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The complete amino acid sequence of bovine milk angiogenin

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The amino acid sequence of angiogenin isolated from bovine milk was deduced by gas-phase sequencing of the protein and its fragments. The protein contains 125 residues and has a calculated molecular mass of 14 577 Da. The sequence is highly homologous (65% identity) to the sequence of human angiogenin, most of the differences being the result of conservative replacements. Like human angiogenin, the bovine protein is also homologous to bovine pancreatic RNase A (34% identity) and the three major active site residues known to be involved in the catalytic process, His-14, Lys-41 and His-115, are conserved. When tested against conventional substrates for RNase A activity, bovine angiogenin displays the same selective ribonucleolytic activity as human angiogenin. The sequence of bovine angiogenin contains the cell recognition tripeptide Arg-Gly-Asp which is not present in the human protein.

Protein sequence; Ribonuclease; Angiogenesis; Angiogenin; (Bovine milk)

1. INTRODUCTION

Human angiogenin, isolated from HT-29 tumor conditioned media [1] and from human plasma [2] by the group of Vallee is able to induce blood vessel growth in the chick chorioallantoic membrane and the rabbit cornea.

The primary structure of this small single-chain protein (M_r = 14400) has significant homology (35% identity) with human pancreatic RNase A [3]. This homology was shown to be biologically functional as human angiogenin displays specific ribonucleolytic activities, catalyzing the cleavage of 28 and 18 S rRNA [4]. Both angiogenic and RNase activities are selectively inhibited by placenta ribonuclease inhibitor [5].

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Abbreviations: RNase, ribonuclease; HPLC, high-pressure liquid chromatography; CNBr, cyanogen bromide; A, C, G and U, adenylic, cytidylic, guanylic and uridylic acids, respectively

Bovine angiogenin was recently isolated from bovine serum [6] and, in 10-fold larger quantities, from bovine milk [7]. We report in this paper the complete amino acid sequence of the bovine protein and its comparison with human angiogenin and bovine RNase A.

2. MATERIALS AND METHODS

2.1. *Materials*

Angiogenin was purified from bovine milk as described [7].

2.2. *Sequence determination*

For reduction and alkylation, 1 nmol angiogenin purified from bovine milk [7] was dissolved in 1-propanol and 0.5 M sodium bicarbonate (1:1, v/v) reduced with 10 μl of a 5% solution of tributylphosphine under N_2 for 1 h at room temperature and then alkylated with 9 μg of iodoacetamide. After 2 h, the product was desalted by reversed-phase chromatography using an octadecylsilane column eluted by two-step gradient.

The *S*-aminocarboxymethylated protein was dissolved in 0.1 M ammonium acetate, pH 4, and digested with *S. aureus* V8 protease at 37°C for 24 h at an enzyme/substrate ratio of 1:50 (w/w).

Cleavage at Asn-Gly bonds was performed by treatment with 2 M hydroxylamine hydrochloride in 0.2 M potassium car-

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bonate containing 6 mg guanidine hydrochloride per μg of sample for 18 h at 23°C. In both cases, the resulting peptides were separated by reversed-phase HPLC (Nucleosil RP C18 column, 0.1% trifluoroacetic acid/acetonitrile, 0–60% gradient over 120 min).

A sample was also submitted to CNBr cleavage for 10 h on glass fiber and the resulting fragments were sequenced without separation. Peptides were sequenced on an Applied Biosystems 470 A gas phase sequencer. PTH amino acid derivatives were identified by HPLC using an Applied Biosystems 120 A on-line PTH analyzer.

2.3. *Ribonucleolytic activity*

Determination of the ribonucleolytic activity of bovine milk angiogenin was carried out according to the procedures described in [4] the following substrates were used: polyribonucleotides [poly(A); poly(U); poly(G); poly(C)]; cytidine cyclic 2',3'-phosphate (C>p); 2',5'-dinucleotides (ApN, CpN, GpN, UpN, where N is A, C, G or V) from Sigma, wheat germ RNA from Calbiochem and 18 and 28 S RNA isolated from calf liver from Pharmacia.

