Reaction of Antithrombin with Proteases. Nature of the Reaction with Trypsin†

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ABSTRACT: The reaction of plasma antithrombin with pancreatic trypsin was studied as a model for the reaction with coagulation proteases. Further evidence, supporting work in the literature, that the reaction with trypsin is specific and analogous to the physiologic reaction is as follows: antithrombin inhibits esterase and amidase activity of trypsin; antithrombin–trypsin complexes are observed on disc gel electrophoresis, and hydroxylamine causes dissociation of the complex; antithrombin displaces the active-site dye proflavin from trypsin unless the inhibitor has been preincubated with thrombin; the trypsin–antithrombin complex does not inhibit thrombin-catalyzed clotting of fibrinogen. The reaction of trypsin with antithrombin has the following characteristics. The rate of formation of the complex is more than an order of magnitude faster, in the absence of heparin, than the thrombin reaction. Active trypsin dissociates spontaneously from the complex with a half-time of about 15 min. The antithrombin species that is generated under these conditions is a modified inhibitor which has virtually no inhibitory activity toward thrombin or trypsin but which has retained the ability to bind to heparin–agarose. Under appropriate conditions, it is possible to observe kinetics that show the sequential inhibition and regeneration of trypsin, presumed to correspond to the two-step hydrolysis of antithrombin. This reaction demonstrates the behavior of the inhibitor as a virtual substrate as described recently by Danielsson & Bjork (1980) [Danielsson, A., & Bjork, I. (1980) *FEBS Lett. 119*, 241] for the antithrombin–thrombin reaction, although much longer reaction times are required to observe thrombin regeneration. Although modified antithrombin is the major product of trypsin reaction, some active inhibitor is present in the reaction mixture, and stable complexes also appear to exist.

Protein inhibitors of proteolytic enzymes are widespread in biological systems, and many can be classified according to their mechanism or structure (Laskowski & Kato, 1980). The relation of the plasma inhibitor antithrombin (AT)[1] to the major classes of protease inhibitors, however, is not well understood. The physiologic targets for this inhibitor are the serine proteases of the coagulation system [for a review, see Harpel & Rosenberg (1976)]. The ability of other enzymes to react with antithrombin has been poorly studied, although several workers have reported reaction with trypsin (Figarella et al., 1974; Learned et al., 1976; Feinman & Chang, 1977; Mahoney et al., 1980), papain (Valeri et al., 1980) and with γ-thrombin, a proteolyzed derivative of α-thrombin that has lost clotting activity (Chang et al., 1979). It has also been difficult to determine the extent to which antithrombin follows the "standard mechanism" as described by Laskowski & Kato (1980). In its most general form, this mechanism (eq 1)

$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^* \qquad (1)$$

proposes the formation of covalent complexes C and X in equilibrium with noncovalent complexes L and L* formed from enzyme and native (virgin) inhibitor, or enzyme and a specifically proteolyzed (modified) inhibitor (I*).

Many inhibitors of serine proteases follow this mechanism to a greater or lesser extent, the most well characterized being the soybean trypsin inhibitor [see Laskowski & Kato (1980) and references cited therein]. The major features of this mechanism are that labile noncovalent intermediates (L and L*) are formed, a proteolyzed derivative of the inhibitor appears, and the reaction is reversible, the equilibrium distribution being pH dependent. Since the demonstration by Rosenberg & Damus (1973) of the probable covalent nature of the antithrombin reaction, and their suggestion that this resembled the soybean trypsin inhibitor mechanism, the relevance of eq 1 has been an underlying theme in AT research. It has been difficult to detect intermediates of the type L or L* (Griffith & Lundblad, 1981; Feinman, 1979; Jesty, 1979a). With regard to the existence of proteolyzed derivatives (I*), several groups have detected modified antithrombin species (Chandra & Bang, 1977; Fish et al., 1979a,b; Fish & Bjork, 1979; Jesty, 1979a; Griffith & Lundblad, 1981) although there is no evidence to date that such modified inhibitors can react with protease. The existence of these inactive proteolyzed antithrombin forms has complicated the understanding of whether the reaction is reversible. In a preliminary report, we presented evidence for regeneration of active enzyme from antithrombin–trypsin complexes (Feinman & Chang, 1977), and similar results have been found for Xa and thrombin (Jesty, 1978, 1979a,b; Fish & Bjork, 1979; Danielsson & Bjork, 1980). To date, however, Griffith & Lundblad (1981) are the only workers who have been able to regenerate an active antithrombin species.

