ELSEVIER

FEBS Letters 343 (1994) 223–228

FEBS LETTERS

FEBS 13948

Primary structure of bovine α_2-antiplasmin

Søren Christensen[a], Lars Berglund[b], Lars Sottrup-Jensen[a,*]

[a]*Department of Molecular Biology, University of Aarhus, Building 130, DK-8000 Århus C, Denmark*
[b]*Protein Chemistry Laboratory, Department of Molecular Biology, University of Aarhus, The Science Park, Gustav Wieds Vej 10, DK-8000 Århus C, Denmark*

Received 17 March 1994; revised version received 25 March 1994

Abstract

The primary structure of bovine α_2-antiplasmin (α_2AP) has been determined from cDNA and partial peptide sequencing. Mature bovine α_2AP contains 470 residues and is 6 residues longer than human α_2AP. Alignment of the two protein sequences show that 81% of their amino acid residues are identically located. Bovine α_2AP has 5 N-linked carbohydrate groups, of which four are found in human α_2AP (Asn105, 274, 288 and 295). Asn227 is the fifth carbohydrate attachement site in bovine α_2AP. The 3 Cys residues of bovine α_2AP are present as an unpaired residue (Cys131) and as a pair in a disulfide bridge (Cys49–Cys122). The assignment of the bridge in bovine α_2AP is at variance with the previous assignment of the two disulfide bridges in human α_2AP [Lijnen, H.R. et al. (1987) Eur. J. Biochem. 166, 565–574].

Key words: α_2-Antiplasmin; Serpin; Plasma protein; cDNA

1. Introduction

α_2-Antiplasmin (α_2AP) is the primary inhibitor of the plasma protease plasmin [1–3]. It is a member of the serpin family of proteins which has been identified in viruses, plants, insects and higher animals [4,5]. Most mammalian plasma serpins participate in the control of thrombolysis, fibrinolysis, complement activation and inflammation. They interact with their target protease(s) through a particularly exposed reactive site loop (RSL) [4].

α_2AP diverged early from the line leading to the inflammatory serpins α_1PI and α_1ACT [5–7]. In several species the latter proteins form multigene families [8–10] in which the RSL sequences show great diversity, and in which the distinction between orthologous and paralogous proteins cannot always be made.

However, as discussed recently [10], orthologoues of α_1PI are indeed strongly related throughout their entire sequences, and it can be expected that the sequences of other orthologous serpins, including their RSLs, will be very similar. This is also apparent from a comparison of the sequences of human and bovine ATIII and PAI-1 [11–14]. While the sequence of human α_2AP has been determined [15,16] only partial sequence data for bovine α_2AP have been available [17].

Here we report the amino acid sequence of bovine α_2AP deduced from cDNA and peptide sequencing. Bovine α_2AP contains 5 sites of N-linked glycosylation, 1 disulfide bridge and an unpaired Cys residue which all have been located. The data add further strength to the view that orthologous serpins are strongly related throughout their sequences.

2. Materials and methods

2.1. Enzymes and reagents

Restriction endonuclases, T4 DNA ligase, nick translation kit and trypsin were from Boehringer Mannheim. As revealed by the digestion pattern of bovine α_2AP (see below) trypsin was contaminated with chymotrypsin. Plasmid pPAAP, with an insert encoding human α_2AP [18] was a kind gift from R. Lijnen and L. Nelles, University of Leuven, Belgium. [α-^{32}P]dATP and [^{35}S]dATP were from Amersham. The bovine liver λgt-11 phage library was from Clontech. DNA sequence reactions were carried out using Sequenase V2.0 (United States Biochemical). Antibodies were from Dako, and Protein-A Sepharose was from Pharmacia.

2.2. Screening, cloning and sequencing of bovine α_2AP cDNA

About 400,000 plaques from an adult male bovine liver λgt-11 phage

*Corresponding author. Fax: (45) 8942 2679.

