Arrangement of the disulphide bridges in human low- M_r kininogen

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The arrangement of the disulphide bridges in human low- M_r kininogen has been elucidated. Low- M_r kininogen contains 18 half-cystine residues forming nine disulphide bridges. The first and the last half-cystine residues of the amino acid sequence form a disulphide loop which spans the heavy- and the light-chain portion of the kininogen molecule. The other 16 half-cystine residues are linked consecutively to form eight loops of 4–20 amino acids; these loops are lined up in the heavy-chain portion of the kininogen molecule. In this way, a particular pattern of disulphide loops is formed which seems to be of critical importance for the inhibitor function of human kininogen.

INTRODUCTION

Kininogens are the biosynthetic precursors of vasoactive kinins. Three types of kininogen have been characterized in detail: high- M_r and low- M_r kininogen, which are present throughout the mammalian lineage, and T-kininogen which has only been found in the rat so far (for recent review see [1]). Principally, the kininogens are synthesized in the liver and secreted into the blood plasma where they serve a variety of physiological functions such as kinin delivery, inhibition of cysteine proteinases, promotion of blood coagulation, and mediation of the acute phase response [1].

The human kininogens are single-chain glycoproteins of apparent M_r 114 000 (high- M_r kininogens) and 68 000 (low- M_r kininogens). They share a major part of their primary structure, i.e. the heavy-chain region preformed at their *N*-termini, followed by the kinin segment and a short stretch of 12 amino acid residues located at the *N*-terminal end of their preformed light chain [2,3]. These common regions encompass the structural units for cysteine proteinase inhibition [4–7] and kinin delivery [1]. The sequences of the *C*-terminal portions of kininogen light chains are highly different; in this region high- M_r kininogen confers the structural elements for contact phase binding [2].

Sequence analysis of the human kininogens revealed that their heavy-chain portions harbour 17 half-cystine residues [8], while their light-chain portions contain only a single half-cystine residue [2,9]. Free thiol groups are not present in the human low- M_r kininogen [10] thus suggesting that the 18 half-cystine residues are engaged in the formation of nine disulphide bridges. A model for the disulphide structure of human low- M_r kininogen based on proteolytic cleavage patterns of the molecule [10] predicts that eight disulphide loops are formed by pairs of adjacent half-cystine residues, whereas a single disulphide bridge spans from the extreme *N*-terminal to the extreme *C*-terminal half-cystine residue. This proposal is in agreement with preliminary findings [11] on the disulphide loop structure of bovine high- M_r kininogen.

Here we communicate the complete disulphide loop structure of the human low- M_r kininogen. We demonstrate that the single-chain molecule has a characteristic arrangement of nine disulphide loops. Maintenance of this loop structure seems to be a major prerequisite for the expression of the inhibitor function by the kininogen.

EXPERIMENTAL

The strategy for the identification of the protein-disulphide linkage pattern of human low- M_r kininogen employs proteolytic cleavage of the protein and isolation of those fragments containing disulphide bridge(s). Fragments representing a single peptide with a single internal disulphide loop are directly subjected to amino acid sequence analysis. Fragments consisting of two peptides connected via a single disulphide bridge are reduced and the constituting peptides are isolated by h.p.l.c. Finally, the purified peptides are identified by amino acid sequence analysis.

Isolation of human low- M_r kininogen

Human low- M_r kininogen was isolated from pooled human plasma [12,13]. Homogeneity of the resultant preparations was monitored by discontinuous SDS electrophoresis in 10–20% (w/v) polyacrylamide gradient gels [14].

