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The Complete Amino Acid Sequence of Bovine Antithrombin (ATIII)

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Bovine antithrombin (ATIII) is a glycoprotein of M_r 56,600. Its primary structure was established using peptide sequences from five different digests. Bovine ATIII exhibits four glycosylation sites as well as human ATIII. The primary structures of bovine and human ATIII were compared: all the residues required for the integrity of the heparin-binding domain are strictly conserved. However, there are differences in the secondary structures of both proteins, bovine and human ATIII.

KEY WORDS: bovine antithrombin (ATIII); amino acid sequence; homologies with human ATIII; secondary structure.

1. INTRODUCTION

Bovine antithrombin (ATIII), a serpin protein, is a glycoprotein of M_r 56,600 found in mammalian plasma (Kurachi *et al.*, 1976). ATIII is the principal protease inhibitor of the coagulation cascade, in particular it specifically inhibits thrombin (Travis and Salvensen, 1983) and factors IXa, Xa, XIa, and XIIa (Rosenberg, 1977). The inhibition of proteases by ATIII is relatively slow in the absence of heparin, a sulfated polysaccharide, but is enhanced as much as 10,000-fold in its presence (Jordon *et al.*, 1980). It has been shown that a specific sequence is responsible in heparin for the binding and activation of ATIII (Choay *et al.*, 1983). Amino acid and carbohydrate composition of bovine ATIII revealed striking similarities to human ATIII, while immunological analyses failed to demonstrate any cross-reactivity (Nordenman *et al.*, 1977). The formation of the inactive complex between antithrombin and thrombin is accompanied by the production of a proteolytically modified form of antithrombin. It has been shown that the thrombin cleavage site of bovine ATIII is at an Arg–Ser bond near the C-terminal end of the chain (Jörnvall *et al.*, 1979). The cleavage site in human ATIII was also identified between Arg393–Ser394 [i.e., at a position homologous to that observed for the bovine protein (Björk *et al.*, 1981].

The present paper deals with the determination of the complete amino acid sequence of bovine ATIII. This allowed a comparison with the known sequences of human ATIII (Chandra *et al.*, 1983) and α1-antitrypsin, a key plasma serpin (Carrell *et al.*, 1982). Besides the knowledge of the primary structure of bovine ATIII is a necessary step in the elucidation of its complete tertiary structure, which has been undertaken at our Institute in Dr. Moras' laboratory (Samama *et al.*, 1989; Delarue *et al.*, 1990).

2. MATERIALS

Bovine ATIII was prepared as described (Thaler and Schmer, 1975) and provided by the Choay–Sanofi laboratory (Gentilly, France). It purity was checked by disc-electrophoresis. Pepsin and endoproteinase Asp-N were obtained from Boehringer (Mannheim, Germany) and *Staphylococcus aureus* V8 protease

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from Miles Laboratory (Slough, United Kingdom). Cyanogen bromide was purchased from Merck Schuchardt (Hohenbrunn, Germany), 2-(2-nitrophenylsulfenyl)-3-methyl-3'bromoindolenine (BNPS-skatole) from Pierce (Rockford, United States). All chemicals used on HPLC columns were analytical grade products of Merck (Darmstadt, Germany) and of Applied Biosystems (Roissy, France) for the Edman degradation. Chromatography of peptides was performed on TSK G2000 SW column (Pharmacia LKB, Uppsala, Sweden) and on a HPLC apparatus (Millipore-Waters Associates, United States), using LichroCart superspher 100 RP-18 (Merck), Delta Pak C18 microbore (Waters), and Ultrabase C4 (SFCC, Neuilly, France) columns.