Abbreviations: α_2AP, human and bovine α_2-antiplasmin; ATIII, antithrombin III; PAI-I, plasminogen activator inhibitor I; α_1PI, α_1-antitrypsin; α_1ACT, α_1-antichymotrypsin; TGA, thioglycollic acid; TFA, trifluoroacetic acid; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis. Solutions: 20 × SSPE: 3.0 M NaCl, 0.3 M sodium phosphate, 0.02 M EDTA, pH 7.4; 1 × SSC: 150 mM NaCl, 15 mM trisodium citrate; 100 × Denhard: 2% bovine serum albumin, 2% polyvinylpyrrolidone and 2% Ficoll-400.

The nucleotide sequence presented here has been submitted to the EMBL/Genbank database under accession no. X78436.

SSDI 0014-5793(94)00333-Q

library were screened with a mixture of the two nick translated *Eco*RI fragments of plasmid pPAAP [18]. Together, these probes contained the entire coding sequence for human α_2AP. Hybridisations to nitrocellulose filters were done in 5 × SSPE, 2 × Denhardt, 0.5% SDS and 100 μg/ml salmon sperm DNA overnight at 68°C. The filters were washed two times (15 min each) with 2 × SSC, 0.1% SDS at 68°C and autoradiographed. DNA from purified plaques was digested with restriction endonuclease *Eco*RI and separated in 1% agarose and transferred to nitrocellulose by Southern blotting [19]. The DNA fragments which hybridized to the insert of pPAAP were subcloned in plasmid pUC19. The DNA sequence was determined by using primers close to the *Eco*RI cloning site in pUC19 and primers complementary to the successively determined stretches of sequence on both strands.

2.3. Northern blot analysis

RNA was isolated from homogenized tissue by CsCl gradient centrifugation and size-fractionated by formaldehyde/agarose gel electroforesis [20,21]. After transfer to nitrocellulose the filter was hybridised with the nick translated *Eco*RI fragment of clone AP11 (see below).

2.4. Localization of N-linked carbohydrate and disulfide bridge

Bovine α_2AP was purified as described [17]. Three hundred μg protein was dissolved in 400 μl 70% HCOOH and incubated for 20 h in the dark with 5 mg of CNBr. After lyophilisation the material was dissolved in 600 μl 0.1 M NH_4HCO_3 and incubated with 5 μg trypsin for 5 h at 37°C. The peptides were separated on a C-18 Nucleosil column (4 × 250 mm) using a gradient of acetonitrile in 0.1% TFA as in [17]. The amino acid composition of all fractions were determined and peptides containing half-cystine or glucosamine selected for sequence analysis.

2.5. Amino acid and sequence analysis

Prior to hydrolysis (6 M HCl/0.1% phenol for 16 h at 110°C) samples were oxidised with 1 M performic acid for 20 min at room temperature. From these hydrolysates HPLC fractions containing half-cystine and glucosamine were identified. To determine cysteine in non-oxidised samples hydrolysis was carried out in the presence of 5% TGA. Amino acids were determined by cation-exchange chromatography [22,23].

Sequence analysis was done by Edman degradation on an AB 477 A instrument with on-line PTH-amino acid analysis. The Normal-1 reaction and conversion cycles were used.

3. Results and discussion

3.1. Cloning, Northern blot analysis and sequencing of bovine α_2AP

From a λgt-11 bovine liver library two overlapping clones (AP11 and AP20) (Fig. 1A) were isolated which together contained the coding sequence for bovine α_2AP, most of the 3′-untranslated region including the polyadenylation signal, and 108 bases located 5′ of the N-terminal Phe residue of mature bovine α_2AP [17]. The cDNA sequence encodes a 492 amino acid precursor of bovine α_2AP (Fig. 1B).

The *Eco*RI fragment of clone AP11 was used as probe in Northern blot analysis of mRNA from different bovine tissues (mammary gland, skeletal muscle, heart, liver). As seen previously [16], hybridisation was found mainly to liver RNA. One band was seen with the size of approx. 2.2 kb. No hybridisation was found to heart RNA, but weak signals were seen in muscle and mammary gland RNA (not shown).

