SLOW, SPONTANEOUS DISSOCIATION OF THE ANTITHROMBIN–THROMBIN COMPLEX PRODUCES A PROTEOLYTICALLY MODIFIED FORM OF THE INHIBITOR

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Received 13 August 1980

1. Introduction

The inhibition of thrombin by the plasma protease inhibitor antithrombin has been extensively studied as a model for the action of antithrombin as an inhibitor of all the serine proteases of the intrinsic coagulation system. The inactivation reaction, which is greatly accelerated by heparin, involves the formation of an inactive, stable, equimolar complex between enzyme and inhibitor [1,2]. This complex resists dissociation by protein-denaturing agents, such as sodium dodecylsulphate (SDS) or guanidine–HCl [2,3]. The integrity of the denatured complex may be due to a covalent bond [3], most likely an acyl bond between the active site serine of the enzyme and the arginine in the active site of the inhibitor (see below). However, the interactions stabilizing the native complex are essentially unknown.

The antithrombin–thrombin complex can be dissociated by certain chemical agents, e.g., hydroxylamine, ammonia or high pH in the presence or absence of SDS [3–5]. Dissociation by these agents does not release intact antithrombin from the complex, but a disulfide-linked, two-chain form of the inhibitor [4,5]. This proteolytically modified antithrombin, which is inactive, is also formed free in solution during the reaction between antithrombin and thrombin [6]. It is cleaved at a single site, an Arg–Ser bond near the C-terminal end of the chain [7]. The cleavage site has been suggested to constitute the active site of antithrombin [5,7,8].

This paper demonstrates that purified antithrombin–thrombin complex slowly dissociates also under physiological conditions, i.e., in the absence of any denaturing or dissociating agents. This spontaneous dissociation also produces only the modified form of the inhibitor and no intact antithrombin. However, active thrombin is released concurrently from the complex.

2. Materials and methods

Bovine antithrombin was prepared as in [9]. Heparin was purified and coupled to cyanogen bromide-activated agarose as detailed in [10]. Bovine α-thrombin was a generous gift from Dr Craig Jackson, Washington University, St Louis, MO.

Bovine antithrombin–thrombin complex was prepared by a modification of the procedure in [5]. Bovine antithrombin (in 0.05 M Tris–HCl (pH 7.4) + 0.1 M NaCl) was mixed with heparin in protein: polysaccharide, 10:1 (w/w). Thrombin was then added to a final 4-fold molar ratio of inhibitor to enzyme. The mixture was kept at +4°C for 15 min then applied to a heparin–Sepharose column in 0.05 M Tris–HCl (buffer (pH 7.4) + 0.1 M NaCl. A gradient to the same buffer containing 0.8 M NaCl was started, and the complex was eluted at an ionic strength of ~0.5. Further purification was achieved by gel chromatography on Sephacryl S200 [5]. The final product contained $\gtrsim$95% of intact complex. Some proteolytically degraded complex [11] and minor amounts of intact and modified antithrombin were evident (see fig.1).

A synthetic, reversible, thrombin inhibitor, 4-(3-nitrophenoxymethyl) benzamidine–HCl (compound 10 of [12]), was a kind gift from Dr J. D. Geratz, Department of Pathology, University of North Carolina at Chapel Hill, NC. This inhibitor has a K_i value with bovine thrombin of 3.3×10^{-6} M at pH 8.1 and 37°C (J. D. Geratz, personal commu-

nication). Another synthetic, irreversible, thrombin inhibitor, D-Phe–Pro–$ArgCH_2Cl$ [13] was generously donated by Dr E. Shaw, Brookhaven National Laboratory, Upton, NY.

SDS–polyacrylamide gel electrophoresis under reducing conditions essentially followed the method in [14]. Samples were boiled for 60 s in 1% SDS and 0.45 M β-mercaptoethanol, and were rapidly chilled. Gels were stained with Coomassie brilliant blue R250 and were scanned at a wavelength of 570 nm.

