Temporary inhibition of papain by hairpin loop mutants of chicken cystatin
Distorted binding of the loops results in cleavage of the Gly^9-Ala^{10} bond

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Abstract Temporary inhibition of the cysteine proteinases papain and cathepsin L was observed with several hairpin loop mutants of recombinant chicken cystatin at enzyme concentrations above nanomolar. Kinetic modelling of inhibition data, gel electrophoresis and amino acid sequencing revealed that reappearance of papain activity is due to selective cleavage of the Gly^9-Ala^{10} bond in the N-terminal binding area of the chicken cystatin variants, resulting in truncated inhibitors of lower affinity. Cleavage of the same bond by contaminating papaya proteinase IV was ruled out by previous purification of papain and suitable control experiments. According to the proposed kinetic model, cleavage occurs within the enzyme–inhibitor complex with first order rate constants k_{temp} of 2.3×10^{-3} up to 5×10^{-1} s^{-1}. A similar k_{temp}/K_m ratio was found for all mutants (0.7×10^6–2.1×10^6 s$^{-1} \cdot$M^{-1}); it is almost identical with the k_{cat}/K_m ratio of the peptide substrate Z-Phe-Arg-NHMec. These results suggest that distorted contacts of one of the hairpin loops affect binding of the N-terminal contact area in a way that covalent interaction of the Gly^9-Ala^{10} bond with the active-site Cys residue of papain can occur and the bond is cleaved in a substrate-like manner.

Key words: Cysteine proteinase inhibitor; Papain; Cathepsin L; Chicken cystatin variant; Inhibition kinetics; Temporary inhibition

1. Introduction

Temporary inhibition, i.e. the reappearance of enzymatic activity after initial formation of an enzyme–inhibitor complex, has been frequently observed with trypsin-like serine proteinases and their protein-type inhibitors [1–3]. For the first time we have also observed temporary inhibition of the cysteine proteinases papain and cathepsin L during interaction with recombinant chicken cystatin variants bearing substitutions or deletions in one of the two hairpin loop binding regions [4]. In this report we present kinetic and structural data describing the temporary inhibition of papain and propose a putative model for its molecular mechanism.

Earlier observations of a loss of inhibitory capacity of natural chicken cystatin and cystatin C during interaction with papain [5] were later explained as the effect of contaminating papaya proteinase IV (PPIV), which is not inhibited by cystatins but cleaves specifically the conserved glycyl bond within their N-terminal portion [6]. In order to exclude artifacts due to PPIV contamination, the papain used in this work was carefully repurified and control experiments with purified PPIV were performed.

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Abbreviations: Boc-, *t*-butyloxycarbonyl-; Bz-, benzoyl-; ΔV55, AEF-[S1M, M29I, ΔV55, M89L] chicken cystatin; ΔV55-S56, AEF-[S1M, M29I, ΔV55, ΔS56, M89L] chicken cystatin; ΔP103-L105, AEF-[S1M, M29I, M89L, ΔP103, ΔW104, ΔL105] chicken cystatin; ΔI102-Q107, AEF-[S1M, M29I, M89L, ΔI102, ΔP103, ΔW104, ΔL105, ΔN106, ΔQ107] chicken cystatin; E-64, L-3-carboxy-2,3-trans-epoxy-propionyl-leucylamido-(4-guanidino)butane; -NHMec, 7-(4-methyl)-coumaryl-amide; -NHPhNO$_2$, *p*-nitroanilide; PPIV, papaya proteinase IV (glycyl endopeptidase, EC 3.4.22.25); rCC, AEF-[S1M, M29I, M89L] chicken cystatin, (recombinant chicken cystatin); Suc-, succinyl-; Z-, benzyloxycarbonyl-.

2. Materials and methods

2.1. Materials

AEF-[S1M, M29I, M89L] chicken cystatin (rCC) is a recombinant chicken cystatin molecule which behaves like the unphosphorylated form of the natural inhibitor [7]. Molecular cloning and expression of recombinant chicken cystatin variants have been described elsewere [4,7–9]. Human cathepsin B (EC 3.4.22.1) and human cathepsin L (EC 3.4.22.15) were purchased from Medor (Herrsching) or Calbiochem (Bad Soden/Ts.) and used without further purification.