3. METHODS

3.1. Alkylation of Cys Residues (Okazaki *et al.*, 1985)

Before enzymatic or chemical cleavage ATIII was alkylated. Samples of ATIII (1.4 mg in 125 μl Tris-HCl, 50 mM, NaCl 150 mM, *p*H 7.4) were denatured in 375 μl guanidium-HCl 8 M, 125 μl Tris-HCl 1 M, EDTA 4 mM, *p*H 8.5, and 2.5 μl 2-mercaptoethanol 10% (v/v) during 2.5 hr at 25°C under N_2. S-pyridylethylation of cysteine residues was performed by adding 4 μl of vinylpyridine. The reaction was stopped after 2 hr by lyophilization.

3.2. Enzymatic Cleavage

3.2.1. Digestion with Pepsin

One milligram of ATIII was dissolved in 400 μl of 20% HCOOH and hydrolyzed with a protease/protein ratio of 1/20 (w/w).

3.2.2. Staphylococcus aureus V8 Protease Digestion (Houmard and Drapeau, 1972)

Samples of ATIII (2 mg) were hydrolyzed in 450 μl N-methylmorpholine buffer 0.1 M, *p*H 8, at 37°C for 20 hr with V8 protease 3% (w/w) added at 0 hr and 6 hr.

3.2.3. Endoproteinase Asp-N Digestion (Drapeau, 1980)

Digestion with endoproteinase Asp-N was performed with 0.6 mg ATIII dissolved in 350 μl ammonium acetate 50 mM, *p*H 8. The protease/protein ratio was 1/10 (w/w), and the reaction was allowed to proceed for 5.5 hr at 37°C.

3.3. Chemical Cleavage

3.3.1. Cleavage of ATIII by BrCN (Gross and Witkop, 1962)

One milligram ATIII was dissolved in 600 μl of 70% HCOOH and 10 mg of BrCN (500-fold excess over methionine residues) were added in two steps at 0 hr and after 24 hr. The reaction was carried out at 25°C in the dark for 48 hr and was stopped by the addition of water.

3.3.2. Cleavage of ATIII by 2-(2-Nitrophenylsulfenyl-)3-methyl-3'-bromoindolenine (BNPS-skatole)

The cleavage of ATIII by BNPS-skatole was done as described earlier (Fontana, 1972) with slight modifications. 2.5 mg of ATIII was dissolved in 200 μl of CH_3COOH 50% (v/v). The cleavage was started at 25°C by adding 8 mg of BNPS-skatole (200-fold excess over tryptophane residues) in 500 μl of a 1 : 1 mixture of acetic acid and ethanol. The solution was protected from light. After 30 hr of incubation, the excess of BNPS-skatole was extracted by benzene.

3.4. Isolation of Peptides, Amino Acid Analysis, and Sequencing

A combination of gel filtration on TSK column and chromatography on HPLC column was used to separate the peptides obtained from the different cleavages. Large peptides obtained by BNPS-skatole cleavage were fractionated on a TSK G2000 SW column (60 × 0.75 cm) in a volatile buffer (TFA 0.05%, H_2O 50%, acetonitrile 50%, (v/v)) eluted at a flow rate of 24 ml/hr). Peptides were monitored at 220 nm. All the other peptides were purified using different commercially available columns (see Materials) with linear gradients of volatile buffers (TFA 0.05% and acetonitrile).

Amino acid analyses were performed on a Pico-Tag analyzer (Waters) and on a 420A-130A Derivatization and Analysis System (Applied Biosystems). Protein and peptides were sequenced by automated Edman's degradation, using an Applied Biosystems 470A protein sequencer equipped with a PTH 120A analyzer (Hewick *et al.*, 1981).

4. RESULTS AND DISCUSSION

The results of our study are summarized in Fig. 1, which shows (i) the complete amino acid sequence, and (ii) the peptides covering this sequence. Five different digests were used to achieve maximum coverage. Bovine ATIII consists of a single polypeptide chain of 433 amino acids, giving a calculated M_r of 49,126.

