Human low- M_r kininogen contains three copies of a cystatin sequence that are divergent in structure and in inhibitory activity for cysteine proteinases

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We point out that human low- M_r kininogen contains three cystatin-like sequences, rather than two, as had previously been thought. The protein was purified by affinity chromatography on carboxymethyl-papain-Sepharose, and subjected to limited proteolysis by trypsin and chymotrypsin. Fragments were isolated, and three corresponding to the individual cystatin-like domains were identified. By comparison with the known amino acid sequence of the protein they were numbered 1 to 3 from the *N*-terminus. Domain 1 was not found to have any inhibitory activity for cysteine proteinases, which is consistent with the absence of residues that are highly conserved in inhibitors of the cystatin superfamily, and have previously been suggested to be essential for activity. Domain 2 was a good inhibitor of chicken calpain, and also papain and cathepsin L. Domain 3 showed negligible inhibition of calpain, but inhibited papain and cathepsin L strongly. The probable arrangement of disulphide bonds in the heavy chain of low- M_r kininogen is deduced from the homology with the cystatins and other evidence contained in the present paper.

INTRODUCTION

The physiological activities of human cysteine proteinases are presumed to be controlled by their interactions with the several protein inhibitors present in the body (Barrett & Salvesen, 1986). These are α_2 -macroglobulin, a general inhibitor of endopeptidases not specific for cysteine proteinases, the calpastatins, which are thought to be selective for calpains, Ca^{2+} -dependent cysteine proteinases (Murachi *et al.*, 1983), and the cystatins, proteins of M_r about 12000 that are considered specific for the papain-like cysteine proteinases including the lysosomal enzymes cathepsins B, H and L (Barrett, 1985; Turk *et al.*, 1985), to which calpains are only distantly related (Ohno *et al.*, 1984). Exceptional amongst the specific protein inhibitors of cysteine proteinases has been plasma α -cysteine-proteinase inhibitor, the only one reported to inhibit the calpains and also the papain-like cysteine proteinases (Sasaki *et al.*, 1983).

Ohkubo *et al.* (1984) sequenced a cDNA encoding human α -cysteine-proteinase inhibitor, and found the protein to be so similar to bovine low- M_r kininogen as to leave little doubt that α -cysteine-proteinase inhibitor was human low- M_r kininogen. Further work confirmed the identity of α -cysteine-proteinase inhibitor with low- M_r kininogen and the other product of the kininogen gene, high- M_r kininogen (Nawa *et al.*, 1983), in human, bovine and rat plasmas (Müller-Esterl *et al.*, 1985; Sueyoshi *et al.*, 1985). Since the low- M_r form of α -cysteine-proteinase inhibitor (apparent M_r about 64000; Gounaris *et al.*, 1984) is identical with low- M_r plasma kininogen, we here use the term 'low- M_r kininogen' for both.

The amino acid sequence of human low- M_r kininogen

was shown by Ohkubo *et al.* (1984) to contain two segments (about 120 residues each) that, although not identical, are clearly homologous with each other, and also with chicken cystatin. Those authors reported that low- M_r kininogen has 1.7 binding sites per molecule for papain. Our examination of the sequence reveals that the *N*-terminal part of the molecule comprises a previously unrecognized third cystatin-like segment (Fig. 1 and Table 1). In the present study we have examined the inhibitory activities of each of the three cystatin-like segments of the low- M_r kininogen molecule. This has been done by dissecting the molecule by limited proteolysis, isolating the segments, and determining their inhibitory activities against cysteine proteinases of the papain/lysosomal and calpain types.

EXPERIMENTAL

Materials

Human low- M_r kininogen was purified as described by Gounaris *et al.* (1984), except that Brij 35 was omitted from the buffers. Papain, Tos-Lys- CH_2Cl - ('TLCK'-) treated chymotrypsin, Tos-Phe- CH_2Cl - ('TPCK'-) treated trypsin, pig pancreatic elastase, *Staphylococcus aureus* V8 proteinase and clostripain were from Sigma (London) Chemical Co. Bromelain (stem) was from Boehringer Corp. (London) Ltd. Z-Phe-Arg-NHMec and Suc-Leu-Tyr-NHMec were obtained from Bachem (U.K.), Saffron Walden, Essex, U.K. Azocasein was prepared as described by Barrett & Kirschke (1981). Dip-F was from Sigma Chemical Co. and was stored as a 0.2 M solution in dry propan-2-ol at -20°C .