2.2. Purification of papain

Papain (Sigma Type III) was applied to a cation-exchange column (S-Sepharose, HiLoad 16/10, Pharmacia) in 0.05 M sodium acetate buffer, pH 5.0 [10]. The proteins were eluted with a linear gradient of 0.0022 M sodium acetate/ml from 0.05 M to 0.5 M at a flow rate of 5 ml/min. A similar procedure was used for the purification of papain from Boehringer (Mannheim). Papaya proteinase IV (PPIV) activity was assayed in 0.1 M phosphate buffer pH 6.8, 2 mM EDTA, 0.015% Brij 35, using the substrate Boc-Ala-Ala-Gly-NHPhNO$_2$, and discriminated from papain activity by its inhibition with E-64 and lack of inhibition with chicken cystatin [11,12]. Active papain concentration was determined by titration with E-64 [13].

2.3. SDS-PAGE

SDS-PAGE was performed in a 10–20% polyacrylamide gel following the procedure of Laemmli [14]. The amount of protein was determined by densitometry with an UltroScan XL (Pharmacia LKB).

2.4. Amino acid sequencing

Amino acid sequence analysis was done with a gas-phase sequencer 473A (Applied Biosystems GmbH, Weiterstadt, Germany) following the instructions of the manufacturer.

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2.5. Inhibition kinetics

Cysteine proteinase activity was measured in continuous kinetic assays using the fluorogenic substrates Z-Phe-Arg-NHMec, Bz-Arg-NHMec and Suc-Leu-Tyr-NHMec (Bachem, Heidelberg) in a Kontron spectrofluorometer SFM-25 equipped with a 4-cell changer and controlled by an IBM-compatible personal computer [15]. Product concentration data were collected digitally for subsequent evaluation and kinetic modelling (see section 2.6). All assays were performed at 30°C in 1.2 ml of 0.3 M sodium acetate buffer pH 5.5 containing 1 mM dithiothreitol, 2 mM EDTA and 0.015% Brij 35. Appropriate substrates and substrate concentrations were selected allowing long-time continuous kinetic assays with neglegible substrate depletion (<5%). Rate constants and K_i values were corrected for competition with the substrate. Active concentrations of the inhibitor variants were determined from inhibition experiments under equilibrium or incomplete titration conditions performed with defined concentrations of papain (titrated with E-64) or cathepsin L (titrated with standardized chicken cystatin) as described [4].

2.6. Kinetic modelling

Testing of kinetic models for consistence with the progress curves obtained under different experimental conditions was performed with the simulation program FLUSIM [15]. Briefly, the proposed mechanism (see Fig. 1) is described by the following equations:

$$d[EI_a]/dt = k_{ona}[E][I_a] - k_{offa}[EI_a] - k_{temp}[EI_a] \quad (1)$$

$$d[EI_b]/dt = k_{onb}[E][I_b] - k_{offb}[EI_b] + k_{temp}[EI_a] \quad (2)$$

$$[E] = [E_t] - [EI_a] - [EI_b] \quad (3)$$

$$v = v_0[E]/[E_t] \quad (4)$$

$$d[P] = v \cdot dt \quad (5)$$

where [E] is the concentration of free enzyme; $[I_a]$ and $[I_b]$ are the concentrations of free unmodified and free converted (cleaved) inhibitor, respectively, $[EI_a]$ and $[EI_b]$ the concentrations of the corresponding enzyme–inhibitor complexes and k_{ona}, k_{onb}, k_{offa}, k_{offb} the corresponding rate constants; $[E_t]$ is the total enzyme concentration; v the actual reaction rate, v_0 the initial reaction rate (before addition of the inhibitor), and [P] the actual product concentration. The differential equations **(1)**, **(2)**, and **(5)** were integrated numerically to simulate the progress curve [P] = F(t) [16]. The latter was compared with the collected experimental data and unknown parameters were determined by nonlinear regression analysis. K_{ia} and K_{ib} were obtained from the v_{ia}/v_0 and v_{ib}/v_0 ratios (see Fig. 1) as described [4]. If k_{off} was known (see section 3.2), k_{on} was calculated as $k_{on} = k_{off}/K_i$, else the value of k_{on} for rCC ($1.3 \times 10^7 M^{-1} \cdot s^{-1}$) was substituted and k_{off} was calculated as $k_{off} = k_{on}K_i$.