In this paper, we give a complete description of our work with trypsin. We confirm earlier observations that a specific reaction occurs with trypsin and that active protease can be regenerated under a variety of conditions. The predominant form of antithrombin released, however, is an inactive modified derivative. Indeed, the majority of the reaction is most ade-

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[1] Abbreviations used: AT, antithrombin; S-2160, N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide; S-2238, D-phenylalanyl-pipecolyl-L-arginyl-*p*-nitroanilide; pNPGB, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; Tos-Arg-OMe, N^{α}-tosylarginine methyl ester; $NaDodSO_4$, sodium dodecyl sulfate; DipF, diisopropyl phosphofluoridate.

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quately described as the sequential formation of complex and the breakdown to free enzyme and proteolyzed antithrombin; the inhibitor behaves as a substrate as proposed by Danielsson & Bjork (1980) for the thrombin reaction. The reaction is more complicated, however, and some stable complexes are formed, and a small amount of active inhibitor species exists in trypsin–AT solutions.

Materials and Methods

Bovine Trypsin. Twice-crystallized bovine pancreatic trypsin was purchased from Worthington Biochemicals (Freehold, NJ). Either this material was used without further purification or the β form was isolated by the method of Schroeder & Shaw (1968) as described by Luthy et al. (1973).

The unfractionated material showed behavior essentially identical with that of the β form, and most experiments were performed with the commercial product. Enzyme concentrations were determined by pNPGB titration (Chase & Shaw, 1969).

Human antithrombin was prepared by a modification of the method of Thaler & Schmer (1975) as described previously (Chang et al., 1979). Human α-thrombin was the generous gift of Dr. John W. Fenton II of the New York State Department of Health, Albany, NY, and typically had 2300 NIH units/mg.

Chemicals. All chemicals were reagent grade. Specific products and suppliers are as follows: *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (pNPGB) (Vega-Fox Biochemicals); tosylarginine methyl ester and proflavin sulfate (Schwarz/Mann Biochemical Co.); N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide (S-2160) and D-phenylalanylpipecolyl-L-arginyl-*p*-nitroanilide (S-2238) (Ortho, Raritan, NJ).

Assay Methods. Determination of the level of free trypsin in incubation mixtures was performed in the following manner by using the substrates S-2160 or S-2238. Linear standard curves were made for the inital rate of release of the *p*-nitroanilide vs. known trypsin concentrations. Unknown levels of free enzyme were then determined from the standard curve. The clotting test and details of the procedures for kinetic experiments involving proflavin and stop-flow methods have been described (Feinman et al., 1977; Chang et al., 1979).

$NaDodSO_4$ Disc Gel Electrophoresis. $NaDodSO_4$ disc gel electrophoresis was performed essentially according to the method of Weber & Osborn (1969). The gels were stained with Coomassie Brilliant Blue R dye and destained with 7% acetic acid at 65–70 °C.

Hydroxylamine Solutions. In experiments to measure the dissociation of trypsin–AT complexes by hydroxylamine, reaction conditions were found to be critical. In particular, buffering of 2 M hydroxylamine hydrochloride solutions caused salting out of the complex. Concentrated hydroxylamine solutions were prepared by neutralization of the hydrochloride with Bio-Rad anion-exchange resin AG 1-X8, hydroxide form.

Results

Antithrombin Displaces Proflavin from Trypsin. Antithrombin causes a reduction in the characteristic difference absorption spectrum of trypsin–proflavin solutions. The binding of proflavin to trypsin is known to be at the active site, and substances which bind to the site displace the dye, causing an increase in free proflavin and loss of the difference spectrum (Bernhard et al., 1966). Figure 1 shows the decrease in the visible difference absorption of proflavin–trypsin when AT is added (spectra A and B). This behavior is similar to the effect of antithrombin on the thrombin–proflavin system, although

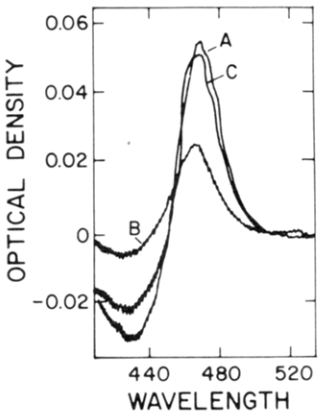

FIGURE 1: Difference absorption spectra of trypsin and antithrombin–trypsin solutions. The reference cuvette contains 30 μM proflavin in 0.05 M sodium barbital, 0.1 M NaCl, and 0.02 M $CaCl_2$, pH 8.3, 25 °C. Samples contain proflavin plus the following additions: (A) bovine β-trypsin (14 μM); (B) trypsin as in (A) plus 13 μM antithrombin; (C) same conditions as (B) except the antithrombin had been preincubated with 11 μM human α-thrombin for 30 min prior to addition to the trypsin solution.

