Quail cystatin: Isolation and characterisation of a new member of the cystatin family and its hypothetical interaction with cathepsin B

Bernd Gerhartz[1,a], Richard A. Engh[b], Reinhard Mentele[a], Christoph Eckerskorn[c], Richardo Torquato[2,a], Josef Wittmann[d], Helmut J. Kolb[e], Werner Machleidt[f], Hans Fritz[a], Ennes A. Auerswald[a,*]

[a] *Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik und Poliklinik, Klinikum Innenstadt, LMU München, Nußbaumstr. 20, D-80336 Munich, Germany*
[b] *Max-Planck-Institut für Biochemie, Strukturforschung, D-82152 Martinsried, Germany*
[c] *Max-Planck-Institut für Biochemie, Proteinanalytik, D-82152 Martinsried, Germany*
[d] *Institut für Physiologische Chemie, Physiolog. Chemie und Ernährungsphysiologie, LMU München, Veterinärstr. 13, D-80539 Munich, Germany*
[e] *Klinisch-Chemisches Institut am Städtischen Krankenhaus Harlaching, Sanatoriumsplatz 1, D-81545 Munich, Germany*
[f] *Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie, LMU München, Goethestr. 33, D-80336 Munich, Germany*

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Abstract **Quail cystatin, a new cysteine proteinase inhibitor protein of the cystatin superfamily, was purified from egg albumen of Japanese quail *Coturnix coturnix japonica*. Amino acid sequencing and mass spectrometry revealed the complete 116 amino acid residue primary structure of a phosphorylated form (13 173 Da). The inhibitor has a 90% sequence identity with chicken cystatin. Its interaction with papain is rapid and tight ($K_i = 4.4$ pM; $k_{on} = 1.8 \times 10^7$ M^{-1} s^{-1}; $k_{off} = 0.8 \times 10^{-4}$ s^{-1}) and very similar to that of chicken cystatin. Surprisingly, however, cathepsin B was inhibited 15-fold more strongly by quail cystatin ($K_i = 47$ pM; $k_{on} = 19 \times 10^7$ M^{-1} s^{-1}; $k_{off} = 9 \times 10^{-4}$ s^{-1}) than by chicken cystatin ($K_i = 784$ pM; $k_{on} = 2.9 \times 10^7$ M^{-1} s^{-1}; $k_{off} = 24 \times 10^{-4}$ s^{-1}). Intuitive comparative conformational inspection of related inhibitors and of cognate enzymes suggest that: (i) the 3D structure of quail cystatin is nearly identical to that of chicken cystatin, (ii) quail cystatin can interact with cathepsin B analogous to the stefin B–papain interaction, if the 'occluding loop' of cathepsin B possesses an 'open' conformation, (iii) the greater inhibition of cathepsin B by quail cystatin compared to chicken cystatins probably arises from two additional ionic interactions between residues Arg^{15} and Lys^{112} of the inhibitor and Glu^{194} and Asp^{124} of the enzyme, respectively. The two potential salt bridges are located outside of the known contact regions between cystatins and peptidases of the papain family.**

Key words: Cystatin; Cysteine proteinase inhibitor; Cathepsin B; Inhibition kinetics; Mass spectrometry; Molecular interaction

1. Introduction

Protein-type cysteine proteinase inhibitors are widely distributed in animals and plants. It is generally believed that these inhibitors are involved in the regulation of physiological and pathological processes caused by cysteine proteinases [1–3]. Recently, we described two new proteinases from the japanese quail *Coturnix corturnix japonica* [4,5]. During these studies we detected a cysteine proteinase inhibitory activity comparable to that of chicken cystatin. This quail cystatin is described and characterized here in more detail.

Chicken cystatin is a well characterized member of the cystatin superfamily cystatin family or family 2 [6–14]. The small protein is a reversible, competitive, tight-binding inhibitor of papain. A model for its interaction with papain was proposed [15,16] and confirmed by the structure of the papain–stefin B complex [17] as well as by detailed kinetic studies of variants. The inhibitor N-terminal 'elephant trunk' (Leu^7-Gly^9) binds the (unprimed) specific substrate recognition sites of papain while the hydrophobic first (Gln^{53}-Gly^{57}) and second hairpin loops (Pro^{103}-Leu^{105}) bind in the broad and long U-shaped substrate binding cleft especially via hydrophobic contacts [18–25].

Less well understood is the interaction of cystatins with other cysteine proteinases like cathepsin B. The molecular structure of human liver cathepsin B was solved by X-ray analysis at 2.15 Å resolution [26]. Cathepsin B exhibits both endopeptidase and exopeptidase activity. Cleavage of C-terminal dipeptides (peptidyldipeptidase activity) is dependent on the presence of the specialized 'occluding loop' which presents C-terminal anchoring interactions; this is not present in other cysteine proteinases. Its occlusion of the broad substrate binding site seemed to exclude a papain–cystatin-like interaction because of steric clashes between it and the cystatin second hairpin loop. Our first cathepsin B-chicken cystatin interaction model suggested that either cystatin binds to cathepsin B with a new geometry or that displacement of the occluding loop must accompany binding of cystatin to cathepsin B [26]. Later it was proposed that such an additional conformational change could explain the weaker cathepsin B inhibition by cystatins in general [3].

