme at increasing concentrations of the inhibitor, as detailed earlier [23,31]. The substrates and their concentrations were the same as in the measurements of association rate constants; substrate hydrolysis was less than 5% in all analyses. The inhibitor concentration was varied from $\leq K_{i,\text{app}}$ (the apparent inhibition constant) to $\geq 5 \times K_{i,\text{app}}$, except in the analyses with cathepsin L, in which the lowest concentration was $\sim 2 \times K_{i,\text{app}}$. The enzyme concentration was at least 10-fold lower than that of the inhibitor. For slow inhibition reactions, the equilibrium rates of substrate hydrolysis were obtained by non-linear least-squares regression analysis of the progress curves [23]. The apparent inhibition constants were corrected for substrate competition as described above.

Miscellaneous procedures

SDS/PAGE was performed on 16.5% (w/v) gels with the Tricine buffer system [35]. Analytical isoelectric focusing was performed with the Phast system (Pharmacia Biotech). N-Terminal sequences were determined as in [7]. Molecular mass was analysed in a Kratos Kompact MALDI III instrument (Kratos, Manchester, U.K.) with α -cyano-4-hydroxycinnamic acid as matrix.

Experimental conditions and protein concentrations

All analyses were carried out at $25.0 \pm 0.2^\circ\text{C}$. Reactions of cystatin A with active papain, inactivated papains and actinidin were analysed in 0.05 M Tris/HCl, pH 7.4, containing 0.1 M NaCl and 100 μM EDTA. The buffers in studies of reactions with other enzymes were: for cathepsin B, 0.05 M Mes, pH 6.0, containing 0.1 M NaCl, 100 μM EDTA, 0.5 mM dithiothreitol (DTT) and 0.1% (w/v) poly(ethylene glycol); for cathepsin C, 0.1 M sodium phosphate, pH 6.5, containing 0.05 M NaCl, 1 mM EDTA and 1 mM DTT; for cathepsin H, 0.1 M sodium phosphate, pH 6.0, containing 1 mM EDTA and 1 mM DTT; for cathepsin L, 0.1 M sodium acetate, pH 5.5, containing 1 mM EDTA, 1 mM DTT and 0.005% (w/v) Brij 35.

Most protein concentrations were obtained by absorption measurements at 280 nm. A specific absorption coefficient of $0.80 \pm 0.01 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (mean $\pm$ S.E.M.; $n = 3$) was determined for cystatin A by quantitative amino acid analysis [26]. The molar absorption coefficient of $8800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ calculated from this value and a relative molecular mass of 11000 [27,29] was used also for ATVAQA-cystatin A and Ser⁷-cystatin A.

Absorption coefficients and relative molecular masses for papain, actinidin and cathepsin B have been given previously [8,23]. The molar concentration of active sites of sheep cathepsin L was determined by titration with E64 [24]. The concentrations and relative molecular masses of human cathepsins B and H and of bovine cathepsin C were provided by the manufacturers.

RESULTS

Expression, isolation and general properties

The expression vector was constructed to express the cystatin A polypeptide chain [27,29] fused N-terminally to the OmpA signal sequence (Figure 1). The latter sequence was designed to transport the fusion protein to the periplasmic space with concomitant cleavage of the signal peptide, thus facilitating correct folding of the protein and minimizing inclusion body formation as well as risk of proteolytic degradation.

Isolation of the recombinant cystatin A by affinity chromatography on matrix-linked *S*-(carboxymethyl)papain yielded 25–30 mg of active protein/litre of bacterial culture. However, this preparation was a mixture of three forms of the inhibitor, which could be best separated by hydrophobic interaction chromatography. The major peak, eluted at ~ 0.95 M $(\text{NH}_4)_2\text{SO}_4$ and comprising about half of the material, had the N-terminal sequence Met-Ile-Pro-Gly-Gly-Leu-, i.e. that reported for human cystatin A [29]. The two other peaks, eluted at ~ 0.83 M and ~ 0.64 M $(\text{NH}_4)_2\text{SO}_4$, were elongated by six and nine residues respectively of the OmpA signal sequence (Figure 1), reflecting aberrant cleavage by the signal peptidase. The final yield of cystatin A was 4–8 mg/l of bacterial culture.

