

Hairpin loop mutations of chicken cystatin have different effects on the inhibition of cathepsin B, cathepsin L and papain

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Received 1 February 1995

Abstract Five recombinant hairpin loop variants of chicken cystatin ($\Delta V55$, $\Delta V55$ -S56, $\Delta P103$ -L105, $\Delta I102$ -Q107, loop2-KD2) were constructed by cassette mutagenesis, expressed in *E. coli*, purified to homogeneity, characterized by protein-chemical means and by their inhibitory properties. The variant forms, modified in two of the three postulated cysteine proteinase binding regions, were inhibitorily active. However, the equilibrium dissociation constants of the complexes between papain as well as human cathepsin B or L and the cystatin variants show a weaker affinity for all three enzymes compared with recombinant chicken cystatin. These results prove the contribution of both hairpin loops to complex formation with the three enzymes. Furthermore, the kinetic constants indicate discrete differences in the molecular mechanism of interaction between chicken cystatin and papain, cathepsin B, and cathepsin L. Inhibition of cathepsin L was much less affected than inhibition of papain or cathepsin B by the modifications achieved in the five variants. Remarkably, at high enzyme concentration (above 0.5 nM) inhibition of papain by these variants was 'temporary', that means, active papain was released from the enzyme-inhibitor complex within minutes to hours (compare [1]).

Key words: Cysteine proteinase inhibitor; Cysteine proteinase; Chicken cystatin variant; Protein engineering; Inhibition kinetics

1. Introduction

Chicken cystatin, a small protein-type inhibitor of cysteine proteinases, represents a well studied member of the cystatin superfamily (for reviews see [2,3]). It was shown to be a reversible tight-binding inhibitor of papain and papain-like enzymes

[4–9]. Synthetic genes of chicken cystatin and variants of them have been expressed and characterized [10–12]. Crystal and solution structures of native and recombinant chicken cystatin are known [13,14] and a model of the interaction of chicken cystatin with papain has been proposed [7,13]. According to the so-called 'elephant-trunk model', the enzyme-inhibitor complex is formed mainly by hydrophobic interactions between chicken cystatin and the complementary active-site cleft of papain, whereby cystatin contributes the N-terminal 'trunk' (Leu⁷-Gly⁹), the first hairpin loop (Gln⁵³-Gly⁵⁷) and the second hairpin loop (Pro¹⁰³-Leu¹⁰⁵). The validity of this model was confirmed by elucidation of the structure of the complex formed between S-carboxymethylated papain and stefin B [15] as well as by kinetic data obtained for the interactions of native and/or modified cystatins with different cysteine proteinases [7,8,12,16].

Detailed analyses of the N-terminal segments of chicken cystatin and human cystatin C indicate a substrate-like interaction of this region with residues of the subsites S3 and S2 of the proteinase. Especially the amino acid residues preceding the conserved Gly⁹ of chicken cystatin or Gly¹¹ of human cystatin C have distinct but different effects on the binding to papain, actinidin, ficin, cathepsin B or cathepsin L [17–20].

The contribution of the first hairpin loop of cystatins to complex formation has not yet been investigated in such detail. From studies with KVVAG- and QVTAG-mutants of cystatin A, Nikawa et al. [21] proposed that the first hairpin loop is not essential for cysteine proteinase inhibition. But the importance of this region is strongly suggested by (i) the structural data of the crystallized stefin B-papain complex [15], (ii) its conserved primary structure (QxVxG-region) [2] and (iii) inhibition properties measured with substitution variants of oryzacystatin [22] and chicken cystatin [12]. The latter data provide first evidence that amino acid residues of this region are partially responsible for the distinct affinities of cystatins for different cysteine proteinases. However, no data have been presented so far on variants of the first hairpin loop missing conserved amino acid residues.

Little is known about the importance of the second hairpin loop for the stability of the molecule itself or its contribution to complex formation. Using spectroscopic methods, Lindahl and co-workers [6] demonstrated that Trp¹⁰⁴ of chicken cystatin must be involved in the interaction with papain. These early data were confirmed indirectly by the interaction model [13] and by the stefin B papain complex structure [15].