3. Results and discussion

3.1. Repurification of papain

Papain (Sigma or Boehringer) repurified by ion-exchange chromatography yielded a homogeneous N-terminal sequence and contained virtually no cystatin-resistant activity due to PPIV as assayed with the substrate Boc-Ala-Ala-Gly-$NHPhNO_2$. PPIV activity (not inhibited by chicken cystatin) was found in a well separated peak and was isolated for use in control experiments (data not shown).

3.2. Kinetic model of temporary inhibition

The preparation and characterization of hairpin loop variants of recombinant chicken cystatin is described elsewhere [4]. The temporary nature of papain inhibition by these variants (Table 1) was discovered in experiments with enzyme concentrations above 0.5 nM. Inhibition experiments with lower enzyme concentrations for determination of K_i and rate constants did not reveal any reappearance of enzymatic activity even when observed over several hours. No temporary inhibition was seen with wild type-like recombinant chicken cystatin (rCC), the parent molecule of the variants [7].

A typical experiment showing temporary inhibition of papain by the ΔV55-S56 (first hairpin loop) deletion variant of chicken cystatin is presented in Fig. 1. After rapid initial inhibition, papain activity reappears and raises slowly until a second, stable equilibrium is reached. Using a simulation program (see section 2.6), several kinetic mechanisms have been tested for their consistence with the experimental data. One mechanism that was able to describe all experimental data properly is the rapid formation of an initial enzyme–inhibitor complex, EI_a, (equilibrium dissociation constant K_{ia}) followed by its slow (i.e. rate limiting) conversion into a less stable complex, EI_b, which dissociates rapidly towards a new equilibrium (K_{ib}). The first order rate constant of the conversion, k_{temp}, was determined by nonlinear regression analysis on the basis of the postulated mechanism. Table 1 summarizes the results for variants that have been found to be temporary inhibitors of papain. With three of the variants, V55D, ΔP103-L105 and ΔI102-Q107, significant inhibition by the converted complex (EI_b, K_{ib}) was not detected at the applied inhibitor concentrations; in these cases restoration of full initial reaction rate was observed.

The proposed kinetic mechanism (Fig. 1) is consistent with the observed dependance of inhibition on enzyme concentration [4] and on inhibitor concentration (Fig. 2). Reappearence of enzyme activity showed a characteristic sigmoidal course as it has been observed earlier with temporary synthetic inhibitors of trypsin [17]. The initial 'lag' phase is explained by the saturating effect of inhibitor concentrations much higher than K_{ia}; its duration increased with inhibitor concentration as expected

Table 1
Temporary inhibition of papain by hairpin loop variants of chicken cystatin

Inhibitor variant	K_{ia} (nM)	k_{offa} (s^{-1})	k_{temp} (s^{-1})	K_{ib} (nM)	k_{temp}/K_{ia} ($s^{-1} \cdot M^{-1}$)
V55D	0.92	1.2×10^{-2}	1.96×10^{-3}	n.d.	2.13×10^6
ΔV55	361	n.d.	4.80×10^{-1}	3012	1.33×10^6
ΔV55-S56	741	n.d.	4.90×10^{-1}	10,020	0.66×10^6
ΔP103-L105	2.15	1.1×10^{-2}	1.78×10^{-3}	n.d.	0.83×10^6
ΔI102-Q107	2.00	n.d.	2.28×10^{-3}	n.d.	1.14×10^6
Substrate	K_m (μM)		k_{cat} (s^{-1})		k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Z-Phe-Arg-NHMec	52.4		9.16×10^1		1.75×10^{-6}

Data of the fluorogenic peptide substrate Z-Phe-Arg-NHMec determined in our laboratory were included for comparison (lower part of the table). See main text and Fig. 1 for definition of constants. n.d., not determined (see text for explanation).

