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Determination of the disulphide bridge arrangement of bovine histidine-rich glycoprotein*

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Histidine-rich glycoprotein (HRG) was purified from bovine plasma and the disulphide bridge arrangement established. Disulphide-bridged peptides were obtained from peptic and tryptic degradation of native bovine HRG Twelve half-cystine residues were found in bovine HRG (compared to sixteen cysteines in human HRG), all involved in the formation of six disulphide bridges connecting Cys-1 to Cys-12, Cys-2 to Cys-3, Cys-4 to Cys-5, Cys-6 to Cys-11, Cys-7 to Cys-8, and Cys-9 to Cys-10. Additional sequence analysis of ^{14}C-carboxymethylated chymotryptic and *Staphylococcus aureus* V8 protease generated peptides and CNBr-fragments of bovine HRG yielded a partial amino acid sequence of bovine HRG constituting 78% of the sequence when compared to the human cDNA sequence.

Histidine-rich glycoprotein; Disulphide bond; Cystatin domain; Homology

1. INTRODUCTION

Histidine-rich glycoprotein (HRG) is a single-chain glycoprotein of M_r 75,000–81,000 as estimated by SDS-PAGE [1,2]. The protein is synthesized in the liver and is a negative acute-phase protein [3,4]. HRG was first isolated by Haupt and Heimburger in 1972 as a human serum protein with high affinity for CM-cellulose [5]. Since then, human HRG has been characterized from plasma [6], serum [7,8], platelets [9] and milk [10]. In addition, HRG has been isolated from other species such as mouse [11], rabbit [12], hog [13], and cow [14]. Although the exact function of HRG has not been defined, a number of biological properties of HRG have been revealed, establishing the concept of HRG as a multifunctional protein. Many of these properties imply that HRG physiologically may be a modulator of several plasma proteins involved in the blood coagulation and fibrinolysis. HRG has been shown to interact with heparin [15–18], plasminogen [6], thrombospondin [19], and fibrinogen [20]. HRG is capable of binding heme, dyes, and many divalent metal ions [7] and the protein is reported to inhibit autorosette formation [2] as well as inhibiting in vitro contact activation in human plasma [14].

At present, the amino acid sequence of HRG from only one species has been determined, which is the sequence of human HRG derived from the cDNA sequence [21]. The mature human protein contains 507 amino acids, including sixteen cysteine residues. N-terminally, human HRG contains two cystatin-like domains, based upon which HRG has been proposed to belong to the cystatin superfamily [22].

To gain information about conserved functional domains of HRG, comparison of the protein sequence and the organization of disulphide bonds between different species is useful. We have determined the disulphide bridge arrangement of bovine HRG in order to elucidate the conservation of the conformation and tertiary structure of this multifunctional protein.

2. MATERIALS AND METHODS

2.1. *Materials*

Staphylococcus aureus V8 protease, porcine pepsin, and bovine TPCK-treated trypsin were supplied by Worthington (N.J.). Bovine TLCK-treated chymotrypsin was from Sigma (MO). Bovine pancreatic trypsin inhibitor was a kind gift from L. Thim (Novo-Nordisk, DK). Iodo-[2-^{14}C]acetic acid (52 mCi/mmol) was supplied by Amersham International (UK). Sepharose CL-6B, Sephadex G-50 (superfine), and S Sepharose fast flow were all from Pharmacia (Sweden). Vydac C_{18} resin was from The Separations Group (CA) and Nucleosil C_{18} resin was from Macherey-Nagel (FRG). ProBlott PVDF membranes and reagents for sequence analysis were from Applied Biosystems (CA).

2.2. *Purification of bovine HRG*

Bovine HRG was purified from plasma by the method described [14], except that benzamidine, phenylmethanesulphonyl fluoride, and

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Abbreviations: HRG, histidine-rich glycoprotein; DTE, dithioerythritol; Cya, cysteic acid; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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bovine pancreatic trypsin inhibitor were added to all buffers to a final concentration of 1 mM, 1 mM and 10 mg/l, respectively. Purified HRG was concentrated in an Amicon cell using a PM10 filter and quantified by measuring A_{280} using the specific extinction coefficient $E^{1\%\,1\,cm}_{280} = 2.6$ for bovine HRG determined by amino acid analysis. The average yield was 36–90 mg HRG/liter plasma. The isolated HRG was found to be pure by N-terminal sequence analysis. The purified protein was stored at −20°C.