In this report we present data on construction and expression

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Abbreviations: E-64, L-3-carboxy-2,3-trans-epoxy-propionyl-leucyl-amido-(4-guanidino)butane; NH-Mec, 7-(4-methyl) coumaryl-amide; Z, benzylloxycarbonyl; rCC, AEF-[S1M, M29I, M89L] chicken cystatin, (recombinant chicken cystatin); $\Delta V55$, AEF-[S1M, M29I, $\Delta V55$, M89L] chicken cystatin; $\Delta V55$ -S56, AEF-[S1M, M29I, $\Delta V55$, $\Delta S56$, M89L] chicken cystatin; loop2-KD2, AEF-[S1M, M29I, M89L, S101I, I102D, P103I, W104Q, L105, N106R, Q107I, I108A] chicken cystatin; $\Delta P103$ -L105, AEF-[S1M, M29I, M89L, $\Delta P103$, $\Delta W104$, $\Delta L105$] chicken cystatin; $\Delta I102$ -Q107, AEF-[S1M, M29I, M89L, $\Delta I102$, $\Delta P103$, $\Delta W104$, $\Delta L105$, $\Delta N106$, $\Delta Q107$] chicken cystatin; N-del 2, [S1-P11, V12A, P13E, V14F, D15M, M29I, M89L] chicken cystatin.

of deletion and substitution hairpin loop variants of chicken cystatin, on their purity and their altered inhibition behaviour towards papain and human cathepsin L and B. In addition, we present evidence for temporary inhibition of papain by these hairpin loop variants. The mechanism of this new and unexpected behaviour is described in more detail in an accompanying paper [1].

2. Materials and methods

2.1. Materials

All chemicals used were obtained from Sigma, St. Louis, USA; Merck, Darmstadt, Germany; Serva, Heidelberg, Germany; Biomol, Hamburg, Germany; Fluka, Buchs, Switzerland; they were all of analytical grade if not otherwise specified. Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer, Mannheim, Germany and New England Biolabs, Beverly, USA. Natural chicken egg white cystatin was a generous gift of V. Turk, Ljubljana, SLO. AEF [S1M, M29I, M89L] chicken cystatin (rCC) is a recombinant variant which behaves like the natural unphosphorylated form of chicken cystatin [11] and has the same conformation as natural chicken cystatin [14]. Anti-chicken cystatin antibodies and the variants V55D and N-del 2 are described elsewhere [12,20].

2.2. Molecular cloning, cassette mutagenesis, and expression of the variants

Standard techniques of molecular cloning were performed according to Sambrook et al. [23]. The constructions of $\Delta V55$, $\Delta V55$ -S56, $\Delta P103$ -L105, $\Delta I102$ -Q107 and loop2-KD2 were performed by cassette mutagenesis using the synthetic cystatin gene. Briefly, the large *XbaI*-*PstI* fragment of pGG 8.1.1, [10] was substituted by appropriate deleted DNA sequences for $\Delta V55$ and $\Delta V55$ -S56. The large *DraIII*-*XhoI* fragment was substituted by fragments with deletions for $\Delta P103$ -L105 and $\Delta I102$ -Q107 or with the fragment for loop2-KD2 having the sequence 5' g tgc acc ttc gtt gtt tac ATC GAC ATC CAG CTG CGT ATC GCT aaa ctt c 3' (capital letters indicate the substituted nucleotides). The construction of periplasmic expression vectors, the transformation of *E. coli* TG1, the analysis of transformants (restriction analysis of plasmids, DNA sequencing, expression analysis of *E. coli* lysates by SDS-PAGE) were performed as described [12].

2.3. Purification of the inhibitor variants and determination of concentration

All variants were isolated from periplasmic *E. coli* fractions [24]. The

loop2-KD2 variant was purified by carboxymethylpapain affinity chromatography [25] whereas the $\Delta V55$, $\Delta V55$ -S56, $\Delta P103$ -L105 and $\Delta I102$ -Q107 variants were purified by immunoaffinity chromatography using sheep antibodies directed against chicken cystatin (100 mg IgGs) and loaded on CNBr-activated Sepharose 4B (5 g) [20]. Protein concentration was determined from absorbance at 280 nm using A_{280} (1%) values calculated from the content of aromatic residues and cystines according to Mach et al. [26].