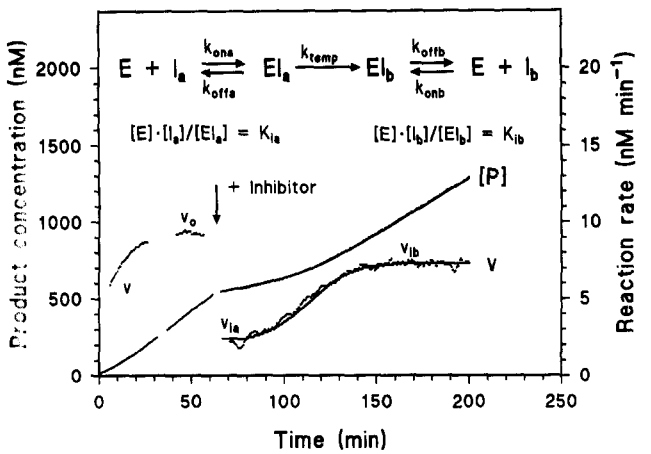

Fig. 1. Temporary inhibition of papain by the ΔV55-S56 deletion variant of recombinant chicken cystatin: Proposed kinetic mechanism and fit of simulated progress curve to experimental data. Continuous assay of papain activity with the substrate Bz-Arg-NHMec (10 μM). After thiol activation of papain (2.4 nM), the ΔV55-S56 variant (2.6 μM) was added (arrow). [P], concentration of the fluorescent reaction product aminomethyl coumarin; v, reaction rate; v_o, initial reaction rate before addition of the inhibitor; v_{ia}, reaction rate after formation of the initial enzyme–inhibitor complex; v_{ib}, reaction rate at the end of the conversion. On the basis of the proposed mechanism (upper part of the panel), simulated progress curves for [P] and v (solid lines) were fitted to the experimental data (represented by dots, 2 samples/min).

from the simulations. Very similar values of k_{temp} were obtained from experiments with varying enzyme and inhibitor concentrations (see Fig. 2). With two of the variants, the dissociation rate constants of the initial complex, k_{offa}, could be determined under conditions of low enzyme concentration when temporary inhibition is not significant (see Table 1). k_{offa} was found to be higher than the corresponding k_{temp}, indicating that the conversion step is rate-limiting rather than dissociation of the initial enzyme–inhibitor complex.

3.3. Cleavage of the inhibitor variants

The predicted conversion of the inhibitor into a 'modified' form with changed structure was confirmed by SDS-PAGE of mixtures of papain with the inhibitor variants, as shown for the V55D (first hairpin loop) variant in Fig. 3A. The time course of disappearance of the unmodified inhibitor determined by densitometry of the SDS gel correlated well with that predicted on the basis of inhibition kinetics (Fig. 3B). The initial rate of disappearance of EI_a, i.e. the slope of the straight line in Fig. 3B, is expected to be first order with respect to the concentration of unmodified complex, $v = k_{temp}[EI_a]$. As the experiment was performed under titration conditions ($[E_t]/K_{ia} = 400$), the enzyme binds stoichiometrically and, as long as $[I_a] > [E_t]$, then $[EI_a] = [E_t]$. The value of k_{temp} calculated from this approach to 1.94×10^{-3} s^{-1} was in agreement with the value of 1.96×10^{-3} s^{-1} calculated from inhibition experiments (see Table 1).

N-terminal sequencing of incubated mixtures of papain with the inhibitor variants revealed that the conversion was always due to the cleavage of a single peptide bond, Gly^9-Ala^{10}, giving rise to a truncated form of the cystatin variant beginning with Ala^{10}, which is expected to be a weaker inhibitor than the full-length form of the inhibitor variant [18,19]. Cleavage goes to completion; after long incubation times, however, additional minor cleavages were observed at various exposed sites of the inhibitor molecule (data not shown). Cleavage of the Gly^9-Ala^{10} bond by contaminating papaya proteinase IV (PPIV), which is not inhibited by cystatins [6], can be excluded after careful purification of papain by ion exchange chromatography followed by negative assay of PPIV activity (see section 3.1). Addition of purified PPIV to the inhibition assays did not change the progress curves up to 15% PPIV (molar ratio). Further increase of the PPIV/papain ratio shifted the progress curves gradually towards a pattern predicted for a mechanism where the free inhibitor is cleaved by a second, non-inhibited enzyme (data not shown).