Limited proteolysis of human low- M_r kininogen

To generate kininogen fragments by limited proteolysis, the procedure of Salvesen *et al.* was applied [10]. Briefly, 10 mg of low- M_r kininogen and 25 μ g of trypsin (treated with *N*-tosyl-L-phenylalanine chloromethane, Worthington) were incubated for 20 min at 30 °C in 4 ml of 0.2 M-Tris/HCl, pH 8.0 (weight-ratio of enzyme to substrate 1:400). The resulting digest was subjected to gel filtration on a Superose 12 HR 10/30 column using a buffer of 50 mM-Tris/HCl/0.5 M-NaCl, pH 7.5 in a fast protein liquid-chromatography system (Pharmacia, Uppsala, Sweden). Three major protein peaks were resolved, collected and dialysed against 10 mM-ammonium formate and then lyophilized. Analytical

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SDS electrophoresis indicated that the fractions contained kinin-free low- M_r kininogen (M_r 68000), double domain 1–2 of M_r 45000 (denoted F1–2) and domain 3 of M_r 21000 (denoted F3), respectively (for nomenclature of the kininogen domains, see [1,10]).

Cleavage of kininogen domains with trypsin

For the generation of smaller fragments, proteolysis of fragments F1–2 and F3 (see above) was carried out with trypsin, using a weight ratio of enzyme to substrate of 1:50. The reaction was performed in 0.1 M- NH_4HCO_3 /10 mM- CaCl_2 , pH 7.0 for 8 h at 37 °C. Proteolysis was stopped by acidifying the solutions with formic acid. The resulting cleavage mixtures were separated by reversed-phase h.p.l.c.

Limited proteolysis with thermolysin

To generate smaller peptides, fragments isolated (see below) from the tryptic digest were incubated with thermolysin in 0.1 M- NH_4HCO_3 , pH 8.0 for 2 h at 37 °C, using a weight ratio of enzyme to substrate of 1:50 [15]. Proteolysis was stopped by adjusting the pH to 2.0 with formic acid. The cleavage mixture was then subjected to reversed-phase h.p.l.c.

H.p.l.c. of the kininogen fragments

Portions of the digests (100 to 300 μl) from the proteolysis with trypsin or thermolysin were subjected to h.p.l.c. (LKB, Bromma, Sweden) on a reversed-phase column (Hibar LiChrospher) using a 0.1% (v/v) trifluoroacetic acid/ H_2O (A)/0.1% (v/v) trifluoroacetic acid/acetonitrile (B) solvent system. The flow rate used was 1.0 ml/min, and the absorbance was monitored at 206 nm. A linear gradient from 0 to 55% (v/v) B in A was applied over a period of 60 min at room temperature. Peaks were collected manually.

Determination of cystine-containing fragments

Portions (0.1–1 ml) from each h.p.l.c. fraction were pipetted into Eppendorf tubes and evaporated to dryness with nitrogen. Disulphide groups were reduced by incubation with 100 μl of a freshly prepared solution of 2.5% (w/v) NaBH_4 at 50 °C for 1 h [16]. The excess of borohydride was then destroyed by addition of 10 μl HCl (1 M). After incubation for 30 min at room temperature, the free thiol groups were reacted with 100 μl of a freshly prepared solution of 0.13% (w/v) 5,5'-dithio-bis-(2-nitrobenzoic acid) in 40 mM- NaH_2PO_4 , pH 8.1 [17]. The absorption of the reaction mixture was then measured at 410 nm. Blanks were processed as given above except peptide was omitted and used to correct for background absorption.

Manual amino acid sequence analysis

Each fraction of the tryptic digest eluted from h.p.l.c. (see above) was characterized by manual degradation of the peptides applying the diaminoazobenzene isothiocyanate method introduced by Chang *et al.* [18].

Oxidation of cystine groups with performic acid

Performic acid was freshly prepared by mixing 100 μl of 30% (v/v) H_2O_2 and 900 μl of formic acid and incubating for 1 h at room temperature. A portion (50 μl) was removed, added to 10 μg of peptide and incubated for 1 h at 0 °C [19]. The reaction mixture was then evaporated to dryness with nitrogen.

Fig. 1. The tryptic digest of fragment F(1–2) from human low- M_r kininogen is resolved by h.p.l.c. into 16 major fragments

The digest was applied to a reversed-phase column of Hibar LiChrospher using an 0.1% (v/v) trifluoroacetic acid/ H_2O (A)/0.1% (v/v) trifluoroacetic acid/acetonitrile (B) solvent system. The flow rate was 1.0 ml/min and absorbance was monitored at A_{206} . A linear gradient [0 to 55% (v/v) B in A] was applied over a period of 60 min at room temperature. Numbers denote the various fractions containing peptide(s) used for further analysis.