2.4. SDS-PAGE, Western blot analysis and isoelectric focussing

SDS-PAGE of the proteins was performed in 10–20% polyacrylamide gels following the procedure of Laemmli [27]. Immunoselected anti(chicken cystatin) IgGs were used for Western blot analysis [28]. Isoelectric focussing was performed with the PhastSystem (Pharmacia) using the calibration kit pH 3–10.

2.5. HPLC analysis and amino acid sequencing

Purified samples of all variants (usually 2–3 nmol) as well as tryptic digests of selected variants were analysed by reversed phase-HPLC as detailed previously [11]. Amino acid sequence analyses of the N-termini of all variants and of the modified Tp14 peptide [11] of the $\Delta I102$ -Q107 variant were done with a gas-phase sequencer 473A (Applied Biosystems GmbH, Weiterstadt, Germany) following the instructions of the manufacturer.

2.6. Mass spectrometry

Solutions of the purified variants $\Delta V55$, $\Delta V55$ -S56, $\Delta P103$ -L105 and loop2-KD2 were infused (5 μ l/min) into an atmospheric pressure ionization source fitted to the tandem quadrupole instrument API III (Sciex, Thornhill, Ontario, Canada). The average molecular mass values of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiple charged ions [29,30].

2.7. Determination of kinetic constants

Kinetic constants for the interaction of the inhibitor variants with cysteine proteinases were determined applying kinetic enzyme assays at 30°C and pH 5.5 with the fluorogenic substrate Z-Phe-Arg-NH-Mec as described in detail elsewhere [1,31]. Rate constants, k_{on} and k_{off} , were obtained by presteady-state analyses [32]. Equilibrium dissociation constants (K_i) were measured directly in equilibrium inhibition experiments or calculated ($K_i = k_{off}/k_{on}$) from presteady-state experiments, and were corrected for substrate competition. Substrate consumption was less than 5% in all experiments.

All calculations are based on inhibitorily active concentrations of the variants determined in separate kinetic assays under equilibrium conditions ($E_i/K_i = 2$ –60) using E-64-titrated papain (4 nM) [33] and Bz-Arg-

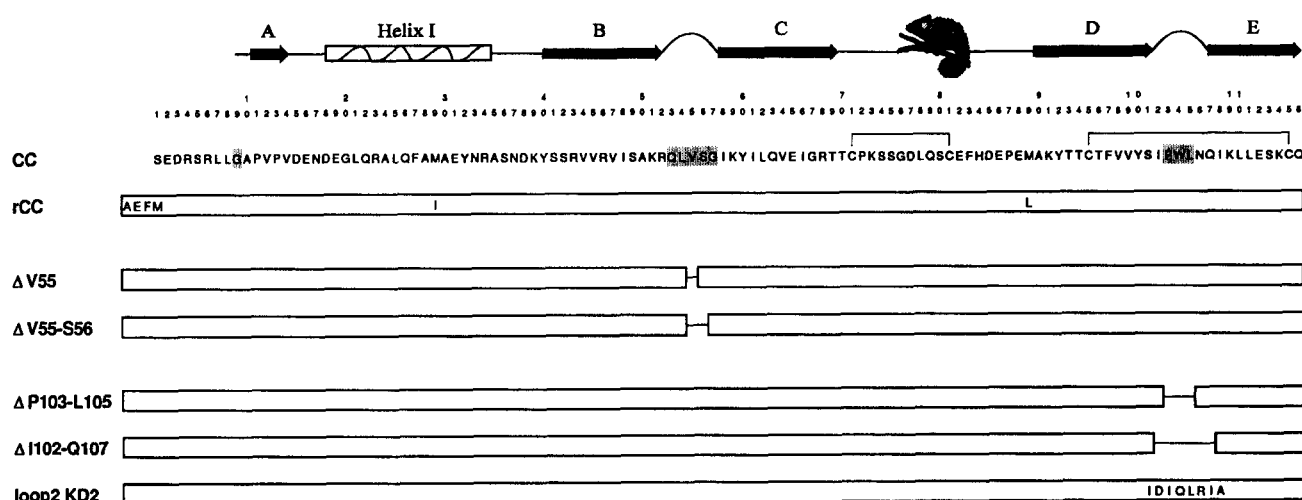


Fig. 1. Scheme and amino acid sequences of chicken cystatin and the designed recombinant variants. Amino acids are given by the one letter code. Boxes indicate identical amino acids (if not otherwise stated) and lines between boxes represent an internal deletion, shaded areas indicate the conserved cystatin sequences of the N-terminus, the first and the second hairpin loop. Secondary structural elements seen in the crystal and in solution are indicated above the sequences; β sheets are identified by letters; the 'chameleon' symbolizes the C71–M89 region whose structure apparently changes according to its environment [13,14].

