The disulphide bridges of human cystatin C (γ-trace) and chicken cystatin

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Human cystatin C, a powerful physiological protein inhibitor of cathepsins B, H and L, contains two disulphide bridges, at least one of which is essential to its inhibitory activity. The positions of these bridges in the single polypeptide chain of the protein have been determined by diagonal paper electrophoresis. The cysteine residues at positions 73 and 83 form one bridge, and those at positions 97 and 117 form the other. In the homologous cystatin of chicken egg-white, the disulphides are Cys 71–Cys 81, and Cys 95–Cys 115.

Cathepsin *Cystatin* *Cystatin C* *Cysteine proteinase* *Plasma protein* *Proteinase inhibitor*

1. INTRODUCTION

Cystatin C (previously called γ-trace) is a tight-binding inhibitor of the lysosomal cysteine proteinases, cathepsins B, H and L; it occurs in human cells and extracellular fluids, and is thought to contribute to physiological control of the proteinases [1]. The amino acid sequences of the single polypeptide chains of human cystatin C and the homologous chicken cystatin have been determined [2,3], but the disulphide bridges have not been assigned, and nothing is known of their contribution to the inhibitory activity of the proteins.

2. EXPERIMENTAL

2.1. *Human cystatin C and chicken cystatin*

Cystatin C was isolated from the urine of a patient with renal failure as in [4], and subjected to additional chromatography on Bio-Gel P-60 in 0.5 M NH_4HCO_3 before being freeze-dried. The N-terminal amino acid sequence was that of the undegraded protein, SSPGKPPRLVGGPMD [5]. Chicken cystatin was the mixed forms of the protein from egg-white [6].

2.2. *Inactivation of cystatin C by reduction*

Cystatin C (1.3 mg/ml) in 0.025 M Tris–HCl buffer (pH 8.1) was made 1 mM dithiothreitol and 1 mM EDTA, and held at 25°C. Samples were removed after 0, 1, 2, 3 and 4 h for assay of inhibitory activity against papain, as in [7]. The thiol groups generated by reduction of disulphide bonds were quantified by the Ellman reaction after blocking of dithiothreitol with sodium arsenite [8].

2.3. *Enzymatic digestion of cystatin C and chicken cystatin*

Five mg cystatin in 0.25 ml of 5% (v/v) formic acid was digested at 37°C for 16 h with 0.1 mg pepsin (Worthington), lyophilized, redissolved in 0.25 ml of 0.2 M NH_4HCO_3 and digested at 37°C for 5 h with 0.1 mg trypsin-TPCK (Worthington).

2.4. *Diagonal paper electrophoresis*

The enzyme-digested material was freeze-dried, redissolved in 0.05 ml water, streaked on What-

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man 3MM paper at a loading of 1.7 mg/cm and subjected to high voltage electrophoresis in pyridine–acetic acid–water (1:10:190, by vol., pH 3.5) for 1.5 h at 50 V/cm. A strip 3 cm wide containing the separated peptides was cut out and used to prepared a diagonal fingerprint after performic acid oxidation exactly as described in [9]. The electrophoresis in the second dimension was for 4 h at 20 V/cm. The peptides were detected with fluorescamine as described [10], and those lying off the diagonal were eluted from the paper with 0.2 M NH_4OH and freeze-dried.

2.5. *Structural analysis*

The amino acid compositions of the peptides off the diagonal were determined with a Beckman 119CL amino acid analyzer after hydrolysis in 6 M HCl at 110°C for 20 h in evacuated and sealed tubes. Liquid phase sequencer degradations were performed in a Beckman 890C instrument in the presence of polybrene [11]. The phenylthiohydantoin amino acid derivatives were identified by high performance liquid chromatography [12].

3. RESULTS AND DISCUSSION

Treatment of cystatin C with 1 mM dithiothreitol caused a loss of inhibitory activity in parallel with the reduction of disulphide bonds. After 4 h, 0.14 mM disulphide bonds had been reduced (0.19 mM maximum predicted for complete reduction), and inhibitory activity had fallen to 9%. In similar experiments, chicken cystatin has been found also to be inactivated by mild reduction, although less rapidly than cystatin C (unpublished).

Diagonal fingerprint analysis of the performic acid oxidized pepsin-trypsin digest of human cystatin C revealed 7 peptides lying off the diagonal in 3 groups (fig.1a). The yields and amino acid compositions of these peptides are given in table 1. The amino acid compositions of the peptides identified them with a high degree of certainty in the known sequence (table 1). Moreover, sequence analysis of the peptides confirmed their identities (table 2). The results allow us to conclude that human cystatin C contains two disulphide bridges. One is formed by cysteine residues 73 and 83 and gives rise to the peptides 2a, 2b, 3a and 3b upon enzymatic digestion of the protein, and the other is formed by cysteine residues 97 and 117, and gives rise to the peptides 1a, 1b and 1c (fig.2a).