Amino acid analysis

For amino acid analyses, 0.1–0.5 μg samples of peptide material recovered from h.p.l.c. were subjected to gas-phase hydrolysis applying 200 μl of a mixture of trifluoroacetic acid/7.2 M-HCl (1:10 v/v) at 159 °C for 20 min. The amino acid mixture was derivatized by adding 20 μl of 0.1% (w/v) *o*-phthalaldehyde/0.1% (v/v) mercaptopropionic acid in 0.5 M-sodium borate, pH 10 and resolved by reversed-phase h.p.l.c.

Automated amino acid sequence analysis

The Edman degradation method in the sequenator version was employed [20] using the gas-phase sequencer 470A (Applied Biosystems, Foster City, U.S.A.). The phenylthiohydantoin derivatives were analysed by an h.p.l.c. system which separates all components isocratically [21].

RESULTS

Isolation of low- M_r kininogen fragments

Human low- M_r kininogen was first cleaved by limited proteolysis with trypsin to generate bradykinin and two major fragments of M_r 45000 and 21000 (results not shown). The larger fragment, termed F1–2, contained the two *N*-terminal domains spanning from amino acid residue 1 through to residue 244 of the kininogen molecule. This polypeptide is connected via a disulphide bridge to the light chain (running from position 372 to position 409) of the kininogen molecule. The smaller fragment, denoted F3, represents the third domain of the kininogen heavy chain and spans from residue 245 to residue 362.

To produce smaller fragments, F1–2 and F3 were

subjected to further tryptic digestion, and the resulting cleavage mixtures were resolved by h.p.l.c. (Figs. 1 and 2). Each eluted peak was collected separately and the peptide(s) recovered. A total of 26 fragments (16 derived from F1-2 and 10 from F3) were isolated (Table 1).

Fig. 2. The tryptic digest of fragment F(3) from human low- M_2 kininogen is resolved by h.p.l.c. into 10 major fragments

The conditions of the experiment are as in Fig. 1. Numbers denote the various protein-containing fractions used for further analysis.

Portions of each fragment were subjected to *N*-terminal amino acid sequence analysis by manual Edman degradation and further analysed for the presence of disulphide groups. In this way, seven fragments containing single disulphide bridges were identified, i.e. T(F1-2)7, T(F1-2)9, T(F1-2)13, T(F1-2)15, T(F3)4, T(F3)5 and T(F3)10 (Table 1). These fragments correspond to peaks 7, 9, 13, and 15, respectively of the h.p.l.c. pattern of the tryptic digest of F1-2 (see Fig. 1), and to peaks 4, 5, and 10, respectively of the h.p.l.c. pattern of the tryptic digest of F3 (see Fig. 2). Moreover, a single fragment, T(F1-2)16 corresponding to peak 16 of Fig. 1, was found to contain two disulphide groups. This fragment was further cleaved with thermolysin and then fractionated by h.p.l.c. to yield two smaller fragments, denoted TL-T(F1-2)16-1 and TL-T(F1-2)16-2, which contained a single disulphide loop each.

Identification of cystine-containing fragments

The nine distinct fragments which had been identified to contain a single disulphide bond each were rechromatographed by h.p.l.c. They were then characterized by their amino acid composition and further analysed for their first three *N*-terminal amino acid residues by automated Edman degradation (Table 1). Identification of half-cystine residues as their 1,4-dithiothreitol adducts was made taking advantage of the fact that cysteine residues are (partially) reduced to cystine by 1,4-dithiothreitol (present in most of the solvents of the

Table 1. Amino acid compositions of the low- M_2 kininogen fragments derived from F1-2 and F3 by proteolysis with trypsin and thermolysin

Peptides isolated from the digest with trypsin are identified by the prefix 'T', and those derived from digestions with trypsin and thermolysin by the prefix 'TL-T'. Amino acid analyses were performed by pre-column derivatization with *o*-phthalaldehyde. Proline, which does not react with *o*-phthalaldehyde, and tryptophan were not determined. Results from amino acid sequence analysis are indicated in parentheses. *N*-Terminal amino acid sequences were determined by automated Edman degradation. Cys, fragments containing half-cystine residues [15] are identified by +; *, peptides derived from the initially separated fragments by oxidation with performic acid and rechromatography by h.p.l.c.