Recently, the tertiary structures of rat and human procathepsin B were solved to shown how the proregion interacts with its parent protease and blocks the access to the active site

*Corresponding authors: E.A. Auerswald [Fax: (49) (89) 5160 4735. E-mail: auerswald@clinbio.med.uni-muenchen.de] and B. Gerhartz at his present address.

[1] *Present address:* Department of Clinical Chemistry, University of Lund, University Hospital, S-221 85 Lund, Sweden.

[2] *Present address:* Departamento de Bioquimica, Escola Paulista de Medicina, UNIFESP – Sao Paulo, S.P., Brazil.

Abbreviations: catB, cathepsin B; cc, chicken cystatin; CNBr, cyanogen bromide; E-64, L-3-carboxy-2,3-trans-epoxy-propionyl-leucyl-amido-(4-guanidino)butane; NH-Mec, 7-(4-methyl) coumaryl-amide; Z, benzyloxycarbonyl; qc, quail cystatin. *Enzymes:* papain (EC 3.4.22.2); cathepsin (EC 3.4.22.1).

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[27,28]. This structure demonstrated the flexibility possible for the 'occluding loop' with its 'open' conformation as opposed to the more compact 'close conformation' found in mature cathepsin B [26].

In this paper, we describe the purification, primary structure and the inhibitory kinetics of a new cystatin. Furthermore, we present evidence for the occurrence of an 'open-like' conformation of the occluding loop during binding of cystatins by cathepsin B similar as found within the proenzyme to occur and show that such a conformation could be stabilized by salt bridges formed between quail cystatin with cathepsin B.

2. Material and methods

2.1. Material

All chemicals used were obtained from Sigma, St. Louis, MO, USA; Merck, Darmstadt, Germany; Serva, Heidelberg, Germany, and were of analytical grade. Natural chicken cystatin was a generous gift from V. Turk, Ljubljana, Slovenia. Human cathepsin B was purchased from Medor, Herrsching, Germany and used without further purification. Papain was repurified as described [29]. Japanese quail eggs were from a random-bred strain.

2.2. Purification of quail cystatin

About 400 ml albumen were obtained from unbred quail eggs (around 100 eggs). After removing ovomucoid [12] the material was alkaline (pH 12) and heat (65°C) treated [11]. The resulting supernatant (10 000 × *g*, 15 min) was dialyzed and applied to a weak cation exchange chromatography (CM-sepharose FF, 26 × 200 mm, Pharmacia, 20 mM acetate buffer pH 5.5) and separated with a salt gradient from 0 to 0.5 M NaCl. Papain inhibitory fractions were pooled and adjusted to 1.2 M $(NH_4)_2SO_4$ for a hydrophobic interaction chromatography (Phenyl-Sepharose FF high substitute, 16 × 180 mm, Pharmacia), elution was performed with a linear gradient to O M $(NH_4)_2SO_4$. The inhibitor containing fractions were concentrated (2 ml) and separated further by gel filtration (Superose 12, 16 × 500 mm, Pharmacia). Final purification was done by cation exchange chromatography on a Mono S (5 × 50 mm, Pharmacia, 50 mM acetate buffer pH 5.5, elution by gradient from 0 to 0.5 M NaCl). Inhibitory fractions were pooled and stored in aliquots at −20°C.

2.3. SDS-PAGE and determination of protein concentration

SDS-PAGE of proteins was performed with 5–22.5% polyacrylamide gels [30] and protein bands were stained with silver according to the method of Blum [31]. Protein concentrations were determined by the method of Lowry et al. [32] or by absorption measurements at 280 nM, using the extinction coefficients for aromatic residues and cystines of Mach et al. [33] resulting in E_{280} 12 570 M^{-1} for the purified inhibitor.

2.4. HPLC analysis and amino acid sequencing

Purified quail cystatin samples as well as CNBr fragments or material of tryptic digests of quail cystatin were analyzed by reversed phase HPLC as detailed previously [34]. Automated amino acid sequencing was performed using a gas-phase sequenator 473A (Applied Biosystems GmbH, Weiterstadt, Germany) following the instructions of the manufacturer.

2.5. Mass spectrometry

Quail cystatin and CNBr cleaved material or peptides were applied to a reversed phase HPLC coupled on-line to an atmospheric pressure ionization source fitted to the tandem quadruple instrument API III (Sciex, Thornhill, Ontario, Canada). The instrument m/z scale was calibrated with ammonium adducted ions of polypropylene glycol. 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 [35]. Theoretical masses of the peptides were calculated with the GCG DNA/Protein Analysis Software [36].

2.6. Determination of kinetic constants

Kinetic constants for the interaction of quail and chicken cystatin with cysteine proteinases were determined applying enzyme assays at 30°C (papain) or 25°C (cathepsin B) and pH 5.5 with the fluorogenic substrate Z-Phe-Arg-NH-Mec as described [23] using 50 mM sodium acetate buffer. Rate constants, k_{on} and k_{off}, were obtained by pre-steady-state analysis [37]. Equilibrium dissociation constants were measured directly in equilibrium inhibition experiments or calculated ($K_i = k_{off}/k_{on}$) from pre-steady-state experiments and were corrected for substrate competition. Substrate consumption was less than 5% in all experiments. All calculations are based on the active concentration of the inhibitor determined by titration E-64-standardized papain (4 nM) and Bz-Arg-NH-Mec (10 μM) as described [34].