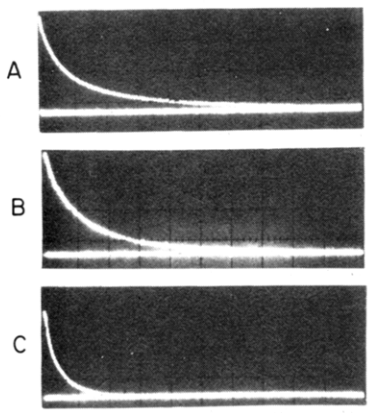

FIGURE 2: Stop-flow kinetics of trypsin–antithrombin reaction. [Trypsin] = 13.1 μM. (A) AT/trypsin ratio = 0.97 (50 ms/division); (B) AT/trypsin ratio = 1.95 (20 ms/division); (C) AT/trypsin ratio = 4.89 (20 ms/division). All solutions contain 30 μM proflavin in 0.5 M sodium barbital, 0.1 M NaCl, and 0.02 M $CaCl_2$, pH 8.3, 25 °C.

spectral changes are much larger in the thrombin system because of the greater affinity of thrombin for proflavin (Chang et al., 1979). If the AT is first incubated with a stoichiometric amount of thrombin, then there is little change in the trypsin–proflavin spectrum when the AT solution is added (Figure 1C).

Stop-Flow Kinetics of the Trypsin–Antithrombin Reaction. The decrease in absorption at 469 nm which accompanies release of proflavin from trypsin as AT is bound can be used to follow the kinetics of the reaction. Figure 2 shows the results of a stop-flow kinetic experiment in which similar concentrations of reactants were mixed in the presence of proflavin (curves A and B). The reaction is observed to be second order, and the calculated rate constant is 4.5×10^5 M^{-1} s^{-1} (corrected for proflavin); this value can be compared to the second-order rate constant for the thrombin reaction at 25 °C, pH 8.0, of 1.7×10^4 M^{-1} s^{-1}.[2] Thus, the reaction of antithrombin with

[2] In our previously published values for rate constants in the AT–thrombin reaction, we neglected to correct for inhibition by proflavin. Becuase human thrombin has an unusually high affinity for proflavin (Chang et al., 1979), this can introduce a significant error. The corrected values for the association rate constant are 4.4×10^3 (pH 7.5, 25 °C) and 1.7×10^4 M^{-1} s^{-1} (pH 8.0, 25 °C).

trypsin is more than an order of magnitude faster than the reaction with thrombin. We attempted to carry out this reaction under pseudo-first-order conditions. The stop-flow reaction with AT in 5 M excess is shown in Figure 2. The pseudo-first-order rate constant is 19.3 s^{-1}. If the true order were second, the calculated second-order rate constant would be $k_{obsd}/[AT]_0 = 3.0 \times 10^5$ M^{-1} s^{-1}. This constant is somewhat lower than the constant measured under second-order conditions. On the other hand, if the true mechanism involved prior formation of an ES-type complex (L in eq 1), then the approximate K_a for such a complex would be k(pseudo first order)/k(second order) = 4.3×10^{-5} M. This value is similar to the dissociation constants K_L and K_{L*} (2.2×10^{-5} M) reported by Luthy et al. (1973) for the formation of the intermediates L and L* in reaction of trypsin with virgin and modified soybean trypsin inhibitor.

The rate of reaction of trypsin with AT is increased severalfold by the presence of heparin. By comparison, the thrombin reaction is accelerated by at least 2 orders of magnitude (Li et al., 1976; Jordan et al., 1979), although, as noted above, the trypsin reaction is substantially faster in the absence of heparin than the thrombin reaction. A detailed description of the heparin effect will be published elsewhere (R. F. Wong et al., unpublished experiments).

Effect of Hydroxylamine on the AT–Trypsin Complex. One of the characteristics of the thrombin–antithrombin association is its dissociability by hydroxylamine under denaturing conditions (Owen, 1975). In addition, Longas & Finlay (1980) have shown that labeled methoxylamine is incorporated into a modified AT with a new C-terminal arginine. These results suggest that the enzyme–inhibitor bond is an ester, at least in the presence of denaturant. Similar generation of proteolyzed inhibitor fragment by NH_2OH has been reported for α_2-antiplasmin (Wiman & Collen, 1979) and α_1-antitrypsin (Johnson & Travis, 1976). Figure 3 shows that the trypsin–AT complex can also be dissociated by hydroxylamine. As a control, a sample of thrombin–AT complex was treated in the same manner. It is significant that the major band reappearing after hydroxylamine treatment is approximately the same—at least at this resolution—as free AT; that is, the dissociated material is not drastically proteolyzed. The results shown in Figure 3 are in disagreement with a report of Mahoney et al. (1980), who did not find dissociation in the presence of hydroxylamine, although the differences may be due to different experimental conditions. In any case, under conditions similar to those in Owen's original experiment, we find it is possible to dissociate both thrombin and trypsin complexes of AT with hydroxylamine.