4.1. N-Terminal Sequence of Alkylated ATIII

The enzyme was alkylated as described in Methods and dialyzed against water. We could establish unambiguously the N-terminal sequence: His1 to Ala31. There is a little microheterogeneity observed at the N-terminal part of ATIII: indeed, we also identified the sequences starting at Arg2 and Ser3, but only to a ratio of 10% as compared to the major N-terminal sequence: His1 → Ala31. This observation could also be made with BrCN peptides accounting for the N-terminal part of ATIII.

4.2. N-Terminal Sequence of Nonalkylated Crystallized ATIII

The enzyme was not alkylated and dialyzed, but directly subjected to the sequencer. Two major sequences could be aligned: (i) His1 to Ala31 and (ii) Leu396 to Asp433, the latter corresponding to the C-terminal part of ATIII. We could determine that at least 80% of ATIII was cleaved in the crystal. It must be stressed that the cleavage site observed in the crystal Ser395-Leu396 is next to thrombin cleavage site of bovine ATIII: Arg394-Ser395 (Jörnvall *et al.*, 1979). Indeed, in redissolved crystals, bovine ATIII exhibits no activity at all (Samama *et al.*, 1989). The respective amounts of the two N-termini species determined by sequencing agree very well with each other, within the limits of error, with the finding that no detectable activity was measurable in redissolved crystals. As to alkylated ATIII (i.e., not crystallized), no cleavage should occur at position Ser395-Leu396, as found in the crystal, since the protein displays normal activity against thrombin (Samama *et al.*, 1989).

4.3. Determination of the Sequence of ATIII

In order to determine the sequence and the alignment of the peptides within ATIII, we used various chemical and enzymatic cleavages of ATIII. In each case, only those peptides obtained in sufficient yields were purified and sequenced, thus avoiding the time-consuming process of purifying all the peptides. The combined chemical and enzymatic cleavages gave us all the necessary overlapping fragments.

Only a few peptides available for sequencing resulted from the peptic cleavage, but they were of great help to align other peptides—for instance, the peptic peptides 64-88, 275-284, and 364-372. Some peptides arising from BNPS-skatole cleavage could be isolated on a TSK column. We sequenced an interesting overlapping fragment from residue: Leu309 to residue Arg325, and also some minor peptides due to partial acid hydrolysis during BNPS-skatole cleavage. The knowledge of their sequence enabled us to establish unambiguously the alignment of the fragments encompassing the following residues: 149-161, 153-166, 373-385, 393-415, and 396-415. The last one is of particular interest: indeed, as seen above during the crystallization process of bovine ATIII, most if not all enzyme molecules were cleaved between Ser395 and Leu396. This bond must be extremely labile in the ATIII sequence.

4.4. Cyanogen Bromide Fragments

All the cyanogen bromide fragments could be isolated and sequenced. There were additional cleavages, respectively, at Trp50, Trp190, Trp226, and Ala392. Among these, it must be mentioned that cleavage at Ala392 also occurs with *Staphylococcus aureus* V8 protease, strongly suggesting that this area of ATIII is highly sensitive to proteolysis. Six cyanogen bromide fragments were used to overlap the following regions: residues 22-50, 105-142, 227-249, 254-282, 283-304, and 340-365. Unfortunately, we never could sequence fragment 227-249 up to its C-terminal residue Met253, the material being completely lost on the sequencer at position Val250. This is probably due to the higher degree of hydrophobicity of this C-terminal part.

4.5. *Staphylococcus aureus* V8 Digestion

As seen in Fig. 1, peptides from this last set are evenly distributed along the polypeptide chain. Thus, they were used to establish the sequence and to get overlapping fragments. Most of them are due to cleavage of glutamoyl bonds and some arising from splitting of aspartoyl bonds. This is consistent with the well-known enzyme specificity (Houmard and