2.7. Structural inspection and modelling

The coordinates of chicken cystatin [15], human stefin B-papain [17] cathepsin B [26] and procathepsin B [27] were taken from the Protein Data Bank [38] and displayed and manipulated using the program O [39]. Docking models were prepared by fitting binding site residues of cathepsin B, procathepsin B and chicken cystatin to the corresponding residues of papain in the papain stefin complex.

3. Results

3.1. Purification and protein chemical characterization of quail cystatin

After having identified a papain inhibitory activity in the albumen of quail eggs [4] two classical chicken cystatin purification steps were used to enrich inhibitor: alkaline extraction (30 min at pH 12) and heat treatment (65°C at pH 6.0 for 10 min) [10,11]. Affinity chromatography (with Cm-Papain-Sepharose) did not increase the yield in active material (data not shown) although this step has been successfully applied for isolation of several other native and recombinant cystatins [12,24,39]. Therefore, a new purification scheme was established comprising cation exchange chromatography (CM-sepharose FF), hydrophobic interaction chromatography (Phenyl-sepharose FF), gel filtration (Superose 12) and cation exchange chromatography (Mono S). Each step was checked by papain inhibition tests and SDS-PAGE analyses. The purification scheme and the resulting data are given in Table 1. From 400 ml albumen 200 μg pure inhibitor were obtained, corresponding to an enrichment of specific activity of about 30 000-fold. The purified material migrates as a homogenous

Table 1
Purification of quail cystatin from albumen

Purification step	Protein (mg)	Active material (units)	Specific activity (units/mg)	Purification (-fold)
Homogenate	46 000	30.3	0.0007	
Ovomucoid precipitation	39 000	25.4	0.0007	1
Heat precipitation	10 166	18.3	0.0018	3
CM-sepharose	549	41.6	0.0750	107
Phenyl-sepharose	108	15.7	0.1450	207
Superose 12	1.0	10.5	10.2	14 571
Mono S	0.2	4.9	23.3	33 000

Protein was determined by the method of Lowry [31]; inhibitory activity was tested as papain inhibition with Z-Phe-Arg-NH-Mec as substrate; one inhibitory unit is defined as the inhibition of one unit papain activity; homogenate was obtained from 400 ml albumen.