Human cathepsin L was a gift from Dr. R. Mason of

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with IUPAC-IUB recommendations [Biochem. J. (1984) 219, 345–373]. Additional abbreviations are: NHMec, 4-methyl-7-coumarylamide; Dip-F, di-isopropyl phosphorofluoridate.

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Fig. 1. Alignment of the amino acid sequences of the three cystatin-like domains of the kininogen heavy chain with those of some known cystatins

The sequences are those of (a) human cystatin A (also known as stefin; Machleidt *et al.*, 1983), (b) human cystatin B (Ritonja *et al.*, 1985), (c) human cystatin C (Grubb & Löffberg, 1982), (d) human cystatin S (Isemura *et al.*, 1984), (e) chicken cystatin (Schwabe *et al.*, 1984), (f) kininogen domain 1 (residues 19–127), (g) kininogen domain 2 (residues 128–249) and (h) kininogen domain 3 (residues 250–373) (all from Ohkubo *et al.*, 1984). The sequences of small cystatins are grouped into those lacking disulphide bonds (a and b) and those with disulphide bonds (c, d and e), and the positions indicated for the disulphide bonds in the latter are those established for human cystatin C and chicken cystatin (Grubb *et al.*, 1984). The disulphide bonds indicated for the kininogen domains are those proposed on the basis of analogy with the cystatin structures and other evidence in the present paper. Underlined residues are those identified in the kininogen domain 1 sequence and at least one of the other sequences, and a bold underline is used to mark the residues identified as described in the Results section.

Table 1. Identity matrix of cystatins and kininogen domains

The values are the percentages of residues identical between pairs of sequences aligned as in Fig. 1.

b	53						
c	19	18					
d	19	15	58				
e	14	19	44	43			
f	10	8	15	15	25		
g	15	12	17	17	19	20	
h	19	16	23	26	25	19	32
	a	b	c	d	e	f	g

this laboratory and was prepared as described by Mason *et al.* (1985). Calpain was isolated from chicken gizzard smooth muscle as described by Parkes *et al.* (1985).

Determination of protein

Concentrations of solutions of low- M_r kininogen and its fragments were determined by assuming $A_{280}^{1\%} = 5.9$ (Ryley, 1979).

Quantification of inhibitory activity

The concentration of active enzyme in preparations of papain, cathepsin L and calpain was determined by titration with L-3-carboxy-2,3-epoxypropionyl-leucyl-amido-(4-guanidino)butane as described by Anastasi *et al.* (1983), with Z-Phe-Arg-NHMec as substrate for papain and cathepsin L, and azocasein as substrate for calpain (Parkes *et al.*, 1985).

Molar concentrations of inhibitory reactive sites in preparations of the low- M_r kininogen fragments were determined by titration with the standardized enzyme preparations. K_i values were determined by use of continuous-rate fluorimetric assays, and a method for analysis of tight-binding inhibition kinetics (Henderson, 1972; Gounaris *et al.*, 1984). For calpain, the continuous-rate fluorimetric assays were done at 22 °C in 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-CaCl₂, 1 mM-dithiothreitol and 0.1% Triton X-100. The substrate was Suc-Leu-Tyr-NHMec (Sasaki *et al.*, 1984) at 250 μ M final concentration. The assays were started by the addition of enzyme (31 nM final concentration), and the inhibitor was added 3 min later.

Purification of low- M_r kininogen fragments

Low- M_r kininogen was subjected to limited proteolysis with trypsin, 10 mg being incubated with 30 μ g of trypsin in 4.0 ml of 0.20 M-Tris/HCl buffer, pH 8.0, for 20 min at 30 °C. The digestion was stopped by the addition of Dip-F to a concentration of 2 mM. The digest was run in gel chromatography in a column (1.5 cm $\times$ 40 cm) of Sephadex G-75 (superfine grade) in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl, at a flow rate of 10 ml/h. Fractions (3 ml) were collected, and assayed for inhibitory activity. The 44000- M_r tryptic peptide (see the Results section) was concentrated by ultrafiltration and further digested with chymotrypsin (5 mg with 0.15 mg of chymotrypsin in 4 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 0.15 M-NaCl) for 20 min at 30 °C, followed by the addition of Dip-F to a concentration of 2 mM.