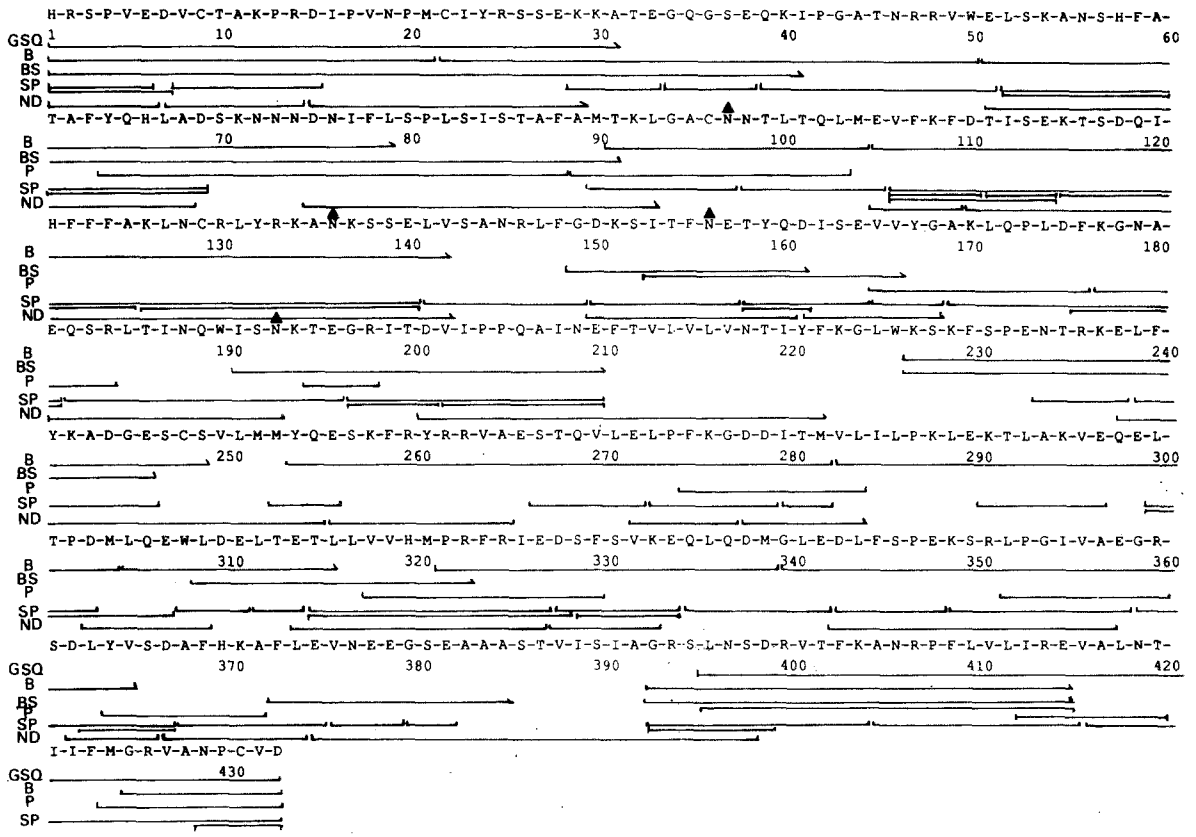

Fig. 1. The complete primary structure of bovine ATIII. GSQ: sequencing of the N- and C-terminal regions of bovine ATIII in an Applied Biosystems gas-phase sequencer. Peptic peptides are abbreviated P, cyanogen bromide fragments B, BNPS-skatole fragments BS, those arising from staphylococcal aureus V8 protease digestion SP and those from endroproteinase Asp-N cleavage DN. Symbol ▲ indicates the glycosylation sites of bovine ATIII. ——→ means that the sequencing proceeded unambiguously to this residue.

Drapeau, 1972). However, among the peptides identified in our digest, some are obviously due to secondary cuts: 5 after an Ala residue, 3 after a Gly residue, 2 after a Met residue, and, more surprisingly, 1 after Lys404 and 1 after Asn97, which is glycosylated.

