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# Cystatin inhibition of cathepsin B requires dislocation of the proteinase occluding loop. Demonstration by release of loop anchoring through mutation of His110

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**Abstract Cystatins A and C were both shown to inhibit cathepsin B by a two-step mechanism, involving an initial weak interaction followed by a conformational change. Disruption of the major salt bridge anchoring the occluding loop of cathepsin B to the main body of the enzyme by mutation of His110 to Ala converted the binding to an apparent one-step reaction. The second step of cystatin binding to cathepsin B must therefore be due to the inhibitor having to alter the conformation of the enzyme by displacing the occluding loop to allow a tight complex to be formed. Cystatin A was appreciably less effective in displacing the loop than cystatin C, resulting in a considerably lower overall inhibition rate constant.** **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

*Key words:* Cysteine proteinase; Cysteine proteinase inhibitor; Cathepsin; Cystatin; Stopped-flow kinetics

## 1. Introduction

Cystatins are inhibitors of papain-like cysteine proteinases and act by binding tightly to the active site cleft of the enzyme (reviewed in [1–5]). The cystatin superfamily contains three subfamilies. Cystatins of family I, also termed stefins, are single domain proteins of about 100 amino acids, having no disulphide bonds. The inhibitors of family II are somewhat longer, about 120 amino acids, and have two internal disulphide bonds. Cystatins of family III, termed kininogens, are high molecular mass, multi-domain glycoproteins.

Most papain-like cysteine endoproteinases have an open, easily accessible active site [5–7]. The kinetics of cystatin inhibition of these enzymes is consistent with a simple, one-step reaction mechanism [8–14], in agreement with computer docking studies [15]. In contrast, the dominating lysosomal cysteine proteinase, cathepsin B, has an 'occluding loop' that partially blocks the active site, thereby being responsible for the dipeptidase activity of this enzyme [16,17]. Studies of the kinetics of binding of cystatin C, a family II cystatin, to cathepsin B have indicated that the reaction occurs in two steps, an initial weak binding being followed by a conformational change in the second step [18]. It has been proposed that this change is due to the necessity for the inhibitor to alter the conformation of the enzyme by displacing the occluding loop so that a tight complex can be formed [16,18].

In this work we show that cystatin A (also called stefin A), a family I inhibitor, also binds to cathepsin B by a two-step reaction mechanism with a conformational change in the second step, consistent with this mechanism being common for all cystatins. Moreover, we show that increasing the mobility of the occluding loop of cathepsin B by disruption of the salt bridge between His110 and Asp22 through mutation of His110 to Ala [16,19] transforms the two-step binding of both cystatins A and C to an apparent one-step reaction. This observation strongly supports the notion that the transition between the initial weak and final tight cathepsin B–cystatin complexes involves the inhibitor dislocating the occluding loop of cathepsin B, thereby inducing a conformational change in the enzyme.

## 2. Materials and methods

### 2.1. Proteins

Human recombinant cystatins A and C and papain (≥95% active) were produced and isolated as described earlier [12,20–23]. The inactive papain derivative, *S*-(methylthio-)papain, was prepared as in previous work [23]. Human recombinant cathepsin B and the C29A and H110A/C29A mutants of the enzyme, constructed by site-directed mutagenesis, were expressed as proenzymes [17,18]. The wild-type proenzyme was activated as described previously [17,18]. C29A-procathepsin B was processed by incubation with a catalytic amount of wild-type human cathepsin B for 4 h at 37°C in 20 mM sodium acetate, 1 mM dithiothreitol (DTT), pH 5.0, resulting in complete conversion to the mature form. The wild-type enzyme was then inactivated by addition of [*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amido-4-guanidobutane to 15 μM. Processing of H110A/C29A-procathepsin B occurred on storage of the purified proenzyme for 2 months at 4°C in 20 mM sodium acetate, pH 4.7. The processed proteins were purified by ion-exchange chromatography on CM Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden).

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*Abbreviations:* C29A-cathepsin B, cathepsin B variant in which Cys29 is replaced by Ala; DTT, dithiothreitol; H110A/C29A-cathepsin B, C29A-cathepsin B variant in which His110 is replaced by Ala; $K_d$, overall dissociation equilibrium constant; $K_i$, inhibition constant; $k_{obs}$, observed pseudo-first-order rate constant; $k_{off}$, overall dissociation rate constant; $k_{on}$, overall association rate constant; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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Protein concentrations were obtained by absorption measurements at 280 nm. The molar absorption coefficients used were: 8800 $M^{-1}$ $cm^{-1}$ for cystatin A [12], 11 100 $M^{-1}$ $cm^{-1}$ for cystatin C [10], 55 900 $M^{-1}$ $cm^{-1}$ for papain and *S*-(methylthio-)papain [23] and 63 000 $M^{-1}$ $cm^{-1}$ for wild-type cathepsin B and its two mutants [18].