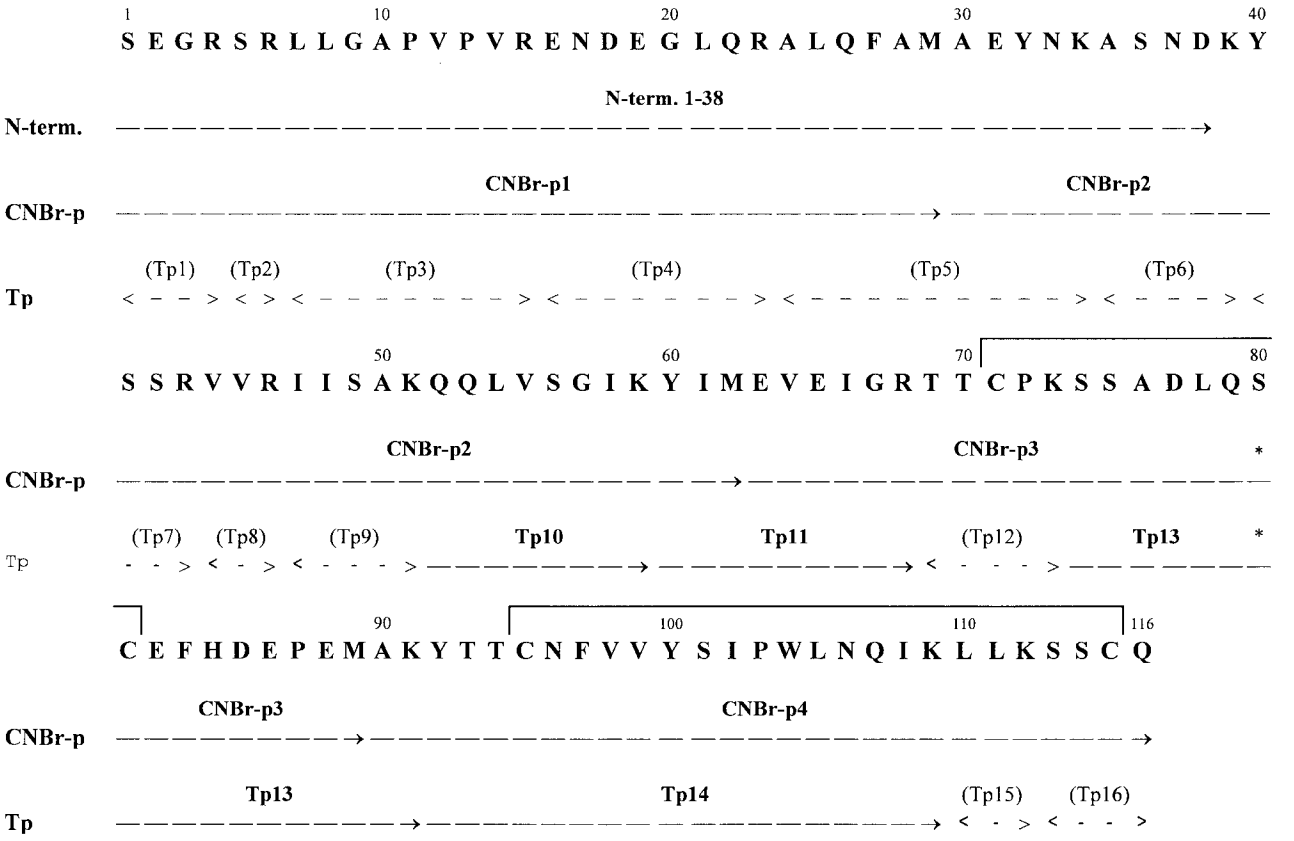

Fig. 1. Amino acid sequence of quail cystatin and its tryptic and CNBr peptide map. The complete primary structure of quail cystatin is given. Numbers denote the positions of amino acid residues in the peptide chain of native quail cystatin. Peptides isolated from the tryptic digest are identified with the prefix Tp, in ( ) brackets peptide names and in < > brackets amino acid positions are marked which were not determined. N-term. indicates the N-terminal sequenced region; CNBr-p indicates the CNBr fragments 1–4; — and → residue identified by Edman degradation or last residue of a peptide; *, phosphorylated serine.

15-kDa protein in SDS-PAGE (data not shown). HPLC analyses by reverse phase chromatography confirmed the homogeneity of the purified inhibitor (data not shown).

3.2. Protein sequencing and mass spectrometry

In order to determine the complete primary structure of the inhibitor, we used the following overall strategy: (1) N-terminal amino acid sequencing of the inhibitor; (2) tryptic cleavage and sequencing of selected peptides; (3) preparation and sequencing of CNBr fragments; and (4) mass determination of CNBr fragments and of tryptic peptides. The resulting primary structure of quail cystatin, schemes of sequenced peptide fragments and the order of the hypothetical tryptic fragments are displayed in Fig. 1.

In more detail, N-terminal sequencing allowed the identification of the first 38 amino acid residues. This sequence showed a high homology to chicken cystatin and facilitated the preparation, selection and identification of tryptic peptides in comparison to an approach performed earlier for recombinant chicken cystatin [34]. The sequences of the tryptic peptides Tp10, Tp11, Tp13, Tp14 confirmed and extended the high similarity to chicken cystatin. The tryptic peptides were aligned to chicken cystatin and a methionine substitution (M62) instead of L62 in chicken cystatin was identified. This directed us to perform CNBr cleavage. By sequencing CNBr fragments, the gaps between D38-Q52, R68-S74 and K109-Q116 could be filled and the positions and sequences of the peptides Tp10, Tp11, Tp13, Tp14 overlapped and verified consistently (Fig. 1). In order to confirm the primary structure unequivocally, masses of CNBr fragments and of the purified inhibitor were determined (Fig. 2 and Table 2). The sequence correctness of non-overlapping gap borders, the C-terminus and the presence of the two S-S bridges (Cys^{71}-Cys^{81} and Cys^{95}-Cys^{115}) were deduced by these data.

As expected, not only single masses of CNBr fragments containing homoserine lactone or homoserine residues (see Fig. 2 and Table 2, CNBr-p1, CNBr-p2, CNBr-p3) were detected (mass difference of 18 Da). Additional peptides were identified and their masses could be interpretated as lysine-formylated forms (CNBr-p1, CNBr-p2, CNBr-p4 with a mass difference of 28 Da) or as oxidated forms of CNBr-p4 (probably first and second oxidation of Trp^{108} with mass differences of 15 and 31, respectively). The C-terminal CNBr-p4 fragment did not contain a methionine but its first oxidized form seemed to be formylated, too (peak 3403.0, Fig. 2). Taking these considerations together, the detected profiles of the mass spectrometric measurements of all four CNBr fragments can be resolved unequivocally into four subgroups CNBr-p1, -p3, -p4 and -p2 (Fig. 2).

The measured masses of the CNBr-p3 subgroup (E63-M89) differ by approx. 80 Da from the theoretical expected masses of a homoserine and a homoserine-lactone form. The same difference has been detected by mass analysis of the complete inhibitor. This variation is probably due to phosphorylation of a serine residue. Leading to a gap in Edman degradation of Tp13 and the CNB3-p3 at Ser^{80}. Therefore, it is most likely