Figure 2 CD spectra of recombinant human cystatins A and C and of Ser⁷-cystatin A

(a) Far-UV wavelength region. The cell pathlength and protein concentrations were 0.1 cm and 15–19 μM respectively. (b) Near-UV wavelength region. The cell pathlength and protein concentrations were 1 cm and 70–75 μM respectively. —, Cystatin A; ---, cystatin C; -.-, Ser⁷-cystatin A. The data for cystatin C, shown for comparison, are from [26].

Figure 3 Spectroscopic changes on interaction of recombinant human cystatins A and C with papain

(a) Near-UV absorption difference spectra; (b) near-UV CD difference spectra; (c) fluorescence emission difference spectra. —, Cystatin A; ---, cystatin C. The proteinase concentrations were 15 μM in (a) and (b) and 1 μM in (c). The molar ratio of cystatin to papain was 1.2, giving > 99% saturation of the enzyme. The unit on the ordinate in (b) is the difference in molar ellipticity. The data for cystatin C, shown for comparison, are from [26].

The isolated cystatin A showed one band with an apparent molecular mass of about 11 000 Da in SDS/PAGE under reducing conditions. Its isoelectric point was 4.8 ± 0.1 (mean $\pm$ S.E.M.; $n = 3$), consistent with results for the inhibitor isolated from human tissues [10,11]. The molecular mass, as determined by matrix-assisted laser-desorption mass spectroscopy, was 11 006 Da, in good agreement with the expected value of 11 007 Da [29]. These observations, together with the N-terminal analyses, indicate that the recombinant cystatin A had the correct sequence and length. Titrations, monitored by fluorescence, of active papain and its inactive derivative, *S*-(methylthio)papain, with the inhibitor gave binding stoichiometries of 1.00 ± 0.05 (mean $\pm$ S.E.M.; $n = 6$) and 0.91 ± 0.01 ($n = 3$) respectively. The recombinant cystatin A thus was fully active in proteinase binding.

The far-UV and near-UV CD spectra of the inhibitor (Figure 2) were similar to those reported previously for human cystatin

Table 1 Association rate constants (k_{ass}), dissociation rate constants (k_{diss}) and dissociation equilibrium constants (K_d) at 25 °C for the interaction between human recombinant cystatin A and cysteine proteinases

Measured values are given as means $\pm$ S.E.M., with the number of measurements in parentheses. The methods and buffers used in the determinations are given in the Materials and methods section. Calculated values are given without errors.

Proteinase	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_d (M)
Papain	$(3.1 \pm 0.05) \times 10^6$ (15)	$(5.5 \pm 0.5) \times 10^{-7}$ (5)	$1.8 \times 10^{-13*}$
Actinidin	$(1.6 \pm 0.1) \times 10^5$ (13)	$7.7 \times 10^{-4}\dagger$	$(4.8 \pm 0.7) \times 10^{-9}$ (10)
Cathepsin B (human)	$(3.9 \pm 0.2) \times 10^4$ (10)	$3.5 \times 10^{-5}\dagger$	$(9.1 \pm 1.0) \times 10^{-10}$ (6)
Cathepsin B (rat)	$(3.7 \pm 0.05) \times 10^4$ (10)	$3.6 \times 10^{-5}\dagger$	$(9.7 \pm 0.3) \times 10^{-10}$ (9)
Cathepsin C (bovine)	$(2.3 \pm 0.1) \times 10^5$ (15)	$(1.5 \pm 0.1) \times 10^{-5}$ (3)	$4 \times 10^{-10*}$
Cathepsin H (human)	$(5.9 \pm 0.2) \times 10^5$ (14)	$9.4 \times 10^{-3}\dagger$	$(4.1 \pm 0.2) \times 10^{-8}$ (15)
Cathepsin L (sheep)	$(5.2 \pm 0.05) \times 10^6$ (10)	$(6.3 \pm 0.2) \times 10^{-3}$ (4)	$2.7 \times 10^{-8*}$
		$5.1 \times 10^{-4}\dagger$	$(8.7 \pm 0.1) \times 10^{-10}$ (14)
		$(4.3 \pm 0.2) \times 10^{-4}$ (4)	$7.3 \times 10^{-10*}$
		$9.9 \times 10^{-5}\dagger$	$(1.9 \pm 0.4) \times 10^{-11}$ (9)