4.6. Endoproteinase Asp-N Cleavage

This endoproteinase specifically splits aspartyl bonds at the NH_2 site of Asp. We also isolated and purified peptides beginning with Glu. All the peptides were purified on a HPLC column (Fig. 2). This enzymatic digestion was used in the final stage of our work and enabled us to confirm sequences and to establish definitely overlapping fragments. In particular, we purified three peptides that had never been isolated and sequenced before. Thus, we sequenced unambiguously the fragment from residue 238 to residue 255. Two other peptides of ATIII were only obtained by this endoproteinase cleavage, namely fragments 201-230 and 375-398.

4.7. Glycosylation Sites

With regard to the structural feature of human ATIII, an important point concerns the localization of the glycosylation sites. It is well-known that the tripeptide N-X-T/S represents the code sequence for N-glycosidically linked sugar groups. In bovine ATIII, there are four code sequence: N-N-T (97-99), N-K-S (136-138), N-E-T (156-158) and N-K-T (193-195). According to our sequence studies, all of them should be glycosylated: indeed, whenever an asparagine is glycosylated, there is a blank at the corresponding sequencing step, as already reported by Metz-Boutigue *et al.* (1980). These results agree well with those of human ATIII, where the same

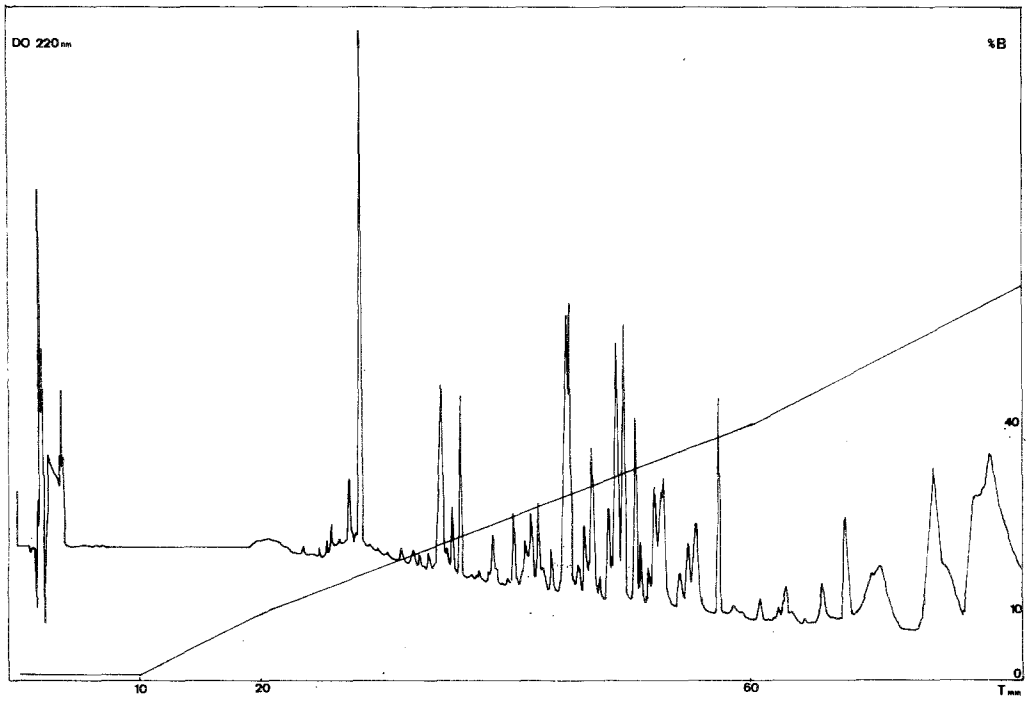

Fig. 2. Separation of the peptides arising from endoproteinase Asp-N digestion of bovine ATIII. Reverse-phase HPLC on a RP-18 Superspher 100 Merck, 24.4 × 0.4 cm. Flow-rate 0.6 ml/min at 25°C. Solvent A: 0.05% trifluoroacetic acid; solvent B: acetonitrile. The gradient is indicated by the line. Monitoring at 220 nm, detector scale: 0.2. Sample injected, 5 nmol of digested bovine ATIII.

residues are fully glycosylated (Franzen *et al.*, 1980; Brennan *et al.*, 1987). Secondary structure predictions of bovine ATIII suggested that the prosthetic sugar groups are linked to asparagine residues located either within, or at the end, or beginning of β turns (see Fig. 4).