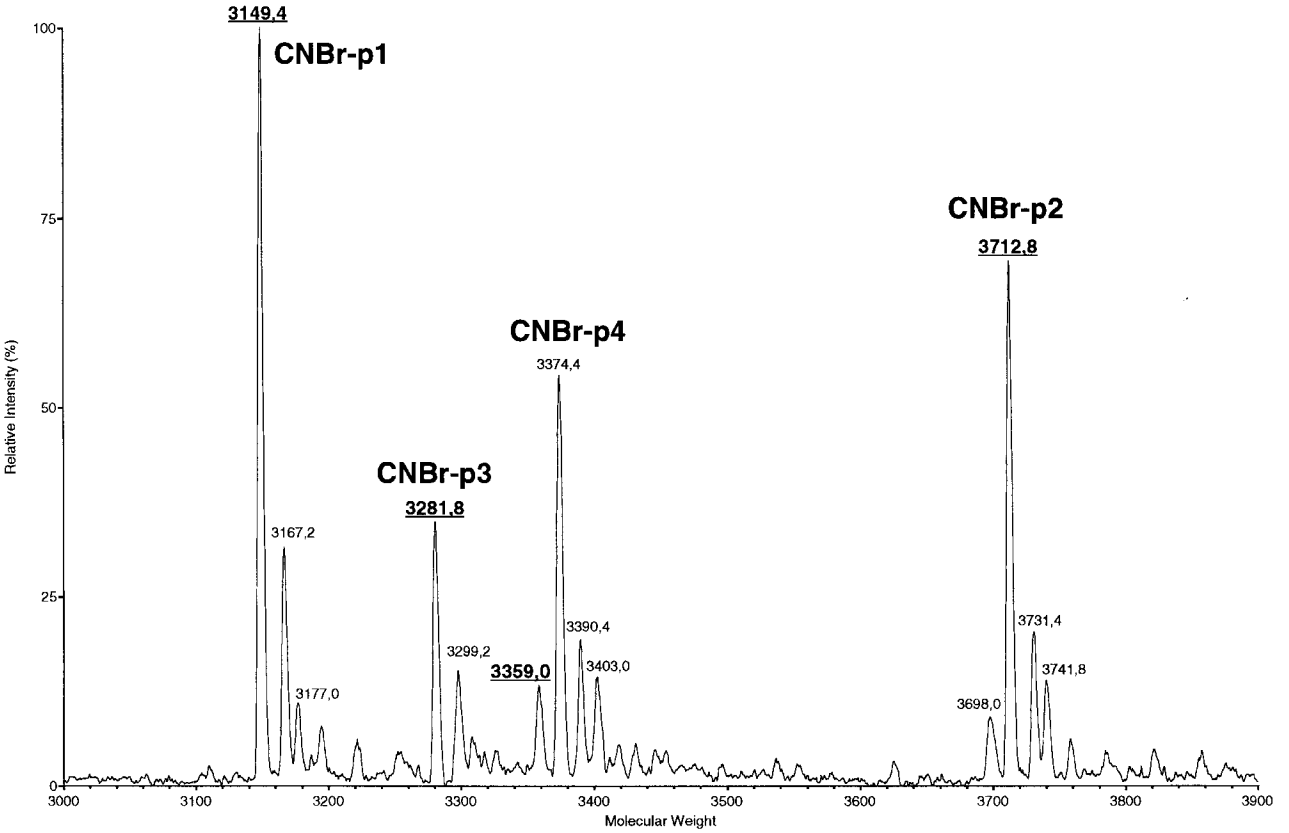

Fig. 2. Mass spectrometry of CNBr-cleaved fragments of quail cystatin. The deconvoluted mass spectra gives final estimated masses of the main products after CNBr-cleavage. CNBr-p1–CNBr-p4 indicates the subgroups which are build by fractions of modified CNBr fragments. Posttranscriptional or artificial modification result in mass difference of 80 Da (phosphorylation), 16 Da (oxidation), and 28 Da (formylation). Numbers denoted the calculated masses from the m/z peaks in the charge distribution profiles. Bold and underlined are the homoserine lactone forms, in case of CNBr-p3 a possibly phosphorylated homoserine lactone form, and in case of CNBr-p4 the unmodified C-terminal fragment. 3167.2 indicates a homoserine form, and 3177.0 a formylated homoserine lactone form of CNBr-p1; 3299.2 indicates the homoserine form of phosphorylated CNBr-p3, 3374.4 the first Trp-oxidized form, 3390.0 the second Trp-oxidized form, and 3403.0 the first Trp-oxidized and formylated form of CNBr-p4; 3698.0 is unidentified, 3731.4 indicates the homoserine form, and 3741.8 a formylated homoserine lactone form of CNBr-p2.

that quail cystatin is completely phosphorylated at Ser^{80}. Whereas chicken cystatin has been found to be partially phosphorylated at Ser^{80} [41]. This is supported by the complete absence of an unphosphorylated peptide within the range of 3201.5 Da (Fig. 2).

3.3. Amino acid sequence alignment

An alignment of the primary structures of quail cystatin, chicken cystatin and human cystatin C is given in Fig. 3. It shows an overall identity of 90% between quail cystatin and chicken cystatin and of 44% with human cystatin C. There are no sequence differences to chicken cystatin within the highly conserved contact regions of the cystatins, the N-terminal segment with the conserved glycine (G9), the first hairpin loop (QLVSG, QxVxG-region) and the second hairpin loop (PW-region). The high sequence identity including cysteine spacing demonstrates clearly that quail cystatin belongs to family 2 of the cystatin superfamily. The identified 11 amino acid substitutions as compared to chicken cystatin are distributed over the whole molecule but are notably absent from regions known to contact cysteine proteases (see below). Most remarkable are the exchanges from basic side chains to acidic ones $Arg^{15}qc \rightarrow Asp^{15}cc$, $Lys^{112}qc \rightarrow Glu^{112}cc$ and the exchanges from uncharged residues to an acidic one $Gly^{3}qc \rightarrow Asp^{3}cc$ or to basic ones $Gln^{52}qc \rightarrow Arg^{52}cc$, $Ser^{114}qc \rightarrow Lys^{114}cc$.

3.4. Inhibition kinetics

Equilibrium dissociation constants (K_i) were determined for the complexes of quail cystatin with papain and cathepsin B as well as for the corresponding complexes of their enzymes with chicken cystatin. The calculated K_i-value for the papain–quail cystatin complex is 4.4 pM and very similar to that of the papain–chicken cystatin complex (1.4 pM, see Table 3) Rate constants of complex formation (k_{on}) and complex dissociation (k_{off}) were determined in pre-steady-state experiments. As shown in Table 3, the k_{on} and k_{off} values of papain inhibition by chicken and quail cystatin are quite similar.