* Calculated from k_{ass} and k_{diss} .

† Calculated from k_{ass} and K_d .

A [36], but differed appreciably from those of cystatin C [26]. Analysis of the far-UV spectrum indicated a high content ($\sim 45\%$) of β -structure and a low α -helix content ($\sim 15\%$), in reasonable agreement with previous analyses [36] and with structural data [6,18]. The near-UV CD spectrum was typical of tyrosine residues, cystatin A lacking tryptophans. The maximum at 276 nm and the shoulder at 282 nm are characteristic of phenyl groups of tyrosines exposed to solvent [37].

Spectroscopic changes on interaction with papain

The binding of cystatin A to papain was accompanied by appreciable changes in both near-UV absorption and fluorescence emission (Figure 3). The general characteristics of the difference spectra were those of a shift of the absorption or fluorescence maximum to shorter wavelengths. In contrast, only small changes in near-UV CD were induced by the interaction (Figure 3). The spectroscopic changes were markedly different from those caused by the binding of the related cysteine proteinase inhibitor, cystatin C, to papain.

Kinetics and affinity of interaction with active proteinases

Association and dissociation rate constants, as well as dissociation equilibrium constants, were measured for the binding of cystatin A to several plant and mammalian cysteine proteinases (Table 1). The methods used were governed by the properties of the various interactions studied and the amounts of enzyme available. The association rate constant for binding of cystatin A to papain was measured by stopped-flow fluorescence, but reactions with other proteinases were monitored by the loss of enzyme activity. Dissociation rate constants for most interactions were calculated from measured association rate and dissociation equilibrium constants. In several cases, dissociation rate constants were also measured by trapping the dissociated enzyme (for rate constants $\leq \sim 10^{-5} \text{ s}^{-1}$) or inhibitor (for higher rate constants) and monitoring the appearance of trapped enzyme and liberated enzyme activity respectively. Calculated and measured dissociation rate constants agreed reasonably well in all cases, providing a check of the validity of the data. Dissociation equilibrium constants were determined as inhibition constants

Figure 4 Observed pseudo-first-order rate constants (k_{obs}) for the binding of cystatin A to papain at 25 °C as a function of cystatin A concentration

The molar ratio of inhibitor to proteinase was maintained at 10:1 in all reactions. The vertical bars represent ± 2 S.E.M. computed from four to eight individual experiments.

or, in the case of papain, were calculated from the association and dissociation rate constants. The binding of cystatin A to both human and rat cathepsin B was compared, revealing similar characteristics for the interactions with the two species variants of the enzyme.

In the stopped-flow fluorescence kinetics studies of the binding of cystatin A to papain, the cystatin concentration was increased as high as possible, the limit being set by the dead time of the stopped-flow instrument (Figure 4). The approach of the fluorescence signal to its final value was a first-order process at all cystatin concentrations. Moreover, as in the corresponding analyses of the interaction of chicken cystatin or human recombinant cystatin C with several proteinases [7–9], the observed pseudo-first-order rate constant increased linearly with cystatin concentration throughout the concentration range covered.