Protein homogeneity was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and non-reducing conditions with the tricine buffer system [24].

*2.2. Binding stoichiometries*

The fraction of active inhibitor in the cystatin A and C preparations was checked by stoichiometric titrations, monitored by the fluorescence decrease induced by the binding of 1 μM papain with inhibitor [23]. The fraction of protein in the recombinant cathepsin B preparations that was active in binding cystatins was assessed by analogous titrations of 1 μM enzyme with cystatin C. The titrations of the wild-type enzyme were monitored by activity measurements [18] and those of the inactive C29A and H110A/C29A mutants by the increase in tryptophan fluorescence accompanying the interaction with cystatin C (see Section 3).

*2.3. Affinity and kinetics of inhibition of wild-type cathepsin B*

The inhibition constant, $K_i$, for the interaction of cystatin A with wild-type human cathepsin B was determined from the equilibrium rates of cleavage of the fluorogenic substrate, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan), at different concentrations of the inhibitor [25,26]. The overall association rate constant, $k_{on}$, for the same interaction was evaluated under pseudo-first-order conditions by continuous measurements of the loss of enzyme activity against the fluorogenic substrate by conventional fluorometry [25].

*2.4. Stopped-flow kinetics*

The rates of association of cystatin A with wild-type cathepsin B and of cystatins A and C with the C29A-cathepsin B (cathepsin B variant in which Cys29 is replaced by Ala) and H110A/C29A-cathepsin B (C29A-cathepsin B variant in which His110 is replaced by Ala) mutants were analysed in a SX-17 MV stopped-flow fluorimeter (Applied Photophysics, Leatherhead, UK) by monitoring the resulting tryptophan fluorescence change [18]. The molar ratio of cystatin to the fraction of the cathepsin B variants active in binding inhibitor was at least 10-fold. Observed pseudo-first-order rate constants, $k_{obs}$, were obtained by non-linear least-squares regression of the progress curves.

*2.5. Dissociation kinetics*

The rates of dissociation of the complexes of cystatins A and C with the C29A-cathepsin B mutant were measured by displacement of the inhibitor from 1 μM complex with an excess (2.5–7 μM) of *S*-(methylthio-)papain [12], which forms much tighter complexes with both cystatins than cathepsin B does [10,12,25]. The overall dissociation rate constants, $k_{off}$, were obtained by non-linear least-squares regression analysis of the exponential decrease of tryptophan fluorescence accompanying formation of the complexes between the liberated cystatins and *S*-(methylthio-)papain. The fluorescence decrease is due to these complexes having lower fluorescence than the corresponding complexes with cathepsin B [10,12,18].

*2.6. Experimental conditions*

All experiments were carried out at 25.0 ± 0.2°C. Wild-type cathepsin B was activated with 1 mM DTT for 5 min before measurements and analysed in 50 mM Mes, 100 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1% (w/v) poly(ethylene glycol) 6000, pH 6.0. The experiments with C29A-cathepsin B and H110A/C29A-cathepsin B were performed in the same buffer without DTT.

## 3. Results

*3.1. Homogeneity and activity of the proteins*

As in earlier work, recombinant cystatins A and C and recombinant wild-type cathepsin B were >97% homogeneous in SDS–PAGE [17,20,22]. In further accord with this work, the two cystatins were fully active in binding to papain, whereas wild-type cathepsin B was 48 ± 1% (range, $n = 2$) active in binding to cystatin C.