2.3. *S-carboxymethylation and fragmentation of HRG*

Purified bovine HRG dissolved in 0.02 M NaH_2PO_4, 1 M NaCl pH 6.3 was made 8 M in urea, 0.3 M in Tris-HCl and pH was adjusted to 8.3. Following denaturation, the sample was reduced with DTE, ^{14}C-carboxymethylated with iodo-[2-^{14}C]acetic acid corresponding to 50–100 μCi and finally alkylated with non-labelled ICH_2COOH. ^{14}C-carboxymethylated HRG was dialyzed extensively against 0.1 M NH_4HCO_3 before being stored at −20°C.

Cleavage of bovine HRG was carried out in 70% formic acid with CNBr (1/1 w/w). Degradation was allowed to occur in the dark at 20°C for 20 h. The CNBr-degraded sample was diluted with 14 volumes of water and freeze-dried. Enzymatic fragmentation of reduced and ^{14}C-carboxymethylated bovine HRG was performed in 0.1 M NH_4HCO_3. Digestion was carried out either with *Staphylococcus aureus* V8 protease (1/20 w/w, 18 h, 37°C) or with chymotrypsin (1/100 w/w, 19 h, 37°C.).

Disulphide-bridged peptides of HRG were obtained by degradation of native HRG with porcine pepsin (1/200 w/w, 4 h, 37°C) in 1% formic acid followed by digestion with trypsin (1/200 w/w, 2 h, 37°C) in 50 mM NaH_2PO_4, pH 6.5. All degradations were stopped by freeze-drying.

2.4. *Peptide isolation*

Peptide mixtures were initially fractionated by gel filtration, and further purification was carried out by reverse-phase HPLC using a Hewlett-Packard 1084B liquid chromatograph connected to a HP 79850A LC terminal or a Pharmacia LKB 2249 HPLC.

Peptides of ^{14}C-carboxymethylated bovine HRG were separated on a Sephadex G-50 column in 0.1 M NH_4HCO_3. Absorbance was measured at 230 nm, and aliquots of each fraction were analyzed in a liquid scintillation counter. The resulting radioactive pools were further fractionated by reverse-phase HPLC with a linear gradient of 0–96% ethanol in 0.1% TFA at 60°C using a Vydac C_{18} column (300 Å, 10 μm). Absorbance was measured at 220 nm. During repurifications, a Nucleosil C_{18} column (300 Å, 7 μm) and different solvents (80% acetonitrile, 70% methanol, 75% 2-propanol in 0.1% TFA) were used. Aliquots from each fraction were analyzed by liquid scintillation counting and radioactive peptides were sequenced.

Disulphide-bridged peptides of bovine HRG were separated on a Sephadex G-50 column in 1% formic acid. Aliquots of every second fraction were subjected to performic acid oxidation and amino acid analysis to determine the amount of Cya in the fractions. The obtained cystine-containing pools were further separated on an S Sepharose fast flow column with a linear gradient from 10 mM to 1 M of CH_3COONH_4 pH 5.0. Absorbance was measured at 229 nm. Aliquots of each of the resulting pools were measured for Cya-content and cystine-containing pools were subjected to reverse phase HPLC. Initially, a Nucleosil C_{18} column (300 Å, 7 μm) was used and peptides were eluted with a linear gradient of 0–95% acetonitrile in 0.1% TFA at 20°C and monitored at 229 nm. Peptides were repurified on a Vydac C_{18} column (300 Å, 10 μm) with a linear gradient of 0–96% ethanol in 0.1% TFA at 60°C. Aliquots of all obtained fractions were analyzed for Cya content and cystine-containing peptides were sequenced. CNBr-fragments of HRG were separated on a Sepharose CL-6B column in 8 M urea, 0.1 M CH_3COONH_4 pH 5.0.