Chymotrypsin-generated fragments were isolated by anion-exchange chromatography on the Mono Q HR 5/5

column of the Pharmacia fast protein liquid-chromatography apparatus. Samples were diluted 4-fold with water before application to the column, and the chromatogram was developed with a linear gradient (1 ml/min; 34 min) from 10 mM-sodium acetate buffer, pH 5.5, to the same buffer containing 0.3 M-NaCl. The flow rate was 1 ml/min, and 0.5 ml fractions were collected.

SDS/polyacrylamide-gel electrophoresis

This was as described by Bury (1981) in gels of 12.5% polyacrylamide, with calibration and sample treatment as described by Gounaris *et al.* (1984).

Thiol content of low- M_r kininogen

This was measured by use of 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Glazer *et al.* (1975).

Amino acid sequence determination

Automated Edman degradation (Edman & Begg, 1967) was performed with an Applied Biosystems 470 A gas-liquid solid-phase sequenator (Hewick *et al.*, 1981) with the chemicals and programme (02NRUN) recommended by the manufacturer. The anilinothiazolinone derivatives of amino acids were converted into phenylthiohydantoin derivatives by incubation with 1 M-HCl in methanol in the automatic conversion flask of the sequenator. The amino acid phenylthiohydantoin derivatives were identified on a reverse-phase h.p.l.c. system based upon a Nova Pak C₁₈ column (Waters) eluted as described by the manufacturer with propan-2-ol-containing buffers.

RESULTS

Low- M_r kininogen

Excision of the kinin peptide results in a two-chain low- M_r kininogen in which the N-terminal heavy chain, of M_r about 60000, remains joined to the C-terminal light chain, of M_r about 5000, by a disulphide link (Maier *et al.*, 1983). Low- M_r kininogen purified by the method of Gounaris *et al.* (1984) is largely deficient in the kinin peptide. This can be seen in Fig. 2, lane 2, where the free light chain (M_r about 5000) is visible. The same conclusion was reached by Müller-Esterl *et al.* (1985) for the product of a similar purification procedure, and this is not surprising, because plasma kallikrein has been found to be closely associated with the kininogen during the procedure (Gounaris *et al.*, 1984).

Isolation and identification of fragments

Low- M_r kininogen was digested with a number of proteinases in an attempt to establish conditions for the isolation of inhibitory domains. Of those proteinases tested (including clostripain, bromelain, pancreatic elastase and *S. aureus* V8 proteinase), trypsin and chymotrypsin were found to be the most useful.

Treatment of low- M_r kininogen with trypsin (as described in the Experimental section) produced fragments of M_r 21000 (fragment A) and 44000 (Fig. 2). These were isolated by gel chromatography, and that of M_r 44000 was further digested with chymotrypsin. The resulting fragments (fragment B of M_r 25000 and fragment C of M_r 19000) were purified by anion-exchange chromatography. The homogeneity of fragments A, B and C was examined by polyacrylamide-gel electrophoresis (Fig. 2,

Fig. 2. SDS/polyacrylamide-gel electrophoresis of low- M_r kininogen and fragments produced by limited proteolysis

Low- M_r kininogen (lane 2) was treated with trypsin, and the resulting digest (lane 3) was fractionated by gel chromatography to yield fragment A (lane 4) and the 44000- M_r fragment (lane 6). The further digest of the 44000- M_r fragment with chymotrypsin (lane 7) was fractionated by anion-exchange chromatography to give fragments B (lane 8) and C (lane 9). All samples were reduced before electrophoresis, and standards were run in lanes 1 and 5. Note the presence of the light chain of low- M_r kininogen (M_r approx. 5000) in several lanes.

lanes 4, 8 and 9 respectively) and by *N*-terminal sequence analysis (Table 2).

Automated Edman degradation of about 2 nmol of each of the unreduced fragments A and B (Table 2) allowed them to be located in the sequence of the low- M_r kininogen molecule (Ohkubo *et al.*, 1984) shown schematically in Fig. 3. Thus it was clear that fragment B comprises a segment of low- M_r kininogen starting at position 141 [or (g) position 6 in the alignment shown in Fig. 1]. Similarly, fragment A was found to represent a segment of low- M_r kininogen beginning at position 263 [or (h) position 6 in the alignment shown in Fig. 1].

The *C*-termini of the peptides were not identified directly, but their sizes, as indicated by SDS/polyacrylamide-gel electrophoresis (Fig. 2), were consistent with their being the complete domains. We therefore regard fragments A and B as corresponding to domains 3 and 2 respectively, and fragment C as domain 1 (by a process of elimination), for the purpose of determining the inhibitory activities of the individual domains.