Inhibition of human cathepsin L by several hairpin loop mutants was also found to be temporary, suggesting that temporary inhibition by certain cystatin variants is a general property of cysteine proteinases rather than being restricted to papain. However, both K_{ia} and k_{temp} were 1–2 orders of magnitude lower than with papain (e.g. 0.046 nM and 1.1×10^{-4} s^{-1} for the ΔV55-S56 variant or 0.0029 nM and 2.8×10^{-5} s^{-1} for the

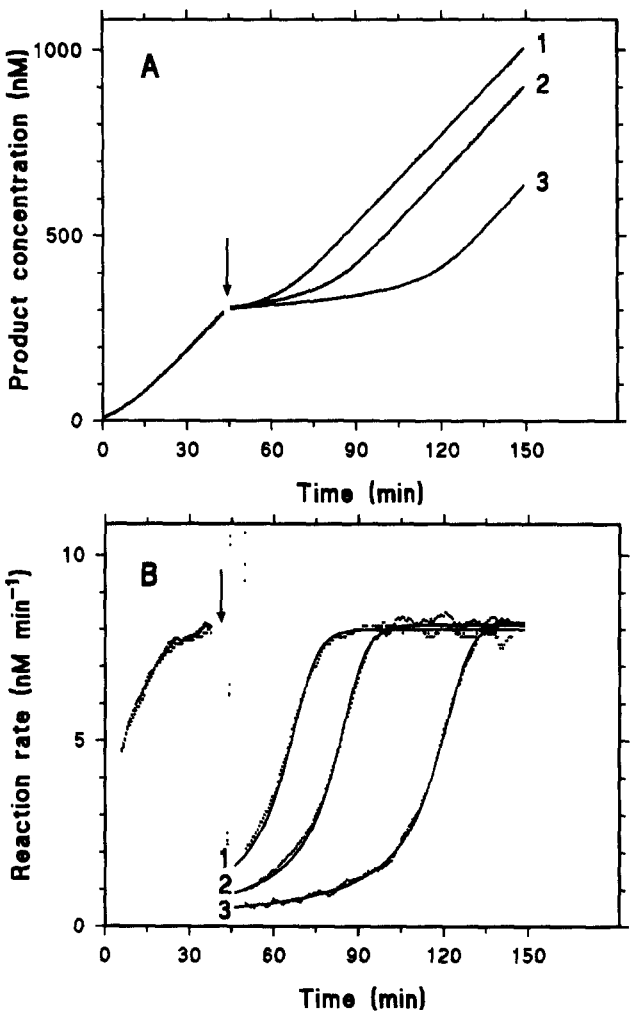

Fig. 2. Temporary inhibition of papain by the ΔP103-L105 deletion variant of recombinant chicken cystatin: Effect of inhibitor concentration. Continuous assays of papain (4.4 nM) with increasing concentrations of the ΔP103-L105 variant: 7.3 nM (1), 14.6 nM (2), 29.1 nM (3). (A) concentrations of the fluorescent reaction product aminomethyl coumarin; (B) reaction rates. Simulated progress curves (solid lines) were fitted to the experimental data (dots) on the basis of the proposed mechanism (see Fig. 1). k_{temp} estimated from these fits were 1.77×10^{-3} s^{-1} (1), 1.80×10^{-3} s^{-1} (2), and 1.74×10^{-3} s^{-1} (3).