A hydrophobic signal peptide of 22 residues was found to precede mature α_2AP. A Gln and Ser in position −1 and −3, respectively, is in accordance with the −1 to −3 rule for signal peptidases, though Gln at −1 is rare [24].

The sequence of bovine α_2AP predicted from its cDNA differs at three positions from that determined from N-terminal sequence analysis [17]: At position 6 (Thr vs. Gln), 18 (Gln vs. Pro) and 21 (Gln vs. Glu). A re-examination of the N-terminal sequence of α_2AP revealed two PTH-amino acids in step 6 (Thr and Gln) and 18 (Gln and Pro). This is likely to be due to heterogeneity or breed differences as the protein was purified from pooled plasma. The residue at position 21 was previously determined as Glu, possibly the result of deamidation of Gln during sequencing. The sequences of 229 residues were obtained from 17 peptide fragments (Fig. 2) two of which were reported before [17]. No additional differences were found for the stretches determined from peptide sequencing.

3.2. Comparison of the sequences of bovine α_2AP and human α_2AP

The sequences of bovine and human α_2AP have 81% residues in common (Fig. 2). This is lower than the score obtained by comparing the sequences of human and bovine ATIII (89%) [11,12] and PAI-1 (86%) [13,14], but higher than the value found when comparing human and bovine α_1PI (72%) [25,26]. This range of scores is typically seen when the sequences of orthologous human and bovine plasma proteins are compared.

As the sequence similarity extends through the signal peptide to the initiator Met residue, processing would be expected to occur at the same site. However, mature bovine α_2AP is 6 residues longer than human α_2AP [17]. This can be explained by the deletion in human α_2AP of Gln(−1) found in bovine α_2AP, hence changing the processing site 6 residues towards the C-terminus. Gln20 of bovine α_2AP is presumably the counterpart of Gln14 of human α_2AP (revised numbering [17]), which is important for transglutaminase-mediated cross-linking of α_2AP to fibrin [27].

Residues P15–P′15 of the RSL are particularly well conserved in bovine and human α_2AP (87% identity) indicating the requirement of a strictly specified structure for interaction with the active site of plasmin. In contrast, the C-terminal extensions of bovine and human α_2AP (starting at residue 414 in bovine α_2AP) have only 69% residues in common. This is surprising since this region is known also to be important for the interaction of α_2AP and plasmin [28]. However, Lys448 and the C-terminal Lys464 of human α_2AP previously shown to interact of with plasmin [28,29] are conserved in bovine α_2AP as well as 4 other Lys residues of unknown functional role (Fig. 2). Tyr463 of human α_2AP is sulfated [30], and this residue as well as the preceding 3 acidic residues commonly associated with sulfation [31] are conserved in bovine α_2AP, suggesting that it is modified in the same way.

