Dissociation of Antithrombin III–Thrombin Complex. Formation of Active and Inactive Antithrombin III†

Michael J. Griffith* and Roger L. Lundblad

ABSTRACT: Dissociation of the complexes formed during the reaction of thrombin with antithrombin III was studied by determining the kinetics of release of thrombin and active antithrombin III from the complexes. The rate of thrombin release increased gradually between pH 7.0 and 8.5 and then sharply increased at pH 9.0. The rate of active antithrombin III release did not change significantly over the same pH range. The results suggested that thrombin is released after reaction with antithrombin III via two pathways which are differentially affected by the pH of the solution. At pH 7.0 thrombin is released predominantly by dissociation of a non-acylated enzyme–inhibitor complex since active antithrombin III was found to be released in nearly stoichiometric amounts. At pH 9.5 thrombin is released predominantly by the rapid deacylation of an acylated enzyme–inhibitor complex since active antithrombin III was released in relatively low amounts. The results suggested that thrombin inhibition by antithrombin III does not require the formation of an acyl bond between the active-site serine of thrombin and a carbonyl carbon of antithrombin III. The release of thrombin by acylation–deacylation appeared to be limited by the rate of enzyme acylation which was favored by more alkaline pH. This further suggests that acyl bond formation is a secondary reaction that can occur during thrombin inhibition by antithrombin III but is not necessary for thrombin inhibition.

The inhibition of thrombin by antithrombin III appears to be typical of protease–antiprotease reactions in which the active site of the enzyme becomes physically blocked by stoichiometric amounts of the inhibitor (Rosenberg & Damus, 1973). The antithrombin III–thrombin reaction follows second-order kinetics over a range of inhibitor concentration which would suggest that if a Michaelis–Menten complex precedes thrombin inhibition, the K_m value for the complex must be $>10^{-5}$ M (Jesty, 1979b; Griffith, 1979). Thermodynamic evidence indicates that the reaction is not diffusion controlled, however (Machovich & Aranyi, 1978). This could indicate that the rate-limiting step for thrombin inhibition is subsequent to the collision between active sites on the inhibitor and enzyme. Several studies have been reported which suggest that a covalent bond is formed between thrombin and antithrombin III during the reaction (Rosenberg & Damus, 1973; Owen et al., 1976; Owen, 1975; Chandra & Bang, 1977). This could represent the rate-limiting step in the antithrombin III–thrombin reaction. The evidence for covalent bond formation comes from two general observations: (1) treatment of the antithrombin III–thrombin complex with $NaDodSO_4$[1] (Rosenberg & Damus, 1973) or guanidine hydrochloride (Owen, 1975; Owen et al., 1976) does not result in dissociation of the complex; (2) cleavage of antithrombin III appears to accompany reaction with thrombin (Fish et al., 1979; Jesty, 1979a; Chandra & Bang, 1977). Fish and co-workers have also reported that antithrombin III, modified by cleavage, does not retain inhibitor activity (Fish et al., 1979).

Recently, Jesty reported the dissociation of the antithrombin III–thrombin complex (Jesty, 1979b). This study was based on the determination of thrombin dissociation. The results suggested that if the covalent structure of antithrombin III is altered during reaction with thrombin, the modified inhibitor, which codissociates with thrombin, retains inhibitor activity. However, it was also reported by Jesty that the initial reversible antithrombin III–thrombin complex appears to "decay to a more stable form" with time (Jesty, 1979b). This observation might reconcile the data if it were presumed that the reversible antithrombin III–thrombin complex forms an acyl enzyme–inhibitor complex within a finite time.

The present investigation was undertaken to study the dissociation of both thrombin and antithrombin III from the antithrombin III–thrombin complex. The results reported by Jesty and others have indicated that high concentrations of tripeptide substrates effectively block the association of thrombin and antithrombin III (Jesty, 1979b; Odegard & Lie, 1978) and can be used to study the dissociation of thrombin from the antithrombin III–thrombin complex. Hydrolysis of the substrate occurs relatively rapidly and therefore cannot be used to study the release of antithrombin III. As an alterntive, the present study uses the very reactive $IleProArgCH_2Cl$ (Kettner & Shaw, 1977) to rapidly inactivate thrombin as it dissociates from the complex. Trapping of thrombin in this manner favors dissociation of the complex and permits the study of the antithrombin III which codissociates with thrombin.

Experimental Procedures

Materials

TosGlyProArgNaN[1] was purchased from Boehringer-Mannheim. Poly(ethylene glycol) (M_r = 6000–7500) was purchased from J. T. Baker Chemical Co. Prothrombin complex concentrate and antithrombin III were obtained from the American Red Cross National Fractionation Center.[2] The antithrombin III was judged to be ≥95% homogeneous by

† From the Dental Research Center, Department of Pathology and Department of Biochemistry, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514. *Received March 14, 1980; revised manuscript received August 11, 1980.* This work was supported by National Institutes of Health Research Grants DE-02668, RR-3344, and HL-07225.