3. RESULTS AND DISCUSSION

3.1. *Sequence determination*

The complete amino acid sequence of bovine angiogenin is shown in fig.1, together with the peptides used for the sequence determination. The primary structure is made devoid of any glycosylation signal sequence (Asn-X-Ser/Thr) and no evidence for posttranslational modification was observed from the sequencing data. At the difference with human angiogenin whose N-terminal amino acid was blocked [3], extensive N-terminal sequencing of the intact protein allowed the determination of 47 residues. This sequence perfectly fits the amino acid sequence recently reported [6] for angiogenin isolated from bovine serum.

The complete sequence was deduced primarily based on the complete set of 8 peptides generated by *S. aureus* V8 protease digestion of the *S*-carboxymethylated protein, which were isolated by reversed-phase HPLC. Except for residues 96–99 which were determined by sequencing fragment HA-2, because peptide V8-4 could not been sequenced further than Tyr 95, all the residues shown in fig.1 were identified at least twice in the sequence analysis. The V-8 peptides were aligned by sequencing overlapping peptides generated by hydroxylamine cleavage of Asn-Gly bonds and CNBr cleavage.

3.2. *Characteristics of the sequence*

Bovine angiogenin contains 125 residues cor-

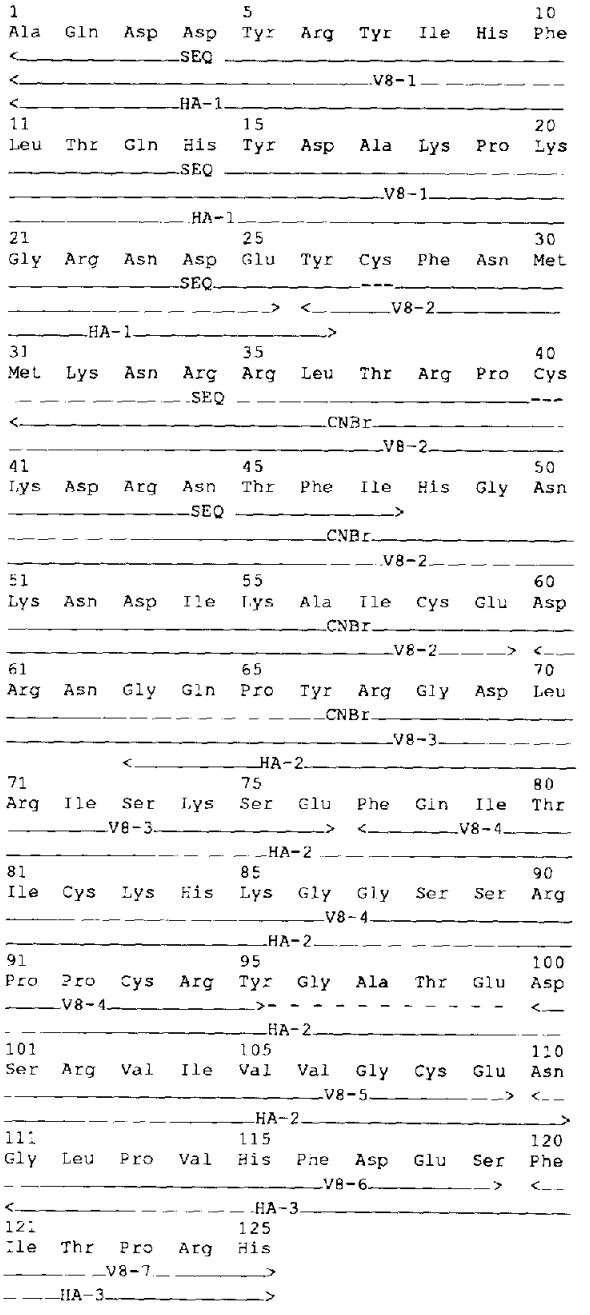

Fig.1. Amino acid sequence of bovine angiogenin and peptides used for sequence determination. SEQ, degradation of the intact protein; V8, peptides from digestion with *S. aureus* V8 protease; CNBr, cyanogen bromide peptides; HA, peptides generated by cleavage with hydroxylamine. Dashed lines denote tracts of sequences inferred from comparison with sequences of different peptides.

