THE PRODUCTION OF AN INACTIVE FORM OF ANTITHROMBIN THROUGH LIMITED PROTEOLYSIS BY THROMBIN

Wayne W. FISH, Kerstin ORRE and Ingemar BJÖRK

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

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1. Introduction

It is now well established that antithrombin can act as an inhibitor of all the serine proteases of the intrinsic coagulation system [1–7]. The inhibition, which is accelerated by heparin, involves the formation of an inactive, extremely stable (possibly covalent [8]), equimolar complex between the inhibitor and the serine protease. In spite of the fact that the equimolarity of the inactive complex has been convincingly demonstrated [2], studies of the antithrombin–thrombin reaction have shown that total inhibition of the protease is not achieved until a molar excess of antithrombin is present [9]. It has been postulated that the reason for this is the proteolytic inactivation of free inhibitor by the enzyme. Evidence for a proteolytic modification of antithrombin by thrombin can be found [2,10], but no detailed studies of the products of this reaction have been made. This report deals with the preliminary characterization and with various conditions for the formation of such a proteolytically modified form of antithrombin, which is rapidly formed in considerable amounts in the presence of thrombin. This form has lost the ability to inhibit thrombin and has reduced affinity for heparin.

2. Materials and methods

Bovine antithrombin, human antithrombin and heparin, coupled to Sepharose 4B, were prepared by methods detailed in [11,12]. A preparation of bovine α-thrombin that was a generous gift from Dr Craig Jackson, Washington University, St Louis, MO, was used in most experiments. However, comparable results were obtained with commercial thrombin purified on heparin–Sepharose [13].

Disc gel electrophoresis or gradient-pore electrophoresis in dilute buffer, and gel electrophoresis in the presence of sodium dodecyl sulfate followed methods detailed in [11,14]. Gel electrophoresis at pH 3.3 in 8 M urea on 7.5%/20% step-gradient polyacrylamide gels was performed essentially as in [15].

Spectrophotometric assays for antithrombin and thrombin activities essentially followed the methods in [9,16].

Protein concentrations were measured spectrophotometrically. A pre-determined specific absorption coefficient ($A^{1\%}_{280}$) of 6.7 was employed for bovine antithrombin [14], while a specific absorption coefficient of 17.5 was measured for bovine thrombin [17].

3. Results

The putative thrombin-modified bovine antithrombin was prepared from native bovine antithrombin by the following procedure. A suspension of heparin–Sepharose, equilibrated in 0.05 M Tris · Cl + 0.20 M NaCl (pH 7.5) was saturated with bovine antithrombin, and the excess protein removed by washing. About 3.5 mg protein was found to bind per ml settled gel. Bovine thrombin, dissolved in the equilibrating buffer, was added to the gel suspension in an amount representing half the number of moles of antithrombin bound to the gel. The gel was washed

with the above buffer, and all protein which was released from the heparin–Sepharose at this ionic strength was combined. At this stage, > 80% of the protein in the preparation was the putative thrombin-modified antithrombin as judged by SDS–gel electrophoresis. The preparation was further purified by subsequent gel chromatography on Sephacryl S-200 followed by rechromatography on heparin–Sepharose. In the latter procedure, elution was effected by a linear gradient from 0.02 M Tris · Cl (pH 7.4) to 0.5 M NaCl in the same buffer. The final yield of putative thrombin-modified antithrombin by this procedure was ~20%, while SDS–gel electrophoresis suggested that its actual production may be up to 40% of the native antithrombin originally bound to the heparin–Sepharose.

In dilute buffer, the isolated protein eluted from a Sephacryl S-200 column at the same position as native bovine antithrombin and could not be differentiated from the native protein either by disc-gel electrophoresis, gradient-gel electrophoresis or immunoelectrophoresis. A reaction of immunological identity with native bovine antithrombin was obtained in immunodiffusion analyses. The protein exhibited no thrombin activity, and no antithrombin activity either in the presence or absence of heparin. It eluted from heparin–Sepharose at ~0.25 ionic strength during gradient elution. Thus, the protein's binding of heparin is much weaker than that of native bovine antithrombin, which elutes from heparin–Sepharose at ~0.8 ionic strength [11]. Together these data suggest that the isolated protein is bovine antithrombin which has been modified in some manner in the presence of thrombin.