2.5. *Electroblotting, amino acid and sequence analysis*

Discontinuous SDS-PAGE was performed in 10–20% gradient gels with a 4% stacking gel [23]. Gels were stained with Coomassie brilliant blue R. For electroblotting, proteins were transferred from an unstained SDS-PAGE gel to a ProBlott PVDF membrane [24]. The membrane was stained with Coomassie brilliant blue R, destained in 50% methanol, and washed in distilled water.

The amino acid composition of peptides, or intact protein, was determined by amino acid analysis [25] after hydrolysis at 110°C for 16 h in evacuated, sealed tubes containing 50 μl 6 M HCl, 0.05% phenol and 5 μl thioglycollic acid. Cystine was determined as Cya, generated by performic acid oxidation prior to hydrolysis.

Automated Edman degradation was performed using an Applied Biosystems model 477A pulsed liquid sequencer connected on-line to a model 120A PTH-derivative analyzer HPLC. Typically, 100–500 pmol of the peptide was applied per run. The amino acid sequence of a disulphide-bridged peptide was determined by simultaneous Edman degradation of the two linked peptide chains. The position of the first half-cystine in a disulphide bridge was observed as a missing PTH-derivative in the actual step, whereas the second half-cystine was released as PTH-cystine. This derivative was observed as a peak eluting just in front of PTH-Tyr in the HPLC separation of PTH-amino acids.

2.6. *Alignment of protein sequences*

Protein sequences were aligned using a progressive, pairwise alignment method from the program Pileup and displayed by the program PrettyPlot in the Genetics Computer Group Sequence Analysis Software Package, Version 7.1 [26]. All numbered residues in bovine HRG refer to the corresponding position in human HRG.

3. RESULTS

3.1. *Isolation of ^{14}C-carboxymethylated cysteine-containing peptides*

The amount of cysteines in bovine HRG was determined by amino acid analysis after performic acid oxidation with porcine insulin as a parallel standard. Unexpectedly, bovine HRG yielded only twelve cysteine residues compared to the sixteen cysteines predicted from the human cDNA sequence. Therefore, *Staphylococcus aureus* V8 protease and chymotrypsin digests of reduced and ^{14}C-carboxymethylated bovine HRG were performed in order to determine the positions of the cysteine residues. The peptide mixtures were separated by gel filtration and the fractions obtained were screened for radioactivity. Radioactive pools were further separated by reverse-phase HPLC. Sequencing of the purified peptides yielded all the twelve carboxymethylcysteines. To establish more of the amino acid sequence of bovine HRG some non-radioactive peptides were also isolated and their sequence determined. The resulting partial amino acid sequence of bovine HRG is shown in Fig. 1.

3.2. *Isolation of disulphide-bridged peptides*

After the positions of the twelve half-cystines had been established, disulphide-bridged peptides of native bovine HRG were isolated in order to determine the disulphide bridge arrangement of the protein. To generate relatively small peptides for disulphide bridge assignment, HRG was degraded successively with pepsin and trypsin. The risk of disulphide bridge rearrangement was minimized by the relatively low pH at which pepsin and trypsin treatment of HRG was carried out.