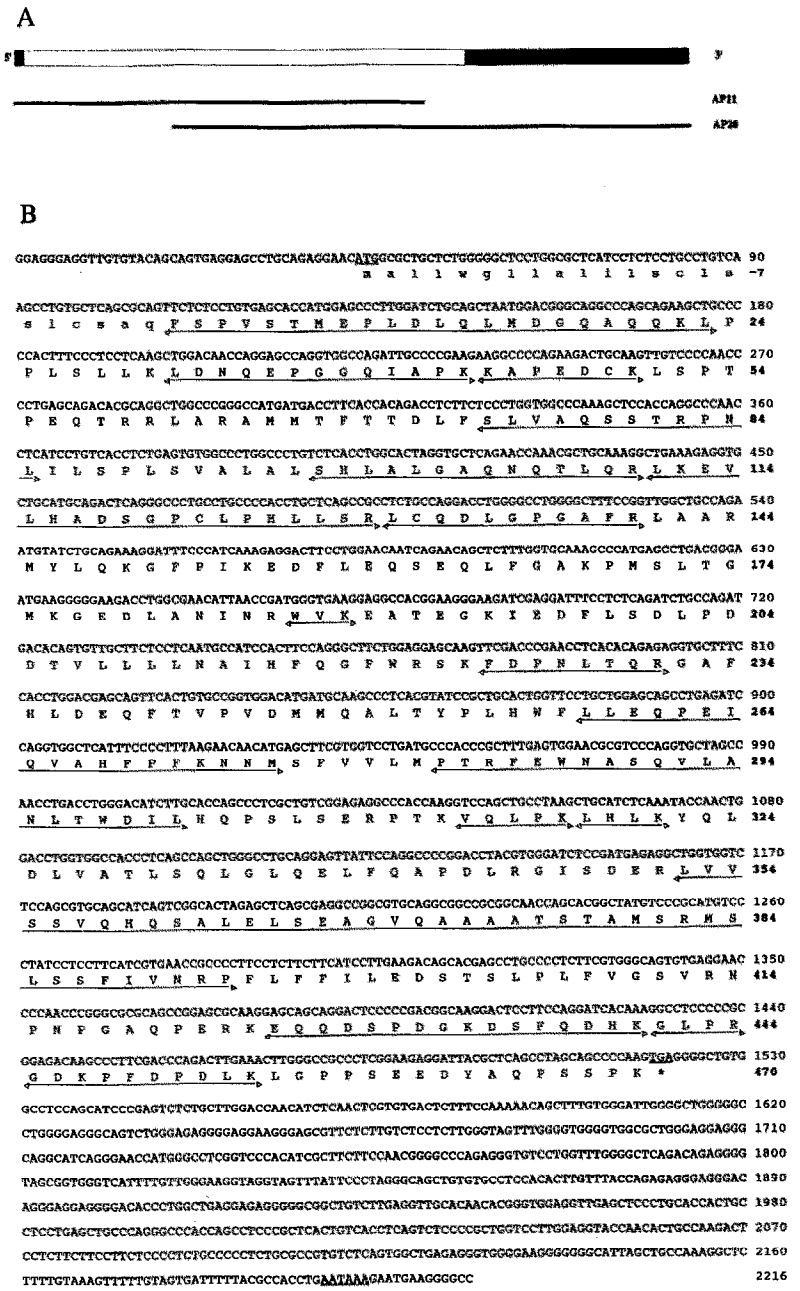

Fig. 1. (A) Schematic representation of the complete cDNA for bovine α_2AP and the two clones AP11 and AP20. The filled areas represent the 5'- and 3'-untranslated regions. (B) The complete nucleotide sequence and the translated protein sequence of bovine α_2AP are shown. The initiation codon (ATG) for translation, the stop codon (TGA) for translation and the polyadenylation signal (AATAAA) are underlined. Protein numbering is in bold. Stretches of bovine α_2AP determined from amino acid sequencing of protein and peptides are indicated by arrows.

3.3. *Localization of carbohydrate attachment sites in bovine α_2AP*

Bovine α_2AP contains 3.1% (w/w) glucosamine [17] and its cDNA sequence predicts 5 potential *N*-glycosylation sites (Figs. 1B and 2). Peptides containing glucosamine were identified, from a CNBr/tryptic digest (Fig. 3). Upon sequence analysis of these, a gap at the potential glycosylation site was taken as evidence for the attachment of carbohydrate. The results showed that the 5 potential sites, Asn105, Asn227, Asn274, Asn288 and Asn295, were all glycosylated in bovine α_2AP. Apart from Asn227, these sites are the same as in human α_2AP [15], emphasizing the strong similarity of the 2 orthologous proteins.

3.4. *Disulfide bond arrangement of bovine α_2AP*

Bovine α_2AP contains 3 Cys residues (Fig. 1A) and these residues (positions 49, 122 and 131) correspond to the Cys residues 43, 116 and 125 of human α_2AP. The counterpart of Cys76 in human α_2AP is Arg82 in bovine α_2AP (Fig. 2).

