THE THROMBIN CLEAVAGE SITE IN BOVINE ANTITHROMBIN

Hans JÖRNVALL, Wayne W. FISH[+] and Ingemar BJÖRK[+*]

Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm and [+]*Department of Medical and Physiological Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, The Biomedical Centre, Box 575, S-751 23 Uppsala, Sweden*

Received 22 August 1979

1. Introduction

It has recently been shown that the formation of the inactive complex between thrombin and the plasma protease inhibitor antithrombin is accompanied by the production of a proteolytically modified form of antithrombin [1]. This modified inhibitor has lost all or most of the ability to inhibit thrombin and has a reduced affinity for heparin. In urea/polyacrylamide gel electrophoresis of the modified antithrombin, only two polypeptide fragments with apparent molecular weights of ~50 000 and 5000 were seen [1]. This indicated that the modification by thrombin which produces this inactive antithrombin species is restricted to the scission of one or at most a few adjacent peptide bonds near one of the ends of the antithrombin polypeptide chain. The two peptide chain segments which result from this cleavage are held together by one or more disulfide bonds. Subsequent investigations showed that a protein which electrophoretically was indistinguishable from the free thrombin-modified form of antithrombin could be released from the antithrombin–thrombin complex by various procedures [2,3]. It was therefore suggested that the thrombin-sensitive bond or bonds of antithrombin are located in the active site region of the molecule. This proposition is further supported by the results presented here. We show that the free modified form of bovine antithrombin has only a single thrombin cleavage site, which is at an Arg–Ser bond near the C-terminal end of the chain. This thrombin cleavage site is identical to the site of chain cleavage in the modified form of the inhibitor which can be released from the antithrombin–thrombin complex.

* To whom correspondence should be addressed

2. Materials and methods

Free thrombin-modified bovine antithrombin and bovine antithrombin–thrombin complexes were prepared as described in [1,3].

The antithrombin–thrombin complex was dissociated by 1 M hydroxylamine (pH 7.6) in 0.1% sodium dodecyl sulphate under non-reducing conditions [3,4]. The modified antithrombin released from the complex by this treatment was separated from undissociated complex and liberated thrombin by gel chromatography on Sephacryl S-200 in 0.05% dodecyl sulphate [3].

Modified antithrombin, free or released from the antithrombin–thrombin complex, was reduced at 5–10 g protein/l by 10 mM dithiothreitol in 0.1 M Tris buffer (pH 8.4) in the presence of dodecyl sulphate. The amount of dodecyl sulphate used was 3-times the weight of the protein. After reduction overnight, the solution was carboxymethylated in the dark by the addition of iodo-[1-^{14}C]acetate (spec. act. ~4000 dpm/nmol) to 25 mM final conc. Excess iodoacetate was destroyed after 1 h by additional dithiothreitol, and the solution was dialysed overnight against 0.05 M Tris buffer (pH 7.5) containing 0.1 M NaCl, 0.05% dodecyl sulphate and 5 mM β-mercaptoethanol.

Samples for amino acid analyses were hydrolyzed in evacuated tubes for 24 h or 72 h at 110°C with 6 M HCl containing 0.1% phenol. Amino acids were

determined on a Beckman 121 M amino acid analyzer.

N-Terminal amino acids were determined with the dansyl method, and the dansyl derivatives were identified on polyamide layers in 4 chromatographic systems, as in [5].

A Beckman 890C instrument was used for liquid-phase sequencer analyses. Polybrene [6,7] was added as a carrier, together with glycine, and 2 cycles were then performed before peptide application. A 1 M Quadrol protein program was used for the large peptide of modified antithrombin, and a 0.1 M Quadrol peptide program was employed for the small peptide. High-performance liquid chromatography of phenylthiohydantoin derivatives was carried out in a Hewlett-Packard 1084 B instrument on a RP-8 reverse phase column with a gradient of 0.01 M sodium acetate (pH 4.5) and acetonitrile [8]. Thin-layer chromatography was done on fluorescent-coated silica gels in xylene:isopropanol (7:2, v/v). After a first identification of the derivatives, their positions were additionally checked by staining the plate with 0.1% ninhydrin in ethanol:collidine (95:5, v/v) [9].

3. Results and discussion

The large and small polypeptide chains of either free modified bovine antithrombin, or the modified form of the protein which was released from the antithrombin–thrombin complex and subsequently purified, were separated by gel chromatography. The reduced and [^{14}C]carboxymethylated proteins (5–10 mg) were applied to a 1.6 × 90 cm column of Sephacryl S-200 in 0.05 M Tris buffer (pH 7.5) containing 0.1 M NaCl and 0.05% sodium dodecyl sulphate. Three radioactive peaks were obtained by this procedure. Two of these appeared at the expected elution positions of polypeptides with mol. wt ~50 000 and 5000. Moreover, the material in these peaks migrated as the large and small polypeptides of modified antithrombin, respectively, in dodecyl sulphate/polyacrylamide and urea/polyacrylamide gel electrophoresis [1,3]. A third minor gel chromatographic peak eluted at the total volume of the column and contained no material that could be identified in either type of electrophoresis. It was therefore concluded that this peak consisted only of residual reaction products between iodoacetic acid and dithiothreitol. The two polypeptide-containing fractions were pooled separately, dialysed for 24 h against several changes of water and lyophilised. The lyophilised material may have contained some residual dodecyl sulphate.

