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Two-step mechanism of inhibition of cathepsin B by cystatin C due to displacement of the proteinase occluding loop

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Abstract Stopped-flow kinetics showed that the inhibition of the lysosomal cysteine proteinase, cathepsin B, by its endogenous inhibitor, cystatin C, occurs by a two-step mechanism, in which an initial, weak interaction is followed by a conformational change. The initial interaction most likely involves binding of the N-terminal region of the inhibitor to the proteinase. Considerable evidence indicates that the subsequent conformational change is due to the inhibitor displacing the occluding loop of the proteinase that partially obscures the active site. The presence of this loop, which allows the enzyme to function as an exopeptidase, thus complicates the inhibition mechanism, rendering cathepsin B much less susceptible than other cysteine proteinases to inhibition by cystatins.

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Key words: Cysteine proteinase; Cysteine proteinase inhibitor; Cathepsin; Cystatin; Kinetics

1. Introduction

Cathepsin B is a mammalian, papain-like lysosomal cysteine proteinase, the main function of which is to degrade proteins inside lysosomes. The extralysosomal action of cathepsin B, as well as that of other lysosomal cysteine proteinases, is regulated by protein inhibitors of the cystatin superfamily. Cystatin C, an important such inhibitor in most tissues, is a non-glycosylated protein with M_r ~13 400, containing two disulfide bonds [1–3]. Like other cystatins, cystatin C inhibits its target proteinases by trapping them in a tight complex, in which a binding region of the inhibitor comprising three different regions of the polypeptide chain interacts with the proteinase active-site cleft [4,5].

All rapid-kinetics studies at high inhibitor concentrations done so far of the binding of cystatin C and other cystatins to cysteine proteinases are compatible with the enzyme-inhibitor complexes being formed by a one-step, reversible bimolecular reaction [6–12]. This behavior is in agreement with computer docking experiments based on the X-ray structures of chicken cystatin and papain, which show that a complex can be formed with negligible conformational adaptations of either protein [4]. In contrast, the X-ray structure of cathepsin B [13] suggests that cystatins may react in a different manner with this enzyme. Cathepsin B thus has an occluding loop, comprising residues 104–126, that partially blocks the active site, thereby modulating the substrate- and inhibitor-binding properties of the enzyme [14]. In particular, by limiting access of substrates to the primed sites of the active-site cleft, this loop enables cathepsin B to function as an exopeptidase, removing C-terminal dipeptides, in addition to the usual endopeptidase action of papain-like cysteine proteinases [13,14]. Docking experiments with chicken cystatin suggested that the occluding loop would interfere with the binding of cystatins but that this interference could be reduced by the loop being displaced during the binding [13]. Analyses of the interaction of N-terminally truncated or engineered variants of cystatin C and chicken cystatin with cathepsin B further indicated that this displacement is facilitated by initial binding of the N-terminal region of the inhibitor to the S_2 and presumably also the S_3 subsite of cathepsin B [15–21]. Both these observations thus imply that the mechanism of cystatin C binding to cathepsin B is more complicated than a simple one-step reaction.

In this work we show by stopped-flow fluorimetry that the binding of human recombinant cystatin C to human recombinant cathepsin B is indeed best described by a two-step reaction mechanism, involving an initial weak interaction followed by a conformational change. We also present evidence that the occluding loop is displaced during the binding by studies of the interaction of cystatin C with a mutant of cathepsin B, in which a fluorescent probe had been introduced in the loop by replacement of His-111 with Trp.

2. Materials and methods

2.1. Proteins

Human recombinant cystatin C was produced and isolated as described in [8,22]. Human wild-type procathepsin B and the pro-form of the H111W mutant were expressed as alpha factor fusion constructs in the methylotropic yeast, *Pichia pastoris*. In this system the proenzymes are secreted into the culture medium. Autoprocessing was initiated by dialysis of the medium to pH 5.0, and the resultant mature, active forms were purified by ion exchange chromatography [14]. The substitution H111W was introduced by site-directed mutagenesis. As previously [17], for both the wild-type and mutant proteins the consensus sequence for asparagine-linked oligosaccharide substitution was removed through the modification S115A (NGS to NGA). Both enzyme forms were activated with 0.5 mM dithiothreitol for 5 min at 25°C before measurements. The purification, storage and activation of papain have been reported elsewhere [23]. The inactivated papain forms, *S*-(methylthio)-papain and *S*-(carbamoylmethyl)-papain, were obtained as in previous work [8,24].