Dissociation of the Antithrombin–Trypsin Complex. Final values for the change in proflavin absorption in the AT–trypsin reaction were invariably less than that expected for stoichiometric formation of the complex; that is, there was always free enzyme at the end of the reaction. In order to study this effect at lower concentrations of reactant, we utilized a rate assay based on inhibition by antithrombin of the trypsin-catalyzed hydrolysis of the anilide substrate S-2160 (Feinman & Chang, 1977; Feinman, 1979). We found, in fact, that free trypsin can be titrated in solutions that contained an excess of AT. In our original report, we considered that the existence of free trypsin in such solutions of antithrombin was a reflection of the reversibility of complex formation (Feinman & Chang, 1977), and we calculated a dissociation constant in the nanomolar range (Feinman, 1979). However, as we studied this phenomenon under a variety of conditions, we were unable to obtain a consistent constant, and it became obvious that

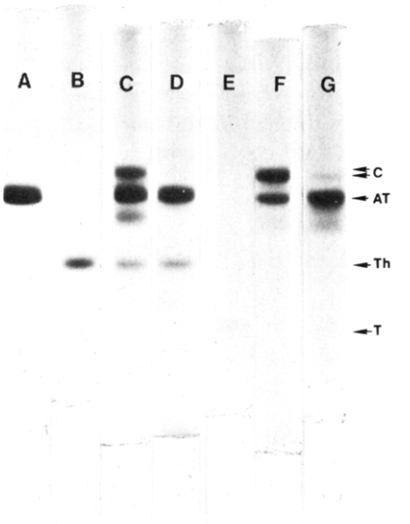

FIGURE 3: Treatment of the trypsin–antithrombin complex with hydroxylamine. Thrombin–AT and trypsin–AT complexes were prepared by incubating AT (31 μM) with thrombin (24 μM) for 3 min and with trypsin (24 μM) for 0.5 min in 0.02 M imidazole, 0.15 M NaCl, and 0.02 M $CaCl_2$, pH 7.50. At the end of each incubation period, DipF was added (0.24 mg/mL final concentration) to stop the reaction, followed by addition of 0.1 volume of 10% $NaDodSO_4$ with heating at 90 °C for 10 min. Equal volumes of 2 M hydroxylamine, pH 7.5, were added to treated samples and incubated for 17 h at 25 °C. After overnight dialysis against 0.1 M sodium phosphate, 0.05 M NaCl, and 0.1% $NaDodSO_4$, pH 6.5, the samples were analyzed by $NaDodSO_4$ gel electrophoresis. (A) AT; (B) thrombin; (C) untreated thrombin–AT complex [The bands migrating slightly faster than AT in bands C and G and in Figure 8 appear frequently in incubation mixtures containing thrombin in our work and in the literature. They are usually attributed to degradation products resulting from proteolysis by free thrombin [e.g., see Fish et al. (1979b)].]; (D) thrombin–AT complex + 1 M hydroxylamine; (E) trypsin; (F) untreated trypsin–AT complex; (G) trypsin–AT complex + 1 M hydroxylamine.

equilibrium is not always attained, and trypsin is still being released during the time of measurement. Figure 4 shows the kinetics of the AT–trypsin reaction followed by S-2160 hydrolysis. At equimolar concentrations of reactants (curve B), there is a rapid loss in enzyme activity followed by a slower reappearance. At the earliest time point that can be measured by this method, there is about 60% inhibition although it is assumed that complete inhibition occurs and the beginning of the reappearance of the enzyme is missed in the first minute. The final point in curve B shows that the enzyme is still about 35% inhibited. Similar results are found with experiments in which the trypsin:AT ratio is 2:1 (curve A, maximum inhibition would be 50%). If a large excess of antithrombin is used (Figure 4, curve C), there is complete inhibition and no apparent return of enzymatic activity. The spontaneous release of enzyme from the AT complex is similar to that observed by Danielsson & Bjork (1980) for the thrombin reaction although in that case the time for release is very much longer. The lack of regeneration of enzyme seen in curve C of Figure 4 is presumably due to recycling of the released trypsin which can react with the excess AT in the solution. The apparent first-order rate constant for release is 3.7×10^{-4} s^{-1}. This is in agreement with the value of 4.0×10^{-4} s^{-1} previously determined by the increase in the postburst rate in the pNPGB titration (Feinman, 1979). If the reactant concentration is lowered to the nanomolar range, the rate of formation of complex becomes comparable with its breakdown, and the two reactions can be seen on the same time scale. Figure 5A is a kinetic curve which shows sequential complex formation and reappearance of enzyme. The very end of this reaction can

