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INTERACTION OF CYSTATIN C VARIANTS WITH PAPAIN AND HUMAN CATHEPSINS B, H AND L

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Recombinant human cystatin C and two of its mutants were expressed in *Escherichia coli*. The recombinant inhibitor was found to be identical to authentic cystatin C as judged by isoelectric focusing (pI 9.2) and kinetics of inhibition of papain and human cathepsins B, H and L. N-terminal truncation of 8 residues resulted in a decrease of isoelectric point (pI 7.8), but the inhibitory properties were similar to those of recombinant cystatin C, suggesting that Leu9 is a critical residue for the inhibition. The mutation of Trp106 to Ser, however, resulted in a decreased affinity of the inhibitor for the enzymes tested, with the largest effect on cathepsin B inhibition (~100-fold increase in K_i).

Keywords: Cathepsins B, H, L; Human cystatin C; Inhibition; Papain; W106S and L9-cystatin C mutants

Abbreviations: Recombinant human cystatin C, cystatin C with N-terminus elongated for Gly-Ser-Met segment; L9-cystatin C, truncated form of cystatin C starting with Leu in position 9; W106S-cystatin C, cystatin C with the Trp106 substituted by Ser; IPTG, isopropyl β-D-thiogalactoside

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INTRODUCTION

Human cystatin C is the representative of the cystatin family and, together with other evolutionarily related inhibitors of cysteine proteinases, belongs to the cystatin superfamily.[1,2] Cystatin C is a non-glycosylated basic protein containing 120 amino acid residues and two disulphide bonds.[3,4] It is a tight binding inhibitor of papain-like cysteine proteinases[5] and is found in nM concentrations in various body fluids.[6] It appears to be the physiologically most important extracellular inhibitor of papain-like cysteine proteinases.

Based on the 3-D structures of chicken cystatin and of the stefin B-papain complex three regions of the inhibitor interact with the enzyme: the N-terminal part and two hairpin loops, the major contribution being from the first loop containing the QVVAG motif.[7,8] Deletion up to eleven residues in the N-terminal part of cystatin C from different species results in affinities for the target proteinases which are lower by several orders of magnitude.[9–17] By contrast, removal of first 6 residues in chicken cystatin has only a minor effect on papain inhibition.[11,17] Furthermore, a variant of cystatin C, isolated from the urine of patients with nephrological disorders and lacking the 8 N-terminal residues, showed no significant differences in inhibition of cysteine proteinases compared to the full-length form.[18]

Our study was aimed at elucidating the role of a truncation shorter than 10 N-terminal residues in the proteinase inhibition and verifying the importance of the second hairpin loop in cystatins, which has been previously demonstrated by both chemical modification[19,20] and site-directed mutagenesis.[16,21–23]

MATERIALS AND METHODS

Production and Purification of Recombinant Human Cystatin C Variants

The chemically synthesized cystatin C gene, with or without W106S substitution, was cloned in the pUC 18 cloning vector between *Bam*HI and *Sal*I restriction sites.[24] Both inserts with the verified DNA sequence were subcloned into the pMS103 expression vector by the same pair of enzymes. Cystatin C was expressed under the control of the *Escherichia coli* alkaline phosphatase signal sequence and secreted into the periplasmic space of the bacteria. *E. coli* DH5α harbouring expression plasmid was propagated in a 10 l fermenter containing 20 μg/ml ampicillin. The complex medium (pH 7.2) was composed of 1% yeast extract, 1% casein hydrolysate, 2% Na

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lactate, and 0.1% antifoaming agent. Stirring (400 rpm) and aeration (1 l/min) at 37°C were kept constant. Expression of the inhibitor was induced by addition of IPTG (0.1 mM final concentration) when the culture reached an A_{600} of 0.6.

The scheme outlined in Table I represents the main steps of the purification procedure. If not otherwise specified all steps were performed at 4°C. Bacteria were harvested from the fermentation broth by centrifugation and suspended in an equal volume of 0.2 M Tris buffer, pH 9.0, containing 20% (w/v) sucrose and 0.1 M EDTA. After 30 min of incubation on ice the periplasmic cell lysate was obtained by centrifugation. Nucleic acids and some bacterial proteins were removed by precipitation with 0.3% (w/v) polyethyleneimine. The inhibitory active material was separated from non-inhibitory proteins by affinity chromatography on a CM-papain Sepharose column. Concentrated and dialysed samples (0.02 M piperazine buffer, pH 9.1) were chromatographed on DEAE-cellulose (Whatmann, USA), equilibrated with the same buffer. Two peaks were eluted with the starting buffer. The first peak was the recombinant cystatin C with N-terminus elongated for Gly-Ser-Met segment. The second peak was L9-cystatin C, truncated form of cystatin C starting with Leu in position 9. Both forms were the result of the construction of the expression vector.[24] W106S-cystatin C was expressed and purified in the same way.

Homogeneity of the proteins was analysed by isoelectric focusing, performed on the PhastSystem (Pharmacia LKB), and by automated N-terminal sequencing (Applied Biosystems protein sequencer).