Steady-state experiments with human cathepsin B resulted in a K_i-value of 47 pM. This value differs significantly from the published K_i-values of the chicken cystatin–human cathepsin B complexes (0.8–2.9 nM) [12,40,41]. Therefore, we compared cathepsin B inhibition of chicken and quail cystatin in the same set of experiments. Steady-state as well as pre-steady-state experiments confirm the stronger inhibition of quail cystatin (47 pM) comparing to chicken cystatin (828 pM). This difference is due to a 7-fold faster association (k_{on}: 19 and 2.9×10^6 M^{-1} s^{-1}, respectively) and a 3-fold

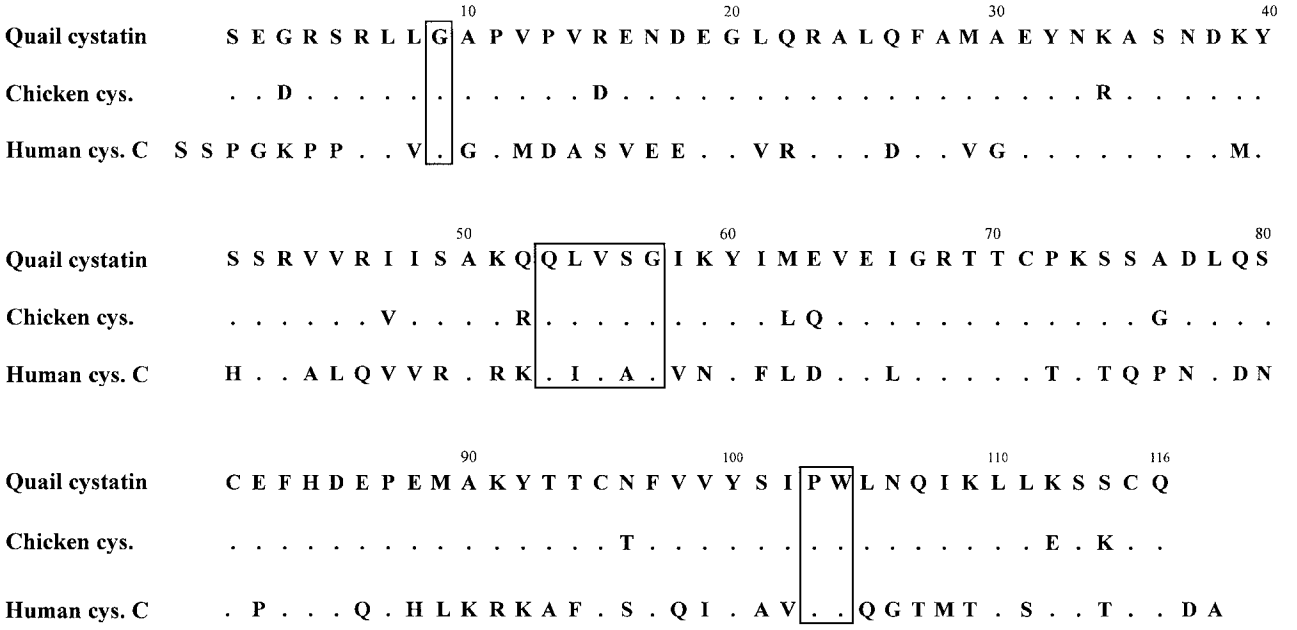

Fig. 3. Alignment of amino acid sequences of quail cystatin with chicken cystatin and human cystatin C. Amino acid residues are given in the one-letter code and numbers denote the position within quail cystatin. Sequences of chicken cystatin (Chicken cys.) are form [11] and of human cystatin C (Human cys. C) are from [48]. Identical residues in corresponding positions are indicated by points. The conserved contact regions of cystatins with glycine in the N-terminus, QLVSG in the first hairpin loop (QxVxG-region) and PW second hairpin loop are boxed. Amino acid residues which probably stabilize the interaction with cathepsin B outside of the conserved contact region are shaded.

slower dissociation (k_{off}: 9 and 24×10^{-3} s^{-1}, respectively). Initially it was not clear why quail cystatin was such a strong cathepsin B inhibitor especially since the amino acid substitutions compared to chicken cystatin were not found in regions known to contact cysteine proteases. Computer-aided model building and structural inspections suggested in particular that the stronger inhibition may arise from salt bridge interactions.

3.5. Structural inspection (Modelling)

The known crystal structures of chicken cystatin [15], human cathepsin B [26]; human procathepsin B [27] and the stefin B–papain complex [17] were used for modelling and docking experiments. Substitution of the 11 amino acid side chains mutated in quail cystatin relative to chicken cystatin showed no evidence that the tertiary structure forming interactions of chicken cystatin are altered; the fold is expected to be identical to that of chicken cystatin (Fig. 4). Furthermore, the docking model shows no substitutions in quail cystatin which should significantly alter interactions with papain compared to chicken cystatin. This is consistent with the observed kinetic data for the papain–quail cystatin complex.

However, because of the lack of substitutions in known cystatin–cysteine protease contact regions, the strong inhibition of human cathepsin B by quail cystatin was totally unexpected. Inspection of the naive (superposition without refinement) docking model of cystatin immediately reveals, however, that two substitutions which swap negative for positive charges are positioned adjacent to acid side chains of cathepsin B (Figs. 4 and 5). The potential salt bridges (Arg^{15}I-Glu^{194}; Lys^{112}I-Asp^{124}), although accessible to solvent, could contribute considerable binding energy to the interaction. These interactions are not available in papain due to the lack of the 'occluding loop' (Asp^{124}) and neutral residues in a different loop conformation (Glu^{194}).