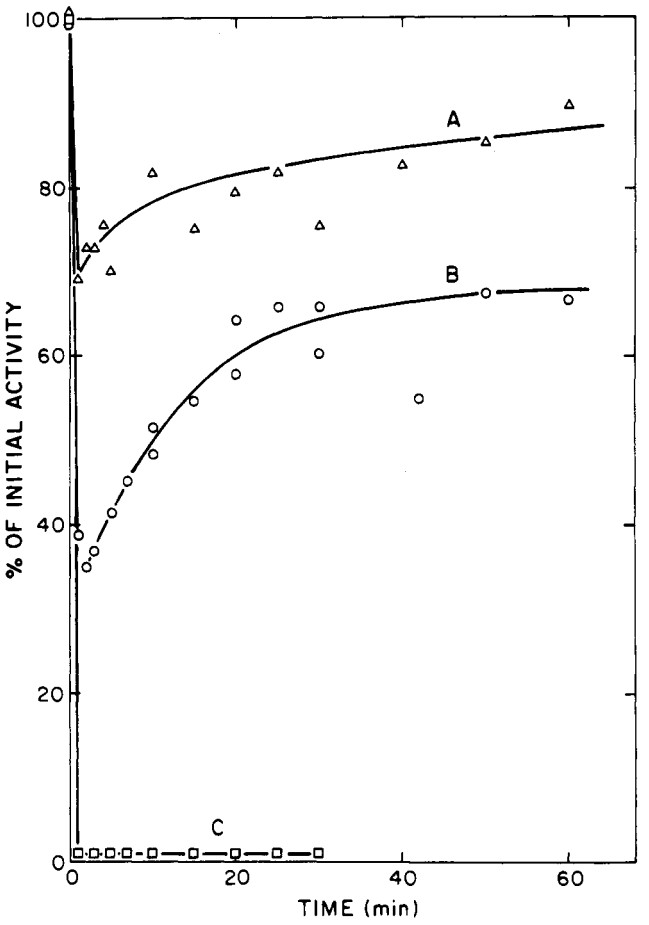

FIGURE 4: Release of trypsin from the AT–trypsin complex. Antithrombin was incubated with trypsin at various concentrations. After different times, aliquots were removed from the incubation mixtures and diluted 1:33.3, and the free trypsin levels were assayed with S-2160. (A) [AT] = 0.12 μM, [trypsin] = 0.22 μM; (B) [AT] = 0.40 μM, [trypsin] = 0.40 μM; (C) [AT] = 1.3 μM, [trypsin] = 0.14 μM.

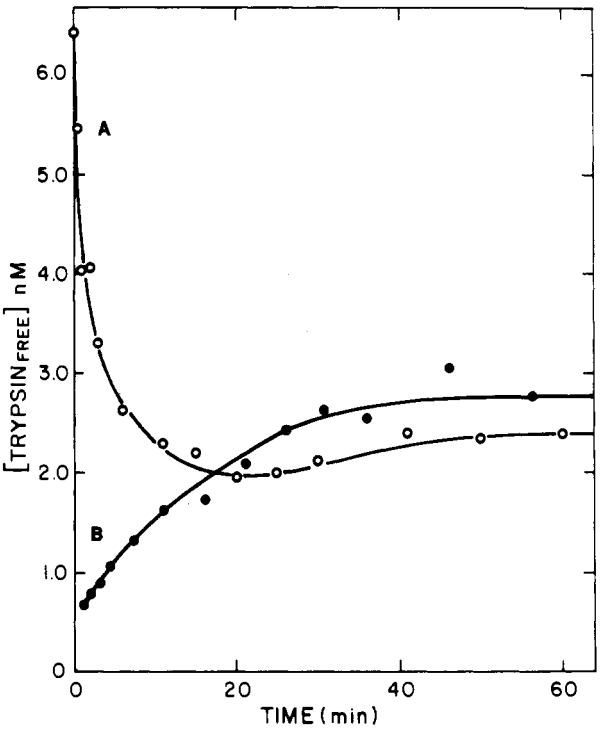

FIGURE 5: Time course for the dissociation of trypsin from the complex. (A) Stock solutions of 0.21 μM trypsin and 0.36 μM antithrombin were diluted separately (1:33.3) and then mixed. Free enzyme levels were assayed at the indicated times by adding 30 μM S-2160. All solutions were in 0.5 M sodium barbital, 0.0625 M NaCl, and 0.02 M $CaCl_2$, pH 8.3. (B) Same as (A) except trypsin and antithrombin were incubated at the stock (0.1 μM range) concentrations for 5 min then diluted 1:33.3.

be compared with the regeneration of enzyme from complex formed (rapidly) at a high concentration with the same AT/T ratio (curve B of Figure 5).