Kinetics and affinity of interaction with inactivated papains

The second-order rate constants for binding of cystatin A to *S*-(methylthio)papain or *S*-(carbamoylmethyl)papain, determined

Table 2 Association rate constants (k_{ass}), dissociation rate constants (k_{diss}) and dissociation equilibrium constants (K_d) at 25 °C for the interaction between human recombinant cystatin A and inactivated papains

Measured values are given as means $\pm$ S.E.M., with the number of measurements in parentheses. The buffer was 0.05 M Tris/HCl, pH 7.4, containing 0.1 M NaCl and 100 μ M EDTA. Calculated values are given without errors. N.D., Not determined.

Inactivating group	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_d (M)
S-(Methylthio)-	$(3.4 \pm 0.05) \times 10^6$ (8)	$(8.8 \pm 0.1) \times 10^{-4}$ (8)	$2.6 \times 10^{-10*}$
S-(Carbamoylmethyl)-	$(2.3 \pm 0.1) \times 10^6$ (7)	0.16†	$(7.1 \pm 0.6) \times 10^{-8}$ (4)
S-(Carboxymethyl)-	N.D.	N.D.	$(3.4 \pm 0.6) \times 10^{-6}$ (3)

* Calculated from k_{ass} and k_{diss} .

† Calculated from k_{ass} and K_d .

by stopped-flow fluorimetry, were similar to that for the binding of the inhibitor to active papain (Tables 1 and 2). The dissociation rate constant for the S-(methylthio)papain interaction, measured by a displacement technique, was considerably higher than that for active papain, resulting in ~ 1000 -fold lower affinity (Tables 1 and 2). The affinity for S-(carbamoylmethyl)papain, measured by fluorescence titrations, was a further ~ 200 -fold lower, again the result of a higher dissociation rate constant (Tables 1 and 2). S-(Carboxymethyl)papain had only a weak affinity for cystatin A (Table 2); no rate constants were determined for this interaction.

N-Terminal truncation and elongation

Attempts to generate an N-terminally truncated form of cystatin A by proteolytic cleavage were made as a means to elucidate the contribution of the N-terminal region to proteinase binding, as in previous studies with chicken cystatin and cystatin C [9,23,38,39]. Prolonged treatment of cystatin A with the exopeptidase, leucine aminopeptidase, of cytosolic or microsomal origin failed to remove any amino acids from the N-terminus, as evidenced by sequence analyses. Moreover, of several endopeptidases tested, only digestion with subtilisin produced a truncated inhibitor. It had the N-terminal sequence Ser-Glu-Ala-Lys-Pro-Ala- and showed one band in SDS/PAGE under reducing conditions. Its molecular mass, as determined by mass spectroscopy, was 10435 Da, in good agreement with the expected value of 10438 Da. A single cleavage of the protein had thus occurred between Leu-6 and Ser-7. The cleaved inhibitor (Ser⁷-cystatin A) had near- and far-UV CD spectra that differed somewhat from those of the intact inhibitor (Figure 2). Ser⁷-cystatin A bound more weakly to papain than intact cystatin A, with a dissociation constant of $\sim 1.2 \mu\text{M}$, as determined by fluorescence titrations. The fluorescence decrease on saturation of papain with the cleaved inhibitor was only $\sim 6\%$, i.e. appreciably lower than that produced by intact cystatin A, making this value somewhat uncertain.

The interaction of the form of cystatin A having six extra amino acids from the OmpA signal peptide at its N-terminus, ATVAQA-cystatin A (Figure 1), and two proteinases, papain and cathepsin H, was characterized by the same methods as used for the authentic inhibitor. The elongated inhibitor bound to papain with about half the affinity of authentic cystatin A, because of a decreased association rate constant (Tables 1 and 3). The affinity of ATVAQA-cystatin A for cathepsin H was about

Table 3 Association rate constants (k_{ass}), dissociation rate constants (k_{diss}) and dissociation equilibrium constants (K_d) at 25 °C for the interaction between ATVAQA-cystatin A and cysteine proteinases

Measured values are given as means $\pm$ S.E.M. if more than one measurement was performed, with the number of measurements in parentheses. The methods and buffers used in the determinations are given in the Materials and methods section. Calculated values are given as means.