Half-cys containing peptides generated by CNBr and

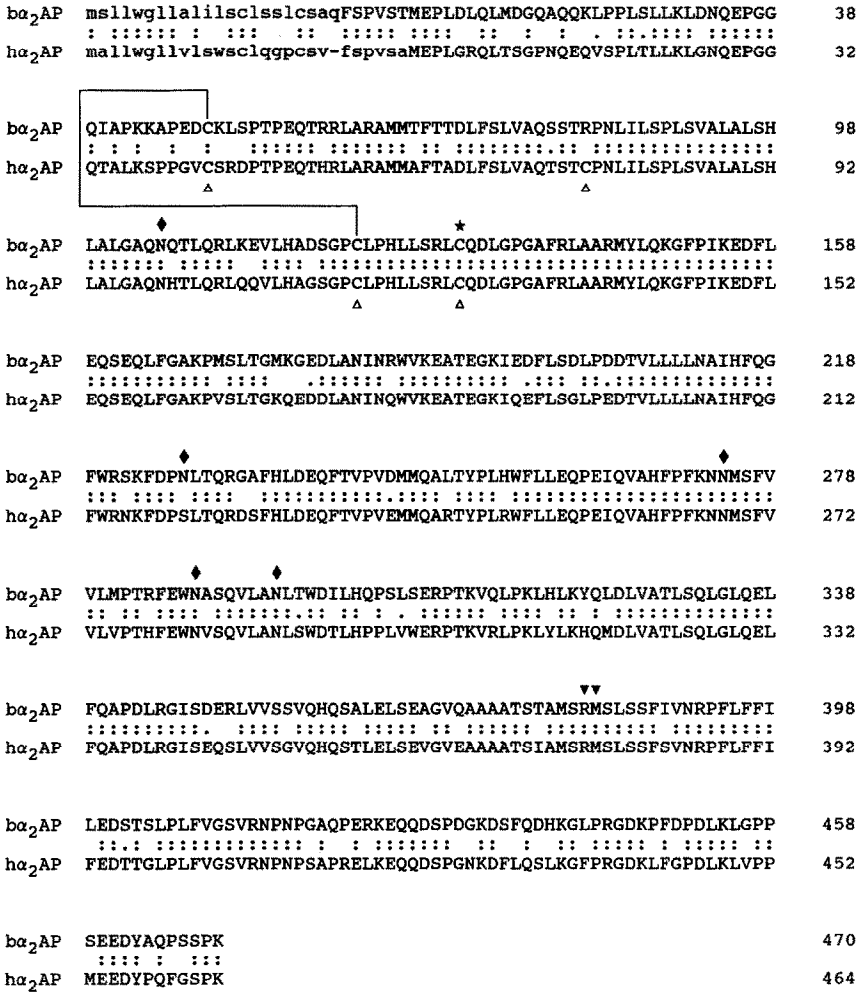

Fig. 2. Alignment of the protein sequences of bovine (b) and human (h) α_2AP. Two dots (:) indicate identical and one dot (·) chemically similar residues. The reactive site P_1–P_1' residues are shown with (▼). The 5 glycosylation sites are shown with (◆). The paired Cys residues in bovine α_2AP are connected by a line, and the unpaired Cys residue is marked with an (★). The 4 Cys residues of human α_2AP are shown with (△).

tryptic digestion of bovine α_2AP were detected in fractions 51–67 (Fig. 3). Peptide 53 gave a single sequence Leu-Xaa-Gln-Asp-Leu-Gly-Pro-Gly-Ala-Phe-Arg corresponding to residues Leu130–Arg140 of bovine α_2AP (Fig. 2). Since the composition of performic acid oxidised peptide 53 (Cya, 0.9; Asx, 1.0; Glx, 1.1; Pro, 1.0; Gly, 2.0; Ala, 1.1; Leu, 1.8; Phe, 1.1; Arg, 0.9) agreed with that expected from residues 130–140, the results indicated that Cys131 is unpaired. Curiously, by conducting hydrolysis of peptide 53 (which had not been treated with performic acid) in the presence of 5% TGA Cys131 was also recovered as cysteic acid, possibly the result of oxidation of cysteine by CNBr as previously described [32]. As expected, peptide 53 did not incorporate radioactivity when treated with ^{14}C-labelled ICH_2CONH_2 at pH 8.0 for 2 h (not shown). Since native bovine α_2AP likewise did not incorporate radioactivity in the same conditions, Cys131 is probably present as a buried unpaired Cys residue in native bovine α_2AP.