Dilution of the Antithrombin–Trypsin Reaction Mixture. The experiment in Figure 5B can be done in two ways. Curve B shows the increase in trypsin activity after a sample of complex formed at high concentration has been diluted. A second method for carrying out the reaction is to take samples from the concentrated reaction and dilute these into the assay mixture. We found that the two methods gave essentially identical kinetics. This means that the appearance of trypsin is due predominantly to the first-order breakdown of complex and is not significantly affected by re-formation of complex; i.e., dilution of reactants does not affect the rate of release of trypsin.

Regeneration of Antithrombin Species from the Antithrombin–Trypsin Complex. To investigate whether an active AT species is released from the complex when trypsin dissociates, we added additional trypsin at various times in the reaction and assayed after a 20-min incubation. The reaction mixtures initially contained an excess of antithrombin, but in all cases (additions at 20 min to 5 h), there was less AT activity even than the amount in excess. In other words, the trypsin had recycled and attacked more than a single AT molecule. On a molar basis, however, there appeared to be about 10–20% active AT left in these reaction mixtures. Thus, it appeared that the major product released with active trypsin was a modified AT, although some trypsin-inhibiting activity remained. Because these are not ideal conditions for characterizing the product, i.e., long incubation times in the presence of free protease, we tried to dissociate the complex by trapping trypsin as it was released. We first used soybean trypsin inhibitor which was added to the reaction mixtures, in large molar excess over AT, at various times. Under these conditions of excess soybean inhibitor, the association between regenerated trypsin and AT becomes negligible, allowing the dissociation of the complex to be monitored. Figure 6 shows the composition of incubation mixtures at different times after the addition of trypsin inhibitor as analyzed by $NaDodSO_4$ gel electrophoresis. After 30 s, most of the trypsin and AT are still in the form of the complex (Figure 6B), but after 45 h, almost all of the complex has been dissociated (Figure 6C). The quantitative increase in intensity of the bands corresponding to trypsin and AT indicates that the dissociated complex was essentially completely recovered as the initial reactants. In separate experiments, we found that it was possible to observe the dissociation of the complex, as seen in lane C after 1 or after 17 h of incubation.

To determine whether the antithrombin species released from the complex was active, we added soybean trypsin inhibitor to equilibrium mixtures of trypsin and AT, and the thrombin-inhibiting capacity of the mixture was measured at various times. This approach utilized the fact that the soybean inhibitor does not inhibit thrombin (Fenton et al., 1979). Figure 7 shows the results obtained when thrombin activity was monitored in a clotting test. No significant amount of thrombin inhibition was generated in the mixtures incubated with trypsin inhibitor up to 15.5 h (Figure 7B,C). Antithrombin alone was found to be stable under the incubation conditions. Figure 7D shows the predicted loss in thrombin activity if active antithrombin were released from the complex