For chicken cystatin, diagonal electrophoresis showed 4 peptides off the diagonal (fig.1b), and

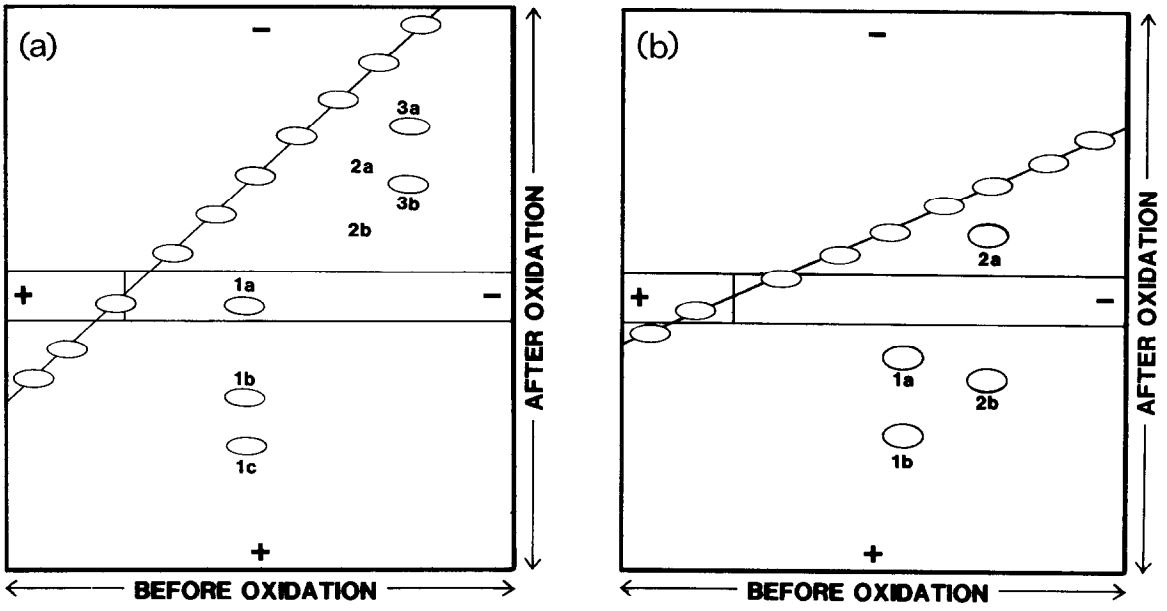

Fig.1. Drawing of the diagonal peptide maps of performic acid oxidized pepsin-trypsin digests of: (a) native human cystatin C; (b) ovocystatin, showing the major (solid line) and minor (dotted line) fluorescamine-positive spots.

Table 1

Amino acid composition of peptides with cysteic acid residues from a performic acid oxidized digest of native human cystatin C and of the corresponding segments of its intact polypeptide chain. Residue numbering according to [2]

	Peptide or polypeptide chain segment															
	1a[a]	95–101	1b[a]	95–100	1c[a]	115–120	2a[a]	71–75	2b[a]	76–91	3a[a]	76–92	3b[a]	71–75		
$CySO_3H$	0.5	1	0.8	1	0.8	1	0.7	1	0.9	1	0.8	1	0.9	1		
Asp	0.3		0.2		1.0	1	0.6		4.0	4	3.9	4	0.2			
Thr	0.4		0.2		1.0	1	2.5	3	1.5	1	0.9	1	2.8	3		
Ser	0.9	1	0.8	1	1.0	1	0.3		0.5							
Glu	1.1	1	1.2	1	1.0	1	0.5		2.2	2	2.0	2	0.1			
Pro	0.2		0.2				0.4		2.9	3	3.2	3				
Gly	0.4		0.4		0.1		0.3		0.6							
Ala	1.0	1	0.8	1	1.0	1	0.2		0.3							
Val	0.3		0.2				0.3		0.4							
Ile	0.7	1	0.1						0.1							
Leu			0.2				0.4		1.8	2	2.0	2				
Phe	1.5	2	1.6	2	0.2		0.1		0.9	1	0.9	1				
Lys							1.0	1	0.3		0.9	1	1.0	1		
His									1.8	2	1.9	2				
Arg									0.1							
Yield (nmol)	35		45		56		13		7		84		81			

[a] Expressed as mol amino acid residues per mol peptide. Figures below 0.1 are not reported

Table 2

Amino acid sequences of cysteic acid-containing peptides from human cystatin C

Peptide	Amino acid sequence
1a	Ala-Phe-X[a]-Ser-Phe-Gln-Ile
1c	Ser-Thr-X[a]-Gln-Asp-Ala
3a	Thr-Gln-Pro-Asn-Leu-Asp-Asn-X[a]-Pro-Phe-His-Asp-
3b	Thr-Thr-X[a]-Thr-Lys

[a] The cysteic acid derivative is not extractable with butyl chloride

amino acid analysis of these (table 3) unambiguously located them in the sequence (fig.2b). As expected, they matched those in the homologous cystatin C.