[1] Abbreviations used: TosGlyProArgNaN, N^{α}-*p*-tosyl-L-glycyl-L-prolyl-L-arginine *p*-nitroanilide; $IleProArgCH_2Cl$, L-isoleucyl-L-prolyl-L-arginine chloromethyl ketone; PEG 6000, poly(ethylene glycol); TEA, triethanolamine; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[2] Antithrombin III and prothrombin complex concentrate, the latter used for the preparation of thrombin, were provided by the American Red Cross National Fractionation Center with the partial support of NIH Grant HL-13881.

0006-2960/81/0420-0105$01.00/0

sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentrations were determined spectrophotometrically by using an extinction coefficient value of 0.61 mL·mg^{-1}·cm^{-1} at 280 nm for human antithrombin III (M_r = 65000) (Miller-Andersson et al., 1974). In the presence and absence of heparin, 1 mol of antithrombin III was required to inactivate 1 mol of human α-thrombin indicating essentially fully active antithrombin III. L-Isoleucyl-L-prolyl-L-arginine chloromethyl ketone (IleProArgCH$_2$Cl) was a gift from Drs. C. Kettner and E. Shaw, Brookhaven National Laboratory. Heparin (160 USP units/mg, M_r 14000) was obtained from Erwin Coyne, Cohlefred Laboratories.

Human α-thrombin was isolated from prothrombin complex concentrates as described previously (Lundblad et al., 1976). Fibrinogen clotting activity was determined by adding 0.1 mL of enzyme solution to 0.2 mL of 0.15 M Tris-HCl (pH 8.0) containing 5.0 mg/mL fibrinogen at 37 °C. Clotting times were converted to NIH units from a standard curve prepared against NIH thrombin standard Lot 3B. Specific activities of 3000–3500 NIH units/mg were obtained for the purified enzyme. Protein concentrations were determined spectrophotometrically by using an extinction coefficient value of 1.75 mL·mg^{-1}·cm^{-1} at 280 nm for human α-thrombin (M_r = 36600) (Fenton et al., 1977). Enzyme solutions contained 0.1% poly(ethylene glycol) to prevent adsorption to surfaces (Wasiewski et al., 1976).

Methods

Hydrolysis of TosGlyProArgNaN by thrombin was determined as described previously (Griffith et al., 1979). Briefly, thrombin was added to a solution containing 6.0 × 10^{-5} M TosGlyProArgNaN, 0.1 M NaCl, 0.1% PEG 6000, and 0.1 M TEA at the pH being investigated. The complete hydrolysis of substrate was followed spectrophotometrically at 400 nm (ϵ_{400} = 1.16 × 10^{-4} M^{-1}·cm^{-1}; *p*-nitroaniline). K_m and V_{max} values were determined by using the integrated rate equation (Segel, 1975).

k_a Determination. The rate of thrombin inhibition by antithrombin III was determined under pseudo-first-order reaction conditions, i.e., antithrombin III in a 20-fold molar excess relative to thrombin. Antithrombin III was added to a thrombin solution containing 0.1 M TEA (pH variable), 0.1 M NaCl, and 0.1% PEG 6000. The final concentrations of thrombin and antithrombin III were 5.0 × 10^{-8} and 1.0 × 10^{-6} M, respectively. At timed intervals samples were removed from the reaction solution and assayed for residual thrombin activity by adding the sample to a solution containing 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG 6000, and 6.0 × 10^{-5} M TosGlyProArgNaN. The hydrolysis of substrate was terminated by adding $^1/_{10}$ volume of glacial acetic acid to the solution. The amount of *p*-nitroaniline formed was determined by absorbance at 400 nm. Duplicate samples varied by <5%. A plot of ln (% activity remaining) vs. time of reaction was made, and the value of k_a was determined by using the equation

$$\ln (T/T_0) = -k_a[\mathrm{AT}]t \qquad (1)$$

where AT is antithrombin III, T_0 is the initial thrombin activity and T is the thrombin activity at time t, and k_a is the observed second-order rate constant for thrombin inhibition by antithrombin III.

k_d Determination. Thrombin and antithrombin III were mixed at equimolar concentrations (10^{-5} M) and allowed to react for 10 min at room temperature. IleProArgCH$_2$Cl was then added to a final concentration of 1.0 × 10^{-4} M, and the solution was incubated for 10 min. A 1.0-mL sample of the solution was then filtered through a Sephadex G-25 medium column (1.5 × 40 cm) equilibrated with 0.1 M TEA at the pH being investigated. Protein concentration was estimated by absorbance at 280 nm. An E_{280} value of 1.18 mL·mg^{-1} for AT–T was empirically derived by determining the absorbance at 280 nm of an equimolar solution of thrombin and antithrombin III after ≥98% inactivation of thrombin. Within the limits of experimental error, total recovery of protein was obtained from gel filtration. Exactly 30 min after the initial mixing of thrombin and antithrombin III a sample of the gel filtered solution was assayed for thrombin dissociation essentially as described by Jesty (1979b). Briefly, a 0.1-mL samples was added to 0.9 mL of substrate solution (2.5 × 10^{-4} M TosGlyProArgpNaN, 0.1 M TEA, and 0.1 M NaCl at the pH being investigated) and the change in absorbance at 400 nm determined spectrophotometrically over a 10-min period. The rate of change in absorbance was converted into the rate of thrombin dissociation as described in the appendix.