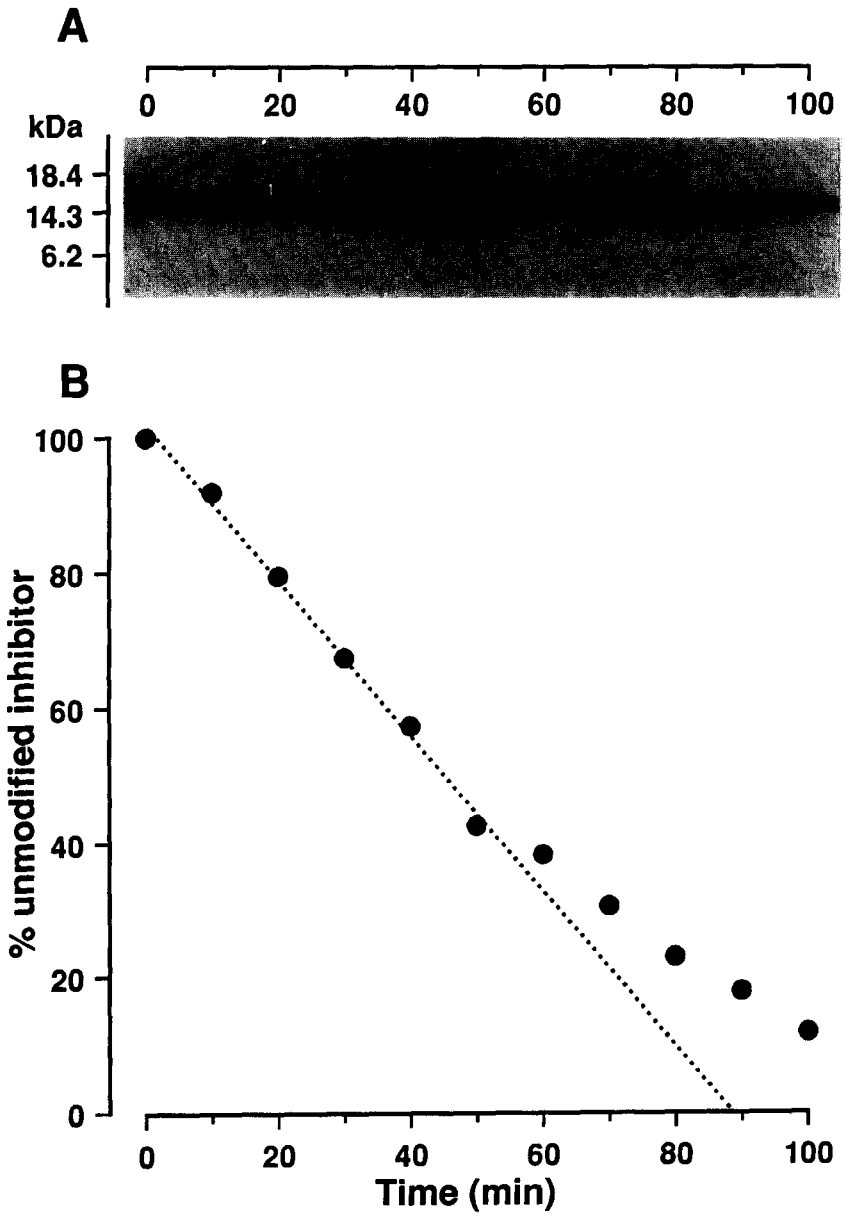

Fig. 3. Cleavage of the V55D substitution variant by papain. Papain (0.4 μM) was incubated with the V55D variant (4 μM) in 0.3 M sodium acetate, 2 mM EDTA, 0.05% Brij 35, 1 mM dithiothreitol, pH 5.5 at 30°C. The reaction was stopped after the indicated times by addition of 0.025 M sodium monochloroacetate. (A) SDS-PAGE of incubation mixtures. (B) Time-dependent disappearance of the unmodified inhibitor (upper band) as determined by densitometry of the SDS gel. The dotted line was obtained by linear regression analysis of the first six data points.

ΔP103-I105 variant), necessitating experiments with high concentrations of cathepsin L and recombinant chicken cystatin variants. For these reasons the peptide bond cleaved by cathepsin L has not yet been identified by sequence analysis. Interestingly, we have not been able to detect temporary inhibition of cathepsin B in preliminary experiments performed with high enzyme concentrations (70 nM) and long incubation times.

The Ala-form of natural chicken cystatin, as it would result from cleavage of the Gly^9-Ala^{10} bond, has a 20,000-fold higher K_i value with papain than the intact inhibitor [18,19]. The same type of N-terminal truncation raised the K_i of the ΔV55 and ΔV55-S56 variants by only a factor of 8.3 and 13.5, respectively (calculated as K_{ib}/K_{ia} from the data in Table 1). This marked discrepancy suggests that the binding mode of the variants containing distorted hairpin loop sequences is different from that of the wild type. The contribution of the N-terminal contact area to complex formation (before cleavage of the Gly^9-Ala^{10} bond) seems to be much less important in the mutants than in the wild type with intact loops. Recently Hall et al. reported a similar low effect on affinity on truncation of variants of cystatin C in which Gly^{11} (corresponding to Gly^9 of chicken cystatin) was replaced by bulky residues, disturbing the conformation of the N-terminal contact area [20].