The exact reason for the dependence of activity of cystatin C on the disulphide loop(s) remains to be established. In many serine proteinase inhibitors, including the soybean Kunitz inhibitor [13], the inhibitory site is within a disulphide loop that is essential for activity. This seems unlikely for cystatin C, however, since the homologous cystatins A and B do not contain the disulphide loops, but nevertheless are also strong inhibitors, and the portions of sequence within the disulphide loops are very poorly conserved between the three human cystatins [1]. More probably, the disulphides simply stabilize the native conformations of cystatin C and chicken cystatin, as they do for many other extracellular proteins. Cystatins A and B are thought to be predominantly intracellular, and intracellular proteins generally do not contain disulphides, which might in any case be unstable in the reducing conditions of the cytosol.

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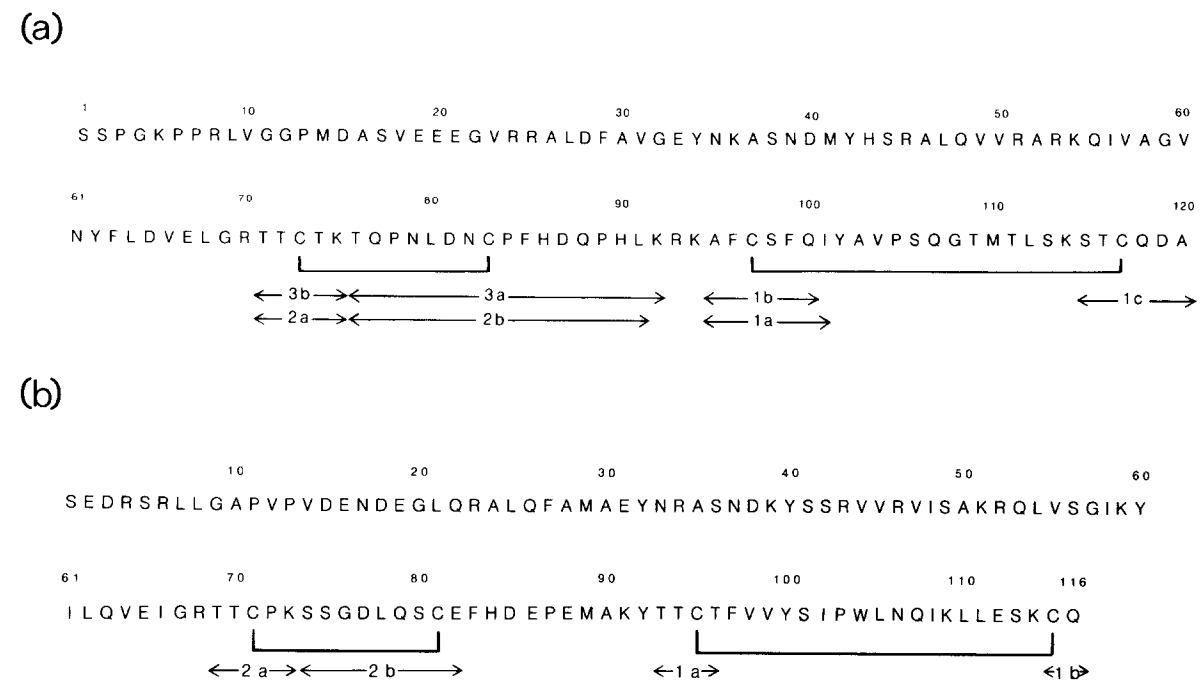

Fig.2. The disulphide bridges of human cystatin C (a) and ovocystatin (b), and the peptides used in their localization. The amino acid sequences are those of [2] and [3], respectively.

Table 3

Amino acid composition of cysteic acid-containing peptides from a performic acid oxidized digest of chicken cystatin, and of the corresponding segments of its intact polypeptide chain. Residue numbering according to [3]

	Peptide or polypeptide chain segment							
	1a[a]	93–96	1b[a]	115–116	2a[a]	69–73	2b[a]	74–82
$CySO_3H$	1.0	1	0.8	1	0.9	1	0.8	1
Asp					0.1		1.0	1
Thr	2.8	3			1.9	2		
Ser					0.2		2.4	3
Glu	0.1		1.0	1	0.2		2.0	2
Pro					1.1	1		
Gly	0.1				0.2		1.0	1
Val					0.2		0.1	
Leu					0.2		1.0	1
Tyr	0.3							
Lys					1.0	1	0.1	
Yield (nmol)	76		61		29		47	

[a] Expressed as mol amino acid residue per mol peptide. Figures below 0.1 are not reported

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