Peptides	Asx	Thr	Ser	Glx	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Cys	Sequ.
T(F1-2)1	-	-	1.1(1)	0.9(1)	-	-	1.2(1)	-	-	-	-	-	-	-	-	-	EVS
T(F1-2)2	-	1.8(2)	-	0.8(1)	-	1.1(1)	-	-	1.2(1)	-	-	-	1.2(1)	-	-	-	ITE
T(F1-2)3	-	0.8(1)	1.2(1)	1.9(2)	1.3(1)	3.1(3)	-	-	-	-	-	-	-	-	0.9(1)	-	AGA
T(F1-2)4	-	1.7(2)	1.2(1)	1.8(2)	-	-	-	-	-	1.0(1)	-	-	-	-	0.7(1)	0.9(1)	EET
T(F1-2)5	0.7(1)	-	-	-	-	3.3(3)	1.2(1)	-	-	1.2(1)	-	-	1.1(1)	-	-	-	AVD
T(F1-2)6	0.7(1)	-	-	-	-	3.2(3)	1.2(1)	-	-	1.1(1)	-	-	2.3(2)	-	-	-	AVD
T(F1-2)7	1.9(2)	0.8(1)	1.1(1)	4.1(4)	2.1(2)	-	1.2(1)	-	-	-	0.7(1)	-	2.2(2)	-	-	-	EGD/TWQ
T(F1-2)7-1*	0.8(1)	-	1.2(1)	1.8(2)	2.2(2)	-	1.1(1)	-	-	-	-	-	1.3(1)	-	-	-	EGD
T(F1-2)7-2*	0.9(1)	0.9(1)	-	2.0(2)	-	-	-	-	-	-	0.8(1)	-	1.2(1)	-	-	-	TWQ
T(F1-2)8	-	-	-	0.9(1)	1.2(1)	-	-	-	2.1(2)	-	-	-	1.1(1)	-	-	-	IGE
T(F1-2)9	2.8(3)	-	3.3(3)	5.6(8)	-	-	-	-	1.1(1)	-	0.8(1)	-	2.1(2)	-	-	-	SCE/---
T(F1-2)9-1*	2.7(3)	-	2.2(2)	4.7(5)	-	-	-	-	1.1(1)	-	-	-	1.2(1)	-	-	-	---
T(F1-2)9-2*	-	-	1.2(1)	0.8(1)	-	-	-	-	-	-	0.7(1)	-	1.2(1)	-	-	-	SCE
T(F1-2)10	3.8(4)	-	2.2(2)	2.8(3)	-	-	1.1(1)	-	-	1.2(1)	1.8(2)	0.9(1)	-	-	0.9(1)	-	YNS
T(F1-2)11	0.8(1)	-	-	0.9(1)	1.2(1)	1.1(1)	2.1(2)	-	-	1.1(1)	-	0.8(1)	-	-	1.0(1)	-	QVV
T(F1-2)12	0.7(1)	1.8(2)	2.3(2)	-	1.1(1)	-	1.1(1)	-	-	-	0.7(1)	1.7(2)	1.1(1)	-	-	-	TVG
T(F1-2)13	2.8(3)	1.8(2)	2.1(2)	1.7(2)	-	-	1.0(1)	-	1.2(1)	2.3(2)	-	1.9(2)	2.1(2)	-	-	-	ENF/SIV
T(F1-2)13-1*	0.7(1)	0.9(1)	2.2(2)	0.8(1)	-	-	1.1(1)	-	1.1(1)	-	-	-	1.1(1)	-	-	-	SIV
T(F1-2)13-2*	1.7(2)	0.8(1)	-	0.9(1)	-	-	-	-	-	2.2(2)	-	1.8(2)	1.2(1)	-	-	-	ENF
T(F1-2)14	3.6(4)	0.9(1)	2.2(2)	2.7(3)	1.2(1)	-	1.1(1)	0.8(1)	1.1(1)	2.1(2)	0.7(1)	1.8(2)	1.1(1)	1.8(2)	-	-	HGI
T(F1-2)15	8.5(7)	1.7(2)	3.2(3)	2.8(3)	3.2(3)	2.3(2)	-	-	4.3(4)	2.1(2)	1.7(2)	0.9(1)	1.0(1)	-	0.8(1)	+	SLW/IAS