Thrombin activity was assayed as in [5]. Protein concentrations were measured spectrophotometrically with the use of the specific absorption coefficients in [5,6,15].

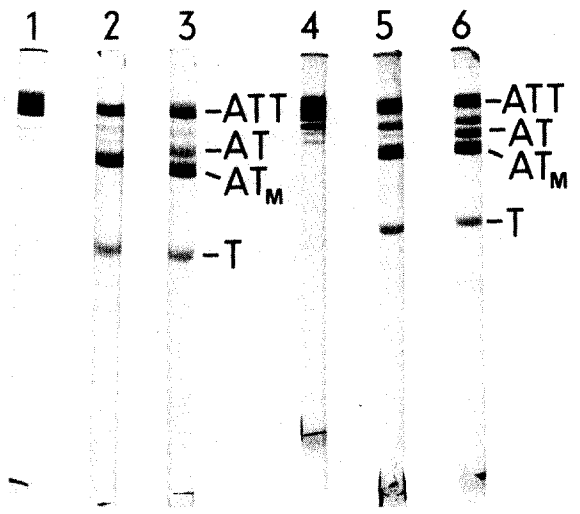

Fig.1. Dissociation of antithrombin–thrombin complex at 37°C, monitored by SDS–polyacrylamide gel electrophoresis under reducing conditions. (1) Antithrombin–thrombin complex. (2) Complex kept at 37°C for 66 h. (3) As in (2) but with intact antithrombin added before electrophoresis. (4) Antithrombin–thrombin complex (this preparation contained more proteolytically degraded complex [11] than that of (1). (5) Complex kept at 37°C for 90 h in the presence of the irreversible inhibitor D-Phe–Pro–$ArgCH_2Cl$. The inhibitor was added at the start of the experiment in a 5-fold molar ratio to the complex, followed by equimolar amounts every 12 h. (6) As in (5) but with intact antithrombin added before electrophoresis. (ATT) antithrombin–thrombin complex; (AT) intact antithrombin; (AT_M) large chain of modified antithrombin; (T) thrombin B-chain. The complex was 3 mg/ml, and the solvent was 0.02 M sodium phosphate buffer (pH 7.4) + 0.18 M NaCl. Amounts of 20 μg (gels 1–3) or 30 μg (gels 4–6) protein were applied to each gel.

3. Results

The spontaneous dissociation of the antithrombin–thrombin complex at 37°C was monitored by removing aliquots from a solution of the complex (in 0.02 M phosphate buffer (pH 7.4) + 0.18 M NaCl) at different times and analysing these samples by SDS–polyacrylamide gel electrophoresis under reducing conditions. Initially, phosphate buffer was used, since Tris, being a weak nucleophile, may dissociate the antithrombin–thrombin complex [4]. However, later experiments showed that similar results were obtained in both buffer systems. Gels 1–3 of fig.1 show that the amount of intact antithrombin–thrombin complex has decreased markedly after 66 h at 37°C, concomitant with an increase in the amounts of the large chain of modified antithrombin and the B-chain of thrombin. The small chain of modified antithrombin and the A chain of thrombin, which have $M_r < 5000$, are not easily visible on the gels. No increase of the small amounts of proteolytically degraded complex or intact antithrombin is evident from the gels. The large chain of modified antithrombin migrates ahead of the intact inhibitor under reducing conditions [6] and was identified by adding intact antithrombin or purified, modified antithrombin [6] to some gels.

The time course of the dissociation is illustrated in fig.2. The proportions of the components were estimated by gel scanning and therefore are only approximate. The disappearance of the antithrombin–thrombin complex is parallelled by a corresponding appearance of the large chain of modified antithrombin and the thrombin B chain, while the small amount of intact antithrombin originally present in the complex preparation remains constant (not shown). Fig.2 also shows the release from the complex of thrombin activity, measured as amidolytic activity against a synthetic tripeptide substrate in a separate dissociation experiment. However, the apparent rate of this release was much lower than would be expected from the rate of appearance of the thrombin B chain in the electrophoretic experiments. A major part of the thrombin liberated from the complex thus most likely was inactivated during the prolonged incubation at 37°C.