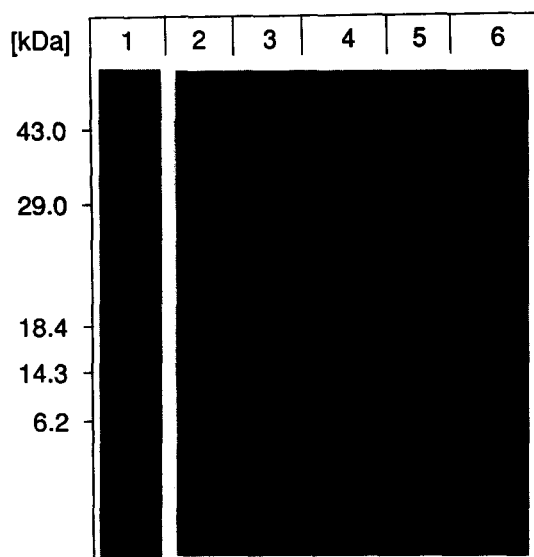


Fig. 2. SDS-PAGE (10–20%) of recombinant chicken cystatins. Purified inhibitor preparations were applied in amounts of 3–5 $\mu\text{g/slot}$. Lanes: 1, rCC; 2, variant ΔV55 ; 3, variant $\Delta\text{V55-S56}$; 4, variant $\Delta\text{P103-L105}$; 5, variant $\Delta\text{I102-Q107}$; 6, variant loop2-KD2; positions of molecular mass protein markers are indicated.

NH-Mec (10 μM) or chicken cystatin-titrated cathepsin L (0.4 nM) and Suc-Leu-Tyr-NH-Mec (60 μM). Briefly, the residual activity at equilibrium was measured after addition of increasing volumes of inhibitor solution. Entering the defined enzyme concentration as fixed parameter, both the concentration of the added inhibitor solution and K_i were obtained by non-linear regression analysis using the general equation describing tight-binding inhibition [34].

3. Results and discussion

3.1. Construction and expression of the hairpin loop variants

The amino acid sequences of chicken cystatin, recombinant chicken cystatin and the hairpin loop variants are shown in Fig. 1 together with a schematic drawing of the secondary structural elements.

The genes of the mutants, constructed by cassette mutagenesis and ligated into an *ompA* expression vector, were expressed

in *E. coli* TG1 cells. Positive transformants were identified after induction of transcription and analysis of *E. coli* lysates by SDS-PAGE and limited DNA sequencing (data not shown). The level of expression was about 2.5–4 mg/liter fermentation broth (estimated after SDS-PAGE) and the titer was 1.4×10^{12} cells/liter.

An additional deletion variant lacking the complete first hairpin loop, $\Delta\text{L54-I58}$, could be expressed only in minor amounts and the purified material was not stable during kinetic measurements. We conclude, therefore, that the five amino acid residues of the first hairpin loop are indispensable for the overall fold and stability of the cystatins.

3.2. Purification and protein chemical characterization

The five variants were isolated after shaker-flask fermentation (1–6 liter) from *E. coli* periplasma fractions. The loop2-KD2 variant (1.5 mg/l fermentation broth) was purified by carboxymethyl papain affinity chromatography. The yields of the variants ΔV55 , $\Delta\text{V55-S56}$, $\Delta\text{P103-L105}$, $\Delta\text{I102-Q107}$, purified by immunoaffinity chromatography, were in the same range. Each variant showed a single, homogeneous protein band in SDS-PAGE. Four variants migrated at a position corresponding to M_r of 15,000 Da, the variant $\Delta\text{I102-Q107}$ migrated at 14,300 Da (Fig. 2). With the exception of the loop2-KD2 variant, distinct and well comparable immunological detection reactions were observed by Western blotting and immunoprinting, but staining was less intense than with chicken cystatin (data not shown). The very weak staining observed with loop2-KD2 variant indicates a high immunoreactivity of the exposed amino acid residues in the second hairpin loop of chicken cystatin with our antibody.