T(F1-2)15-1*	4.5(5)	1.8(2)	1.2(1)	1.7(2)	2.3(2)	1.1(1)	-	-	2.1(2)	2.2(2)	0.8(1)	-	-	-	0.8(1)	-	SLW
T(F1-2)15-2*	1.7(2)	-	2.1(2)	0.9(1)	1.1(1)	1.2(1)	-	-	2.0(2)	-	0.7(1)	0.8(1)	1.1(1)	-	-	-	IAS
T(F1-2)16	1.7(2)	7.5(8)	3.2(3)	8.7(7)	4.3(4)	6.3(8)	5.2(5)	-	3.3(3)	3.2(3)	0.7(1)	0.8(1)	1.3(1)	0.7(1)	0.8(1)	+	AAT/FSV
TL-T(F1-2)16-1*	-	4.8(5)	-	2.7(3)	1.2(1)	4.2(4)	1.1(1)	-	-	-	-	-	-	-	-	-	AAT/VAT
TL-T(F1-2)16-1a*	-	2.8(3)	-	0.8(1)	1.2(1)	3.3(3)	-	-	-	-	-	-	-	-	-	-	AAT
TL-T(F1-2)16-1b*	-	1.7(2)	-	1.8(2)	-	1.1(1)	1.2(1)	-	-	-	-	-	-	-	-	-	VAT
TL-T(F1-2)16-2*	0.8(1)	-	-	-	1.2(1)	-	-	-	-	1.1(1)	0.8(1)	-	-	-	-	-	YDC
T(F3)1	1.7(2)	-	-	-	-	-	1.1(1)	-	1.2(1)	-	-	-	1.1(1)	-	-	-	IDN
T(F3)2	-	-	-	0.9(1)	1.1(1)	1.1(1)	3.2(3)	-	-	-	-	-	1.1(1)	-	-	-	VQV
T(F3)3	-	-	-	0.8(1)	1.1(1)	1.0(1)	3.3(3)	-	-	-	-	-	2.1(2)	-	-	-	VQV
T(F3)4	0.7(1)	3.7(4)	3.2(3)	5.5(8)	-	-	-	-	-	1.2(1)	-	-	2.2(2)	-	-	-	ETT/ESN
T(F3)4-1*	-	1.8(2)	1.1(1)	0.9(1)	-	-	-	-	-	-	-	-	1.2(1)	-	-	-	ETT
T(F3)4-2*	0.8(1)	1.7(2)	2.2(2)	4.7(5)	-	-	-	-	-	1.1(1)	-	-	1.1(1)	-	-	-	ESN
T(F3)5	-	-	-	-	1.1(1)	-	1.2(1)	-	1.0(1)	-	-	-	-	-	0.9(1)	+	ICV
T(F3)6	2.7(3)	0.9(1)	-	0.8(1)	-	2.1(2)	-	-	-	1.2(1)	0.7(1)	1.9(2)	1.1(1)	-	-	-	LNA
T(F3)7	1.8(2)	4.7(5)	1.1(1)	2.7(3)	-	-	-	-	2.2(2)	2.1(2)	-	-	1.1(1)	0.8(1)	-	-	DIP
T(F3)8	0.8(1)	-	-	-	-	1.2(1)	1.1(1)	-	1.0(1)	-	0.7(1)	1.8(2)	1.2(1)	-	0.9(1)	-	KYF
T(F3)9	0.9(1)	-	-	-	-	1.1(1)	1.1(1)	-	1.1(1)	-	0.8(1)	1.9(2)	-	-	0.8(1)	-	YFI
T(F3)10	2.8(3)	0.8(1)	2.1(2)	3.7(4)	2.1(2)	1.0(1)	4.3(4)	1.8(2)	2.1(2)	4.3(4)	1.8(2)	-	2.2(2)	-	-	+	LGQ/IYP
T(F3)10-1*	1.8(2)	-	1.1(1)	2.8(3)	1.2(1)	1.1(1)	3.2(3)	-	-	2.2(2)	0.8(1)	-	1.2(1)	-	-	-	IYP
T(F3)10-2*	0.8(1)	0.9(1)	1.2(1)	0.8(1)	1.2(1)	-	1.1(1)	1.5(2)	2.2(2)	2.3(2)	0.7(1)	-	1.1(1)	-	-	-	LGQ