The composition of performic acid oxidised peptide 63 (Cya, 1.8; Asx, 2.0; Ser, 1.9; Glx, 2.2; Pro, 2.9; Gly, 1.2; Ala, 1.9; Val, 0.9; Leu, 3.9; His, 2.0; Lys, 2.0; Arg, 1.1) was in agreement with that peptide being a disulfide bridged set consisting of the mates Lys44–Lys50 and Glu113–Arg129. Upon sequence analysis of peptide 63 two sequences, Lys-Ala-Pro-Glu-Asp-Xaa-Lys and Glu-Val-Leu-His-Ala-Asp-Ser-Gly-Pro-Xaa-Leu-Pro-His-Leu-Leu-Ser-Arg, corresponding to Lys44–Lys50 and Glu113–Arg129 respectively, were found in equimolar yield. However, no bis-PTH-Cys_2 was observed in cycle 10, due to its instability during extended Edman degradations.

Rechromatography of peptide 63 (retention time 28.2 min) after reduction and carboxamidation, resulted in separation of the alkylated peptides Lys44–Lys50 (retention time 9.9 min) and Glu113–Arg129 (retention time 30.5 min), as determined by amino acid analysis. From the combined results it is concluded that a disulfide bridge connects Cys49 and Cys122 in bovine α_2AP.