Proteins

Human cathepsin B (EC 3.4.22.1), human cathepsin H (EC 3.4.22.16), and human cathepsin L (EC 3.4.22.15) were purified as described previously.[25,26] Twice recrystallized papain (EC 3.4.22.2; Sigma) was further purified by

TABLE I Production and purification of recombinant cystatin C variants

Periplasmic cell lysate from 1l *E. coli* culture	912 mg[a]
↓ *precipitation, centrifugation*	
Supernatant	153 mg[a]
↓ *affinity chromatography*	
Inhibitory active fractions	62 mg[a]
↓ *ion exchange chromatography*	
(a) recombinant cystatin C	6 mg[b]
(b) L9- cystatin C	0.3 mg[b]

[a]Protein concentration determined according to Lowry *et al.*[38]
[b]Protein concentration estimated from A_{280}.

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affinity chromatography.[27] Bovine L10-cystatin C variant was purified as described.[28] The active concentrations of enzymes and inhibitors were determined by titration with Ep-475 (Peptide Research Institute) and active-site titrated papain, respectively.[29]

Kinetic Measurements

The kinetics of inhibition were studied at 25°C under pseudo-first-order conditions by continuous fluorimetric assay as described previously.[29] Papain and cathepsin B were assayed in 0.1 M phosphate buffer, pH 6.0, cathepsin H in 0.1 M phosphate buffer, pH 7.0, and cathepsin L in 0.34 M acetate buffer, pH 5.5. All buffers contained 1.5 mM EDTA and 2 mM dithiothreitol. Five μM Z-Phe-ArgNMec (Peptide Research Institute) was used to assay papain and cathepsins B and L, and 10 μM ArgNMec (Bachem) was used for cathepsin H.

RESULTS AND DISCUSSION

Recombinant cystatin C, which differs from the authentic protein by an N-terminal extension of 3 amino acid residues (Gly-Ser-Met), and its two mutants (L9-cystatin C, W106S-cystatin C) were expressed in *E. coli* as secreted proteins under the control of *tac* promoter and alkaline phosphatase signal sequence.[24] The inhibitors were isolated from bacterial lysates by a relatively simple procedure including precipitation by polyethyleneimine, and affinity and ion exchange chromatographies (Table I). The method took advantage of periplasmic expression, high affinity of recombinant products for papain and their basic isoelectric points.[30] The purity and identity of the proteins were confirmed by SDS-PAGE, isoelectric focusing and N-terminal amino acid sequencing (not shown).

The kinetics of inhibition of papain and cathepsins B, H and L with the three human cystatin C mutants were studied under pseudo-first-order conditions (Table II). All progress curves showed an exponential approach to a final linear rate and were analysed by the least-squares fitting of the appropriate integrated rate equation[31] to the experimental data. The enzymes were stable during the experiments, as judged on the basis of linearity of product formation in separate experiments without inhibitors. A linear dependence of the observed pseudo-first-order rate constant on inhibitor concentration was observed for all enzyme–inhibitor pairs investigated (Figure 1), consistent with a simple, competitive inhibition mechanism.[31]

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TABLE II Kinetic and equilibrium constants for the interaction between recombinant cystatin C variants and bovine L10-cystatin C, and papain and cathepsins B, H and L at 25°C. The values are given together with their standard errors N.D., not determined.

Enzyme	*Inhibitor*	$k_{ass} \times 10^{-6}$ ($M^{-1} s^{-1}$)	$k_{diss} \times 10^{4}$ (s^{-1})	K_i (pM)
Papain	Rec. Cystatin C	32.4 ± 3.6	3.1 ± 0.9	9 ± 3
	L9-Cystatin C	54.4 ± 4.9	6.0 ± 2.0	11 ± 4
	W106S-Cystatin C	9.6 ± 0.5	6.8 ± 2.2	71 ± 26
	L10-Cystatin C (bov.)	N.D.	N.D.	N.D.
Cathepsin B	Rec. Cystatin C	2.1 ± 0.1	7.8 ± 0.2	380 ± 30
	L9-Cystatin C	3.6 ± 0.2	9.5 ± 0.4	270 ± 25
	W106S-Cystatin C	0.036 ± 0.001	8.5 ± 0.9	23 500 ± 3100
	L10-Cystatin C (bov.)	0.013 ± 0.001	5.9 ± 0.7	45 700 ± 8900
Cathepsin H	Rec. Cystatin C	24.1 ± 3.0	30.6 ± 3.5	127 ± 29
	L9-Cystatin C	27.5 ± 0.8	8.2 ± 2.1	30 ± 8
	W106S-Cystatin C	0.7 ± 0.08	N.D.	> 500
	L10-Cystatin C (bov.)	2.3 ± 0.4	41.0 ± 5.0	1790 ± 530
Cathepsin L	Rec. Cystatin C	> 50	N.D.	< 5
	L9-Cystatin C	> 50	N.D.	< 5
	W106S-Cystatin C	> 50	N.D.	< 5
	L10-Cystatin C (bov.)	18.0 ± 1.1	N.D.	< 20