4.8. Homologies with Human ATIII

The primary structures of bovine and human ATIII were compared (Fig. 3). Alignment of the two proteins is based on the BestFit program contained in the sequence analysis software package available from the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). They share a high percentage of identity, about 89%, and about 95% of similarity. Bovine ATIII contains 433 residues (i.e., one residue more than human ATIII). This additional Glu residue is located at position 6 in bovine ATIII. Table I shows the different types of substitution observed between human and bovine ATIII. There are some striking features: for instance, there are rather conservative changes like Arg (in human ATIII) to His (in bovine ATIII), but also nonconservative ones: Asn to Asp and specially Gly to Arg or Ser and Pro to Ser, Leu or Ala. As Gly and Pro are considered to play a role in the formation of β turns, such replacements might provoke some changes in the secondary structure as will be discussed later.

In particular, selective chemical modification at Lys114 (Liu and Chang, 1987), Lys125 (Liu and Chang, 1987; Peterson *et al.*, 1987), and Trp49 (Blackburn *et al.*, 1984), and single amino acid replacement at Pro41 (Chang and Tran, 1986) or Arg47 (Koide *et al.*, 1984) were shown to impair the heparin cofactor activity of human ATIII. Chang (1989) demonstrated that Lys107, Lys125, and Lys136 are situated within the heparin-binding site of human ATIII and that binding of heparin to ATIII causes a conformational change of ATIII that leads to the exposure of Lys236. Furthermore, in human ATIII, the disulfide bridge Cys8-Cys128 is required for the integrity of the heparin-binding domain (Sun and Chang, 1989). It must be pointed out that all these residues are strictly conserved in bovine ATIII (see Fig. 3).

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Bovine ATIII   1 HRSPVEDVCTAKPRDIPVNPMCIYRSSEKKATEGQGSEQKIPGATNRRVW 50
Human ATIII    1 HGSPV.DICTAKPRDIPMNPMCIYRSPEKKATEDEGSEQKIPEATNRRVW 49

              51 ELSKANSHFATAFYQHLADSKNNNDNIFLSPLSISTAFAMTKLGACNNTL 100
              50 ELSKANSRFATTFYQHLADSKNDNDNIFLSPLSISTAFAMTKLGACNDTL 99

             101 TQLMEVFKFDTISEKTSDQIHFFFAKLNCRLYRKANKSSELVSANRLFGD 150
             100 QQLMEVFKFDTISEKTSDQIHFFFAKLNCRLYRKANKSSKLVSANRLFGD 149

             151 KSITFNETYQDISEVVYGAKLQPLDFKGNAEQSRLTINQWISNKTEGRIT 200
             150 KSLTFNETYQDISELVYGAKLQPLDFKENAEQSRAAINKWVSNKTEGRIT 199

             201 DVIPPQAINEFTVLVLVNTIYFKGLWKSKFSPENTRKELFYKADGESCSV 250
             200 DVIPSEAINELTVLVLVNTIYFKGLWKSKFSPENTRKELFYKADGESCSA 249

             251 LMMYQESKFRYRRVAESTQVLELPFKGDDITMVLILPKLEKTLAKVEQEL 300
             250 SMMYQEGKFRYRRVAEGTQVLELPFKGDDITMVLILPKPEKSLAKVEKEL 299

             301 TPDMLQEWLDELTETLLVVHMPRFRIEDSFSVKEQLQDMGLEDLFSPEKS 350
             300 TPEVLQEWLDELEEMMLVVHMPRFRIEDGFSLKEQLQDMGLVDLFSPEKS 349

             351 RLPGIVAEGRSDLYVSDAFHKAFLEVNEEGSEAAASTVISIAGRSLNSDR 400
             350 KLPGIVAEGRDDLYVSDAFHKAFLEVNEEGSEAAASTAVVIAGRSLNPNR 399

             401 VTFKANRPFLVLIREVALNTIIFMGRVANPCVD 433
             400 VTFKANRPFLVFIREVPLNTIIFMGRVANPCVK 432
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Fig. 3. Comparison of the primary structures of bovine ATIII and human ATIII. Bovine ATIII, upper line; human ATIII lower line.