Due to the presence of a slight chymotryptic activity

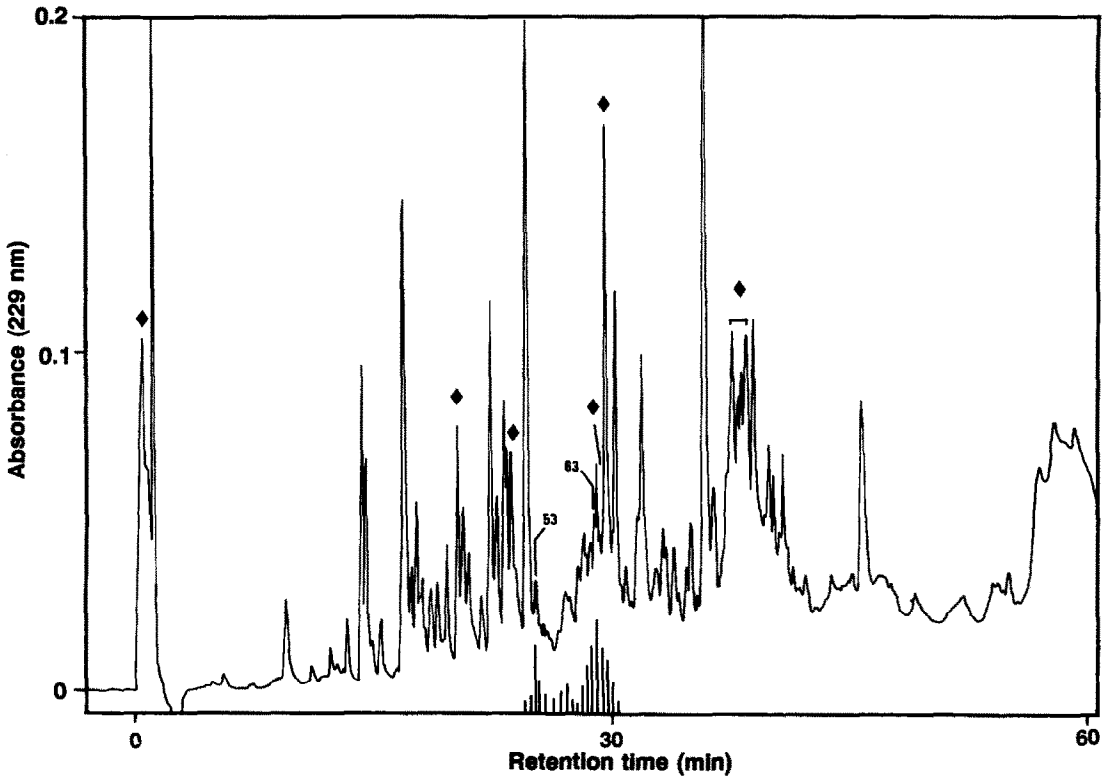

Fig. 3. Reversed-phase HPLC separation of bovine α_2AP tryptic peptides on Nucleosil 100–5 C18. The peptides were eluted (50°C) at a flow rate of 1 ml/min with a linear gradient (0–60% B in 60 min) formed from 0.1% TFA (solvent A) and 90% acetonitrile containing 0.075% TFA (solvent B). Vertical bars indicate the relative amount of cysteic acid. The fractions found to contain glucosamine are marked with (◆). Selected peptides were rechromatographed using shallow gradients of solvent B. The position of peptides 53 with an unpaired cys-residue and peptide 63 with a disulfide bridge are shown.

in the trypsin preparation used, variants of the peptide set represented by peptide 63 were also found in fractions 55–67.

It was suggested previously from compositional analysis that in human α_2AP a disulfide bridge connects Cys76 and Cys116 [15]. Further, it was suggested that Cys43 and Cys125 are disulfide bridged (revised numbering of human α_2AP [17], Figs. 1A and 2). The latter assignment was made by compositional analysis of peptides obtained after reduction and alkylation of insoluble material [15].

The assignment of the bridges Cys76–Cys116 and Cys43–Cys125 in human α_2AP is at variance with the assignment of the bridge Cys49–Cys122 in bovine α_2AP. However, by comparison with the three-dimensional structure of human α_1PI [33] it is found that Cys76 of human α_2AP is located in the loop between helix A and B, and that Cys116 is located in helix D. In effect, these residues are separated by the length of helix A (approx. 40 Å) which precludes connection by a disulfide bond. In contrast, the presence of a disulfide bond between Cys49 and Cys122 in bovine α_2AP is compatible with the three-dimensional structure of α_1PI, where the residue corresponding to Cys49 is located at the N-terminal part of helix A and in proximity to the residue corresponding to Cys122 in helix D. In analogy with bovine α_2AP. Cys43 and Cys116 of human α_2AP could be bridged. Whether Cys76 and Cys125 of human α_2AP are unpaired as Cys131 of bovine α_2AP is not known.

3.5. *Functional aspects of bovine α_2AP*

In human and bovine α_2AP the functionally important RSL sequence, the factor XIIIa cross-linking site and the C-terminal lysine residue are conserved as expected for the cognate inhibitor of plasmin. Indeed, a recent study (S.C., U. Christensen and L.S.-J., submitted) has shown that the association rate for inhibition of bovine plasmin by bovine α_2AP is fast ($k_{ass} = 1.7 \times 10^6$ $M^{-1} \cdot s^{-1}$). The importance of the lysine binding sites in this reaction was also investigated, and a hitherto overlooked role of kringles 4 and/or 5 in bovine plasmin was revealed. In addition to plasma, bovine α_2AP has recently been found in milk (S.C., T. Wiegers, J. Hermansen and L.S.-J., submitted), where it might have a similar function, since it is found to be complexed with midiplasmin (kringles 4 and 5 bridged to plasmin light chain).

Acknowledgements: We thank Drs. Roger Lijnen and Luc Nelles for donating the human α_2AP cDNA clone and Tove Wiegers and Lene Kristensen for technical assistance. This work was supported by the Danish Ministry of Agriculture, the Danish Dairy Board, and the Danish Biomembrane Center.