Figure 1 presents a comparison of the electrophoretic behaviours under denaturing conditions of bovine antithrombin and the putative thrombin-modified antithrombin. In SDS, a difference in electrophoretic mobility between the two could be detected only under reducing conditions (gels 1–3). Electrophoresis of the reduced proteins on step-gradient polyacrylamide gels in 8 M urea (pH 3.3) visualized a small polypeptide, which was released upon reduction only from the putative thrombin-modified antithrombin (gels 4–6). It was later found that the smaller peptide also could be observed as a lightly staining, diffuse band ahead of the marker dye on standard SDS–gels. Comparison of the mobility of the smaller peptide in 8 M urea (pH 3.3) with the

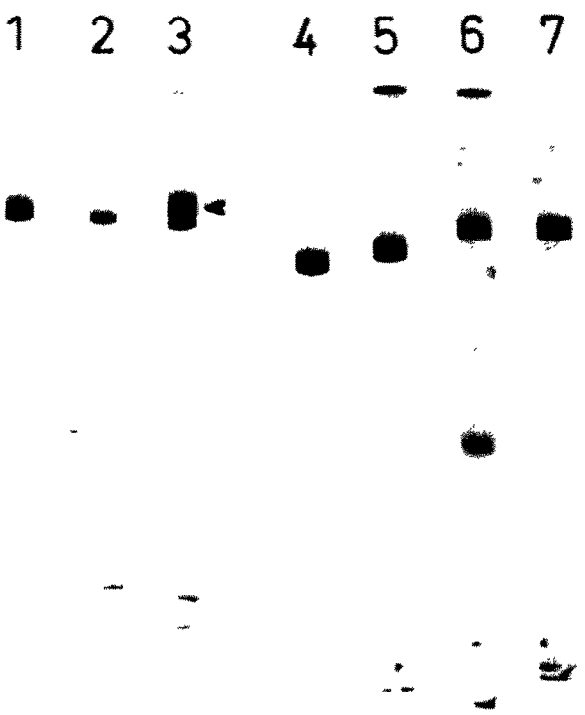

Fig.1. A comparison of the electrophoretic behaviour in denaturing solvents of antithrombin and thrombin-modified antithrombin. Gel 1, a mixture of bovine antithrombin and thrombin-modified antithrombin, unreduced in SDS. Gel 2, thrombin-modified antithrombin, reduced in SDS. Gel 3, a mixture of bovine antithrombin and thrombin-modified antithrombin, reduced in SDS. Bovine antithrombin is marked with an arrow. Gel 4, bovine antithrombin, unreduced in 8 M urea (pH 3.3). Gel 5, thrombin-modified antithrombin, unreduced in 8 M urea (pH 3.3). Gel 6, thrombin-modified antithrombin, reduced and carbaminomethylated, in 8 M urea (pH 3.3). Gel 7, bovine antithrombin, reduced and carbaminomethylated, in 8 M urea (pH 3.3). The position of the tracking dye is indicated by a prick in the gel with India ink.

mobility of the thrombin A-chain suggested that the former has mol. wt 5000. The larger polypeptide of modified antithrombin was estimated to have mol. wt 50 000 by comparison of its mobility in SDS–gel electrophoresis with those of standard polypeptides.

Thrombin was incubated with native antithrombin under various experimental conditions in order to verify that the modified form of antithrombin did not result only as a consequence of the conditions under which it was prepared but that it arises naturally

in the encounter of thrombin and antithrombin in solution. The products of these incubations were monitored for the presence of the larger polypeptide chain of thrombin-modified antithrombin by SDS–gel electrophoresis under reducing conditions. The results are summarily illustrated in fig.2. Within the time that the first sampling could be made (about 10 s), both the thrombin–antithrombin complex and the large polypeptide of thrombin-modified antithrombin were observed. The quantities of these two products increased until the thrombin or antithrombin was depleted. Beyond this point, the quantity of thrombin-modified antithrombin appeared to remain constant (cf. gels 1,2). Within the limits investigated, the appearance of the modified antithrombin was independent of the molar ratio of antithrombin to thrombin used (gels 1,3). The rate of production of modified antithrombin increased in the presence of heparin in a manner qualitatively similar to the rate of formation of the antithrombin–thrombin complex (gel 4). A product similar to that produced from bovine antithrombin was also observed when human antithrombin was incubated with bovine thrombin (gel 5). Finally, the conclusion that this modified antithrombin is a product of thrombin action is supported by the observations that none of it was formed when antithrombin was incubated with thrombin which had been inactivated with phenylmethylsulfonylfluoride, or when thrombin and antithrombin were incubated in the presence of high concentrations of benzamidinium chloride (gel 6).