Proteinase	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_d (M)
Papain	$(1.4 \pm 0.05) \times 10^6$ (5)	6.2×10^{-7} (1)	$4.4 \times 10^{-13*}$
Cathepsin H (human)	$(6.0 \pm 0.2) \times 10^5$ (14)	$2.3 \times 10^{-3}\dagger$	$(3.8 \pm 0.2) \times 10^{-9}$ (14)

* Calculated from k_{ass} and k_{diss} .

† Calculated from k_{ass} and K_d .

5-fold lower than that of authentic cystatin A, although in this case it was due to an increased dissociation rate constant (Tables 1 and 3).

DISCUSSION

Human cystatin A has been expressed previously in *E. coli*, in most cases from synthetic genes. However, several procedures have either been designed to express the inhibitor as a fusion protein or resulted in very low yields of soluble material [12,14,20]. Although our expression system produces elongated forms of cystatin A in addition to the authentic inhibitor, the final yield of the latter is somewhat better than the yields of previous procedures giving soluble cystatin A [13,15]. Moreover, the expression system produces fully active inhibitor, which has not been ascertained in previous work.

The features of the UV absorption and fluorescence difference spectra produced by the binding of cystatin A to papain indicate that these spectroscopic changes arise predominantly from perturbations around tryptophan residues. As cystatin A lacks tryptophan, the most likely candidates are Trp-177 and/or Trp-69 near the active site of papain, in accordance with previous results for chicken cystatin and cystatin C [22]. The spectroscopic changes may be caused by either shielding of these tryptophans from solvent by the inhibitor or local interactions with residues in cystatin A [22]. The different spectral changes induced by the binding of cystatin A and cystatin C to papain presumably reflect mainly interactions involving the single tryptophan residue of cystatin C, Trp-106. In particular, the minimal changes of near-UV CD seen with cystatin A are consistent with the pronounced near-UV CD changes observed for cystatin C being caused almost exclusively by interactions involving this residue [22,26]. The UV absorption and fluorescence changes are appreciably different from those reported for the binding of rat cystatin α to papain [40], indicating that the two species variants of cystatin A may interact somewhat differently with the enzyme.

Reported affinities for the interactions of human cystatin A with cysteine proteinases vary substantially for several enzymes. The inhibition constants for papain and cathepsin L measured in this work are at least 50–100-fold lower than previously reported values, and that measured for cathepsin B is at least 5-fold lower [11–13,19,20]. These differences may reflect difficulties in directly measuring equilibrium constants for tight interactions in the earlier work. The affinities of human cystatin A for the physiological target enzymes cathepsins B and L are thus appreciably higher than previously believed and in approximate agreement

with recently published affinities of these enzymes for bovine cystatin A (stefin A) [17]. However, the inhibition constants for the interactions of human cystatin A with cathepsins C and H measured in this work are comparable with values obtained previously [11,20] and, in the case of cathepsin H, also with the value for the binding to bovine cystatin A [17].

Of the enzymes studied, human cystatin A binds most tightly and rapidly to papain and cathepsin L, i.e. with dissociation equilibrium constants of $\sim 10^{-11}$ – 10^{-13} M and association rate constants of 3×10^6 – 5×10^6 M $^{-1}$ ·s $^{-1}$. The affinity for papain is somewhat lower than those of the family-II inhibitors, chicken cystatin and human cystatin C, but higher than that of human cystatin B [7,26,41]. However, the affinity for cathepsin L is similar to that of cystatin B [41]; the affinities of chicken cystatin and cystatin C for cathepsin L appear to be higher but have not been accurately determined. The rate constant for the binding of human cystatin A to papain is similar to the value reported previously [19]. The rate constants for the association of the inhibitor with papain and cathepsin L are also comparable with values measured for chicken cystatin, human cystatins B and C and bovine cystatin A [7,17,26,38,41]. These rate constants approach those expected for a diffusion-controlled rate. Moreover, the linear increase in the observed pseudo-first-order rate constant for papain inhibition with inhibitor concentration up to the highest concentration that could be studied in the stopped-flow fluorimeter is consistent with a simple reversible bimolecular reaction mechanism. As suggested for the interaction of chicken cystatin and human cystatin C with several proteinases, cystatin A thus also appears to bind to papain with no appreciable conformation adaptation of either protein [5,7,26].

