

Mechanism of inhibition of papain by chicken egg white cystatin

Inhibition constants of N-terminally truncated forms and cyanogen bromide fragments of the inhibitor

Werner Machleidt, Ulrich Thiele, Bernd Laber*, Irmgard Assfalg-Machleidt, Anna Esterl, George Wiegand*, Janko Kos[†], Vito Turk[†] and Wolfram Bode*

*Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, D-8000 München 2, *Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG and [†]Odsek za biokemijo, Institut J. Stefan, Univerza E. Kardelja, YU-61000 Ljubljana, Yugoslavia*

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N-terminally truncated forms of chicken egg white cystatin and its cyanogen bromide fragments were isolated and assayed for inhibition of papain. Truncated forms beginning with Gly-9 and Ala-10 had a 5000-fold lower affinity for papain than the two isoelectric forms ($pI=6.5$ and 5.6) of the full-length inhibitor ($K_i=6$ pM and 7 pM) or a truncated form beginning with Leu-7 ($K_i=6$ pM), indicating the outstanding importance of one or two residues preceding conserved Gly-9 for binding. A weak inhibition of papain ($K_i=900$ nM) was exhibited by the intermediate cyanogen bromide fragment (residues 30–89) containing the chicken cystatin QLVSQ variation of the QVVAG segment which is conserved in almost all members of the cystatin superfamily. The obtained affinity data provide independent evidence for the validity of the proposed docking model of a chicken cystatin-papain complex [(1988) EMBO J. 7, 2593–2599].

Cystatin; Papain; Cysteine proteinase; Enzyme inhibitor; Inhibitor mechanism; (Chicken egg white)

1. INTRODUCTION

Chicken egg white cystatin belongs to family 2 of the cystatin superfamily comprising various protein inhibitors of cysteine proteinases (reviews [1,2]). It forms a tight, reversible 1:1 complex with most known cysteine proteinases [3]. Chicken egg white cystatin has been reported to occur in two major isoelectric forms, form 1 ($pI=6.5$) and form 2 ($pI=5.6$), which can be separated by ion exchange chromatography [4,5]. Both forms represent the full-length inhibitors starting with Ser (Ser-form) and comprising 116 amino acid residues

[4,6]. Also shorter forms starting with Gly-9 (Gly-form) and Ala-10 (Ala-form) have been found [4,7]. The Ala-form was shown to be a 10000-fold weaker inhibitor of papain [7].

Recently we have proposed a model for the interaction of cystatins with cysteine proteinases based on the X-ray crystal structure of the Gly-form of chicken egg white cystatin and subsequent docking experiments [8]. Three parts of the inhibitor are in close contact with the active site cleft of papain: the amino terminus, a first hairpin loop (residues 53–57) containing the chicken cystatin QLVSQ variation of the prototype sequence QVVAG conserved in almost all members of the cystatin superfamily [1,2] and a second hairpin loop (residues 102–107) containing the conserved Trp-104. According to the docking model, Leu-8 of chicken cystatin was predicted to interact specifically with the S_2 -subsite of papain raising the question which residues preceding Ala-10 are

Correspondence address: W. Machleidt, Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestraße 33, D-8000 München 2, FRG

Abbreviations: E-64, 1-(trans-epoxysuccinyl-L-leucylamino)-4-guanidinobutane; FPLC, fast protein liquid chromatography

the most important for the increased inhibitory affinity of the full-length form.

In this paper we describe further separation of isoelectric forms 1 and 2 of chicken cystatin by hydrophobic interaction chromatography. Using this method, we were able to separate up to four components of each isoelectric form differing in the length of the amino terminus. Furthermore, peptide fragments of chicken cystatin were prepared by cyanogen bromide cleavage. The estimated inhibition constants for the interaction of the N-terminally truncated forms and the peptide fragments with papain support the proposed mechanism of inhibition.

2. MATERIALS AND METHODS

2.1. Materials

Chicken egg white cystatin was isolated as described [4]. Papain (EC 3.4.22.2, from papaya latex, type III, Sigma) was repurified by covalent chromatography on an agarose mercurial column [9] leading to a 96% active enzyme as determined by titration with E-64 [10].