The docking complex as depicted in Fig. 4. is unlikely due to the steric hindrance between the second hairpin loop of chicken/quail cystatin and the conformation of the 'occluding loop' of mature cathepsin B [26]. This conformation of the 'occluding loop' blocks the hydrophobic/aromatic surface of the cathepsin B binding cleft formed primarily by residues Trp^{177} and Trp^{181} (papain numbering). However, the 'open-like' conformation of the 'occluding loop' as found in procathepsin B [27] is rotated approx. about the principal axis of the loop. This rotation frees the hydrophobic/aromatic surface

Table 2
Masses of CNBr fragments from quail cystatin

Peptides	Theoretical mass	Measured mass	Δ
cNBr-p1	3149.5[a]	3149.4	−0.1
(S1-M29)	3167.5[b]	3167.2	−0.3
	3176.5[c]	3177.0	0.5
CNBr-p2	3713.3[a]	3712.8	0.5
(A30-M62)	3731.3[b]	3731.4	0.1
	3741.3[c]	3741.8	0.5
CNBr-p3	3201.5[a]	3281.8	80.3
(E63-M89)	3219.5[b]	3299.2	79.7
CNBr-p4	3359.0	3359.0	0.0
(A90-Q116)	3375.0[d]	3374.4	−0.6
	3391.0[e]	3390.4	−0.6
	3403.0[f]	3403.0	0.0
Quail cystatin*	13594.6	13593.8	−0.8

CNBr-p1-CNBr-p4 are subgroups of CNBr-fragments, in brackets amino acid residues and their positions. The theoretical masses were determined according to [35]. Measured masses were determined as outlined in materials and methods.
*S-pyridy ethylated and phosphorylated form; Δ, difference between measured mass and theoretical mass;
Modifications: [a], homoserine lactone; [b], homoserine; [c], formylation of lysine; [d], oxidation of tryptophan; [e], higher oxidation of tryptophan; [f], formylation and oxidation of tryptophan.

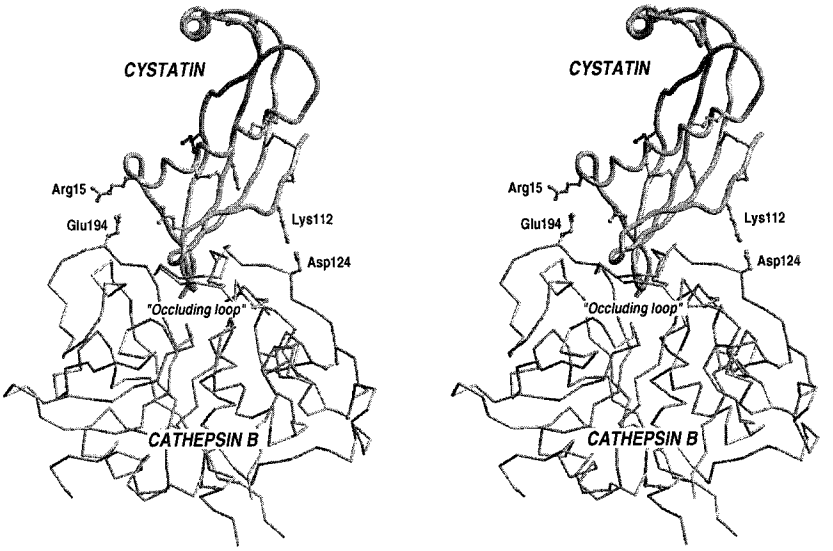

Fig. 4. Stereo depictions of the docking model of cystatin and human cathepsin B. The model is based on the superposition of corresponding binding site residues of cathepsin B and cystatin onto corresponding residues of the stefin–cathepsin B complex without further refinement. Side chains of mutations in quail cystatin relative to chicken cystatin are displayed in a ball and stick representation, as are disulfide bridges. This 'naive' docking model places two charge substitution chicken→quail substitutions (D15→R, E112→K) in ideal positions for salt bridge formation to E194 and D124 of cathepsin B, respectively.

and expands the cleft. Minor adjustments in this loop conformation and/or minor adjustments in the cystatin orientation (Fig. 5) would presumably allow an interaction similar to that of papain with stefin B [17]. The apparent lack of rigidifying interactions of cystatin inhibition of cathepsin B strengthen this hypothesis.

4. Discussion

Avian albumen is a source of various kinds of proteinase inhibitors such as ovomacroglobulin, ovomucoid, ovoinhibitor and cystatin. Although chicken egg white cystatin was discovered already in 1968 by Fossum and Whitaker [9] no further avian cystatins have been described since. This paper reports the purification and characterization of a second avian cystatin, quail cystatin, which is very similar to chicken cystatin from the Japanese quail *Coturnix coturnix japonica*. The cystatin concentration is rather low in the albumen of the quail eggs, approx. 3 μg/ml albumen, whereas Anastasi et al. (1983) reported the presence of more than 80 μg cystatin/ml in the albumen of chicken eggs [12]. This may be one reason why the purification of quail cystatin was not as easy as those of other cystatins; other reasons included the co-migration and co-eluting fractions of lipocalin which were not separated in the first purification steps.