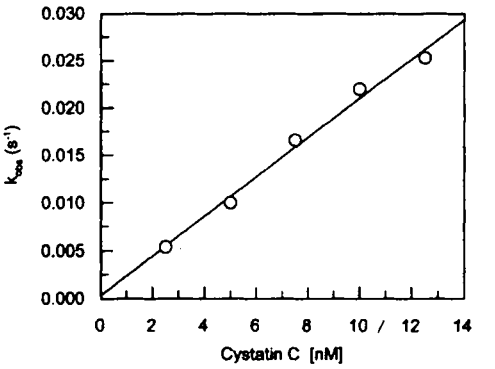

FIGURE 1 Dependence of the observed pseudo first-order rate constant (k_{obs}) on the inhibitor concentration for the interaction between recombinant cystatin C and human cathepsin B. Experimental conditions are described in Materials and Methods. The solid line was generated using the best estimate for apparent k_{ass} and k_{diss} obtained by linear regression analysis according to Morrison.[30]

Apparent second-order association rate constants, k_{ass}, were obtained from the slopes of these plots. The k_{ass} value for papain was corrected for substrate competition with the use of a K_m of 65 μM,[32] whereas no correction for substrate concentration was necessary for cathepsins B and H, as the substrate concentrations were much lower than the corresponding K_m values for these two enzymes (150 and 115 μM, respectively).[28,33]

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K_i values were calculated by linear regression analysis of the initial (v_z) and steady state (v_s) rates of substrate hydrolysis obtained at different inhibitor concentrations according to Morrison.[31] All plots of (v_z/v_s-1) versus [I_o] gave straight lines. The k_{diss} values were calculated from K_i and k_{ass} values ($k_{diss} = K_i \cdot k_{ass}$). Due to extremely tight and rapid interaction of the inhibitors with cathepsin L all the values are only approximate (lower limits for k_{ass} and K_i). All kinetic and equilibrium constants are given in Table II.

Recombinant cystatin C inhibits all four proteinases with constants similar to those of authentic and recombinant cystatin C produced in other expression systems.[6,10,13,14,18,34,35] The N-terminal extension therefore has no effect on the inhibitory activity of recombinant cystatin C. Similar constants were obtained for the inhibition of tested cysteine proteinases by L9-cystatin C, but the K_i for the inhibition of cathepsin H was $\sim$4-fold lower, reflecting the decreased k_{diss}. Our results are in agreement with those obtained with bovine parotid cystatin C, which has an N-terminal leucine, which corresponds to Leu9 in human cystatin C.[36] Bovine parotid cystatin C was shown to have affinities for papain and cathepsin B that are similar to full-length chicken and human cystatin C.[13,17] The alteration of the inhibition profile of cystatin C was observed also by different amino acid substitutions of 9 and 10 N-terminal residues, which are proposed to bind in the S3 and S2 substrate-binding pockets respectively of the enzymes.[23] Moreover, a differently truncated variant of bovine cystatin C, starting with Leu10 (human cystatin C numbering) is seen to have a substantially decreased affinity for cathepsin B ($\sim$100-fold; $K_i = 45$ nM) and a smaller decrease in affinity for cathepsin H (< 10-fold; $K_i = 1.8$ nM) (Table II). On this basis Leu9 in cystatin C is a critical residue for the inhibition of papain-like cysteine proteinases. Any further truncation does result in a substantial loss of affinity. This is also supported by the X-ray structures of chicken cystatin[7] and the stefin B-papain complex,[8] which show that the two residues preceding the highly conserved Gly (Gly11 in human cystatin C) form numerous interactions with the putative S2 and S3 sites of the enzyme.

Replacement of the evolutionarily conserved Trp106, located in the second hairpin loop, with Ser resulted in approximately 4–100-fold lower affinity for the enzymes, cathepsin B inhibition being the most affected. This reduction in affinity for proteinases on Trp106 substitution is in agreement with results on other Trp106 cystatin C mutants, confirming the importance of this residue in the interaction with target proteinases.[16,20,22] W106S-cystatin C demonstrates a lower k_{ass} in the inhibition of papain and cathepsins B and H which probably is a consequence of unfavourable interaction of Ser with the enzyme active site. Trp106 namely lies in a hydrophobic

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environment stacked on the side chains of Trp177 and Trp181 of the enzyme (papain numbering)[7] and a replacement of Trp with a hydrophilic residue could therefore substantially weaken the interaction. However, a local conformational change in the mutant cannot be excluded, since the stability of this mutant was higher than that of the recombinant cystatin C and no changes in the global tertiary structure were observed from the far and near UV CD spectra.[37]

In conclusion, recombinant cystatin C and two of its mutants were expressed in *E. coli*. Recombinant cystatin C exhibits closely similar biochemical properties to the authentic inhibitor. Trp106 of the inhibitor is confirmed as being important for the interaction with target enzymes, whereas Leu9 appears to be a critical residue in the interaction between cystatin C and its variants and papain-like cysteine proteinases.

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