Fig. 3. Nine fragments contain the complete set of disulphide bridges of the human low- M_r kininogen molecule

The complete primary structure of human low- M_r kininogen is given [3,4,9]. Numbers denote the relative positions of the amino acid residues in the peptide chain. Fragments containing a single disulphide bridge are shaded. $\longleftrightarrow$, denotes the length of a fragment; $\rightarrow$, residue identified by manual Edman degradation; $\square$, a disulphide bridge. Peptides isolated from the tryptic digest of fragments F1-2 and F3 are identified by the prefix 'T', and those further treated with thermolysin have the prefix 'TL-T'.

Edman degradation process) and converted into dehydroalanine by β -elimination.

Out of the selected fragments, T(F1-2)9, TL-T(F1-2)13, T(F1-2)15, TL-T(F1-2)16-1, T(F3)4, and T(F3)-sequence each, while six fragments, T(F1-2)7, T(F1-2)13, T(F1-2)15, TL-T(F1-2)16-1, T(F3)4, and T(F3)-10, showed two *N*-terminal sequences (Table 1).

Sequence analysis of the fragments

All fragments containing cystine residues were oxidized by performic acid and rechromatographed by reversed-phase h.p.l.c. (not shown). The six fragments showing two *N*-terminal sequences (see above) were separated

into two peptides each. The resolved peptides were then characterized by analysis of their amino acid sequences (Fig. 3). In this way, disulphide loops bridging cysteine residues in positions 65 and 76 [T(F1-2)7], 89 and 108 [TL-T(F1-2)16-1], 188 and 200 [T(F1-2)13], 211 and 230 [T(F1-2)15], 310 and 322 [T(F3)4], and 333 and 352 [T(F3)10] were established (Fig. 3).

Fragment T(F1-2)9 which showed a single *N*-terminal sequence yielded two peptides upon reduction, one of them representing a peptide derived from the light chain of low- M_r kininogen [T(F1-2)9-2], and the other being the *N*-terminal peptide of the heavy chain blocked by a pyroglutamic acid residue [T(F1-2)9-1]. These peptides

Fig. 4. Four distinct types of disulphide loops are present in the human low- M_r kininogen

Four types of disulphide loops of various length and copy number are present in human low- M_r kininogen: type-A loops spanning 12 to 13 amino acids and present in three copies; type-B loops (20 amino acids; three copies); type-C loops (four amino acids; two copies); type-D loop (380 amino acids; one copy). Numbers identify the relative positions of the amino acids in the sequence of the mature low- M_r kininogen [3,4]. Amino acids in identical positions of the various loops are indicated by shading and boxing.

containing a single half-cystine residue each establish the unique inter-chain disulphide bridge of human low- M_r kininogen which spans from residue 10 to residue 389. Fragments TL-T(F1-2)16-2 and T(F3)5 which had a single *N*-terminal sequence each proved to be single peptides, each containing an internal disulphide loop. These loops span from positions 124 to 127 [TL-T(F1-2)16-2] and from positions 246 to 249 [T(F3)5].