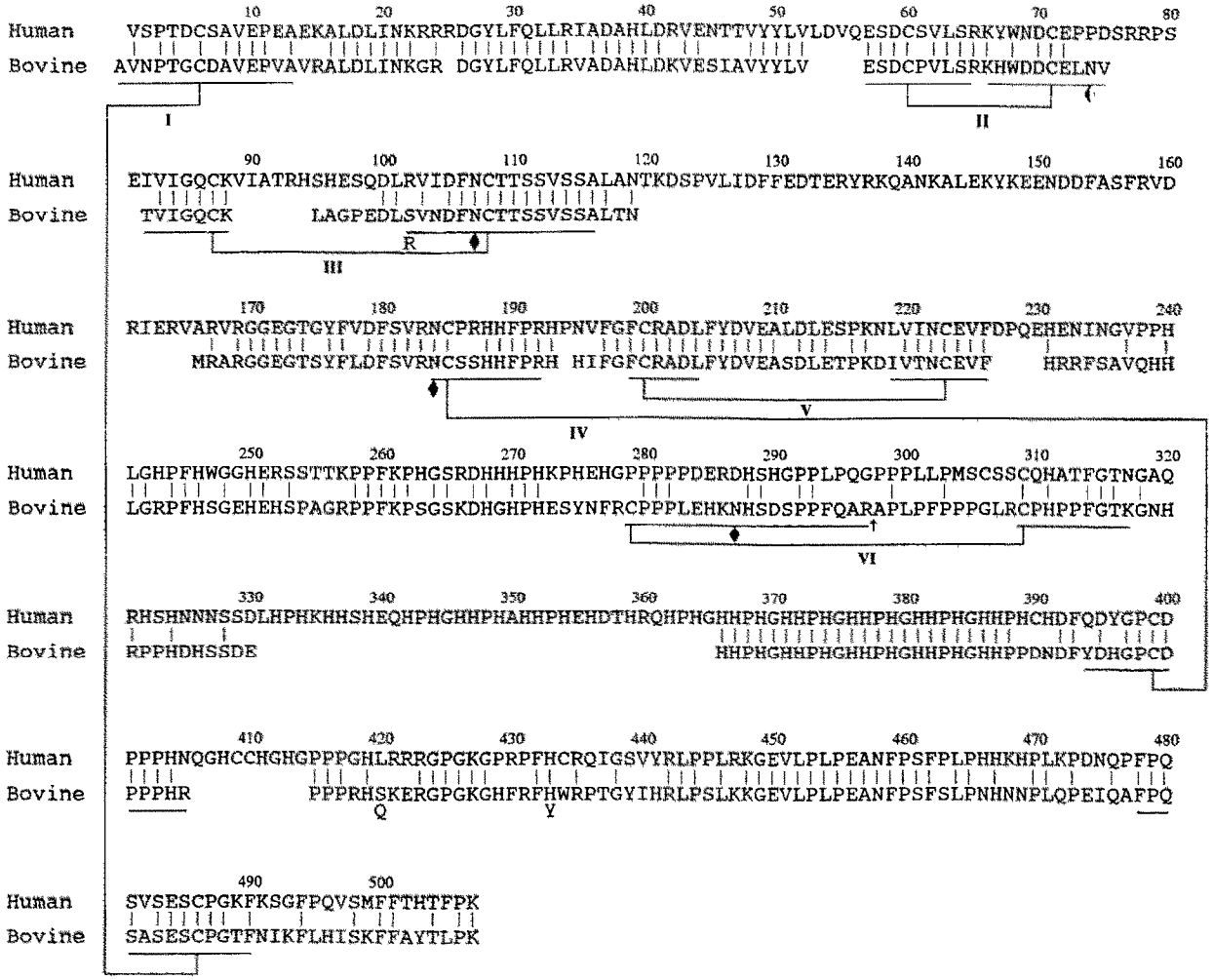

Fig. 1. Alignment of the partial amino acid sequence of bovine HRG with human HRG. The six disulphide bridges (I–VI) in bovine HRG are indicated. The longest obtained disulphide-bridged peptides are underlined. Identical residues are marked by vertical bars. Filled diamonds indicate Asn-linked glycosylation of the amino acid residue; partial Asn-linked glycosylation is represented by a half-filled diamond. Polymorphism with respect to amino acid residues was found at positions 102, 420, and 433. The proteolytic cleavage-site is shown by an arrow. Numbers refer to the amino acid sequence of human HRG [21].