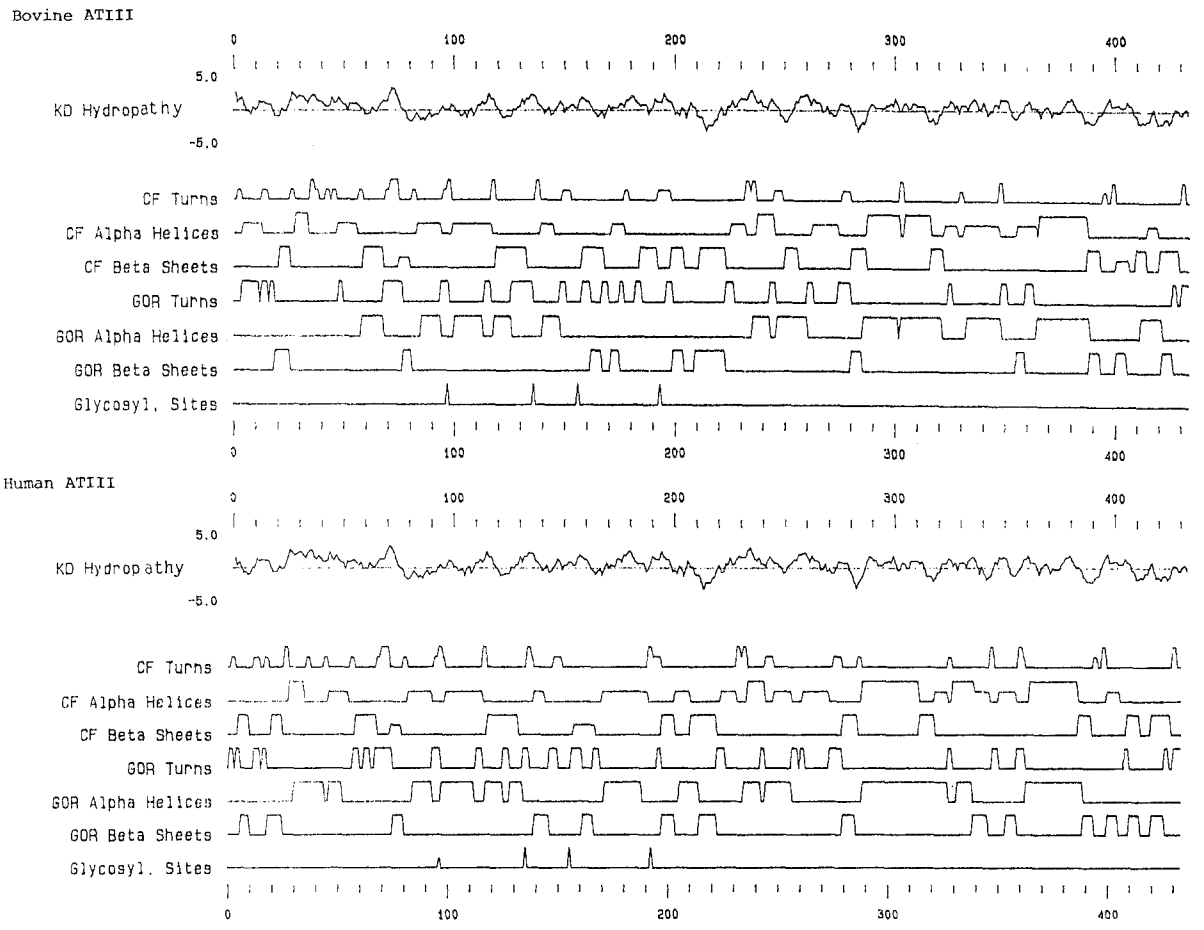

Fig. 4. Secondary structures of bovine and human ATIII. Abbreviations used: CF (Chou-Fasman), GOR (Garnier, Osguthorpe, Robson), KD (Kyte-Doolittle).