Amino-acid analyses of the isolated chains of free modified antithrombin (table 1) show that the sum of the compositions of the two chains agrees remarkably well with the composition of intact antithrombin. The small differences that are seen are within the variation expected from the experimental conditions, especially since the isolated chains were analysed after only a single time of hydrolysis (24 h), whereas the values for intact antithrombin were derived from analyses after different times of hydrolysis (24 h and 72 h). Consequently, the hydrolytic destruction and slow release of certain amino acids are not compensated for in the values for the sum of the chains. The excellent agreement between the amino acid composition of the intact molecule and the sum of the compositions of the two chains suggests that, together, the isolated large and small fragments of modified antithrombin account for the whole antithrombin polypeptide chain. This supports the conclusion from the previous electrophoretic studies that thrombin cleaves the antithrombin molecule at one site only [1,3].

The thrombin cleavage site in free modified antithrombin was localised by N-terminal sequence analyses of the isolated large and small polypeptide chains in a liquid-phase sequencer. Initial coupling in the analyses was ~50% and the repetitive yields ~94%. The results are shown in table 2, which also includes a comparison with the known structures of the homologous parts of intact human antithrombin [10], and with the known N-terminal sequence of the bovine protein [11]. The N-terminal sequence of the large chain of free modified bovine antithrombin was found to be identical to the N-terminal sequence of the intact protein (table 2). Moreover, the N-terminal sequence of the small chain of the modified bovine protein is clearly homologous with the residues which start at the position with tentative no. 386 in the intact human protein (table 2). This shows that free modified antithrombin is produced by thrombin cleavage in the C-terminal end of the antithrombin polypeptide chain. This cleavage liberates Ser-386 as the N-terminus of the small fragment and residue 385, which is Arg in the human [10] and presumably also

Table 1
Amino acid compositions of the large and small polypeptide chains of free modified bovine antithrombin and of intact antithrombin (mol amino acids/mol polypeptide)[a]

Residue	Large chain[b]	Small chain[b]	Sum of large and small chains	Intact antithrombin[c]	Difference between sum of chains and intact antithrombin
CysCm	4.0	0.9	4.9	6.5[d]	−1.6
Asx	42.5	5.4	47.9	47.4	0.5
Thr	23.3	2.0	25.3	26.1	−0.8
Ser	30.7	3.4	34.1	36.2	−2.1
Glx	54.9	1.3	56.2	53.8	2.4
Pro	18.0	2.0	20.0	18.2	1.8
Gly	18.6	2.3	20.9	17.8	3.1
Ala	27.4	2.9	30.3	30.3	0
Val	26.9	3.9	30.8	29.7	1.1
Met	10.0	0.7	10.7	10.7	0
Ile	23.9	1.7	25.6	23.6	2.0
Leu	34.3	3.1	37.4	40.8	−3.4
Tyr	10.7	–	10.7	10.0	0.7
Phe	25.2	2.6	27.8	27.0	0.8
Lys	32.4	1.5	33.9	32.0	1.9
His	5.7	0.1	5.8	6.3	−0.5
Arg	21.5	2.7	24.2	24.0	0.2
Sum	410.0	36.5	446.5	440.4	−8.4 +14.5

[a] The values given are based on mol. wt 56 000 (including 9.8% carbohydrate) for the intact molecule [13] and on fragment sizes estimated from the cleavage site identified in this work. Tryptophan was not determined
[b] Determined after a 24 h hydrolysis
[c] Average of 3 determinations, each after a 24 h and a 72 h hydrolysis. Corrected for destruction of Thr and Ser. 72 h value used for Val and Ile
[d] Value for Cys, determined as Cys–SO_3H after performic acid oxidation

in the bovine protein, as the C-terminus of the large fragment. The position of the cleavage site is consistent with the sizes of the fragments (see above and [1]), with the compositions of the fragments (table 1) in relation to the sequence of the intact protein [10], and with the known specificity of thrombin for cleavage at arginyl bonds.

In order to further verify the cleavage site and to ascertain that small fragments from possible additional cleavages have not escaped detection, free modified bovine antithrombin was submitted to direct sequencer analysis without reduction or separation of the chains. Degradation of ~100 nmol protein was found to produce 2 residues in each cycle, both in similar yield (~60 nmol) and both exactly those shown for the small and large chains in table 2. Moreover, due to the large amount analyzed, all early residues, including Arg in position 2 of the large chain (cf. table 2), were positively identified, and the amino acid sequences could be followed longer than shown in table 2. All residues but 3 were thus identified up to position 22 in both chains. This analysis of the whole modified antithrombin molecule confirms the presence of only 1 cleavage site of the polypeptide chain and verifies the position of this site. It is obvious that direct sequencer analysis of peptide mixtures is a good method to evaluate the number of fragments obtained by limited proteolytic cleavage, especially when a protein with known primary structure is cleaved. The same approach has been used to show as many as 3