3.4. Inhibitor variants as substrates

Combining evidence from inhibition kinetics and amino acid sequencing, the observed temporary inhibition of papain by hairpin loop mutants is explained by cleavage of the Gly^9-Ala^{10}

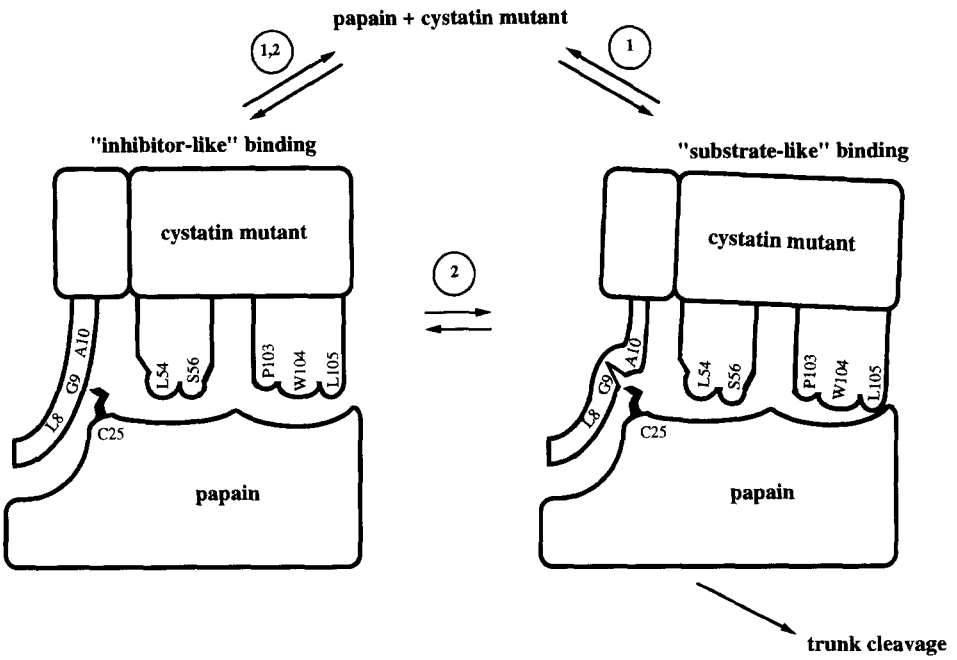

Fig. 4. Temporary inhibition of papain by cystatin mutants in the 'elephant-trunk model' [18,27]. The mutant can adopt 'inhibitor-like' or 'substrate-like' binding modes; the latter brings the Gly^9-Ala^{10} bond within the N-terminal 'trunk' in the vicinity of the active site Cys^{25}, resulting in its cleavage. Two possible mechanisms can be proposed: (1) competition of inhibitor-like and substrate-like complexes for free papain; or (2) rearrangement of the inhibitor-like conformation by 'slippage' in the hydrophobic environment.

bond of the inhibitors. Due to the proposed kinetic model, we suggest that this cleavage occurs within the initial complex of the enzyme with the non-modified inhibitor variant and is followed, depending on K_{ib}, by at least partial dissociation of the modified inhibitor variant from the enzyme.