Protein concentrations were determined by absorption measurements at 280 nm. Molar absorption coefficients of 11 100 and 56 000 M^{-1} cm^{-1} have been determined previously for cystatin C and papain, respectively [8,25]. Molar absorption coefficients of 63 000 and 69 000 M^{-1} cm^{-1} were calculated for wild-type and H111W-cathepsin

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Abbreviations: H111W-cathepsin B, a cathepsin B variant in which His-111 is replaced by Trp

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B, respectively, from the amino acid sequence [26,27] by the method of Pace et al. [28].

2.2. Experimental conditions

All experiments were performed at 25°C in 50 mM MES, 100 mM NaCl, 100 μM EDTA, 0.1% (w/v) poly(ethyleneglycol), 0.5 mM dithiothreitol, pH 6.0.

2.3. Activity of inhibitor and enzymes

The fraction of active inhibitor in the cystatin C preparation used was determined by titration with *S*-(methylthio)-papain, monitored by the decrease of tryptophan fluorescence accompanying the interaction, as described previously [8,24]. The fraction of enzyme in the preparations of wild-type and H111W-cathepsin B that was active in binding to cystatin C was determined by titrations of enzyme with inhibitor, monitored by the residual enzyme activity. Cathepsin B at a concentration of 1 μM was incubated at 25°C for 5 min with cystatin C at concentrations ranging from 0.1 to 2.0 μM. The samples were then diluted 1000-fold into a 10 μM solution of the fluorogenic substrate, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan), and the residual cathepsin B activity was obtained from measurements of the initial fluorescence increase with time at excitation and emission wavelengths of 370 and 440 nm, respectively. The residual activity was plotted against the molar ratio of inhibitor to enzyme, and the apparent inhibition stoichiometry was obtained by extrapolation of the linear portion of this plot to the abscissa.

2.4. Kinetic measurements

The kinetics of association of cystatin C with wild-type or H111W-cathepsin B were analyzed at 25.0 ± 0.1°C in an SX-17MV stopped-flow instrument (Applied Photophysics, Leatherhead, UK), as described previously [6,8,21]. All experiments were done under pseudo-first-order conditions with a 10-fold molar ratio of inhibitor to enzyme, based on total protein concentrations. The reactions were monitored by changes of tryptophan fluorescence, measured at an excitation wavelength of 280 nm and with an emission cutoff filter with 50% transmission at ~320 nm.

The rate of dissociation of complexes between cystatin C and wild-type or H111W-cathepsin B was measured by a displacement method [6,17,19]. Cystatin C dissociating from 200 nM complex was essentially irreversibly trapped by an excess (2–4 μM) of the inactive papain derivative, *S*-(carbamoylmethyl)-papain, which binds about five-fold more tightly to cystatin C than cathepsin B [19]. The rate of the concomitant release of the enzyme was monitored by continuous absorption measurements at 410 nm of the cleavage of the chromogenic substrate, *N*-α-benzoyl-L-arginine *p*-nitroanilinide (350 μM; Sigma, St. Louis, MO, USA).

3. Results

3.1. Activity of inhibitor and enzymes

Titrations, monitored by tryptophan fluorescence, of *S*-(methylthio)-papain with cystatin C gave a stoichiometry of binding of inhibitor to enzyme of 1.01 ± 0.03 (range, $n = 2$), i.e. the inhibitor was fully active in binding papain, as established previously [8]. Wild-type and H111W-cathepsin B were shown by measurements of residual activity in titrations with cystatin C to be 46 ± 1% (S.E., $n = 4$) and 73% ($n = 1$) active in binding to the inhibitor, respectively.