2.2. Isolation of inhibitor forms and fragments

Ion exchange chromatography of the crude inhibitor was performed on a Mono Q FPLC column (Pharmacia) with a 0–0.5 M KCl gradient in 20 mM Tris-HCl, pH 8.0. The pooled peak fractions were adjusted to 0.5 M $(\text{NH}_4)_2\text{SO}_4$ and loaded on a Bio Gel Phenyl-5PW column (Bio Rad). Hydrophobic interaction chromatography on this column was done in 0.1 M potassium phosphate buffer, pH 7.0, using a gradient of decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration from 0.5–0 M at a flow rate of 1.0 ml/min.

Limited proteolysis of chicken cystatin was performed by incubation of the Ser-form 1 with 10% (w/w) of endoproteinase Arg-C (EC 3.4.21.40, Boehringer) in 0.1 M potassium phosphate buffer, pH 8.2, 0.25 M $(\text{NH}_4)_2\text{SO}_4$, 2 mM glycine for 15 h at 37°C. The digest was separated on the Bio Gel Phenyl-5PW column.

Cyanogen bromide cleavage of chicken cystatin (Ser-form 1) was performed with a 200-fold molar excess of CNBr (Serva) in 70% formic acid for 15 h at 25°C. The cleavage products were separated by FPLC on a Superose 12 column (Pharmacia) in 5% (v/v) formic acid (0.2 ml/min).

2.3. Amino acid sequence analysis

Peptides were sequenced by automated solid-phase Edman degradation in a non-commercial instrument [11], or, after desalting on a reverse phase C-8 column, in a Knauer sequencer model 810 [12].

2.4. Determination of inhibition constants

The inhibition constants K_i of the complexes with papain were determined in stopped fluorometric assays as described [13]. The active concentrations of inhibitory peptides were determined by titration with papain. Inhibition of papain was

measured at equilibrium with a set of different concentrations of the inhibitor (usually 10 or more), and K_i was calculated by nonlinear regression analysis using the general equation for tight-binding inhibition [14] in the computer program ENZFITTER (Elsevier-BIOSOFT, Cambridge, England).

3. RESULTS

3.1. N-terminally truncated forms

Ion exchange chromatography of crude chicken egg white cystatin on Mono Q resulted in two ma-

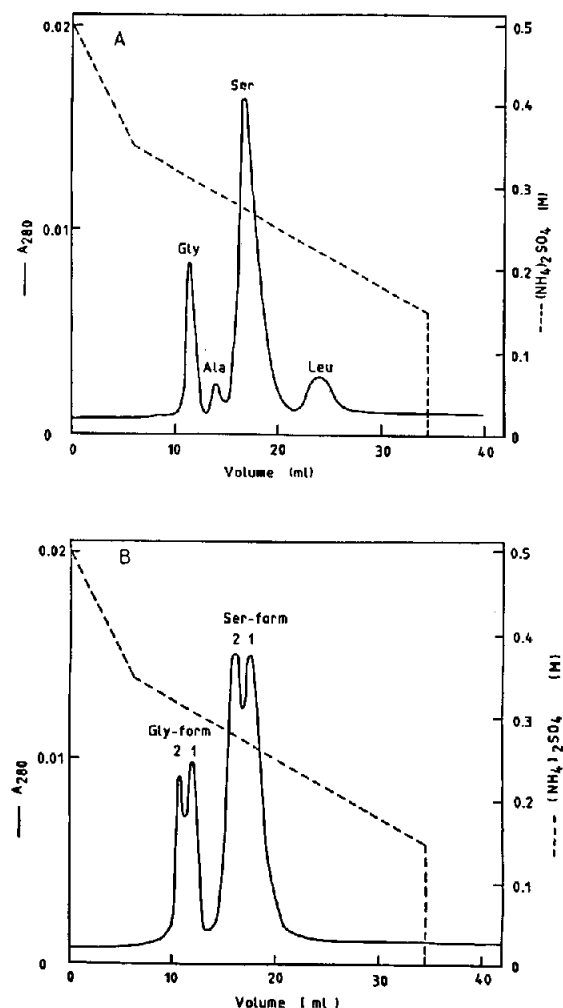


Fig.1. Hydrophobic interaction chromatography of chicken cystatin. (A) Separation of isoelectric form 1 ($pI = 6.5$) into N-terminally truncated isoforms eluting in the order Gly-form, Ala-form, Ser-form and Leu-form (see table 1 for explanation of nomenclature). (B) Separation of a mixture of isoelectric forms 1 ($pI = 6.5$) and 2 ($pI = 5.6$) into the N-terminally truncated Gly- and Ser-forms.