The peptic/tryptic peptide mixture was fractionated on a Sephadex G-50 column and three cystine-containing pools were subjected to further separation by cation-exchange on an S Sepharose fast flow column. A total of 22 cystine-rich pools were obtained, each of which underwent purification by reverse-phase HPLC. The purified disulphide-bridged peptides were subjected to amino acid analysis prior to amino acid sequencing. Six disulphide-bridges (numbered I–VI) were assigned in this way. To confirm that the two peptide-strands of a disulphide-bridged peptide were actually linked to one another, performic acid oxidation of the disulphide-bridged peptide was carried out. The oxidized peptides were separated by reverse phase HPLC and sequenced. A number of the peptides containing identical disulphide bridges were found to elute in different pools during cation-exchange and reverse phase HPLC due to variation caused by lack of pepsin specificity and carbohydrate heterogeneity. The resulting disulphide bridge arrangement of bovine HRG is shown in Fig. 1, with the longest obtained disulphide-bridged peptides underlined.

The assignment of disulphide bridge I in bovine HRG was additionally verified by isolation and sequencing of unreduced fragments obtained by CNBr-degradation of intact bovine HRG. The fragments were fractionated by gel-filtration on a Sepharose CL-6B column resulting in two pools (pool 1 and pool 2). SDS-PAGE of pool 1 revealed a double band of 75 kDa similar to intact HRG, which also appeared as two bands of 75 kDa on SDS-PAGE. Whereas electroblotting and sequencing of each of the two 75 kDa bands of intact HRG revealed a single sequence corresponding to the N-terminus of bovine HRG, each of the two bands from pool 1 showed

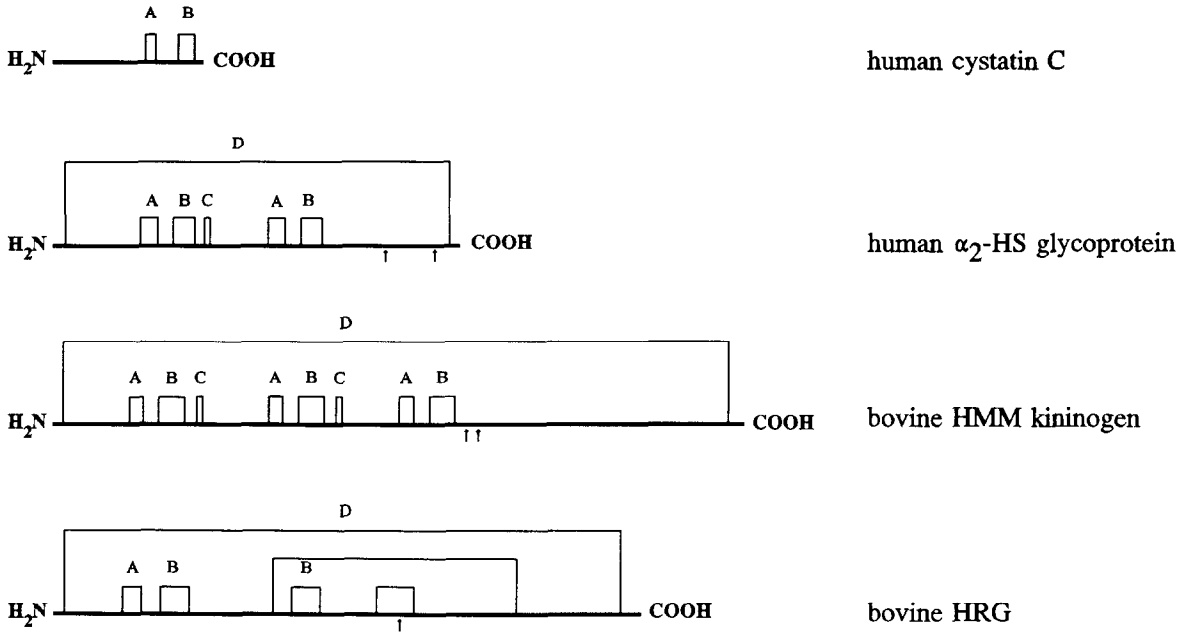

Fig 2. A schematic comparison of the disulphide loop patterns in members of the cystatin superfamily. The disulphide bridge arrangements of human cystatin C [32], human α_2-glycoprotein [30], and bovine HMM-kininogen [31] are shown. The various types of loops are denoted A, B, C, and D, respectively. Arrowheads indicate the cleavage sites for kallikrein in kininogen, and the cleavage sites in α_2HS-glycoprotein and bovine HRG.