responding to a calculated molecular mass of 14577 Da [3]. Comparison of the sequence of human and bovine angiogenin shows (fig.2) a high degree of homology, consistent with usual interspecies variations: 79 of the 123 residues (64% identity) of human angiogenin are conserved, while, in the other positions, most differences are the result from conservative replacements. No gap had to be used for optimal alignment. Contrary to human angiogenin, in which the amino-terminal residue is pyroglutamic acid, in bovine angiogenin an additional alanine residue is found in this position, thus avoiding cyclisation of the glutamine side chain, following the removal of the leader sequence by the signal peptidase. In the C-terminal position, an additional histidine residue is responsible, with the N-terminal alanine residue, for the difference in size between the two proteins. In both angiogenins, the six cysteine residues involved in disulfide bridges are found in the same positions reflecting the highly conserved tertiary structure of these molecules.

As shown previously, one of the most surprising features of the human angiogenin sequence was its homology with the sequence of human pancreatic RNase A [4]. Here we demonstrate (fig.2) that the sequence of bovine angiogenin shares a similar homology (34% identity) with the sequence of bovine RNase A [8]. Human and bovine pancreatic RNases differ, however, from human and bovine angiogenin by the presence of one disulfide bridge (Cys-65–Cys-72 RNase numbering) in addition to the six cysteine residues located at the same positions as in the angiogenin molecule. However, as this bond could be selectively reduced in RNase A without any effect on ribonucleolytic activity [9], it may be concluded that its presence is not critical for the enzymatic activity.

As shown in table 1, among the different residues known to be present in the active region of RNase, most are also found in both angiogenin sequences with the notable exception of Gln-69 which is deleted in the two proteins. Another interesting difference is Phe at position 116 of bovine angiogenin, since the Phe ⟶ Leu substitution in position 120 of RNase was shown to induce a 7-fold decrease in its enzymatic activity [10].

Three amino acid residues are known to be directly involved in the catalytic process of pancreatic RNase A: His-12, His-119 and Lys-41. Interestingly, most of the amino acids surrounding these residues are conserved in the two angiogenins while they differ largely from those found in the corresponding RNase: 11 identical residues among