HPLC analysis on RP- C_{18} columns confirmed the homogeneity of $\Delta\text{P103-L105}$, $\Delta\text{I102-Q107}$ and loop2-KD2 variant (data not shown). The ΔV55 and $\Delta\text{V55-S56}$ variant appeared as two closely related peaks, both being inhibitorily active against papain. Amino acid sequencing and mass spectrometry indicated that each second peak represents the acetylated form of the corresponding variant (data not shown). The first five N-terminal amino acids were confirmed in all variants. Average molecular masses were estimated by mass spectrometry to 13,401 Da (acetylated form: 13,443.8 Da) for variant ΔV55 ; to 13,314 Da (acetylated form: 13,356.8 Da) for variant $\Delta\text{V55-S56}$; to 13,105

Table 1

Equilibrium dissociation constants (K_i) and rate constants (k_{on} , k_{off}) of the complexes between recombinant hairpin loop variants of chicken cystatin and cysteine proteinases

Inhibitor	Cathepsin L			Papain			Cathepsin B
	k_{on} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{off} (s^{-1})	K_i (nM)	k_{on} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{off} (s^{-1})	K_i (nM)	K_i (nM)
rCC	7.8×10^7	6.7×10^{-5}	0.0009	1.3×10^7	1.8×10^{-5}	0.0014	4.5
N-del 2	n.d.	n.d.	1.63	n.d.	n.d.	166	$\geq 50,000$
V55D	3.0×10^7	5.8×10^{-5}	0.0019	1.3×10^7	1.2×10^{-2}	0.923	3,960
ΔV55	9.2×10^7	5.4×10^{-4}	0.0059	n.d.	n.d.	361	18,000
$\Delta\text{V55-S56}$	7.7×10^7	3.5×10^{-3}	0.0455	n.d.	n.d.	741	$\geq 50,000$
$\Delta\text{P103-L105}$	7.9×10^7	2.3×10^{-4}	0.0029	5.4×10^6	1.1×10^{-2}	2.15	254
$\Delta\text{I102-Q107}$	3.9×10^7	3.3×10^{-4}	0.0085	n.d.	n.d.	1.64	293
loop2 KD2	1.7×10^7	5.5×10^{-5}	0.0032	3.8×10^6	2.8×10^{-3}	0.737	5,870

The corresponding data for recombinant wild type chicken cystatin, AEF-[S1M, M29I M89L] chicken cystatin (rCC) [11] and the N-terminal deletion variant [$\Delta\text{S1-P11}$, V12A, P13E, V14F, D15M, M29I, M89L] chicken cystatin (N-del 2) [20] are included for comparison. n.d., not determined (see text for reasons).