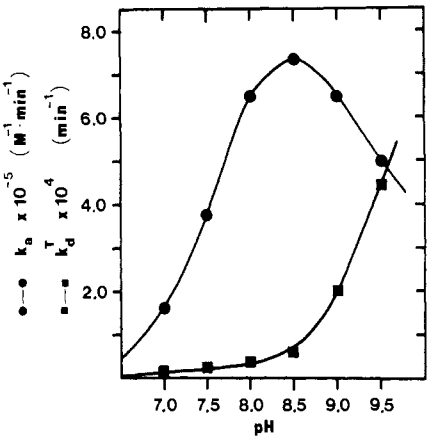

FIGURE 1: pH dependence of antithrombin III–thrombin association and thrombin dissociation. Thrombin and antithrombin III were dialyzed against 0.1 M TEA and 0.1 M NaCl at the pH to be investigated. The values of k_a and k_d^T were determined as described under Methods.

Active Antithrombin III Determination. The amount of active antithrombin III in antithrombin III–thrombin–IleProArgCH$_2$Cl solutions was determined after gel filtration by adding a sample of the solution to a solution containing 0.1 M TEA (pH 8.1), 0.1 M NaCl, 0.1% PEG 6000, and a known concentration of thrombin. After incubation for 15 min, heparin was added (1.0 × 10^{-7} M) and the solution incubated an additional 10 min to ensure complete reaction of antithrombin III with thrombin. Residual thrombin activity was determined by adding a 0.1-mL sample from the solution to a solution containing 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG 6000, and 6.0 × 10^{-5} M TosGlyProArgNaN. Substrate hydrolysis was terminated by adding 0.1 mL of 50% glacial acetic acid, and the absorbance at 400 nm was determined. The amount of active antithrombin III was determined from a standard curve prepared by reacting a known amount of antithrombin III with thrombin as above.

Results

The values of k_a and k_d^T were determined as a function of pH. The results are shown in Figure 1. The optimum pH for inhibition appeared to be around pH 8.5. This can be compared with the pH dependence for TosGlyProArgNaN hydrolysis by thrombin, shown in Figure 2, where a plateau was reached between pH 9.0 and pH 9.5. This difference is not entirely understood at the present time but suggests that the catalytic properties of thrombin are not adversely affected by increasing the pH to 9.5. The decrease in k_a values at pH

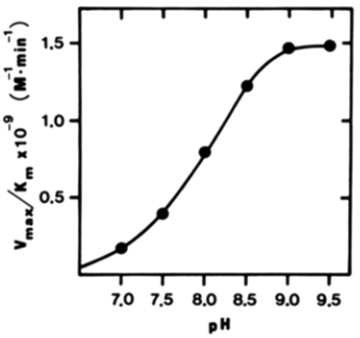

FIGURE 2: pH dependence of TosGlyProArgNaN hydrolysis by thrombin. The hydrolysis of TosGlyProArgNaN by thrombin was determined at the indicated pH values. The values of K_m and V_{max} (1 M thrombin) were determined as described under Methods.

9.0 and pH 9.5 might, therefore, reflect a decrease in the reactivity of functional groups not directly involved in catalysis. The marked increase in k_d^T at pH values above pH 8.5 suggests that the stability of the antithrombin III–thrombin complex decreases at these pH values. However, the continued increase in TosGlyProArgNaN hydrolysis above pH 8.5 suggests that the rate of acylation increases.[3] The extent to which acylation–deacylation contributes to k_d^T was therefore determined at pH 8.1 and pH 9.5 by determining the amount of active antithrombin III released from the complex as a function of time. At pH 8.1 the amount of active antithrombin III released increased linearly over a 5-h incubation of antithrombin III–thrombin in the presence of IleProArgCH$_2$Cl. The k_d^{AT} value was 2.0×10^{-5} min^{-1}. The k_d^T value did not change during the 5-h incubation, $k_d^T = 4.0 \times 10^{-5}$ min^{-1}.

The apparent 2-fold greater rate of thrombin release compared with that of active antithrombin III suggested that a significant amount of thrombin was released via bond cleavage and deacylation as well as via a reversible interaction of thrombin with antithrombin III. At pH 9.5 active antithrombin III could not be detected during the 5-h incubation even though ~12% of the complex had dissociated, based on the rate of thrombin release, $k_d^T = 4.4 \times 10^{-4}$ min^{-1}. These results suggested that the majority of thrombin released at pH 9.5 was via deacylation following peptide bond hydrolysis.