two sequences in equal amounts. The two sequences found in each band of pool 1 corresponded to the N-terminus of HRG and a fragment beginning at Arg-167. Thus, due to disulphide bridge I, CNBr-treated HRG appeared on SDS-PAGE to be identical to intact HRG. Reduction of the 75 kDa CNBr-fragment with DTE resulted in two bands of 25 kDa and 45 kDa. Sequence analysis demonstrated the 25 kDa-fragment to be the N-terminus of HRG and the 45 kDa-fragment to start at position Arg-167. These results are in agreement with the finding of only one methionine in bovine HRG by amino acid analysis.

SDS-PAGE of pool 2 showed two closely spaced bands of about 40 kDa. Electroblotting and sequence analysis of the lower band revealed two sequences in equal amounts, corresponding to the N-terminal of HRG and a fragment starting at Pro-415. Probably, the peptide bond Xaa-414–Pro-415 had been cleaved by formic acid during CNBr-treatment of HRG. Due to disulphide bridge I, the N-terminal-Met-166 fragment and the Pro-145–Lys-507 fragment appeared as one band of 40 kDa. Reduction of this disulphide bridge by DTE produced a 10 kDa-band corresponding to the Pro-415–Lys-507 fragment and a 25 kDa band corresponding to the N-terminal-Met-166 fragment. The other half of the protein is contained in the central Arg-167–Xaa-414 fragment of HRG cut out by CNBr and formic acid cleavage. This fragment was found as the upper band of the 40 kDa double band.

Despite the addition of inhibitors during purification of the protein, bovine HRG was occasionally purified in a partly degraded form, as judged by SDS-PAGE in the presence of DTE. Upon reduction of the disulphide bridges in partly degraded bovine HRG, three bands appeared with an apparent molecular weight of 80 kDa, 50 kDa, and 30 kDa, respectively. Electroblotting and sequencing of each band established that both the 80 kDa and the 50 kDa bands yielded the N-terminal sequence of HRG while the 30 kDa band revealed the sequence APLPFPPP..., corresponding to Ala-298 and forward. Consequently, the peptide-bond between Arg-297 and Ala-298 is sensitive to proteolysis. The degradation of bovine HRG may result from residual plasmin activity although a proline residue (Pro-299) is situated at position P_2', thereby making the site unfavourable for plasmin degradation. The proteolytic cleavage-site is indicated by an arrow in Fig. 1.

3.3. *Asn-linked carbohydrate attachments and polymorphism*

Peptides containing Asn-linked oligosaccharides were identified by observing N-acetylglucosamine in peptide hydrolysates. The position of Asn-linked carbohydrate was identified during sequence analysis as a missing PTH-derivative in the actual step. Asn-linked oligosaccharides were found to be attached to four Asn-residues in positions 74, 107, 184, and 287. All sites of attachment were in the carbohydrate acceptor consensus sequence Asn-Xaa-Ser/Thr. One site, Asn-74, was found to be heterogeneous with respect to carbohydrate

attachment and it seems most likely that this heterogeneity is at least partly responsible for the double band appearance of unreduced HRG in SDS-PAGE.

Three sites were found to be heterogeneous with respect to amino acid residues. Position 102 was either a serine or an arginine, position 420 was either a serine or a glutamine, whereas either a tyrosine or a histidine was situated at position 433.