Hence, a total of nine disulphide bridges could be established for human low- M_r kininogen, and eight of them form loops connecting consecutive half-cystine residues of the heavy chain, whereas a single bridge links the heavy- and the light-chain portion of the molecule.

Fig. 6. Type A and B loops of human low- M_r kininogen are homologous to the disulphide loops of mammalian cystatins

The three copies of type-A loops (a) and type-B loops (b) of human low- M_r kininogen have been aligned with the *N*-terminally-located loops (a) and the *C*-terminally located loops (b) of mammalian cystatins. Abbreviations: hu kin, human kininogen heavy chain [8]; hu cys C, cystatin C (alias γ -trace) from human plasma [26,27]; hu cys S, salivary acidic protein from human saliva [28]; bo cys, cystatin from bovine colostrum [29]; ch cys, cystatin from chicken egg-white [30,31]. Numbers identify the relative positions of the amino acids in the sequence. Gaps have been introduced as in [22]. Amino acid residues found in identical positions of at least one of the kininogen loops and one of the cystatins are boxed and shaded. It should be noted that among the various cystatins, only the disulphide loops of human cystatin G' and chicken cystatin have been established so far [32].

Types of disulphide loops

The nine disulphide loops of the human kininogen can be classified into four types of various length: type-A loops span 12 to 13 amino acids, type-B loops, 20 amino acids, type-C loops, 4 amino acids, and the type-D loop encompasses 380 amino acids (Fig. 4). Human low- M_r kininogen harbours three copies each of type-A and type-B loops, two copies of type-C loops and only a single copy of the type-D loop. Each of the various types of disulphide bridges is characterized by an extensive sequence homology among its members, while the sequence homology among the various types is low (see

Fig. 5. Human low- M_r kininogen possesses a characteristic arrangement of disulphide bridges

The arrangement of disulphide loops in human low- M_r kininogen is schematically shown. A, B, C, and D denote the various types of disulphide loops. Numbers identify the positions of the amino acids in the sequence of mature low- M_r kininogen. †, indicates a carbohydrate attachment site; ‡, indicates the typical cleavage sites for kallikrein which flank the kinin region; *, locates the canonical sequence Q-V-V-A-G which is supposed to form part of the reactive site of the kininogens [4,5].

Fig. 4). This finding reflects the fact that the kininogen heavy chain is built up of three repetitive units (domains) in a head-to-tail orientation, and that each unit contains 2 or 3 disulphide loops [22,23].

Arrangement of disulphide loops

In the low- M_r kininogen heavy chain, the disulphide loops are positioned in such a way that a thrice-repeated pattern of tandemly arranged type-A and type-B loops results, and two type-C loops are interspersed between them (Fig. 5). Thus the loop arrangement can be symbolized by A-B-C-A-B-C-A-B. The linear arrangement of eight consecutive disulphide loops is flanked by two half-cystine residues forming the single type-D loop which spans from the *N*-terminus of the heavy-chain portion to the *C*-terminus of the light-chain portion of the kininogen molecule. In this way, a particular arrangement of disulphide bridges in human low- M_r kininogens is formed.

Homology with mammalian cystatins

The kininogens are classified as a distinct family (family 3) among the superfamily of mammalian cysteine proteinase inhibitors [22], now known as the cystatin superfamily [24]. Among the other families, the stefins (family 1) do not have any disulphide bridges at all, while the cystatins (family 2) contain two tandemly-arranged disulphide loops, spanning 11 and 21 amino acids, respectively [25]. Proper alignment of the kininogen type-A loops with the *N*-terminally-located loop structures of the cystatins demonstrates a considerable sequence homology among the loops from various families of cysteine proteinase inhibitors (Fig. 6*a*). In particular, the segments flanking half-cystine residues have been well conserved among the two families. Similarly, kininogen type-B loops have remarkable sequence identities with the corresponding cystatin loops positioned at their *C*-termini (Fig. 6*b*). Here, both the flanking and the core regions of the loops are well preserved (note a stretch of six consecutive amino acids shared by human kininogen and bovine cystatin, Fig. 6*b*). Thus, type-A and type-B loops are common to human kininogen and cystatins, while type-C loops (as well as the type-D loop) are unique for the kininogen.