The dissociation of the antithrombin III–thrombin complex was also investigated by NaDodSO$_4$–polyacrylamide gel electrophoresis, shown in Figure 3. At pH 8.0 the extent of dissociation was too small to quantitatively determine accurately. After a 17-h incubation of the complex in the presence of IleProArgCH$_2$Cl, there was an estimated 7% decrease in the amount of antithrombin III–thrombin. A 5% decrease was expected at this pH ($k^T = 4.0 \times 10^{-5}$ min^{-1}). There was an apparent 2–4% increase in the amount of protein migrating at a rate equivalent to native antithrombin III and a similar increase in the amount of protein migrating at a rate slightly faster than native antithrombin III.[4] These results provide

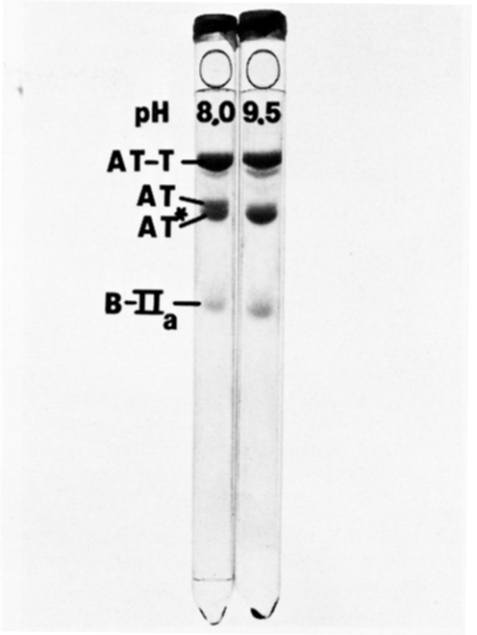

FIGURE 3: Dissociation of antithrombin III–thrombin. Antithrombin III was added to a solution containing 0.1 M TEA (pH 8.0 or 9.5), 0.1 M NaCl, and thrombin. The final concentrations of thrombin and antithrombin III were 7.5×10^{-6} M. After 10 min, IleProArgCH$_2$Cl was added (1×10^{-4} M). After 17 h samples were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis after reduction with 0.1% β-mercaptoethanol, essentially as described by Weber & Osborn (1969). The gels shown in the present figure were 7.5% acrylamide. The running buffer was 0.1 M sodium phosphate (pH 7.0) and 0.1% NaDodSO$_4$. The gels were stained with Coomassie brilliant blue and destained by diffusion with 7.5% acetic acid–25% methanol. The gels were scanned with a Helena quick scan. Antithrombin III–thrombin, AT–T (M_r = 95000); antithrombin III, AT (M_r = 65000); modified antithrombin III, AT* (M_r = 60000); B-chain thrombin, B-IIa (M_r = 32000).

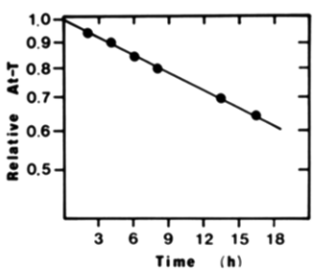

FIGURE 4: Decrease in antithrombin III–thrombin vs. time of incubation at pH 9.5. The relative amounts of protein migrating as antithrombin III–thrombin after NaDodSO$_4$–polyacrylamide gel electrophoresis were determined by integration of densitometric scans of gels similar to those shown in Figure 3. From the slope of the line a k_d^{AT-T} value of 4.5×10^{-4} min^{-1} was determined.

qualitative evidence supporting the conclusions drawn from the kinetic data that both active antithrombin III and inactive antithrombin III are released at pH 8.0. At pH 9.5 the rate of complex dissociation was fast enough to determine the decrease in antithrombin III–thrombin by electrophoresis. These results are shown in Figure 4. The k_d^{AT-T} value was 4.5×10^{-4} min^{-1}, in close agreement with the rate of thrombin dissociation at this pH, $k_d^T = 4.4 \times 10^{-4}$ min^{-1}. There was a concomitant increase in the amount of modified antithrombin III with little evidence for the appearance of native antithrombin III after 17 h (Figure 3).

The change in k_d^T after prolonged incubation in the presence of IleProArgCH$_2$Cl was determined to determine whether acylation or deacylation was rate limiting. A pH 8.0, 9.0, and 9.5, k_d^T decreased according to first-order kinetics suggesting

[3] The rate-limiting step in the hydrolysis of anilide synthetic substrates is the acylation step (Wainberg & Erlanger, 1971).

[4] Small amounts of protein migrating with a rate equivalent that of to AT* is generally observed immediately after the AT–thrombin reaction. The amount of protein is ≤5% of the total antithrombin III and may indicate the presence of trace amounts of AT* contaminating the antithrombin III preparation. In addition, there is a small amount of proteolysis of the antithrombin III–thrombin complex during the 10-min incubation prior to the addition of IleProArgCH$_2$Cl. This results in the appearance of protein bands migrating between AT–T and AT. The extent of proteolysis is not kinetically significant in the present study but can influence the apparent rate of thrombin dissociation when extensive degradation of the complex has occurred (M. J. Griffith and C. M. Noyes, unpublished experiments).