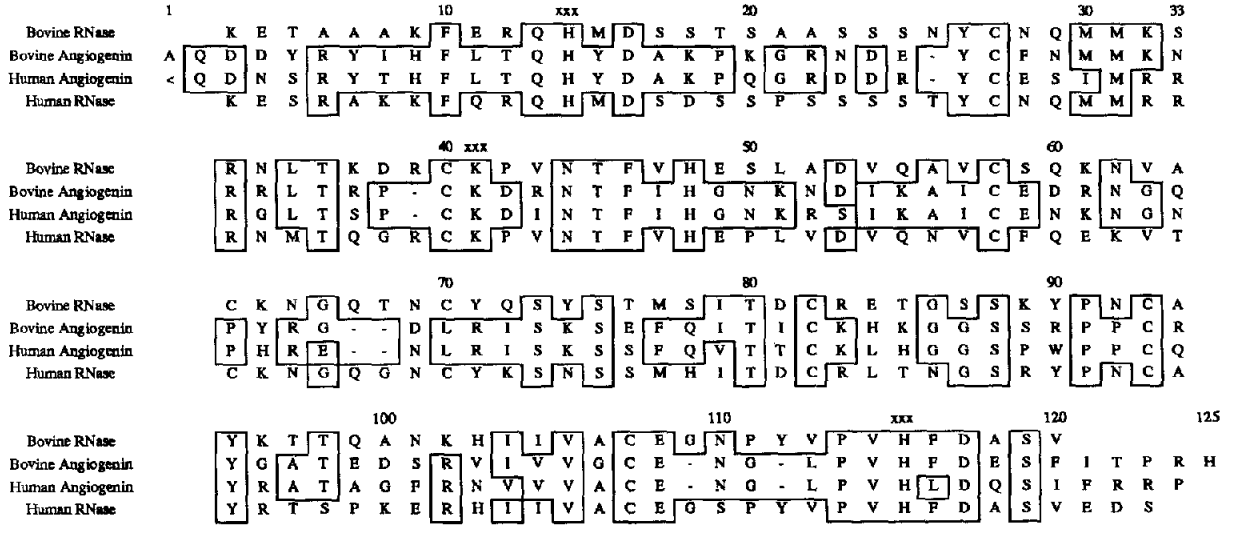

Fig.2. Comparison of amino acid sequences of bovine pancreatic ribonuclease [8], bovine angiogenin, human angiogenin [3], and human pancreatic ribonuclease (Chamoux, M. et al., unpublished). The alignment is numbered according to the bovine angiogenin sequence. Gaps were introduced in four places to retain the highest homologies. Identities between bovine angiogenin and either of the other proteins are boxed. xxx, residues known to be involved in catalysis by bovine RNase.

Table 1

Residues in the active site of RNase and corresponding amino acids in human and bovine angiogenin: RNase residues and proposed roles were compiled by Shapiro et al. [4] from several X-ray and neutron diffraction studies of bovine RNase A and RNase S with inhibitors

RNase residue	Proposed role(s)	Human angiogenin residue	Bovine angiogenin residue
His-12	removes 2'OH proton; H bonds to phosphate	His-13	His-13
His-119	protonates 5'O	His-114	His-115
Lys-41	stabilizes intermediate; H bonds to 2'OH	Lys-40	Lys-41
Gln-11	H bonds from side chain NH to phosphate	Gln-12	Gln-13
Val-43		Ile-42	Arg-43
Asn-44		Asn-43	Asn-44
Thr-45	H bonds from NH and OH to pyrimidine O2 and N3	Thr-44	Thr-45
Gln-69	H bonds from side chain C=O to purine N or O6	deleted	deleted
Asn-71	H bonds from side chain C=O to purine N or O6	Asn-68	Asp-69
Glu-111	H bonds from COO^- to purine N1	Glu-108	Glu-109
Phe-120	H bonds from amide NH to phosphate	Leu-115	Phe-116
Asp-121	H bonds from COO^- to His-119 Im	Asp-116	Asp-117
Ser-123	H bonds from OH to uracil O4	Ser-118	Ser-119

an 11 amino acid stretch (9–19) surrounding His-14 (bovine numbering), 14 identical residues among a 16 amino acid stretch (35–51) surrounding Lys-41, and 10 identical residues among a 12 amino acid stretch (106–119) surrounding His-115.

As these strong local homologies probably reflect similar unusual ribonucleolytic activities as those found with human angiogenin [4], we have determined the catalytic properties of bovine angiogenin. When the protein isolated from bovine milk was tested against polyribonucleotides, dinucleotides and wheat germ RNA no ribonucleolytic activity could be detected. However, when the hydrolysates of 18 and 28 S RNA isolated from calf liver were analysed by agarose gel electrophoresis under denaturing conditions degradation products were detected indicating an endonucleolytic activity of angiogenin. These results are in full agreement with those reported by Bond et al. [6] for the protein isolated from bovine serum and confirm that bovine angiogenin has the same enzymatic activities as human angiogenin.

A major difference between bovine angiogenin and the three other related proteins is the presence in position 67 of an Arg-Gly-Asp sequence. This tripeptidic sequence represents a recognition site in fibronectin and other adhesive proteins from Arg-Gly-Asp directed adhesion receptor localized in endothelial cells [11]. Our preliminary results, which show that bovine angiogenin induces the proliferation of bovine brain capillary endothelial cells at concentrations ranging from 10 to 100 ng, suggest that this tripeptide could be involved in the interaction of bovine angiogenin with the endothelial cells (Chamoux, M. et al., unpublished).

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