The binding of human cystatin A to the other enzymes studied is weaker than to papain and cathepsin L, i.e. with dissociation equilibrium constants of 10^{-8} – 10^{-9} M. The affinity of cystatin A for cathepsin B is comparable with those of chicken cystatin and cystatin C for this enzyme, but appreciably higher than that of cystatin B [11,23,38,41,42]. The weaker binding of human cystatin A to cathepsin B than to papain or cathepsin L is largely due to a considerably reduced association rate constant ($\sim 4 \times 10^4$ M $^{-1}$ ·s $^{-1}$), in contrast with the corresponding weaker binding of the two family-II inhibitors to cathepsin B, which is due more to increased dissociation rate constants [19,23]. This behaviour, also observed for bovine cystatin A [17], is consistent with the 'occluding loop' of cathepsin B [43] restricting the access of cystatin A to the active site of the enzyme to a much larger extent than the access of chicken cystatin or cystatin C.

The affinity of human cystatin A for actinidin is similar to those of chicken cystatin and cystatin C [8,26], whereas the affinity of cystatin A for cathepsin H is up to ~ 10 -fold lower and that for cathepsin C up to ~ 100 -fold lower than those reported for the two family-II cystatins [1,38,42,44]. Cystatin A also appears to bind cathepsins C and H considerably more weakly (50–100-fold) than does cystatin B [11,41]. The lower affinity of cystatin A for actinidin and cathepsins C and H than for papain and cathepsin L is due partly to lower association rate constants (2×10^5 – 6×10^5 M $^{-1}$ ·s $^{-1}$) but primarily to higher dissociation rate constants.

Human cystatin A binds to inactivated papains, but the affinity decreases with increasing size of the inactivating group, due to increasing dissociation rate constants. Substituents on the active-site cysteine of papain can thus be accommodated in the complex with the inhibitor, but progressively displace the latter from the tight fit with the enzyme with increasing size, in a similar manner to that shown for chicken cystatin and cystatin C [25,26]. However, the inactivating groups on papain have much larger effects (≥ 100 -fold) in reducing the affinity for cystatin A

than they have on the affinities for chicken cystatin and cystatin C, because of larger effects on the dissociation rate constants [25,26,31]. This finding indicates that there is appreciably less space around the active-site cysteine of papain in the complex with cystatin A than in the complexes with the family-II cystatins [5].

The N-terminally truncated form of cystatin A, which lacks the first six residues, had an appreciably lower affinity for papain than the full-length inhibitor; this is similar to naturally occurring truncated variants of rat cystatin α [40]. An intact N-terminal region of cystatin A thus appears to be of importance for proteinase binding. This region may interact with the enzyme in a similar manner to that deduced for chicken cystatin and cystatin C [9,23,31,38,39,42]. The CD changes observed on N-terminal truncation of cystatin A are not in conflict with this proposal, as these changes most likely reflect local structural perturbations as a result of the cleavage. A change in the conformation of the remainder of the binding surface of the inhibitor induced by removal of the N-terminal region is less probable in view of the demonstration by NMR that this region is flexible and does not interact with the main body of the protein [18]. Nevertheless, it cannot be excluded that the N-terminal region may also be important for maintaining the correct inhibitory conformation of cystatin A. In contrast with truncation, elongation of the N-terminal region had a minor effect on the affinity of cystatin A for both papain and cathepsin H. This finding is contrary to results obtained for cystatin B, elongation of which, although with different residues from those used in this work, resulted in a markedly weaker binding to cathepsin H [41]. This difference is consistent with the N-terminal regions of the two inhibitors interacting with cathepsin H in somewhat different manners.

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