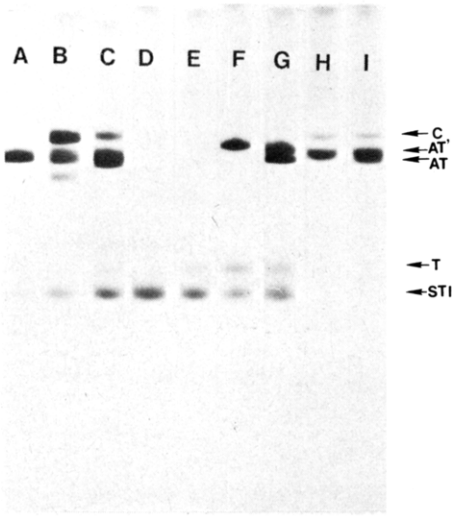

FIGURE 6: Regeneration of the AT species from the trypsin–AT complex. AT (10.9 μM) was incubated with trypsin (8.9 μM) for 20 s, and then soybean trypsin inhibitor (STI) (18.2 μM) was added. The buffer was 0.02 M imidazole, and 0.15 M NaCl, pH 7.35. (A) AT + STI; (B) 30 s after the addition of STI to the incubation mixture; (C) 45 h after STI addition. (D–I) Mixture (C) was loaded onto a heparin–agarose column (0.7 × 16.5 cm) equilibrated with the incubation buffer. The column was washed with the equilibrating buffer until the A_{280} returned to the original base line. Protein bound to the column was then eluted with 0.02 M imidazole and 1.2 M NaCl, pH 6.5. Both the wash and the high-salt elution were collected in 1-mL fractions. Wash fractions: (D) tube 4; (E) tube 7; (F) tube 10; (G) mixtures (A) + (F). High-salt elution: (H) pool of tubes 7–9; (I) mixtures (A) + (H).

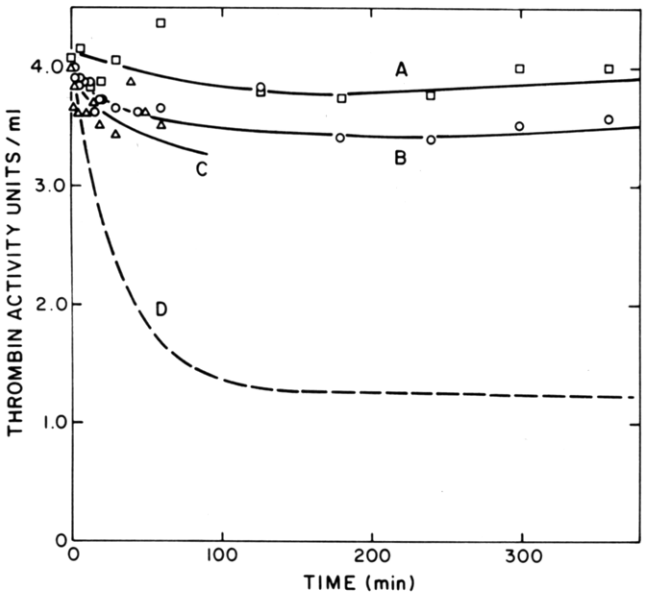

FIGURE 7: Regeneration of AT activity from the trypsin–AT complex. AT (0.55 μM) was incubated with trypsin for 1.5–2.0 min, and then STI (4.4 μM) was added. At different times, aliquots of the reaction mixture were removed and incubated for 5 min with thrombin (0.66 μM) and heparin (2.9 USP/mL). Residual thrombin activity was then assayed by measuring clotting times. (A) Thrombin + heparin alone. (B) AT incubated with 0.48 μM trypsin. (C) AT incubated with 0.37 μM trypsin. (D) Predicted loss in thrombin activity if active AT is regenerated at the same rate trypsin is released from the complex.

at the same rate that trypsin dissociates from the complex. Results similar to these were obtained if 0.45 mM DipF was used in place of trypsin inhibitor in the incubation mixtures. Also, no re-formation of complex was detected on $NaDodSO_4$ gel electrophoresis if thrombin or excess trypsin was added to the incubation mixture after dissociation of the complex.

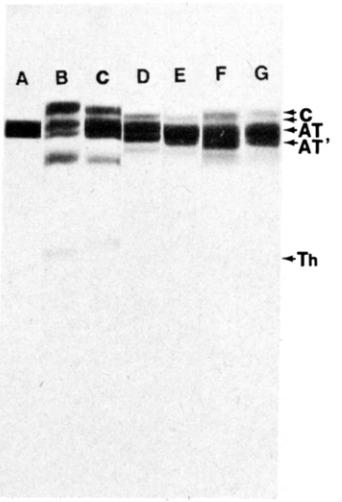

FIGURE 8: AT (24 μM) was incubated with thrombin (18.4 μM) or trypsin (18.4 μM) in 0.0156 M sodium phosphate and 0.12 M NaCl, pH 7.2, at 37 °C. After the indicated times, the samples were heated at 90–95 °C for 10 min in 1% $NaDodSO_4$ and 2.8% 2-mercaptoethanol. (A) AT; (B) AT + thrombin for 2 min; (C) samples (A) and (B) were mixed to identify the native AT band in sample (B); (D) AT + trypsin for 45 s; (E) samples (A) and (D) were mixed to identify the native AT band in sample (D); (F) samples (B) and (D) were mixed to compare the migration of the modified AT species from the thrombin and trypsin–AT reactions; (G) samples (A) and (F) were mixed in order to identify the native AT band in sample (F).