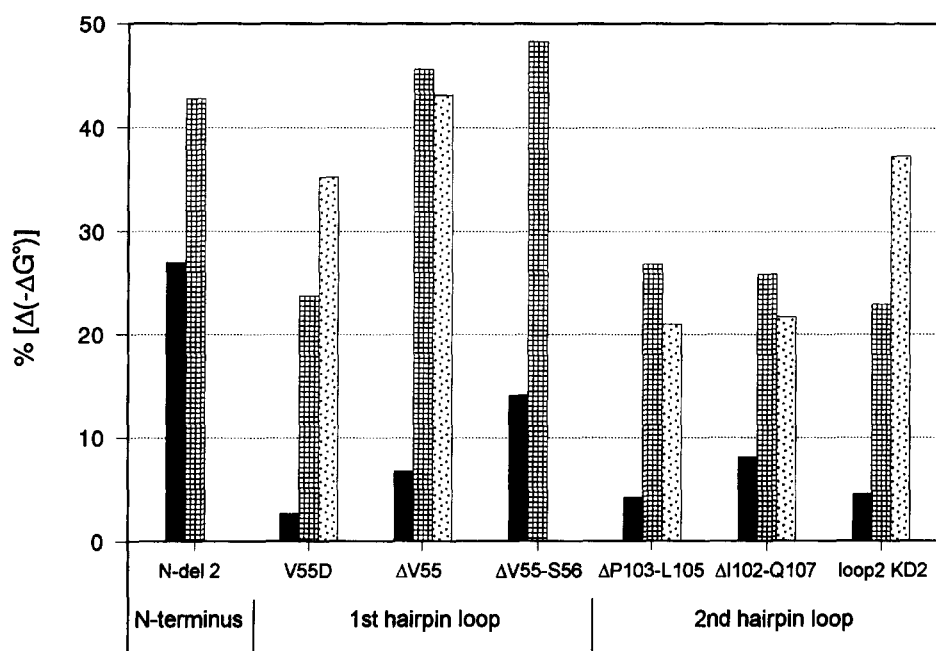


Fig. 3. Effect of mutations on the unitary free energy change (ΔG°) for complex formation. The ΔG° (var) for the binding reaction of the individual variants to the different enzymes were calculated from K_i (var) according to ΔG° (var) = $RT \ln K_i$ (var) and the differences ΔG° (rCC) – ΔG° (var) were expressed in% of ΔG° (rCC), abbreviated as $\Delta(-\Delta G^\circ)$ on the ordinate; black boxes indicate cathepsin L, squared boxes indicate papain and dotted boxes indicate cathepsin B.

Da for variant Δ P103-L105; and to 13,472 Da for variant loop2-KD2. The molecular mass of the Δ I102-Q107 variant was not determined, but the deletion in this variant was confirmed by tryptic peptide mapping and amino acid sequencing of the modified Tp14-equivalent peptide (for comparison see [11]).

Isoelectric focussing of both variants, Δ V55 and Δ V55-S56, resulted in two silver-stained protein bands, each located at pI 5.4 and 5.9, which probably correspond to the acetylated and non-acetylated forms (theoretical pI 5.9). Variant loop2-KD2 showed two strong bands at pI 5.8 and 5.9 (theoretical pI 6.0), whereas each of the variants Δ P103-L105 and Δ I102-Q107 showed one major band at pI 5.9 and pI 5.8, respectively (theoretical pI 5.9) in addition to some minor bands (data not shown). Such heterogeneities in isoelectric focussing are often found with recombinant proteins but are difficult to interpret.

3.3. Inhibitory profile of the variants

The specific inhibitory activity of the variants was found to be between 13–45% compared to 48% of wild type and to 6–52% of other cystatin variants [12], values that are typical for recombinant cystatin inhibitors. However, this relatively low specific inhibitory activity, determined also in material successfully used for NMR spectroscopy [14], cannot be explained satisfactorily. Table 1 summarizes the measured K_i values and rate constants, based on inhibitorily active protein concentrations. Determination of rate constants was not feasible when complex formation was too fast to be analyzed without stopped-flow techniques. Collectively, the analysed mutants differ from chicken cystatin in their reduced affinity for papain and cathepsin B and L. As far as available, the rate constants indicate that the increased K_i values are due to a lower stability of the complexes (increased k_{off}) rather than an impaired association rate. The magnitude of the effect of mutations on K_i

varies with the nature of the affected loop as well as with the target enzyme. From the K_i values, we calculated the unitary free energy changes of complex formation, which represent suitable additive parameters for interpretation of the effects of individual mutations [16]. Fig. 3 visualizes the reduction of the unitary free energy change of complex formation caused by the individual deletions or substitutions, expressed in% of the unitary free energy change of complex formation with wild type chicken cystatin.

Within the first hairpin loop, the effect of mutations on V55 and S56 increases from the substitution (V55D) over the single V55 deletion to the V55-S56 double deletion. This is in good accordance with the structure of the stefin B-papain complex where the corresponding residues (V55, A56) form most of the hydrophobic contacts in this region [15].