4. DISCUSSION

We have identified the six disulphide bridges in bovine HRG, connecting Cys-1 and Cys-12, Cys-2 and Cys-3, Cys-4 and Cys-5, Cys-6 and Cys-11, Cys-7 and Cys-8, and Cys-9 and Cys-10. Also, a partial amino acid sequence of bovine HRG has been obtained, constituting 78% of the sequence if compared to the human counterpart. The identity between human HRG and the sequenced part of bovine HRG is relatively low (65%), and a striking deviation exists in the number of half-cystines in the two proteins. Three half-cystines have mutated in bovine HRG, corresponding to Cys-306, Cys-390, and Cys-434 in human HRG. Furthermore, we suggest that Cys-409 and Cys-410 also have mutated in the bovine protein based upon the amino acid analysis of bovine HRG and the lack of finding peptide(s) containing these cysteine residues. On the other hand, bovine HRG contains an additional half-cystine at position 279, which is absent in the human counterpart. Consequently, the disulphide bridge arrangement determined in this study does not allow us to predict the complete disulphide bridge pattern of human HRG. However, the amino acid sequence in the N- and C-termini of bovine HRG shows an extensive similarity to the human counterpart, including the positions of the half-cystines. It seems very likely that the half-cystines in these areas are involved in identical disulphide bridges in the two proteins. Therefore we suggest that disulphide bridges I, II, III, IV, and V established in this study are present in human HRG, too.

Like in human HRG, a C-terminal lysine was found in bovine HRG, which may account for the ability of HRG to bind plasminogen. In addition, a second lysine is located eight amino acids towards the N-terminal of bovine HRG, a motif which has been suggested to enhance the binding affinity of proteins for plasminogen [27]. Heterogeneity with respect to carbohydrate was identified at position 74 and polymorphism with respect to amino acid residues was found at positions 102, 420, and 433 suggesting that there are more than one allele of the bovine HRG gene giving rise to different allelic forms of the gene-product or alternatively, more than one gene for HRG. An acid hydrolysis caused by formic acid during CNBr-treatment occurs in the C-terminal part of the protein (between Xaa-414 and Pro-415). This observation is in accordance with an Asp-Pro hydrolysis (between Asp-400 and Pro-401) in human HRG caused by formic acid during CNBr degradation [21,28]. However, it is possible that hydrolysis at Asp-400–Pro-401 also occurs in bovine HRG.

The N-terminal region of human HRG (residues 1–225) contains two cystatin-like domains [22]. These domains show homology towards known members of the cystatin superfamily, including the positions of the half-cystines and the amino acid residues surrounding these. Due to this homology, HRG was proposed to belong to the cystatin superfamily, constituting a new fourth family, although no inhibitor function has been demonstrated for HRG [22]. The determination of the disulphide bridge arrangement in bovine HRG confirms the placement of HRG in the cystatin superfamily. Furthermore, the results clarify why the first half-cystine in cystatin-like domain I in HRG (residues 1–112) is not part of a cystatin-domain, since it is involved in disulphide bridge formation with the last half-cystine residue in the molecule. Likewise, the first half-cystine residue in the second cystatin-like domain (residues 113–225) is lacking, which makes this cystatin-like domain different from those of the other members of the superfamily. Consequently, the second half-cystine in this domain must be engaged in disulphide bridge formation with a different half-cystine, shown in this study to be Cys-399.

The disulphide bonds in the members of the cystatin superfamily have been described in general terms. Four different kinds of disulphide loops, A, B, C, and D have been characterized [29,30]. These are schematically outlined in Fig. 2. Type A and B represent the first and second loop of a cystatin domain, respectively, and both are found in the cystatin family (family 2), the kininogen family (family 3), and in human α_2-HS glycoprotein. The latter does not exhibit any inhibitor function and has been placed in family 4 together with HRG [30]. Type C and D loops are found in the kininogens and in human α_2-HS glycoprotein, both of which become two-chained molecules by proteolysis and excision of a peptide. Type C and D loops are not part of the cystatin domains. Type C loops span 4 residues and are placed in between cystatin domains whereas type D loops span the entire molecule by connejcting the extreme N- and C-termini of the proteins.

The present study reveals that bovine HRG likewise contains one type D loop in addition to one type A and two type B loops. Furthermore, the disulphide bridge arrangements of human and bovine HRG are not identical, in contrast to the disulphide bridge patterns found in kininogens of different species (e.g. man and cow) [29,31]. Whether the proteolytic split between Arg-297 and Ala-298 represents a physiological proteolysis and excision of a fragment, by analogy with kininogens and α_2-HS-glycoprotein remains to be shown, but it would explain the presence of a type D loop in the molecule even though two other disulphide bridges are connecting the two parts of the protein.

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