Table I: Rate of Release of Thrombin and Active Antithrombin III from the Antithrombin III–Thrombin Complex[a]

pH	k_d^T ($\times 10^5$ min^{-1}) (0.5 h)	k_d^T ($\times 10^5$ min^{-1}) (17 h)	k_d^T(theor)[b] ($\times 10^5$ min^{-1}) (17 h)	k_d^{AT}[c] ($\times 10^5$ min^{-1})	k_d^{AT}/k_d^T
7.0	0.5			0.5	1.00
8.0	4.0	3.8	3.8	2.0	0.50
9.0	21.0	17.0	17.0	1.7	0.08
9.5	44.0	27.0	28.0	0.5	0.01

[a] The values of k_d^T were determined as described under Methods. [b] The theoretical values of k_d^T at 17 h were calculated by assuming acylation is rate limiting, i.e., eq A5 describes thrombin dissociation. [c] k_d^{AT} values were calculated by determining the amount of active antithrombin III released from the complex in 17 h. The k_d^{AT}(app) values were then corrected to account for the first-order decrease in the antithrombin III–thrombin complex concentration during the 17-h incubation.

that acylation is rate limiting in these experiments (see Discussion). These results are summarized in Table I. The amount of active antithrombin III was also determined after prolonged incubation. The k_d^{AT} value was calculated by assuming the first-order decrease in AT-T concentration. The initial rate of thrombin release was used to determine the rate of decrease of AT-T. The value of k_d^{AT} relative to k_d^T relative to k_d^T decreased from a value of 1.0 at pH 7.0 to 0.01 at pH 9.5, further indicating that the extent of thrombin release via bond cleavage and deacylation increases as the pH increased. The results are also summarized in Table I.

Discussion

The present investigation has made two primary observations. First, active antithrombin III can be released from the antithrombin III–thrombin complex. Second, the amount of active antithrombin III released from the complex, relative to the amount of thrombin released, decreases as the pH is increased from 7.0 to 9.5. This observation suggests that formation of the antithrombin III–thrombin complex is reversible, but there is a pH-dependent, essentially irreversible dissociation of thrombin which appears to occur via hydrolysis of a peptide bond in antithrombin III.

The results also suggest that the rate of thrombin release via acylation–deacylation is limited by the rate of formation of an acylated enzyme–inhibitor complex. This interpretation was based on the assumptions made in describing the kinetics of dissociation of the antithrombin III–thrombin complexes (see Appendix). Briefly, inhibition of thrombin by antithrombin III would appear to be effected by the formation of a nonacylated enzyme–inhibitor complex. This does not preclude a relatively slow rate of thrombin acylation. If the subsequent rate of deacylation were significantly slower than the rate of acylation, there would be a time-dependent increase in the amount of the acylated complex relative to the amount of the nonacylated complex. Experimentally, the initial rate of thrombin release would reflect k_d, the first-order rate constant for dissociation of the nonacylated complex. After prolonged incubation (17 h in the present study) the rate of thrombin release would reflect both k_d and k_d', the first-order rate constant for deacylation and release of thrombin. As a consequence, the observed rate of thrombin release would change according to the relative values of k_d and k_d'. Since there was not a detectable change in the rate of thrombin release, it was possible to conclude that either deacylation is not rate limiting or $k_d = k_d'$. The latter possibility is argued against by the observation that the rate of active antithrombin III dissociation does not change significantly between pH 7.0 and 9.5, whereas the rate of thrombin dissociation increases nearly 100-fold over the same pH range. Since the rate of release of active antithrombin III release reflects k_d, it would appear that k_d' increases relative to k_d. Therefore, the observed rate of thrombin release would be predicted to increase as the concentration of AT-T increased (after 17 h) if deacylation was rate limiting. This was not observed (Table I).

Although the evidence presented is consistent with the model suggested under Appendix, alternative explanations are possible. For example, the antithrombin III has been assumed to be functionally homogeneous. If this assumption was not valid, it would be postulated that the interaction of thrombin with various antithrombin III subpopulations is different and that inhibition by one population requires acylation whereas another population does not. It should be noted however that there is no evidence to suggest this possibility. The second-order rate constant for the antithrombin III–thrombin reaction was constant over the reaction, suggesting a single population of active antithrombin III molecules. Further, the rate of dissociation of active antithrombin III from the complex was constant over a 5-h incubation and consistent with the amount of antithrombin III released after 17 h. If a small population of antithrombin III molecules formed a nonacylated complex with thrombin, the rate of release would not have been linear but would have decreased in a first-order manner according to the decrease in concentration of the nonacylated complex. An estimate of the minimum concentration of such a nonacylated complex which would have gone undetected in the present study would be ~25% of the total complex concentration. However, if 25% of the antithrombin III molecules formed nonacylated complexes with thrombin, the rate of thrombin release via acylation–deacylation would not have followed first-order kinetics as was observed (Table I).