Table 1
Inhibition of papain by N-terminally truncated forms of chicken cystatin

Form	N-terminal sequence			$K_i \pm SD^a$ (nM)
	5	10	15	
Ser-form 1	S E D R S R L L	G A P V P V D E N D ...		0.006 ± 0.0001
Ser-form 2	S E D R S R L L	G A P V P V D E N D ...		0.007 ± 0.0003
Leu-form 1		L L G A P V P V D E N D ...		0.006 ± 0.0003
Gly-form 1		G A P V P V D E N D ...		26.6 ± 3.2
Gly-form 2		G A P V P V D E N D ...		33.9 ± 2.2
Ala-form 2		A P V P V D E N D ...		31.6 ± 2.6

^a Standard deviation of K_i obtained by nonlinear regression analysis

Truncated forms of the inhibitor are named according to their N-terminal amino acid residue followed by a number indicating their origin from isoelectric form 1 ($pI = 6.5$) or 2 ($pI = 5.6$)

for peaks eluting at 0.07 M KCl (form 1, $pI = 6.5$) and 0.14 M KCl (form 2, $pI = 5.6$). Material from both peaks was heterogeneous in isoelectric focusing. Homogeneous isoforms were obtained by subsequent hydrophobic interaction chromatography (fig.1A). Up to four different forms were separated from both isoelectric forms 1 and 2. The separated forms were identified by sequence analysis as Gly-, Ala-, Ser- and Leu-form according to their N-terminal amino acid residue (table 1). The Gly- and Ser-forms were the most prominent isoforms whereas the Ala- and Leu-forms were usually present in minor amounts. In some batches the Ala-forms were missing. Sequencing over at least 10 steps revealed no differences between Gly-form 1 and 2 or Ser-form 1 and 2, respectively (see table 1 for explanation of nomenclature). All four forms could be separated by hydrophobic interaction chromatography (fig.1B) and non-denaturing PAGE (not shown), suggesting that sequence differences between the isoelectric forms with the same amino terminus could exist and should be located behind position 18 of full-length cystatin.

Treatment of native Ser-form 1 with endoprotease Arg-C resulted in selective and almost quantitative cleavage of the Arg-6-Leu-7-bond. The resulting Leu-form 1 was isolated by hydrophobic interaction chromatography.

Titration of papain indicated that 62–75% of the isolated forms (quantitated by their absorbance at 280 nm) were inhibitorily active after the purification procedure. Inhibition constants (K_i) for papain (see table 1) of full-length Ser-form 1

and Ser-form 2 were virtually identical and comparable to those previously reported of native chicken cystatin ($K_i < 0.005$ nM [3]). The truncated Leu-form 1 had virtually the same K_i for papain as full-length chicken cystatin (fig.2), whereas the truncated Gly-form 1, Gly-form 2 and Ala-form 2 had a ~5000-fold lower affinity for this proteinase.

3.2. Cyanogen bromide fragments

Cyanogen bromide cleavage of full-length chicken cystatin (Ser-form 1) followed by FPLC on a Superose 12 gel filtration column resulted in

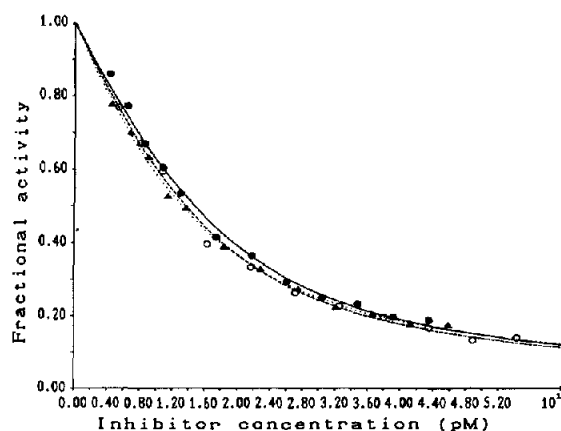


Fig.2. Inhibition of papain (12 pM) by different concentrations of Ser-form 1 (●—●), Ser-form 2 (▲—▲) and Leu-form 1 (○—○) of chicken cystatin. The fractional activity is the reaction rate in the presence of inhibitor divided by the rate in its absence. The curves were calculated from the general equation for tight-binding inhibition [14] using the K_i values determined from the experimental data shown here (cf. table 1).

two peaks (not shown). As revealed by N-terminal sequence analysis, the first peak contained the intermediate fragment (CN-2, residues 30–89), the second peak a mixture of the N-terminal fragment (CN-1, residues (1–29) and the C-terminal fragment (CN-3, residues 90–116) [4]. Only the intermediate fragment (CN-2) inhibited papain weakly with a K_i of 900 nM, compared to a K_i of 10 pM for the uncleaved inhibitor which had been subjected to the same acidic conditions as in cyanogen bromide cleavage and chromatographed on the same column in a control experiment. The mixture of CN-1 and CN-3 showed no inhibitory activity.