The three mutations in the second hairpin loop seem to have a significantly lower effect on the stability of the molecule and on the affinity for papain, cathepsin B and cathepsin L than mutations in the first hairpin loop or the deletion of the N-terminal trunk (see Table 1 and Fig. 3). Complete deletion of the second hairpin loop (Δ I102-Q107) does not further reduce the free energy change observed during complex formation if compared to the deletion of only three residues in this loop (Δ P103-L105). This is an indirect evidence that these amino acid residues do not have a significant influence on the overall structure of the molecule. Interestingly, substitution of the second hairpin loop by the sequence of the second domain of the kininogen heavy chain (loop2-KD2 variant) reduces the free energy change during complex formation with papain by about the same degree as complete deletion of the second hairpin loop. The second hairpin loop of KD2 was selected for substitution because its sequence is markedly different from the corresponding sequences of all other cystatins [3]. Keeping in mind

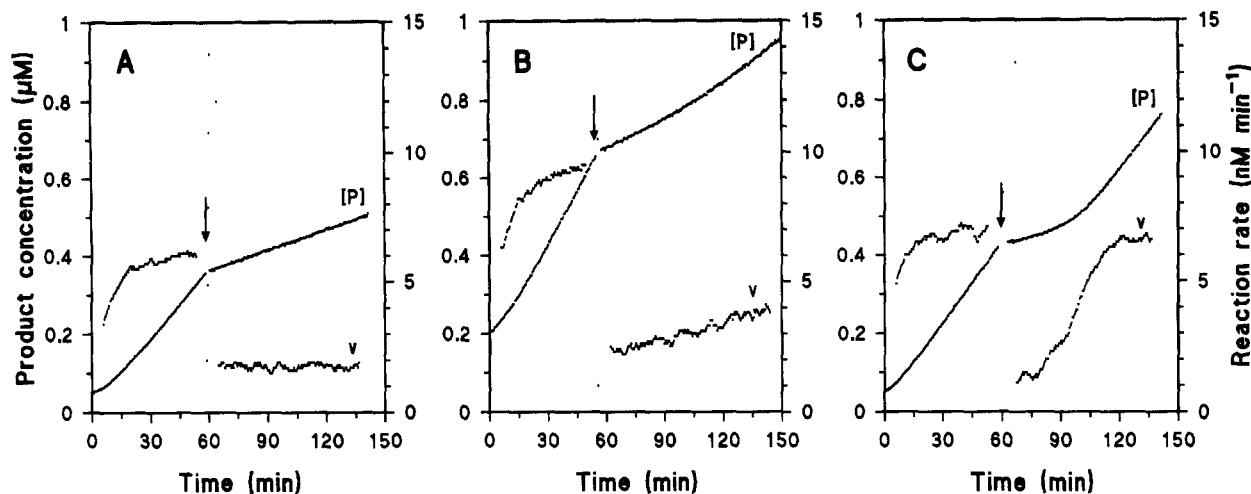


Fig. 4. Temporary inhibition of papain by variant Δ P103-L105: Effect of enzyme concentration. Continuous fluorometric detection of papain activity showing the concentration of reaction product (P) and the actual reaction rate (v) as function of time. After complete thiol activation of enzyme, the variant Δ P103-L105 was added (arrows). (A) 0.006 nM papain assayed with Z-Phe-Arg-NHMec as substrate (10 μ M), 6.3 nM inhibitor; (B) 0.6 nM papain assayed with Bz-Arg-NHMec (100 μ M), 8.5 nM inhibitor; (C) 4.0 nM papain assayed with Bz-Arg-NHMec (10 μ M), 16.8 nM inhibitor.

that the proteolytically isolated KD2 domain is an effective inhibitor of papain ($K_i = 0.017$ nM), this observation suggests that all three binding regions contribute to an optimal fit of the contiguous wedge-like surface into the complementary active-site cleft of papain, and that this fit can be distorted strongly by a 'wrong' combination of loops. Binding to cathepsin B is even more affected by insertion of a 'wrong' second hairpin loop (loop2-KD2) than by its deletion. This may be explained by steric collision with the so-called occluding loop of cathepsin B, which has been predicted to interfere with binding of the second hairpin loop of cystatins [35].

Most surprisingly, inhibition of cathepsin L is only slightly weaker by the five hairpin loop variants if compared with wild type cystatin (see Table). In complex formation with cathepsin L, the contributions of the amino acids mutated in the hairpin loop regions seem to be less important than the deletion of the N-terminal trunk. However, the magnitude of the effect of N-terminal truncation on binding to cathepsin L is also lower than on binding to papain (see Fig. 3). Evidence for this exceptional insensitivity of cathepsin L to cystatin hairpin loop mutations had been obtained already in previous studies with substitution mutants of the first hairpin loop [12].