DISCUSSION

The arrangement of disulphide bridges proposed here for low- M_r human kininogen on the grounds of protein sequence analysis is in perfect agreement with the bridge structure predicted by Salvesen *et al.* [10] from their proteolytic cleavage patterns of human low- M_r kininogen. In view of the fact that human low- M_r and high- M_r kininogen share their entire heavy-chain structure [2,3], and they both have a single half-cystine residue each in their light-chain portion [2,9], it seems reasonable to suggest that human high- M_r kininogen has the same arrangement of disulphide bridges as low- M_r kininogen except that the type-D loop spans 587 residues (instead of 310 in low- M_r kininogen). This view is consistent with the preliminary findings of Sueyoshi *et al.* [11] on the disulphide bridge structure of bovine high- M_r kininogen. Invariance of the positions of the half-cystine residues in the sequences of all known mammalian kininogens (including rat T-kininogens) strongly suggests that the particular arrangement of disulphide bridges is not

Fig. 7. The disulphide loop arrangement of the cystatins is found in triplicate in the human kininogen heavy chain

The human kininogen heavy chain is represented as being composed of three cystatin-like units (not drawn to scale). A, B, C, and D denote the various types of homologous disulphide loops present in mammalian cysteine proteinase inhibitors; K, kinin segment; L, light chain portion of human kininogens. The asterisks locate the consensus sequence Q-V-V-A-G of the inhibitors, whereas the parenthesized asterisk indicates the corresponding region in the first domain of the kininogen heavy chain. Arrows indicate that the gene coding for the kininogen heavy chain might have arisen from a primordial cystatin gene by two successive gene duplication events [10,22] (illustrated here on the level of the translation products).

unique for human kininogens, but characteristic for mammalian kininogens in general.

Interestingly, the kininogens share crucial elements of their disulphide loop structure with other mammalian cysteine proteinase inhibitors. Triplication of the cystatin structure containing a single type-A and type-B loop each, results in the loop arrangement of the kininogen heavy chain except that two additional type-C loops have to be interspersed at the junctions of the various cystatin-like building blocks (Fig. 7). This finding lends further support to the hypothesis that the kininogen heavy-chain gene arose from a primordial cystatin gene by two successive gene-duplication events [10,22,23].

Preservation of the type-A and B loops in the evolution of mammalian cysteine proteinase inhibitors points to the importance of these elements to maintain a proper conformation of the inhibitor molecules. Indeed, selective reduction of the type-D loop in kinin-free human high- M_r kininogen does not interfere with the capability of the isolated heavy chain to inhibit cysteine proteinases [7]. By contrast, reduction and derivatization of all disulphide bonds (including type-A and B loops) renders the kininogen heavy chain completely inactive [7]. Hence, maintenance of (or at least part of) the particular arrangement of disulphide loops in human kininogens is mandatory for proper expression of their inhibitory function. These results are in line with the observation

that reductive cleavage of the (type-A and B) disulphide loops in chicken and human cystatin destroys the inhibitory potential of these molecules [32].

The question arises whether the type-C loops of the kininogens not present in the cystatins, significantly contribute to the inhibitory potential of the kininogens. Comparison of the inhibition constants for human low- M_r and high- M_r kininogen with papain and cathepsin L (K_i ranging from 1.7×10^{-11} to 4.8×10^{-11} M [5]), the isolated heavy-chain domains of low- M_r kininogen (5.0×10^{-12} to 1.4×10^{-10} M [10]), and the human and chicken cystatins (5.0×10^{-12} to 1.0×10^{-11} M [33]), indicates that introduction of type-C loops does not dramatically change the apparent K_i values. Thus, elucidation of the precise role, if any, of the type-C disulphide loops interspersed in the kininogen heavy chain, awaits further experiments.

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