4. DISCUSSION

Sequence comparisons of cystatins provided indirect evidence that the conserved QVVAG sequence motif might be essential for their inhibitory activity [15,16]. This is consistent with our docking model of a chicken cystatin-papain complex where the most intimate contacts are made through the main and side chain groups of the analogous chicken cystatin QLVSG segment with parts of the putative S_1 -subsite of papain [8]. In the N-terminal part of cystatins, a glycine residue corresponding to Gly-9 of chicken cystatin is conserved in all inhibitorily active members of the superfamily [1,2]. According to the docking model, Gly-9 is in the close vicinity of but not in direct contact with the active site Cys-25 of papain. Within the preceding segment, assuming a tight-turn conformation (fig.3), the side chain of Leu-8 could bind to the S_2 -subsite which mainly determines the substrate specificity of papain [17,18]. Our results obtained with N-terminally truncated forms of chicken cystatin strongly support this idea. Whereas the Leu-form exhibits the same inhibitory activity as the full-length Ser-forms, removal of Leu-7 and Leu-8 leads to an approximately 5000-fold lower affinity for papain. This is in agreement with our observation that a truncated form of human cystatin C [19] starting with Leu-Val- before the conserved Gly-11 (corresponding to Gly-9 of chicken cystatin) has virtually the same affinity for papain as the full-length form ($K_i = 5$ pM), whereas the truncated form starting with Gly-12 has been reported to be a more than 1000-fold weaker inhibitor [7]. One or two residues

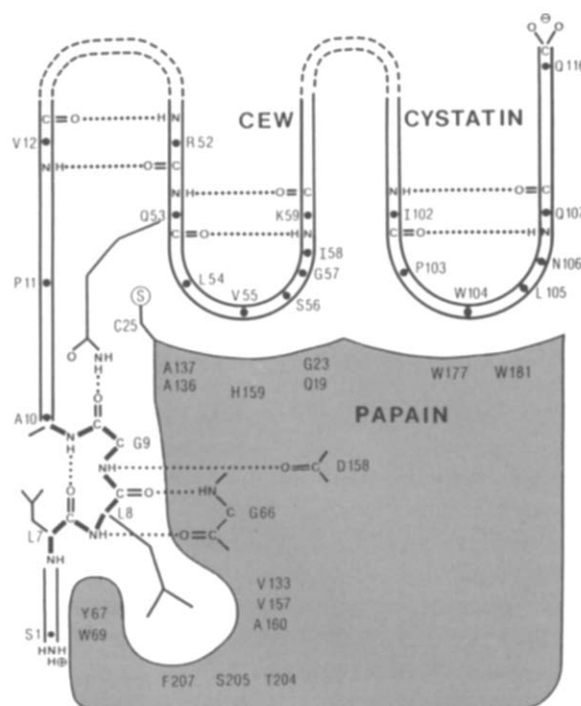


Fig.3. Scheme of the proposed model for the interaction of chicken cystatin with papain. Explanation in text.

preceding the conserved Gly seem to be mainly responsible for the tighter binding of cystatins to papain. Cystatins containing a single residue in front of the conserved Gly which are equally active as the full-length forms have been found in the stefin family [20] and prepared by expression of synthetic genes [21,22].

The selective binding of the intermediate CNBr fragment containing the QLSVG segment is consistent with its central role for the papain-inhibitor interaction. Its significantly increased affinity towards papain compared with short QVVAG-related peptides [23] might be due to intra-peptide stabilisation within a hairpin loop-like structure similar to that observed in intact chicken cystatin [8]. The direct relation between affinity and chain extension in the N-terminal direction beyond Gly-9 is in agreement with the additional contribution of the elongated N-terminal segment to binding. Of great surprise, however, is the stepwise tremendous increase of affinity in going from Gly-9 to Leu-8 and/or Leu-7. In conclusion, the affinity data presented here are independent evidence for the validity of the docking model.

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