In some dissociation experiments the active thrombin released from the antithrombin–thrombin complex was inhibited by synthetic thrombin inhibi-

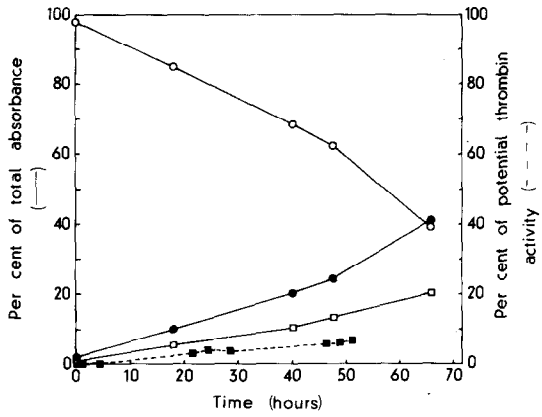

Fig.2. Rate of dissociation of antithrombin–thrombin complex and of appearance of the dissociation products at 37°C. The complex was dissociated as in fig.1 without any inhibitor present. Samples were removed at different times and analysed by SDS–polyacrylamide gel electrophoresis under reducing conditions. The proportions of the components were estimated by gel scanning. Samples were also analysed for thrombin activity by a spectrophotometric assay, using a synthetic substrate, D-Phe–Pip–Arg–pNA [5]. The results of this assay were expressed as % of the maximum potential thrombin activity; the latter was estimated from assays of pure thrombin and from the amount of thrombin calculated to be present in the complex. (○——○) Intact + proteolytically degraded complex. The amount of degraded complex remained at <5% of the total absorbance throughout the experiment; (●——●) modified antithrombin; (□——□) thrombin B chain; (■– – –■) thrombin activity.

tors. Gels 4–6 of fig.1 show that only modified antithrombin and no intact antithrombin was released from the complex also in the presence of the irreversible inhibitor D-Phe–Pro–ArgCH_2Cl. Another dissociation experiment in the presence of 5 mM reversible inhibitor 4-(3-nitrophenoxymethyl) benzamidine–HCl gave an identical result, which is not presented. These observations verify the conclusion in [5] that modified antithrombin is released as such from the antithrombin–thrombin complex and not as intact antithrombin which is subsequently transformed by the active thrombin released concurrently.

4. Discussion

These experiments show that purified antithrombin–thrombin complex spontaneously, but slowly, dissociates to the modified, two-chain form of the inhibitor and to active thrombin. The liberation of modified antithrombin from the complex by dissociating agents in [5] thus is not an artifact produced by these agents. The spontaneous release of modified antithrombin from the antithrombin–thrombin complex adds further support to the proposal that the cleavage site in modified antithrombin is in the active site of the intact inhibitor [5,7,8].

The results further show that after thrombin initially has reacted with antithrombin to form an inactive complex, it is not permanently trapped in this complex. This has often been tacitly assumed because of the marked stability of the complex, notably its resistance to dissociation by protein-denaturing agents. Instead, the antithrombin–thrombin complex can be regarded as an enzyme–substrate complex, which is unusually stable, but nevertheless slowly decomposes to free enzyme and a product in which the active-site peptide bond of the inhibitor has been hydrolysed. The formation and decay of the complex most likely occurs via the same intermediates that are generally acknowledged to occur in normal peptide bond hydrolysis catalysed by serine proteases [16]. The dominant state of the scissile peptide bond of the inhibitor in the complex, which defines the rate-limiting step of this slow enzymatic reaction, has not yet been characterized [5]. However, the slow hydrolysis of the inhibitor is reminiscent of, and may be analogous to, the slow deacylation step in the hydrolysis of those synthetic substrates for serine proteases which show initial-burst kinetics, e.g., *p*-nitrophenyl-*p'*-guanidino benzoate [16,17].