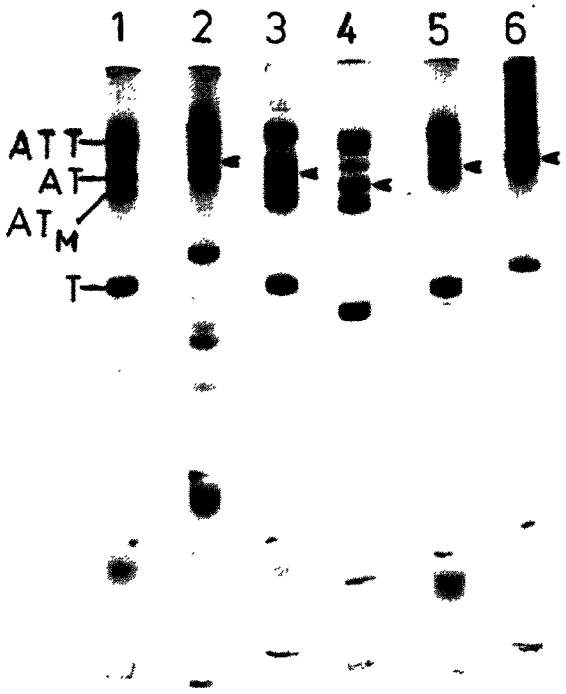

Fig.2. The production of thrombin-modified antithrombin under different sets of experimental conditions. The principal electrophoretic components are antithrombin–thrombin complex (ATT), antithrombin (AT), thrombin-modified antithrombin (AT_M), and the B-chain of bovine α-thrombin (T). Absolute mobilities differ from gel to gel; therefore antithrombin is marked with an arrow. Gel 1, equimolar amounts of bovine antithrombin and bovine thrombin, incubated for 30 s at 25°C (pH 7.4). Gel 2, as in gel 1 except incubation was for 120 min. Gel 3, a 4-fold molar excess of bovine antithrombin over bovine thrombin incubated for 30 s at 25°C (pH 7.4). (Because of the large relative quantity of native antithrombin, the small region of separation between the modified and native antithrombin on this gel is not easily visible on the photograph.) Gel 4, equimolar amounts of bovine antithrombin and bovine thrombin in the presence of heparin (molar ratio of 0.25 relative to antithrombin) incubated 10 s at 25°C (pH 7.4). Gel 5, equimolar amounts of human antithrombin and bovine thrombin incubated for 1 min at 37°C (pH 7.4). Gel 6, equimolar amounts of bovine antithrombin and bovine thrombin in the presence of 1 M benzamidinium chloride incubated 5 h at 25°C (pH 7.4). Samples were heated 5 min in a boiling water bath in the presence of a 5-fold weight excess of SDS and 10% β-mercaptoethanol (pH 7.4). 15 μg protein was applied to each gel.

4. Discussion

These results establish that considerable amounts of an inactive species of antithrombin arise in the presence of thrombin as a result of the proteolytic action of the enzyme. The inactivation appears to involve the scission of one or at most a few adjacent peptide bonds, located 30–60 residues from one of the ends of the antithrombin polypeptide chain. The narrow substrate specificity of thrombin suggests that, in fact, the cleavage may take place at one peptide bond only. The two polypeptides which result remain covalently attached through a disulfide linkage and can be separated only after reduction under denaturing conditions. The localized chain hydrolysis of antithrombin not only renders it incapable of thrombin inactivation but also virtually eliminates its heparin binding capacity. This marked alteration of the func-

tion of the protein indicates that a conformational change may follow the proteolytic event. The inactivation of antithrombin by thrombin concurrent with the formation of the complex between the two proteins offers a quite feasible reason for the finding that greater-than-equimolar quantities of antithrombin are required to completely neutralize thrombin [9].

The thrombin-modified antithrombin was shown to arise under a broad range of in vitro conditions, which suggests that its formation might also occur in vivo. In this regard, it is worth noting that an inactive form of antithrombin was demonstrated [10] in serum produced by the clotting of plasma with exogenous thrombin. The modified antithrombin we have isolated may be identical to this form, although the paucity of the data available renders a comparison difficult. Of further interest is that a perusal of the literature which deals with the inactivation by antithrombin of various coagulation serine proteases [2–6] suggests that formation of modified antithrombin may not be restricted to the thrombin system.

We do not know at this time what the significance may be of this seemingly 'dead end' product in the interaction between thrombin and antithrombin. However, the fact that a protease inhibitor is easily inactivated by what appears to be a single bond hydrolysis catalyzed by its target enzyme is rather puzzling. Comprehensive physical and chemical studies are currently in progress in an attempt to answer this question.

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