The results of the present study are consistent with the results of other investigators with minor qualifications. Jesty (1979b) has shown the reversible formation of antithrombin III–thrombin complexes at pH 7.5. From the results of the present study it is apparent that complex formation is essentially reversible at pH 7.5, with only a small amount of thrombin released via bond cleavage and deacylation. The observation that covalently modified antithrombin III is inactive (Fish et al., 1979) was substantiated by the results of complex dissociation at pH 9.5 in the present study. Since Fish and co-workers (Fish et al., 1979) were not using a thrombin inhibitor analogous to the IleProArgCH_2Cl in the present study, the only form of antithrombin III which would be observed would be the covalently modified form which was generated via bond cleavage.

The stability of the antithrombin III–thrombin complex reported by others (Rosenberg & Damus, 1973; Owen, 1975; Owen et al., 1976; Chandra & Bang, 1977) is evidence that a covalent bond is formed between antithrombin III and thrombin. The results of the present investigation have suggested, however, that the bond is not likely to be an acyl bond involving the active-site serine of thrombin since this appears to result in rapid deacylation and release of thrombin and inactive antithrombin III. Involvement of the active-site serine in formation of a tetrahedral intermediate was not precluded by the present study, but it is not considered likely that this would stabilize the complex to the potent dissociating reagents used by other investigators in attempts to dissociate the complex (Rosenberg & Damus, 1973; Owen, 1975; Owen et al., 1976; Chandra & Bang, 1977).

Owen and co-workers (Owen et al., 1976) have shown that 1 M hydroxylamine will dissociate the antithrombin III–

thrombin complex if the complex is denatured by treatment with 0.1% sodium dodecyl sulfate. These results suggest that an ester bond may be formed between antithrombin III and thrombin. The ester bond, however, would appear to form between a hydroxyl group of one protein and a carboxyl group from the other, rather than by bond cleavage. This possibility was suggested by Owen and co-workers (Owen et al., 1976). A bond of this nature could explain the slow reversible dissociation of the complex measured in the present study and by Jesty (1979b), while maintaining a complex stable to $NaDodSO_4$ treatment. It is also possible, however, that treatment with hydroxylamine results in peptide bond cleavage which destabilizes the complex, favoring dissociation. Hydroxylamine is known to split asparaginyl–glycyl bonds in proteins (Bornstein & Balian, 1977).

The results of the present study suggest that formation of an acylated enzyme–inhibitor complex does not account for the rapid inactivation of thrombin by antithrombin III. This observation is suggestive of a mechanism of thrombin inactivation by antithrombin III which would be analogous to the inhibition of proteases by α_2-macroglobulin. Inhibition of several proteases by α_2-macroglobulin does not appear to involve the formation of a covalently bonded enzyme–inhibitor complex. Instead, enzymes appear to bind to α_2-macroglobulin and then to be "trapped" as a consequence of a conformational change in the inhibitor (Barett & Starkey, 1973). Unlike the antithrombin III–thrombin complex, however, the trapped enzyme can still interact with small substrates. It is possible that "trapping" of thrombin by antithrombin III occurs in such a way that the substrate is no longer accessible to the active site of the enzyme. Involvement of the active-site region of thrombin in the interaction of the enzyme with antithrombin III is strongly argued for, however, by the ability of the substrate to inhibit inactivation of thrombin by antithrombin III (Odegard & Lie, 1978).

Acknowledgments

We thank Drs. Kettner and Shaw for their generous gift of the peptide chloromethyl ketone used in the present study. We also thank Dr. M. Wickerhauser of the National Red Cross Fractionation Center for providing the essential blood derivatives for the present study.

Appendix

Theoretical Considerations for Kinetic Determination. The inactivation of thrombin (T) by antithrombin III (AT) follows second-order reaction kinetics over the range of inhibitor concentration experimentally attainable (Jesty, 1979; Griffith, 1979). Since there is no evidence to indicate the formation of a Michaelis–Menten complex prior to inactivation, the antithrombin III–thrombin reaction will be described by scheme S1 for the present time. AT–T represents a very

$$\mathrm{AT} + \mathrm{T} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{AT\text{–}T} \qquad \text{(S1)}$$

stable complex of the two proteins, i.e., $k_d/k_a \simeq 10^{-10}$ M. The second-order rate constant for association, k_a, and the first-order rate constant for dissociation, k_d, are apparent values and not meant to imply that a single step necessarily accounts for either association or dissociation.

As written (S1) suggests that dissociation of AT–T will yield the original starting proteins, i.e., active antithrombin III and thrombin. Since several studies have suggested that an acylated enzyme–inhibitor complex is formed during thrombin inactivation by antithrombin III, dissociation via deacylation of the complex would yield a covalently modified antithrombin III, which has been shown to be inactive (Fish et al., 1979), and active thrombin. This reaction is shown in scheme S2.

$$\mathrm{AT} + \mathrm{T} \underset{}{\overset{k_a}{\rightleftharpoons}} \mathrm{AT^*\text{–}T} \overset{k_d'}{\rightarrow} \mathrm{AT^*} + \mathrm{T} \qquad \text{(S2)}$$

AT* represents the covalently modified, inactive antithrombin III, and AT*–T represents an acylated antithrombin III–thrombin complex. The difference between (S1) and (S2) is that a nonacylated complex is formed in (S1) and an acylated complex is formed in (S2). Differentiating between (S1) and (S2) is relatively straightforward since active antithrombin III can only be formed by dissociation of the nonacylated complex.