Gly^9 is strictly conserved within the superfamily of cystatins [21]. Various structural and functional evidence has been presented that the residues preceding Gly^9 of chicken cystatin or the homologous Gly^{11} in cystatin C bind in the putative S_2 and S_3 subsites of papain and are essential for effective inhibition [18–20,22]. These findings suggest a formal analogy of the Gly^9-Ala^{10} bond of chicken cystatin with the 'scissile bond' in the reactive site of small serine proteinase inhibitors, which is frequently cleaved in the enzyme–inhibitor complex according to the so called 'standard mechanism' [5,23,24]. Structural data do not support this analogy, however: in the docking model of the papain–chicken cystatin complex and in the experimental structure of the stefin B-papain complex, the Gly^9-Ala^{10} bond is spatially removed from the catalytic Cys^{25} residue of papain and thus seems to be not cleavable [25–27]. Indeed, no experimental evidence has been reported until now for a cleavage of the Gly^9-Ala^{10} or corresponding peptide bond within the complexes of wild type cystatins or stefins or recombinant mutants of these inhibitors. Cleavage of excess chicken cystatin or cystatin C at the Gly^9-Ala^{10} or Gly^{11}-Gly^{12} bond by non-repurified commercial papain [5] has been explained retrospectively by PPIV contamination of the papain used in these experiments [6]. Cleavages due to the attack of excess papain on the complex have been observed recently with cystatin C in exposed regions such as the Gly^4-Lys^5 and the His^{86}-Asp^{87} bond [28]. This type of mechanism, whereby a second free papain molecule acts on the papain–inhibitor complex, can be excluded in our work as cleavage was observed even with a ten-fold molar excess of inhibitor over papain under titration conditions, when virtually no free enzyme is expected (see Fig. 3B).

We hypothesize that severe distortion of binding in one of the two hairpin loop regions can change the overall binding mode of the inhibitor in a way that the Gly^9-Ala^{10} bond moves closer to the active-site Cys^{25} of papain and becomes cleavable in a substrate-like manner. Hydrophobic contacts dominate the papain–cystatin interaction [25,26]; these relatively non-specific contacts are supplemented by a small number of polar contacts that anchor the two components. That certain mutations lead to cleavage of the molecule suggest that these interactions are finely balanced. Temporary inhibition arises as a result of two modes of enzyme–inhibitor binding; one 'inhibitor-like' and one 'substrate-like' (Fig. 4). These modes presumably share comparable interactions at the S_2 and S_3 subsites, fixing the amino terminus. In the case of 'inhibitor-like' binding, the Gly^9-Ala^{10} bond is removed from the active site [25,26]. Upon 'substrate-like' binding, however, this bond approaches Cys^{25} resulting in its cleavage. Unlike the 'canonical inhibitors' of serine proteinases [23,24,27], the cleavage products are free to diffuse away from one another, leading irreversibly to the truncated form. If the two hairpin loops of the truncated mutant are able to make sufficient favorable contacts, then a second inhibition constant will be seen. Attainment of the 'substrate-like' state can occur via two pathways (Fig. 4): (i) inhibitor-like and substrate-like conformations compete for free papain; irreversible cleavage of the substrate form shifts the equilibrium to the truncated form; (ii) after binding in an inhibitor-like fashion, the cystatin mutant 'slips' through the active site cleft, adopts the substrate-like binding mode, and becomes cleaved.

Both pathways seem compatible with the kinetic data of Table 1. In a rough approximation, cleavage of the inhibitor can be described by a Michaelis–Menten mechanism where K_{ia}

is equivalent to K_m and k_{temp} to k_{cat}. Formally the inhibitor variants may be considered as 'substrates' with low K_m and low k_{cat}; therefore inhibition dominates and the cleavage reaction is very slow at low enzyme concentrations. With increasing K_{ia}, i.e. decreasing stability of the initial complex, k_{temp} also increases, suggesting that the putative transition state(s) leading to the cleavage reaction is (are) energetically favored relative to the inhibitor-like state [29]. This is consistent with the observation that for the interaction of cathepsin L with the ΔP103-L105 variant both the K_{ia} (0.0029 nM) and k_{temp} (2.8×10^{-5} s^{-1}) were markedly lower than for the interaction of papain with the same mutant (see Table 1), reflecting the greater 'insensitivity' of cathepsin L to inhibitor mutations [4]. The specificity constant, k_{temp}/K_m, which is considered as a measure of the catalytic efficiency of an enzyme [24], was found to be very similar for temporary inhibition of papain by all inhibitor variants and is close to the value of k_{cat}/K_m for Z-Phe-Arg-NHMec, a good peptide substrate of papain (see Table 1). Assuming a k_{temp}/K_{ia} value of 1×10^6 $s^{-1} M^{-1}$, the k_{temp} of papain and wild type chicken cystatin ($K_i < 1 \times 10^{-12}$ M) can be extrapolated as $<10^{-6}$ s^{-1}, implying that cleavage of the Gly^9-Ala^{10} bond of the natural inhibitor, if there is any, is too slow to be readily detectable.

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