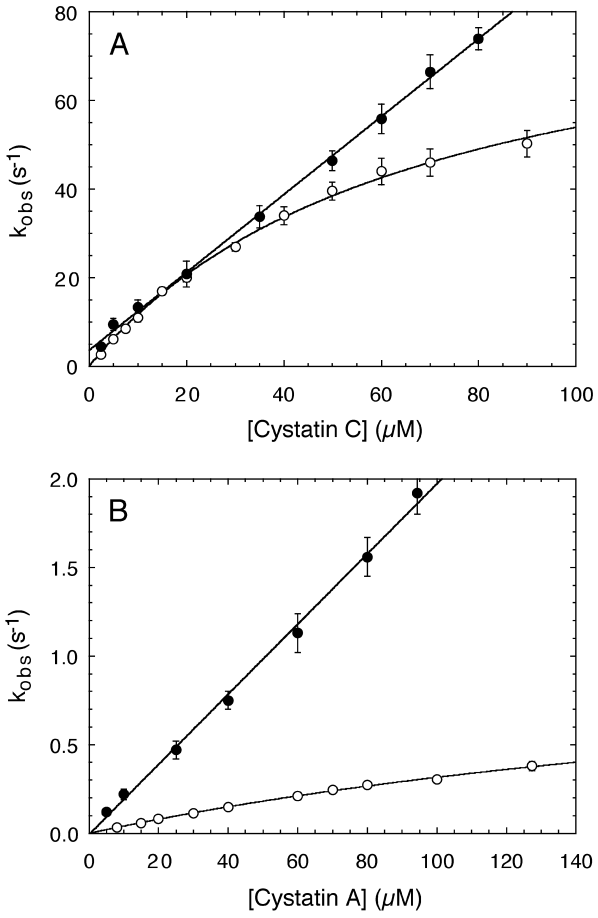



Fig. 1. $k_{obs}$ for the binding of cystatin C (A) or cystatin A (B) to C29A-cathepsin B (○) or H110A/C29A-cathepsin B (●) as a function of inhibitor concentration. The vertical bars represent the standard errors of the mean calculated from 10–20 individual measurements. Error bars not seen lie within the dimensions of the symbols. The solid lines represent non-linear least-squares regression fits to the hyperbolic equation for the two-step mechanism in Scheme 1 [30] in the case of C29A-cathepsin B and linear least-squares regression fits in the case of H110A/C29A-cathepsin B.

In the present work, the mobility of the occluding loop of cathepsin B was increased by mutation of His110 to Ala, disrupting the salt bridge to Asp22 [16,19]. Isolation of sufficient amounts of a stable form of this mutant required that autoproteolysis be prevented by a concurrent mutation of the active site Cys29 to Ala (Krupa and Mort, in preparation). The C29A-cathepsin B control and H110A/C29A-cathepsin B were >97% and ~80% homogeneous in SDS–PAGE, respectively. In the latter case, the unprocessed proenzyme, which is inactive and unable to bind inhibitors [27–29] and therefore should not influence the analyses, was the major contaminating protein. Titrations with cystatin C showed that the C29A and H110A/C29A mutants were 97 ± 4% (S.E.M., $n = 6$) and 79 ± 2% (range, $n = 2$) active in binding cystatins, in good agreement with their purity in SDS–PAGE.

*3.2. Kinetics of cystatin C binding*

The kinetics of binding of cystatin C to C29A- or H110A/

C29A-cathepsin B were monitored under pseudo-first-order conditions by the ~15% increase in tryptophan fluorescence accompanying the interaction with either enzyme form. All progress curves were well fitted to a single exponential function, giving $k_{obs}$. As in similar previous studies with wild-type, active cathepsin B [18], $k_{obs}$ for the binding of cystatin C to the C29A-cathepsin B control varied hyperbolically with the inhibitor concentration (Fig. 1A). This behaviour is characteristic of a two-step binding mechanism with a conformational change occurring in the second step [30]:

$$P + I \overset{K_1}{\rightleftarrows} PI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} PI* \qquad \text{Scheme 1}$$

In this mechanism, a weak complex (PI) between proteinase (P) and inhibitor (I) in a rapid equilibrium, characterised by the dissociation constant $K_1$, is formed in the first step, followed by a conformational change with the rate constants $k_{+2}$ and $k_{-2}$ in the second step to form the final complex (PI*). Fitting of the data for the binding of cystatin C to C29A-cathepsin B to the equation for this mechanism [30] gave values for $K_1$ and $k_{+2}$ (Table 1). However, $k_{-2}$, which in this mechanism is equal to $k_{off}$ and is given by the intercept on the ordinate, was too low to be determined. Instead, $k_{off}$ was measured by displacing the inhibitor from the complex by an excess of a tight-binding, inactive papain derivative (Table 1). Both $K_1$ and $k_{+2}$, as well as $k_{on}$, calculated from the initial slope, i.e. as $k_{+2}/K_1$ [30], were comparable to the values for the binding of cystatin C to active cathepsin B (Table 1). However, $k_{off}$ was ~five-fold higher than for the binding to the active enzyme, thereby being mainly responsible for a somewhat higher overall dissociation equilibrium constant ($K_d$), calculated as $k_{off}/k_{on}$ (Table 1).