A third possibility exists which incorporates both (S1) and (S2) into the reaction between thrombin and antithrombin III. This is shown in scheme S3. This scheme suggests that

$$\mathrm{AT} + \mathrm{T} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{AT\text{–}T} \overset{k_{acyl}}{\rightarrow} \mathrm{AT^*\text{–}T} \overset{k_d'}{\rightarrow} \mathrm{AT^*} + \mathrm{T} \qquad \text{(S3)}$$

thrombin inhibition does not require the formation of an acylated enzyme–inhibitor complex, but acylation, described by k_{acyl}, can occur. The experimental results discussed in the text suggest that (S3) best described the observed reaction between antithrombin III and thrombin.

The present study investigates the dissociation of the complexes formed during thrombin inhibition by antithrombin III. From (S3), the dissociation of thrombin can be derived by assuming that either acylation is rate limiting

$$\frac{dT}{dt} = k_d[\mathrm{AT\text{–}T}] + k_{acyl}[\mathrm{AT\text{–}T}] \qquad \text{(A1)}$$

or deacylation is rate limiting

$$\frac{dT}{dt} = k_d[\mathrm{AT\text{–}T}] + k_d'[\mathrm{AT^*\text{–}T}] \qquad \text{(A2)}$$

Equation A1 indicates that thrombin dissociation will follow first-order kinetics when acylation is rate limiting, whereas eq A2 indicates that thrombin dissociation will appear to follow first-order kinetics only when $k_d = k_d'$.

The dissociation of active antithrombin III can be described by

$$\frac{d[\mathrm{AT}]}{dt} = k_d[\mathrm{AT\text{–}T}] \qquad \text{(A3)}$$

and will follow first-order kinetics only when $k_{acyl} \ll k_d$.

Jesty has shown that the rate of synthetic substrate hydrolysis can be related to the rate of thrombin dissociation by

$$A_t = k_d^{\mathrm{T}}(\mathrm{app})C(t^2/2) + C[\mathrm{T}]t + A_0 \qquad \text{(A4)}$$

where A_t is the absorbance at 400 nm due to *p*-nitroaniline release from the synthetic substrate after a given time interval, t. A_0 is the initial absorbance at 400 nm. The constant C is described as the rate of change in absorbance produced by the hydrolysis of substrate by 1 M thrombin. The value of k_d^{T}(app), the apparent first-order rate constant for thrombin dissociation, will be dependent on the concentrations of the complexes (AT–T and AT*–T) which dissociate in the solution and their associated rate constants. For example, if acylation is rate limiting, as describd in eq A1, then

$$k_d^{\mathrm{T}}(\mathrm{app}) = (k_d + k_{acyl})[\mathrm{AT\text{–}T}] \qquad \text{(A5)}$$

If deacylation is rate limiting, as described in eq A2, then

$$k_d^{\mathrm{T}}(\mathrm{app}) = k_d[\mathrm{AT\text{–}T}] + k_d'[\mathrm{AT^*\text{–}T}] \qquad \text{(A6)}$$

Experimentally, it was observed that the value of k_d^{T}(app) is constant during the length of time required for determination,

10 min. This observation indicated that the concentrations of AT-T and AT*-T do not change significantly during the determination. This is consistent with the very low values obtained for k_d^T(app), which indicate that the total complex concentration would decrease by much less than 1% during assay, an amount which would not be kinetically significant. Further, if deacylation is rate limiting, one of two conditions must apply. Either k_d is approximately equivalent to k_d' or k_{acyl} is small such that the conversion of AT-T to AT*-T is not large enough to significantly alter the value of k_d^T(app). In other words, if one of these conditions were not met, k_d^T-(app) would vary as the concentration of AT*-T increased due to acylation.

The experimental results which are summarized in Table I suggested that acylation is rate limiting for the release of thrombin via deacylation. For this reason, k_d^T(app) was converted to k_d^T throughout the text by dividing k_d^T(app) by the concentration of AT-T (eq A5). The value of k_d^T at any pH will therefore be the sum of k_d and k_{acyl} as shown in eq A7:

$$k_d^T = k_d + k_{acyl} \quad \text{(A7)}$$

References

Barett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709.
Bornstein, P., & Balian, G. (1977) *Methods Enzymol. 47*, 132.
Chandra, S., & Bang, N. U. (1977) *Chem. Biol. Thrombin*, [*Proc. Conf.*], 421.
Fenton, J. W., II, Landis, B. H., Walz, D. A., & Finlayson, J. S. (1977) *Chem. Biol. Thrombin*, [*Proc. Conf.*], 43.
Fish, W. W., Orre, K., & Bjork, I. (1979) *FEBS Lett. 98*, 103.
Griffith, M. J. (1979) *J. Biol. Chem. 254*, 12044.
Griffith, M. J., Kingdon, H. S., & Lundblad, R. L. (1979) *Arch. Biochem. Biophys. 195*, 378.
Jesty, J. (1979a) *J. Biol. Chem. 254*, 1044.
Jesty, J. (1979b) *J. Biol. Chem. 254*, 10044.
Kettner, C., & Shaw, E. (1977) *Chem. Biol. Thrombin*, [*Proc. Conf.*], 129.
Lundblad, R. L., Kingdon, H. S., & Mann, K. G. (1976) *Methods Enzymol. 45*, 156.
Machovich, R., & Aranyi, P. (1978) *Biochem. J. 173*, 869.
Miller-Andersson, M., Borg, H., & Andersson, L.-O. (1974) *Thromb. Res. 4*, 439.
Odegard, O. R., & Lie, M. (1978) *Haemostasis 7*, 121.
Owen, W. G. (1975) *Biochim. Biophys. Acta 405*, 380.
Owen, W. G., Penick, G. D., Yoder, E., & Poole, B. L. (1976) *Thromb. Haemostasis 35*, 87.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley Interscience, New York.
Wainberg, M. A., & Erlanger, B. F. (1971) *Biochemistry 10*, 3816.
Wasiewski, W., Fasco, M. J., Martin, B. M., Detwiler, T. C., & Fenton, J. W., II (1976) *Thromb. Res. 8*, 881.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Adenosine Analogues as Substrates and Inhibitors of *S*-Adenosylhomocysteine Hydrolase†

Andrzej Guranowski,‡ John A. Montgomery, Giulio L. Cantoni, and Peter K. Chiang*

ABSTRACT: In the reaction adenosine + L-homocysteine ⇌ *S*-adenosyl-L-homocysteine, catalyzed by *S*-adenosylhomocysteine hydrolase from beef liver (EC 3.3.1.1), 11 nucleosides are able to substitute for adenosine to generate their corresponding *S*-nucleosidylhomocysteine congeners: 3-deazaadenosine, 2-aza-3-deazaadenosine, nebularine (purine ribonucleoside), formycin, N^6-methyladenosine, 8-azaadenosine, adenosine N^1-oxide, pyrazomycin, 8-aminoadenosine, inosine, and the carbocyclic analogue of adenosine [(±)-aristeromycin]. *S*-Adenosylhomocysteine hydrolase from lupin seeds is able to utilize all of these nucleosides except inosine to synthesize analogues of *S*-adenosylhomocysteine. There is no correlation between the ability of these nucleotides to function as substrates and their inhibitory potencies, except in the case of 3-deazaadenosine. The carbocyclic analogue of adenosine is the most potent inhibitor of *S*-adenosylhomocysteine hydrolase with a K_i of 5×10^{-9} M. When incubated with 3T3-L1 fibroblasts, the carbocyclic analogue of adenosine caused a 10-fold increase in the cellular concentration of *S*-adenosylhomocysteine. The cellular generation of *S*-2-aza-3-deazaadenosylhomocysteine was observed when 3T3-L1 fibroblasts were incubated with 2-aza-3-deazaadenosine.

S-Adenosylhomocysteine (AdoHcy)[1] is a product in all of the biological methylations in which *S*-adenosylmethionine (AdoMet) serves as the methyl donor. AdoHcy hydrolase (EC 3.3.1.1) catalyzes the hydrolysis of AdoHcy in eukaryotes in a reversible reaction first described by de la Haba & Cantoni (1959).

$$\text{AdoHcy} + H_2O \rightleftharpoons \text{Ado} + \text{Hcy}$$

The equilibrium of the reaction favors the synthesis of AdoHcy, but physiologically the reaction proceeds in the hydrolytic direction because Ado and Hcy are efficiently removed by further metabolism (Cantoni & Chiang, 1980). In mammalian tissues, Ado can be deaminated to inosine by Ado deaminase (EC 3.5.4.4) or be phosphorylated by Ado kinase (EC 2.7.1.20) to AMP; in plants, Ado is hydrolyzed to adenine and D-ribose (EC 3.2.2.7). The role of Ado and 2′-deoxy-Ado

† From the Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Maryland 20205 (A.G., G.L.C., and P.K.C.), and the Southern Research Institute, Birmingham, Alabama 35205 (J.A.M.). *Received April 25, 1980.*

‡ Visiting associate from the Institute of Biochemistry, Academy of Agriculture, Poznań, Poland.

[1] Abbreviations used: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; Ado, adenosine; Hcy, L-homocysteine; Ara-A, 9-β-D-arabinofuranosyladenine; C-Ado, carbocyclic analogue of adenosine [(±)-aristeromycin]; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; NucHcy, an analogue of *S*-nucleosidylhomocysteine; NucMet, an analogue of *S*-nucleosidylmethionine.