The position of the trypsin-sensitive bond in the low- M_r kininogen heavy chain is seven residues *N*-terminal to that reported by Müller-Esterl *et al.* (1985), but this can scarcely account for the differences in apparent M_r values of their tryptic fragment (15000) and ours (21000). It is possible that the fragments migrate very differently in the different gel electrophoresis systems, or that Müller-Esterl *et al.* (1985), who unfortunately did not report the conditions of their tryptic digestion, isolated a more extensively proteolysed fragment. Like ourselves, they did not identify the *C*-terminus.

Disulphide bonding in the low- M_r kininogen molecule

Analysis of low- M_r kininogen in 6 M-guanidinium chloride for free thiol groups showed less than 0.1 mol of thiol group/mol, and we therefore conclude that all 18

cysteine residues in low- M_r kininogen (Ohkubo *et al.*, 1984) form disulphide bonds. The fragments of low- M_r kininogen were isolated under non-reducing conditions and run in SDS/polyacrylamide-gel electrophoresis with and without reduction. Neither fragment A (domain 3) nor fragment B (domain 2) contained the light chain of low- M_r kininogen, whereas fragment C (domain 1) did (although not clearly visible in Fig. 2). We conclude that

Table 2. *N*-Terminal amino acid sequences of fragments A and B

Amino acid residues were identified as described in the text. Key: ?, residue not identified; —, residue identified but not quantified.

Cycle no.	Residues identified (nmol)			
	Fragment A		Fragment B	
1	Ile	1.7	Asp	0.9
2	?	—	?	—
3	Val	1.6	Leu	1.9
4	Gly	2.0	Gly	1.2
5	?	—	?	—
6	Pro	0.9	Val	1.0
7	Arg	—	His	—
8	Asp	0.4	Pro	0.45
9	Ile	0.9	Ile	0.69
10	Pro	0.5	Ser	—
11	Thr	0.1	Thr	—
12	Asn	—	Gln	—
13	Ser	—	?	—
14	Pro	—	Pro	—
15	Glu	—	Asp	—
16	Leu	—	Leu	—

Fig. 3. Scheme for the structure of the low- M_r kininogen molecule showing the three cystatin-like domains and the proposed assignment of the disulphide bonds

The numbering of the residues is from the cDNA sequence of the pre-protein reported by Ohkubo *et al.* (1984), residues 1–18 (not shown) comprising the putative signal peptide. Sites of proteolytic cleavage are indicated by gaps; K indicates kinin peptides excised by the action of kallikreins. The pairing of cysteine residues in disulphide bonds (as suggested in the text) is indicated by broken horizontal bars connecting cysteine residues, with the exception of the first and last cysteine residues, which we suggest are also linked together. Primary cleavage sites in the heavy chain (comprising domains 1, 2 and 3) are indicated by arrows.

Table 3. Inhibitory activities of low- M_r kininogen and the three isolated domains

The results are given as K_i values determined as described in the text.

	K_i (nM)			
	Low- M_r kininogen	Domain 1	Domain 2	Domain 3
Papain	0.015	> 100	0.083	0.030
Cathepsin L	0.017	> 100	0.14	0.005
Calpain	~ 1.0	> 100	1.0	> 100

all the disulphides are intra-domain, with the exception of a link between domain 1 and the light chain.

Inhibitory activities of isolated domains

As is explained above, fragments A, B and C are thought to correspond to domains 3, 2 and 1 respectively (Fig. 3), and preparations of the fragments were accordingly used to assess the inhibitory activities of the domains as described in the Experimental section. The K_i values for low- M_r kininogen with papain and cathepsin L (Table 3) are slightly lower than those we previously reported (Gounaris *et al.*, 1984) and in agreement with those of Müller-Esterl *et al.* (1985) for kinin-containing low- M_r kininogen.

Domain 1 shows no inhibition of the enzymes tested above the level that could be accounted for by 1% contamination with other domains. Domains 2 and 3 both inhibit papain and cathepsin L, but both of these enzymes have a higher affinity for domain 3. In contrast with this, only domain 2 inhibits calpain. That the tight-binding nature of the isolated domains 2 and 3 is not an artifact is shown by comparison of their affinities with those of the parent molecule. Treatment of low- M_r kininogen with trypsin or chymotrypsin under the conditions described above did not significantly alter the total inhibitory capacity of the molecule when assayed with papain, cathepsin L or calpain. Taking these data into account, we conclude that domain 1 is not inhibitory, that domain 2 is a multifunctional domain capable of inhibiting the three enzymes that we have studied, and that domain 3 is the tightest-binding inhibitor of cathepsin L and papain. The inhibitory sites of domains

2 and 3 appear to be freely available in the intact low- M_r kininogen.