Although the primary structures of papain and cathepsin L are very similar [36], inspection of the contact region of papain with human stefin B [15] reveals significant differences in the trunk and second hairpin loop binding regions. The trunk binding region of cathepsin L shows a replacement of three basic residues (Arg⁵⁸, Arg⁵⁹ and Arg¹¹¹ of papain) for three acidic residues (Glu⁶³, Asp⁷¹ and Asp¹¹⁴ of cathepsin L). This charge reversal would complement the cystatin amino terminal sequence Arg⁴-Ser⁵-Arg⁶ (P6-P4), and could allow specific salt bridge formation. The results presented here indicate that binding of chicken cystatin and its variants to cathepsin L is also improved in the hairpin loop regions. A hydrophobic/aromatic replacement (papain Phe¹⁴¹-Gln-Leu-Tyr $\rightarrow$ cathepsin L Phe¹⁴³-Leu-Phe-Tyr) is seen in the vicinity of the second hairpin loop; it is therefore conceivable that this segment allows more favourable burial of cystatin Trp¹⁰⁴-Leu¹⁰⁵.

3.4. Temporary inhibition

When the hairpin loop variants were assayed with high concentrations of papain (above 0.5 nM), inhibition was not constant and a time-dependent recovery of enzymatic activity was observed (Fig. 4). This phenomenon has previously been described for serine proteinase inhibitors as temporary inhibition [37]. As shown in detail in an accompanying paper [1], the temporary inhibition by chicken cystatin variants is due to complex formation followed by substrate-like cleavage of the Gly⁹-Ala¹⁰ bond, resulting in truncated inhibitors of much lower or negligible affinity. According to the kinetic model describing this interaction, cleavage is expected and was found to be extremely slow at low enzyme concentration (see Fig. 4) resulting in apparently normal inhibition under these conditions and, therefore, it did not affect determination of the K_i values and rate constants presented in Table 1. However, due to temporary inhibition, true titration experiments ($E_i/K_i > 100$) [34] requiring high enzyme concentrations were not possible, and the active concentration of inhibitors had to be calculated from experiments performed under equilibrium or incomplete titration conditions ($E_i/K_i = 2$ –60). This approach yields reliable results as long as active enzyme and inhibitor concentrations are similar.

3.5. Conclusions

Pure, homogenous and stable deletion as well as substitution variants of both hairpin loops of chicken cystatin were produced. All variants inhibit papain as well as cathepsin B and L, but weaker than chicken cystatin. The general interaction model of chicken cystatin with papain [7,8,13] was confirmed experimentally: as predicted, both hairpin loops as well as the N-terminal trunk of the inhibitor contribute to complex formation. However, the contributions of the three binding regions to the free energy change during complex formation seem to be not simply additive. In the interaction with papain and cathepsin B, the contribution of the second hairpin loop is significantly lower than that of the first hairpin loop and than that of the N-terminal binding region. Furthermore, mutations in

both hairpin loops can affect the binding mode of the inhibitor to papain in a way that the Gly⁹-Ala¹⁰ bond of the inhibitor is cleaved like a peptide substrate (see [1] for details). Inhibition of cathepsin L is only slightly reduced by the selected hairpin loop mutations. Hence, it is shown indirectly that the three contact regions of the inhibitor represent an excellent binding surface, formed complementary to the active-site cleft of cathepsin L, but additional sites or regions, not identified so far, could be important for the strong interaction with cathepsin L.

Acknowledgments: The authors wish to thank Mrs. C. Huber for excellent technical assistance in molecular cloning, Mrs. B. Meisel for isolation of $\Delta V55$, $\Delta V55$ -S56, and Mrs. R. Zauner for performing kinetic measurements. The amino acid sequencing work of R. Mentele performed in the laboratory of Prof. F. Lottspeich, Martinsried and the mass spectrometric determinations by Dr. C. Eckerskorn, Martinsried are gratefully acknowledged. We are indebted to PD Dr. W. Bode and Dr. R. Engh, Martinsried for stimulating discussions. This work was supported by the Sonderforschungsbereich 207 of the LMU Munich (Grants H-9/Machleidt, H-2 /Huber and H-4 /Auerswald).

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