Contrary to what was observed for the C29A-cathepsin B control, $k_{obs}$ for the binding of cystatin C to the H110A/C29A mutant increased linearly with the inhibitor concentration up to the highest concentrations that could be reached with the amounts of protein available (Fig. 1A). This behaviour is indicative of a one-step binding reaction, although it cannot be excluded that it reflects only the initial, approximately linear portion of a hyperbolic curve for a two-step reaction. However, in the latter case both $K_1$ and $k_{+2}$ must be appreciably higher than for the binding to the C29A-cathepsin B control, simulations indicating values for $K_1$ of $\gg 200$ μM and for $k_{+2}$ of $\gg 200$ $s^{-1}$ being compatible with the data. The value of $k_{on}$, which is the only parameter that can be derived from the linear $k_{obs}$ dependence, was similar to those for the binding of cystatin C to active cathepsin B and the C29A mutant (Table 1).

### 3.3. Kinetics of cystatin A binding

Attempts to analyse the stopped-flow kinetics of cystatin A binding to wild-type, active cathepsin B, which have not been previously investigated, were unsuccessful because a ≤4% fluorescence decrease due to complex formation could not be separated from a concurrent slow decrease that most likely was caused by photodecomposition. The binding therefore could only be characterised by monitoring enzyme inhibition in the presence of a fluorogenic substrate by conventional fluorometry (Table 1). The measured $K_i$ and $k_{on}$ and calculated $k_{off}$ values were comparable to those similarly obtained for the interaction of cystatin A with human liver cathepsin B [12].

The kinetics of binding of cystatin A to C29A- or H110A/C29A-cathepsin B could be monitored by an exponential ~5% increase in tryptophan fluorescence. As in the case of cystatin C, $k_{obs}$ for the binding of cystatin A to C29A-cathepsin B showed the hyperbolic dependence on inhibitor concentration characteristic of a two-step binding mechanism with a conformational change occurring in the second step (Fig. 1B; Scheme 1). However, $K_1$ and $k_{+2}$ differed appreciably from those for cystatin C binding (Table 1). $K_1$ was thus ~fourfold higher and $k_{+2}$ almost 100-fold lower, resulting in a ~300-fold lower $k_{on}$. The latter value was ~six-fold lower than that for the interaction of cystatin A with the active enzyme. $k_{off}$, again undeterminable by stopped-flow kinetics and instead measured by displacement experiments, was comparable to the value for the complex with active cathepsin B (Table 1). The somewhat higher $K_d$ for the binding of cystatin A to C29A-cathepsin B than to the active enzyme (Table 1) was thus due mainly to the lower $k_{on}$.

In further agreement with the experiments with cystatin C, $k_{obs}$ for cystatin A binding to H110A/C29A-cathepsin B increased linearly with cystatin concentration (Fig. 1B), again reflecting a transformation to a one-step binding mechanism or a substantial increase in both $K_1$ and $k_{+2}$ of the two-step mechanism. Simulations indicated that $K_1 \gg 500$ μM and $k_{+2} \gg 10$ $s^{-1}$ would be required to account for the observed linear dependence in this case. The H110A mutation thus caused at least ~10-fold increase in $k_{+2}$. $k_{on}$ obtained from the linear $k_{obs}$ dependence was ~five-fold higher than that for

Table 1
Kinetic and equilibrium constants for the interaction of recombinant human cystatins C and A with recombinant human wild-type cathepsin B and its C29A and H110A/C29A mutants

| Inhibitor | Cathepsin B form | $K_1$ (μM) | $k_{+2}$ ($s^{-1}$) | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $10^4 \times k_{off}$ ($s^{-1}$) | $K_d$ (nM) |
|---|---|---|---|---|---|---|
| Cystatin C | wild-type | 48 ± 10 | 118 ± 11 | $2.4 \times 10^6$[a] | 6.7 ± 0.2 | 0.28[b] |
| | C29A | 65 ± 10 | 90 ± 10 | $1.3 \times 10^6$[a] | 34 ± 3 | 2.6[b] |
| | H110A/C29A | | | $(0.88 \pm 0.01) \times 10^6$ | ND | ND |
| Cystatin A | wild-type | ND | ND | $(2.45 \pm 0.05) \times 10^4$ | 0.5[c] | 2.1 ± 0.1 |
| | C29A | 285 ± 35 | 1.2 ± 0.1 | $4.2 \times 10^3$[a] | 1.2 ± 0.1 | 29[b] |
| | H110A/C29A | | | $(1.98 \pm 0.05) \times 10^4$ | ND | ND |