3.2. Association kinetics

The kinetics of binding of cystatin C to wild-type or H111W-cathepsin B were analyzed by stopped-flow measurements, monitored by the changes of intrinsic fluorescence accompanying the binding. The reaction with wild-type cathepsin B resulted in ~4% fluorescence decrease under the conditions of the stopped-flow measurements, a value comparable to the ~6% decrease obtained previously for the reaction of cystatin C with recombinant rat cathepsin B [17]. In contrast, a fluorescence increase of ~11% was observed in the reaction of cystatin C with H111W-cathepsin B. The fluorescence changes were satisfactorily fitted to a single exponential function at all inhibitor concentrations. The resulting pseudo-first-order rate constants showed a hyperbolic dependence on inhibitor concentration for both wild-type and H111W-cathepsin B (Fig. 1), indicating a two-step binding mechanism for both enzyme variants. In the simplest such mechanism compatible with exponential progress curves [29], a weak complex (PI) between proteinase (P) and inhibitor (I) in a rapid equilibrium and with a dissociation constant K_1 is established in the initial step. In the second step, this complex is converted to the final, stable complex (PI*) by a reversible conformational change with rate constants k_{+2} and k_{-2} (Scheme 1).

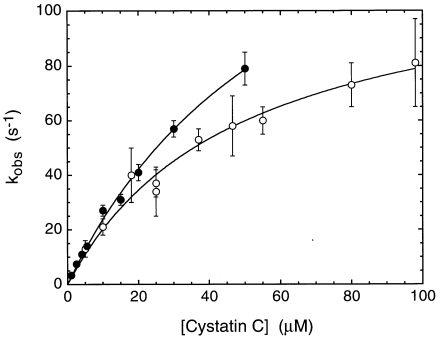

Fig. 1. Observed pseudo-first-order rate constants, k_{obs}, for the binding of cystatin C to wild-type cathepsin B and H111W-cathepsin B as a function of cystatin C concentration. (○) Wild-type cathepsin B; (●) H111W-cathepsin B. Error bars represent ±S.E., calculated from 12–25 individual measurements. The solid lines are the non-linear least-squares regression fits of the data to Eq. 1.

The data for both enzyme forms could be well fitted by non-linear least-squares regression to the equation for this mechanism [29],

$$k_{obs} = \frac{k_{+2}[\mathrm{I}]}{K_1 + [\mathrm{I}]} + k_{-2} \quad (1)$$

(Fig. 1), indicating that the mechanism in Scheme 1 is a satisfactory description of the binding process. Values for K_1 and k_{+2} were derived from these analyses (Table 1), whereas k_{-2} was experimentally indistinguishable from zero for both enzyme forms. The bimolecular association rate constant, k_{on}, which is equal to k_{+2}/K_1 for the mechanism in Scheme 1 [29], was essentially identical for the two enzyme variants (Table 1) and comparable to the values of 1.1×10^6–1.4×10^6 M^{-1} s^{-1} determined previously for the interaction between cystatin C and rat cathepsin B [17,18].

3.3. Dissociation kinetics

The overall dissociation rate constant of the cystatin C-cathepsin B interaction, k_{off}, which is equal to k_{-2} for the mechanism in Scheme 1 [29], was determined by a displacement procedure (Table 1). The value obtained for wild-type cathepsin B is comparable to those of 3.1×10^{-4}–3.5×10^{-4}

$$\mathrm{P} + \mathrm{I} \underset{}{\overset{K_1}{\rightleftarrows}} \mathrm{PI} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} \mathrm{PI}^*$$

Scheme 1.