The slow dissociation of the antithrombin–thrombin complex to active enzyme and inactive inhibitor means that the inactivation of thrombin by antithrombin is a form of so called temporary inhibition [18]. This was, in fact, suggested some time ago [19] from experiments with impure reagents. They observed that thrombin activity regenerated from a mixture of thrombin and antithrombin, while antithrombin activity did not reappear.

The rate of formation and dissociation of the antithrombin–thrombin complex was studied [20]. The kinetics observed could be described by a simple one-step reversible reaction between enzyme and inhibitor, leading to an equilibrium with $K_d \sim 10^{-10}$ M. This mechanism implies that only very low concentrations of the free proteins would be detected in a solution of purified antithrombin–thrombin complex, and also that only intact antithrombin would dissociate from the complex. Both these predictions

are contrary to these observations. However, only the dissociation of the nascent antithrombin–thrombin complex was monitored and a slow conversion of this complex to a more stable form noted [20]. This may be the form of the complex that we have studied, since our purification procedure lasted ≥24 h. Our results thus show that the mechanism of inhibition is more complicated than proposed [20]. Further studies to elucidate this problem by characterizing the kinetics of dissociation of the nascent and mature forms of the antithrombin–thrombin complex are in progress.

Acknowledgements

This research was supported by grants from the Swedish Medical Research Council (project no. 4212) and from the Swedish Society of Medical Research.

References

[1] Abildgaard, U. (1969) Scand. J. Clin. Lab. Invest. 24, 23–27.
[2] Rosenberg, R. D. and Damus, P. S. (1973) J. Biol. Chem. 248, 6490–6505.
[3] Owen, W. G. (1975) Biochim. Biophys. Acta 405, 380–387.
[4] Jesty, J. (1979) J. Biol. Chem. 254, 1044–1049.
[5] Fish, W. W. and Björk, I. (1979) Eur. J. Biochem. 101, 31–38.
[6] Fish, W. W., Orre, K. and Björk, I. (1979) FEBS Lett. 98, 103–106.
[7] Jörnvall, H., Fish, W. W. and Björk, I. (1979) FEBS Lett. 106, 358–362.
[8] Carrell, R. W., Boswell, D. R., Brennan, S. O. and Owen, M. C. (1980) Biochem. Biophys. Res. Commun. 93, 399–402.
[9] Miller-Andersson, M., Borg, H. and Andersson, L.-O. (1974) Thromb. Res. 5, 439–452.
[10] Carlström, A.-S., Liedén, K. and Björk, I. (1977) Thromb. Res. 11, 785–797.
[11] Fish, W. W., Orre, K. and Björk, I. (1979) Eur. J. Biochem. 101, 39–44.
[12] Parrish, R. F., Straus, J. W., Paulson, J. D., Polakoski, K. L., Tidwell, R. R., Geratz, J. D. and Stevens, F. M. (1978) J. Med. Chem. 21, 1132–1136.
[13] Kettner, C. and Shaw, E. (1979) Thromb. Res. 14, 969–973.
[14] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[15] Nordenman, B., Nyström, C. and Björk, I. (1977) Eur. J. Biochem. 78, 195–203.
[16] Walsh, C. (1979) Enzymatic Reaction Mechanisms, pp. 67–71, 94–97, Freeman, San Francisco.
[17] Chase, T. jr and Shaw, E. (1969) Biochemistry 8, 2212–2224.
[18] Laskowski, M. and Wu, F. C. (1953) J. Biol. Chem. 204, 797–805.
[19] Seegers, W. H., Yoshinari, M. and Landaburu, R. H. (1960) Thromb. Diath. Haem. 4, 293–297.
[20] Jesty, J. (1979) J. Biol. Chem. 254, 10044–10050.