Table I. Substitution

Hum ATIII	Bov ATIII
G	R, S
I	V
M	V, T, L
P	S, L, A
D	G, N, S
E	Q, G, D, T
R	H
T	A
Q	T
K	E, Q, R, D
L	I, V, F
A	L, T, V
V	I, M, E, S
S	P, L, T
N	D
F	L

4.9. Structural Homologies Between Bovine ATIII and Human α1-Antitrypsin

Another key plasma serpin is α1-antitrypsin, which is an efficient inhibitor of trypsin, but its prime physiological role is as an inhibitor of the elastase released by leukocytes. The substitution of Met358 in its reactive center by an Arg converts the protein from an inhibitor of elastase to a highly effective inhibitor of thrombin (Owen *et al.*, 1983). Thus, it is tempting to compare its structure to that of bovine ATIII. Huber and Carrell (1989) noted that in the serpin family there are conserved residues important for the structural integrity. As to α1-antitrypsin, they established four segments constituting the core and spine of the molecule. These segments share more than four strictly conserved residues with homologous human and bovine ATIII segments (Table II), suggesting that these segments could also play an important role in the spatial structure of bovine ATIII. This should be verified in a near future when the crystallographic structure of bovine ATIII is known to high resolution (L. Mourey, personal communication).

4.10. Secondary Structure of Bovine ATIII

The secondary structure was predicted and the hydropathy plot was calculated using the above BestFit program (Devereux *et al.*, 1984) and the PEPPLOT program from Gribskov *et al.* (1986). Figure 4 shows the predictions made using the methods of Chou and Fasman (1978) and Garnier *et al.* (1978) for the secondary structure and Kyte and Doolittle (1982) for the hydropathy calculation. Both proteins, bovine (upper section of Fig. 4) and human ATIII are compared. Their hydropathic profiles are practically identical. However, it must be stressed that there are noticeable differences in their secondary structures as far as β turns, α helices, and β sheets are concerned, whatever the prediction method used (CF or GOR, see Fig. 4). Indeed, this result goes along with our above observation that the majority of the replacements between human and bovine ATIII are nonconservative ones.

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Table II. Structural Homologies Between Human α_1-Antitrypsin and Bovine and Human ATIII

α_1-Antitrypsin	53	S	P	V	S	I	A	T	A	F	A	M	L	S	L	G T 68
Bovine ATIII	80	S	P	L	S	I	S	T	A	F	A	M	T	K	L	G A 95
Human ATIII	79	S	P	L	S	I	S	T	A	F	A	M	T	K	L	G A 94
α_1-Antitrypsin	181	V	F	A	L	V	N	Y	I	F	F	K	G	K	W	194
Bovine ATIII	215	V			L	V	N	T	I	Y	F	K	G	L	W	226
Human ATIII	214	V			L	V	N	T	I	Y	F	K	G	L	W	225
α_1-Antitrypsin	369	P	F	V	F	L	M	I	E	Q	N	378				
Bovine ATIII	408	P	F	L	V	L	I	R	E	V	A	417				
Human ATIII	407	P	F	L	V	F	I	R	E	V	P	416				
α_1-Antitrypsin	380	K	S	P	L	F	M	G	K	V	V	N	P	391		
Bovine ATIII	419	N	T	I	I	F	M	G	R	V	A	N	P	430		
Human ATIII	418	N	T	I	I	F	M	G	R	V	A	N	P	429		

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