The classical elucidation of the complete primary structure combined with mass spectrometry was efficient and successful. Conclusive interpretation of data was possible from a crude mixture of un-separated CNBr fragments. All prominent m/z peaks and their determined masses could be identified by calculating and comparing well known artificial (homoserine lactone, homoserine, formylations, oxidations) and posttranslational modifications (phosphorylation) of the fragments. Furthermore, the profile shown in Fig. 2 is a nice example for different modified forms resulting from a CNBr cleavage of a small protein.

The purified quail cystatin is completely phosphorylated at Ser^{80} according to the indirect mass and sequencing data. A similar but partial phosphorylation is described for chicken cystatin and it has been shown that this phosphorylation has no influence on the inhibitory activity [41]. The physiological relevance of such a phosphorylation is not known. Undoubtedly quail cystatin is a new member of the family 2 of the cystatin superfamily.

Surprisingly, a significant difference was found in the inhibition of human cathepsin B by quail cystatin when compared with other cystatins. Quail cystatin seems to be the strongest cathepsin B inhibitor ($K_i = 48$ pM) among the cystatins if compared with Stefin A (4–11.2 nM) [42–44], Stefin B (16–130 nM) [45,46], bovine parotid gland cystatin C (4.4 nM) [40]

Table 3
Comparison of equilibrium dissociation constants (K_i) and rate constants (k_{on} and k_{off}) for the inhibition of papain and cathepsin B by quail and chicken cystatin

Inhibitor	Papain			Cathepsin B		
	K_i (pmol)	k_{on} (10^7 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_i (pmol)	k_{on} (10^6 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})
Quail cystatin	4.4 ± 1.8	1.8 ± 0.4	0.8 ± 0	47 ± 2	19.0 ± 2	9 ± 3
Chicken cystatin	1.4[a]	1.4[a]	0.20[a]	828 ± 34	2.9 ± 0.1	24 ± 2

K_i were calculated as $K_i = k_{on}/k_{off}$ and determined as described in Section 2. Similar values were obtained by direct evaluation of equilibrium inhibition experiments.
[a]From [19].

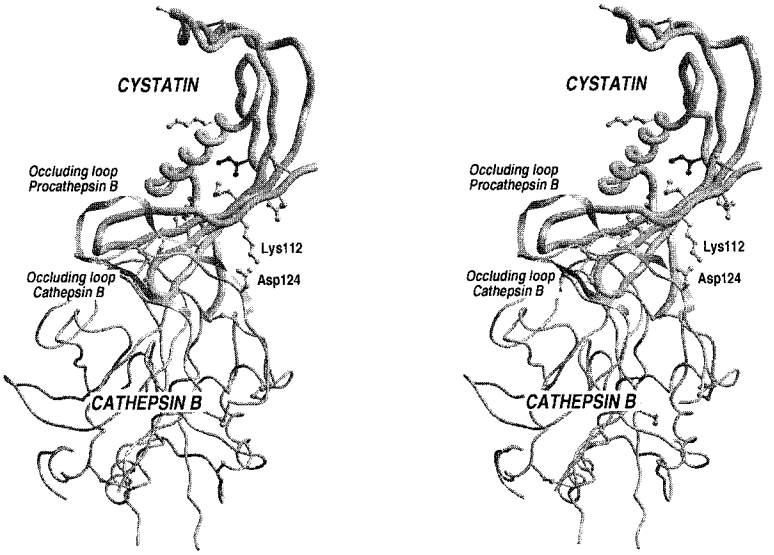

Fig. 5. Stereo view of the docking model of cystatin and human cathepsin B rotated relative to Fig. 4 showing additionally the occluding loop of procathepsin B. In contrast to the closed conformation of the occluding loop, only minor shifts in the occluding loop position in its open conformation and/or minor shifts in the cystatin orientation are required to eliminate steric clashes.

or human cystatin C (0.17–0.26 nM) [22,48], having in mind that comparison of K_i values obtained under different conditions is difficult.

A strong explanation of the observed enzyme-inhibitor interaction is given by computer-aided intuitive docking of the modelled quail cystatin molecule to the structures of cathepsin B and procathepsin B, assuming a stefin B–papain-like interaction [17] for quail cystatin–cathepsin B with an 'open-like' conformation of the 'occluding loop' [27] and its stabilisation by two possible salt bridges.

We speculate further that for the stronger inhibition of cathepsin B by human cystatin C as compared to chicken cystatin a similar situation is present and an equivalent salt bridge (Asp^{124}(catB)-Lys^{114}) is supporting this interaction (K_i 260 pM, [22], K_i 170 pM [48]).

The given observations indicate that selected amino acid residues of the scaffold, which do not belong to the so far known classical contact area (N-terminus, first hairpin loop, second hairpin loop) of cystatin, may have distinct effects on inhibition and that the conformational elucidation of proteinase-inhibitor complexes is of great importance.

Recently it was shown that a deletion variant of cathepsin B lacking 12 central residues of the 'occluding loop' is inhibited by human cystatin C 40-fold stronger than mature cathepsin B and the authors confirmed that the 'occluding loop' restricts access of the inhibitor to the active site [47]. Our model suggest that an 'open-like' position of the 'occluding loop' is mandatory for effective cathepsin B inhibition.

Further detailed interaction analyses and kinetic studies are necessary with cystatin and/or cathepsin B variants to test this hypothesis and to describe the influence of exposed amino acid residues during molecular interaction.

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