DISCUSSION

Low- M_r kininogen contains three homologous segments, or domains (Fig. 1 and Table 1), not two as had previously been noted (Ohkubo *et al.*, 1984). The similarity in primary structure between these three segments, human cystatins C and S and chicken cystatin, particularly in the location of the cysteine residues, makes it clear that the proteins have evolved from a common ancestor. Comparison of the amino acid sequences of domains 1, 2 and 3 (Fig. 1 and Table 1) shows that domains 2 and 3 are much more similar to each other than to domain 1. This suggests that evolution of the molecule involved two duplications of genetic material well separated in time. The first presumably created a two-domain molecule that included the direct ancestor of domain 1 and that of domains 2 and 3. The second event was the duplication of the domain 2/3 ancestor. The foregoing almost certainly applies to high- M_r kininogen also, as it is probably a product of the same gene (Nawa *et al.*, 1983). In light of the obvious ancestry of the three homologous domains of the kininogens, we propose that they be known as cystatin domains.

We have excised the three homologous cystatin domains of low- M_r kininogen in order to investigate the inhibitory activity of the molecule in detail. Only domains 2 and 3 are inhibitory for the enzymes tested, whereas domain 1 appears to have lost all inhibitory activity, possibly through the substitution of amino acids in the part of the sequence most highly conserved in other cystatins, residues Gln-53 to Gly-57 (Fig. 1), proposed as part of the cystatin reactive site (Ohkubo *et al.*, 1984; Barrett, 1985a; Turk *et al.*, 1985). We do not know the function of domain 1, but it is conceivable that its disulphide link to the C-terminal portion of low- M_r kininogen holds the kinin sequence in the correct conformation for excision by kallikrein.

An exceptional characteristic of domain 2 is its capacity to inhibit chicken calpain; no other cystatin is known to inhibit calpains. Low- M_r kininogen has been found to inhibit human (Sasaki *et al.*, 1983) and chicken (Johnson *et al.*, 1984) calpains, and we suggest, in view of the present data, that domain 2 is responsible for this inhibition. Comparison of the structure of domain 2 with those of domain 3 and the other cystatins should ultimately throw light on the structural requirements for calpain inhibition.

Multiplication of reactive sites by gene duplication has occurred in several families of serine proteinase inhibitors (Laskowski & Kato, 1980), but the reasons for this are obscure. Plasma proteins are usually of M_r greater than 40000, which prevents their rapid loss through the kidney. The multiplication of cystatin domains in low- M_r kininogen may have occurred to satisfy this requirement for molecular size, and thus provide kinin precursors with long plasma half-lives. Such a suggestion has been advanced to explain the evolution of the renin substrate angiotensinogen, which, though being a member of the α_1 -proteinase inhibitor superfamily (Doolittle, 1983), has apparently lost its ability to inhibit proteinases (Carrell & Travis, 1985). Whether the cysteine-proteinase-inhibiting capacity of kininogens is of physiological importance is unknown. Gordon & Cross (1981) have suggested a role for a cysteine proteinase, and therefore presumably also its plasma inhibitors, in the control of blood coagulation in malignancy. In addition to possible roles in the control of endogenous enzymes, kininogens may form part of the body's defence against foreign organisms that invade by the use of cysteine proteinases. Clues as to the possible inhibitory functions of kininogens may come from the study of individuals possessing hereditary deficiencies, such as Williams trait (Colman *et al.*, 1975).

Disulphide bonds of low- M_r kininogen

The pairing of cysteine residues to form nine disulphide bonds in low- M_r kininogen is probably as shown in Fig. 3. The two disulphide loops near the C-terminus of each of the three domains are suggested by the conservation of these residues from the homologous chicken cystatin and human cystatin C, for which the arrangement is known (Grubb *et al.*, 1984) (see also Fig. 1). The assignment of the small disulphide loops at the N-termini of domains 2 and 3 is forced by the evidence that there are no inter-domain disulphide bonds, and that all cysteine residues are involved in disulphide bonds (see above). The disulphide bridge linking the heavy and the light chains would therefore involve the first and last cysteine residues in the molecule.

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