References

[1] Collen, D. (1976) Eur. J. Biochem. 69, 209–216.
[2] Moroi, M. and Aoki, N. (1976) J. Biol. Chem. 251, 5956–5965.
[3] Müllertz, S. and Clemmensen, I. (1976) Biochem. J. 159, 545–553.
[4] Huber, R. and Carrell, R.W. (1989) Biochemistry 28, 8951–8966.
[5] Kanost, M.R., Sarvamangala, V.P. and Wells, M.A. (1989) J. Biol. Chem. 264, 965–972.
[6] Bao, J.-J., Sifers, R.N., Kidd, V.J., Ledley, F.D. and Woo, S.L.C. (1987) Biochemistry 26, 7755–7759.
[7] Carrell, R.W. and Boswell, D.R. (1986) in: Proteinase Inhibitors (Barrett, A.J. and Salvesen, G.S., Eds.) pp. 403–420, Elsevier, Amsterdam.
[8] Hill, R.E. and Hastie, N.D. (1987) Nature 326, 96–99.
[9] Inglis, J.D. and Hill, R.E. (1991) EMBO J. 10, 255–261.
[10] Patterson, S.D. (1991) Comp. Biochem, Physiol. 100B, 439–454.
[11] Bock, S.C., Wion, K.L., Vehar, G.A. and Lawn, R.M. (1982) Nucleic Acids Res. 10, 8113–8125.
[12] Mejdoub, H., Le Ret, M., Boulanger, Y., Maman, M., Choay, J. and Reinholt, J. (1991) J. Prot. Chem. 10, 205–212.
[13] Mimuro, J., Sawdey, M., Haitiori, M. and Loskutoff, D.J. (1989) Nucleic Acids Res. 17, 8872.
[14] Loskutoff, D.J., Linders, M., Kiejer, J. Veerman, H., Heerikhuizen, H. and Pannekoek, H. (1987) Biochemistry 26, 3763–3768.
[15] Lijnen, H.R., Holmes, W.E., Van Hoef, B., Wiman, B., Rodriguez, H. and Collen, D. (1987) Eur. J. Biochem. 166, 565–574.
[16] Holmes, W.E., Nelles, L., Lijnen, H.R. and Collen, D. (1987) J. Biol. Chem. 262, 1659–1664.
[17] Christensen, S. and Sottrup-Jensen, L. (1992) FEBS Lett. 312, 100–104.
[18] Holmes, W.E., Lijnen, H.R. and Collen, D. (1987) Biochemistry 26, 5133–5140.
[19] Southern, E.M. (1975) J. Mol. Biol. 98, 503–517.
[20] Chirgwin, J.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[21] Sambrook, J, Fritsch, E.F., and Maniatis, T. (1988) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Lab., Cold Spring Harbor, NY.
[22] Barkholt, V. and Jensen, A.L. (1989) Anal. Biochem. 177, 318–322.
[23] Sottrup-Jensen, L. (1993) Biochem. Mol. Biol. Int. 30, 789–794.
[24] Von Heijne, G. (1984) J. Mol. Biol. 173, 243–251.
[25] Kurachi, K., Chandra, T., Friezner, S.J., White, T.T., Marchioni, T.L., Woo, S.C. and Davie, E.W. (1981) Proc. Natl. Acad. Sci. USA 78, 6826–6830.
[26] Sinha, D., Bakhshi, M.R. and Kirby, E.P. (1992) Biochim. Biophys. Acta 1130, 20 –212.
[27] Kimura, S. and Aoki, N. (1986) J. Biol. Chem. 261, 15591–15596.
[28] Sasaki, T., Morita, T. and Iwanaga, S. (1986) J. Biochem. (Tokyo) 99, 1699–1705.
[29] Hortin, G.L., Beverly, L.T. and Fok, K.F. (1989) Throm. Res. 54, 621–632.
[30] Hortin, G.L., Fok, K.F., Toren, P.C. and Strauss, A.W. (1987) J. Biol. Chem. 262, 3082–3085.
[31] Rosenqvist, G.L. and Nicholas, Jr., H.B. (1993) Protein Sci. 2, 215–222.
[32] Smith, S. and Kyte J. (1989) Biochemistry 29, 3469–3482.
[33] Löbermann, H., Tokuoka, R., Deisenhofer, J. and Huber, R. (1984) J. Mol. Biol. 177, 531–556.