Measured values are given with standard errors of the mean, derived by linear or non-linear regression of the plots in Fig. 1 and similar plots or, in the case of $k_{off}$, obtained from six and three individual displacement experiments with cystatin C and cystatin A, respectively. Calculated values are given without errors. The values for the binding of cystatin C to wild-type cathepsin B are taken from previous work [18]. ND, not determined.
[a]Calculated as $k_{+2}/K_1$.
[b]Calculated as $k_{off}/k_{on}$.
[c]Calculated as $k_{on} \times K_d$.

cystatin A binding to the C29A-cathepsin B control but similar to that for the binding to the active enzyme (Table 1).

## 4. Discussion

The aim of this work was to characterise the effects of increasing the mobility of the occluding loop of cathepsin B on the two-step kinetics of inhibition of the enzyme by cystatins. To this end, the major salt bridge that anchors the loop to the main body of cathepsin B was disrupted by mutation of His110 [16,19]. However, because of the decreased stability of this mutant, the enzyme was inactivated by mutation of the active site. Cystatin C bound to the Cys29 to Ala control mutant in a similar manner as to active cathepsin B, i.e. by a two-step mechanism with a comparable affinity of the first, weak binding step, $K_1$, and a comparable forward rate constant of the conformational change in the second step, $k_{+2}$ (Scheme 1). Only the reverse rate constant of the second step, $k_{-2}$, was somewhat increased, indicating that Cys29 may contribute slightly to the stabilisation of the conformationally altered state. A corresponding comparison of cystatin A binding to the inactivated and active cathepsin B forms was not possible, as a low fluorescence change and a pronounced photodecomposition precluded stopped-flow analyses of the interaction of this cystatin with active cathepsin B. However, the somewhat higher overall association rate constant, $k_{on}$, determined by conventional inhibition kinetics is consistent with slight changes of either or both of $K_1$ and $k_{+2}$, although in this case $k_{-2}$ was essentially unaffected. Nevertheless, it is apparent that mutation of Cys29 of cathepsin B to Ala only minimally affects the interactions of cystatins with the enzyme.

In a previous study it was suggested that the conformational change occurring with the rate constant $k_{+2}$ in the second step of cystatin C binding to cathepsin B involves the inhibitor displacing the occluding loop of the enzyme [18]. The present work provides compelling evidence for this proposal. Increasing the mobility of the occluding loop of cathepsin B by release of loop anchoring thus was found to markedly alter the mode of binding of both cystatins A and C to the enzyme, so that no second step could be discerned. The increased flexibility of the loop thus must have led to cystatin binding in only a single step, as is apparent for cysteine proteinases having easily accessible active sites, like papain [8–14], or to both $K_1$ and $k_{+2}$ of the two-step mechanism having been substantially increased. The first alternative is consistent with the loop having become sufficiently mobile so as not to interfere with the inhibitor entering the active site cleft of the proteinase, whereas the second alternative would mean that the increased mobility of the loop has greatly facilitated its being pushed aside by the inhibitor. Both alternatives strongly indicate that the conformational change in the second step of cystatin binding to cathepsin B with intact loop anchoring occurs in the enzyme and is due to the inhibitor having to dislocate the loop to allow a tight complex to be established. This dislocation is made possible by the initial weak interaction of the cystatin with the enzyme, in which the N-terminal region of the inhibitor may play an important role [25,26,31,32].

Both cystatins C and A were shown in this work to bind to cathepsin B by a two-step mechanism. This behaviour indicates that a conformational change in the enzyme, due to the occluding loop being pushed aside by the inhibitor, is a common feature of the inhibition of cathepsin B by cystatins. However, the kinetics of this change differed markedly for the two inhibitors. In particular, the considerably lower forward rate constant of the conformational change, $k_{+2}$, for cystatin A binding indicates that this inhibitor is much less effective in displacing the occluding loop than cystatin C. Moreover, this lower rate of loop displacement is the major reason for cystatin A inhibiting cathepsin B with a considerably lower overall rate constant, $k_{on}$, than cystatin C. Although all cystatins thus presumably need to move the occluding loop of cathepsin B out of the way to gain access to the active site of the enzyme, their ability to do so apparently varies considerably.

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