Table 1
Kinetic and equilibrium constants for the binding of cystatin C to wild-type cathepsin B and H111W-cathepsin B

Enzyme form	K_1 (μM)	k_{+2} (s^{-1})	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (nM)
Wild-type	48 ± 10	118 ± 11	2.4×10^6	$(6.7\pm0.2)\times10^{-4}$	0.28
H111W	68 ± 10	186 ± 18	2.7×10^6	$(2.1\pm0.1)\times10^{-3}$	0.85

Measured values are given with their standard errors, whereas calculated values are shown without errors. The errors of K_1 and k_{+2} are from the non-linear least-squares fits of the data of Fig. 1 to Eq. 1. The errors of k_{off} represent the range of two measurements.

s^{-1} measured or calculated for the complex between cystatin C and rat cathepsin B [17,18]. The overall dissociation equilibrium constant, K_d, was calculated as k_{off}/k_{on} (Table 1). The K_d for the wild-type enzyme is similar to the values of 0.17–0.26 nM reported previously for the interaction of cystatin C with human liver or recombinant cathepsin B [14,20,30] and also to the K_d of 0.28–0.30 nM measured for the binding of the inhibitor to rat cathepsin B [17,18]. The data for H111W-cathepsin B show that substitution of His-111 by Trp slightly weakened the interaction with cystatin C, predominantly as a result of an increased dissociation rate constant.

4. Discussion

These results show that the inhibition of cathepsin B by its endogenous inhibitor, cystatin C, is best described as a two-step reaction, in which an initial, weak interaction between the two proteins with a dissociation constant of about 50 μM is followed by a conformational change with a rate constant of about 120 s^{-1}, leading to tighter binding. The initial interaction most likely involves binding of the N-terminal region of the inhibitor to the proteinase, in particular to the S_2 and S_3 subsites [4,17,18,20,21]. This conclusion is indicated by the decreased rate of association of cystatin C with cathepsin B, but not with other cysteine proteinases, on removal of the N-terminal region of the inhibitor [17]. In the reaction with cathepsin B, the N-terminal region of cystatin C thus appears to act as a guide directing the inhibitor to the appropriate binding position on the enzyme [21].

The conformational change following the initial binding step presumably is due mainly to cystatin C displacing the occluding loop of cathepsin B that partially obscures the active site of the enzyme. Modelling experiments showed that this loop would interfere substantially with the binding of cystatin C, in particular colliding with the second hairpin loop of the inhibitor [13]. However, much of the steric hindrance could be released by tilting and simultaneous rotation of the inhibitor and displacement of the loop. Such mobility of the occluding loop is demonstrated by the X-ray structure of procathepsin B, in which the loop is pushed aside by the propeptide, which binds to the active site and thereby keeps the enzyme in an inactive state [31–33]. That the occluding loop of cathepsin B is displaced in the reaction with cystatin C is also indicated by the higher forward rate constant of the conformational change step observed in this work for the binding of cystatin C to H111W-cathepsin B than for the binding to the wild-type enzyme. This effect presumably is due to the mutation disrupting interactions between the occluding loop and the rest of the protein [13,14], thereby facilitating movement of the loop. This proposal is in agreement with recent studies showing that elimination of an electrostatic interaction between the occluding loop and the main body of cathepsin B leads to an increased affinity of the enzyme for chicken cystatin, presumably due to loop displacement being facilitated [33]. However, the mutated loop having a Trp residue does not behave substantially differently from the wild-type loop, as indicated by the only moderately decreased affinity of cystatin C for H111W-cathepsin B. The small reduction in affinity most likely is due to the binding of the inhibitor being somewhat hampered by the larger size of the indole ring than of the original imidazole ring, leading to an increased dissociation rate constant. The fluorescence increase observed on binding of H111W-cathepsin B to cystatin C, different from the decrease seen for the wild-type proteinase, is also consistent with the Trp side chain having moved on formation of the complex, probably to a more hydrophobic environment. Further evidence for the need for the loop to be displaced in the reaction with cystatins is provided by the substantially increased affinity of cystatin C for a form of cathepsin B in which the occluding loop had been deleted by protein engineering [14].

Previous rapid-kinetics studies have indicated that reactions of other cysteine proteinases than cathepsin B with cystatin C and similar cystatins proceed in one step and generally result in complexes with higher affinity [6–12]. The evolution of the occluding loop, which allows cathepsin B to function as an exopeptidase [13,14], has thus had the concomitant disadvantage that the enzyme is less efficiently regulated by endogenous inhibitors as a result of a more complicated inhibition mechanism and consequent weaker binding.

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