Table 2
Amino acid sequences of the N-terminal parts of the isolated large and small chains from free modified antithrombin, compared to pre-determined sequences for the intact human and bovine proteins

		1			5			8		
Present analysis of bovine large chain		His	–(Arg)–	Ser	–Pro	–Val	–Glu	–Asp	–Val	–
		H		T	H, T	H, T	H, T	H, T	H, T	
						15			10	
Known structure at positions 1–8 of intact antithrombin	Bovine [12]	1 His–	Arg	–Ser–	Pro	5 –Val	–Glu	–Asp	8 –Val	–
	Human [10]	His–	Gly	–Ser–	Pro	–Val	–Gap	–Asp	–Ile	–

	1				5					10				
Present analysis of bovine small chain	Ser	–Leu	–Asn	–Ser	–Asp	–Arg	–Val	–Thr	–Phe	–Lys	–Ala	–Asn	–	
	D, T	H, T	H, T	H, T	H, T	H	H, T	H, T	H, T	T	H, T	H, T		
		25					20		20		15			
Known structure at tentative positions 385–397 of intact human antithrombin [10]	385				390				395					
	Arg	–Ser	–Leu	–Asn	–Pro	–Asn	–Arg	–Val	–Thr	–Phe	–Lys	–Ala	–Asn	

Letters indicate methods of identification of residues. D, N-terminus by dansyl method; H and T, high-performance liquid chromatography and thin-layer chromatography, respectively, of phenylthiohydantoin derivatives. Figures below arrows show levels of nanomoles recovered. The residue within brackets was not unambiguously determined in the isolated large chain, but was identified on direct analysis of modified antithrombin without chain separation (see text) and is also known before [12]

cleavage sites in a still larger protein, the adenovirus hexon [11].

The results also show that the homology between the bovine and human antithrombins is quite extensive, in agreement with [12]. Two new replacements between the 2 proteins have been characterized in this work, i.e., Pro/Ser and Asn/Asp at positions 389 and 390, respectively (table 2). In addition, a third replacement (not reported in table 2) was found, namely Phe/Leu at position 403. Moreover, the deletion at position 6, as well as the exchanges at positions 2 and 8 [12] were confirmed (table 2). In total, 41 positions in the 2 proteins have now been compared ([12], and this report), which identify 5 amino acid exchanges and one deletion. The exchanges are compatible with one-base mutations, and, if representative for the entire molecule, suggest an 85% identity between human and bovine antithrombin.

The N-terminal sequence of the small chain of the modified form of antithrombin which was released by hydroxylamine from the antithrombin–thrombin complex was also analyzed. It was followed in the sequencer for 8 cycles. The residues obtained were the same as those for the small chain of the free protein shown in table 2. Thus the thrombin cleavage site in free modified antithrombin and the site of chain cleavage in the modified form of the inhibitor released from the complex are identical. This finding lends further support to the proposal that the amino acid sequence around the cleavage site in the modified antithrombin molecule is in the active site region of the intact inhibitor [3].

Acknowledgements

This research was supported by grants from the Swedish Medical Research Council (projects no. 3532, 4212 and 4444) and from Knut and Alice Wallenberg's Foundation. W.W.F., on Sabbatical leave from the Department of Biochemistry, Medical University of South Carolina, Charleston, SC, acknowledges a travel grant and a Visiting Scientist Fellowship from the Swedish Medical Research Council.

References

[1] Fish, W. W., Orre, K. and Björk, I. (1979) FEBS Lett. 98, 103–106.
[2] Jesty, J. (1979) J. Biol. Chem. 254, 1044–1049.
[3] Fish, W. W. and Björk, I. (1979) Eur. J. Biochem. in press.
[4] Owen, W. G. (1975) Biochim. Biophys. Acta 405, 380–387.
[5] Jörnvall, H. (1970) Eur. J. Biochem. 14, 521–534.
[6] Tarr, G. E., Beecher, J. F., Bell, M. and McKean, D. J. (1978) Anal. Biochem. 84, 622–627.
[7] Klapper, D. G., Wilde III, C. E. and Capra, J. D. (1978) Anal. Biochem. 85, 126–131.
[8] Zimmerman, C. L., Apella, E. and Pisano, J. J. (1977) Anal. Biochem. 77, 569–573.
[9] Inagami, T. and Murakami, K. (1972) Anal. Biochem. 47, 501–504.
[10] Petersen, T. E., Dudek-Wojciechowska, G., Sottrup-Jensen, L. and Magnusson, S. (1979) in: The Physiological Inhibitors of Blood Coagulation and Fibrinolysis (Collen, D. et al. eds) pp. 43–54, Elsevier/North-Holland, Amsterdam, New York.
[11] Jörnvall, H. and Philipson, L. (1979) Eur. J. Biochem. submitted.
[12] Kurachi, K., Schmer, G., Hermodson, M. A., Teller, D. C. and Davie, E. W. (1976) Biochemistry 15, 368–373.
[13] Nordenman, B., Nyström, C. and Björk, I. (1977) Eur. J. Biochem. 78, 195–203.