The absence of measurable antithrombin activity after the apparent dissociation of the complex with soybean trypsin inhibitor may be the result of (1) the presence of one or several protein species in the incubation mix which somehow interfere with the assay for AT and, thus, yield erroneous measurements or (2) the AT species that is dissociated from the complex does not inhibit thrombin activity. To distinguish between these two possibilities, we again added soybean trypsin inhibitor to an equilibrium mixture of trypsin and AT. After 48.5 h, the incubation mixture was separated on a heparin–agarose column. As shown in Figure 6 (tracks D–G), the protein species eluted with the equilibrating buffer wash were trypsin and trypsin inhibitor (Figure 6D,E) and a third component which migrates slower than AT (Figure 6F,G). On the other hand, only a single major component migrating slightly slower than AT is eluted at high salt concentration (Figure 6H,I). Similar slower migrating species were observed by Jesty (1979a) in the analysis of the thrombin and Xa reactions with antithrombin. We found no thrombin-inhibiting activity in any of the fractions collected from either the equilibrating or the high salt buffer elutions as measured by the clotting test. In addition, the component eluted at high salt concentration exhibited only low levels of inhibitory activity toward either thrombin or trypsin as measured by hydrolysis of the anilide substrate S-2238. Although only 23% of the original AT incubated was recovered in this manner, it appears that an AT species modified by reaction with trypsin retains the capacity to bind heparin but can no longer function as an inhibitor for enzymes such as thrombin or trypsin.

Modified Antithrombin Species. In order to identify the inactive antithrombin in species generated in the trypsin–AT reaction, gel electrophoresis under reducing conditions was performed on trypsin–AT and thrombin–AT reaction mixtures. Figure 8 demonstrates the existence of a modified AT species in both reactions. Gels D and E indicate that the modified AT from the trypsin reaction migrates faster than the native inhibitor in a manner similar to the modified AT from the thrombin reaction (gels B and C). Gel F shows that, within

the resolution of this system, the two modified AT species comigrate. The released AT molecule in the trypsin reaction would thus appear to be similar to the material reported by other workers for the thrombin reaction [e.g., see Fish et al. (1979a) and Jesty (1979a)]. Figure 8 shows an additional minor band migrating faster than modified AT (gels D and E). Although we did not attempt to identify this material, reactions in which trypsin concentration was in excess showed an increased proportion of this material on electrophoresis. Danielsson & Bjork (1981, and personal communication) have recently studied the trypsin–AT reaction. These workers showed that the modified AT species initially formed is similar to the thrombin-modified AT, in agreement with our results. They found, in addition, that this material (or samples of thrombin-modified AT) could be further degraded to a new modified AT species. This second species may be the same as the minor, rapidly migrating band observed in our gels.

Discussion

The reaction of antithrombin with pancreatic trypsin provides a model for the reaction with coagulation proteases. The conversion to modified inhibitor is relatively rapid, and the sequential formation and breakdown of the complex can be observed on the same time scale. Although it is not possible to give a complete description of the overall process, several points are worthy of discussion. First, we present further evidence that the reaction is specific, analogous to the thrombin reaction. We also show that active protease can be regenerated, although the major AT species released is not active. Finally, the reaction mechanism can be compared with that seen with the serine proteases of the coagulation cascade, and with eq 1 for other protease inhibitors.

Specificity of the Antithrombin–Trypsin Reaction. The AT–trypsin reaction is a specific active-site interaction similar to the reaction with coagulation enzymes. The evidence supporting this idea can be summarized as follows. (1) AT inhibits the trypsin-catalyzed hydrolysis of tosylarginine methyl ester and the anilide substrates S-2160 and S-2238. (2) AT that has been treated with trypsin is no longer capable of inhibiting thrombin-catalyzed clotting of fibrinogen. (3) Trypsin–AT complexes can be observed on disc gel electrophoresis; similar results were reported by Mahoney et al. (1980) and by Learned et al. (1976). (4) AT displaces proflavin from trypsin, unless the inhibitor has been preincubated with thrombin. (5) The AT–trypsin reaction is accelerated by heparin. (6) Hydroxylamine is capable of causing dissociation of the complex.

A crucial question in this work is the extent to which nonspecific proteolysis accounts for the results. The points summarized above which emphasize the similarity of the trypsin reaction to the thrombin association would indicate formation of complex is specific and not due to any interactions between proteolyzed products. On the other hand, one major conclusion is that AT behaves as a substrate and is relatively rapidly (compared to thrombin) converted to a proteolyzed product. That this is not random proteolysis is indicated by the lack of small fragments on disc gel electrophoresis and the fact that, on nonreduced gels, the AT behaves in a manner similar to the product of the thrombin and Xa reactions (Jesty, 1979a). The lack of proteolysis of the AT molecule would also be consistent with the observations of Jesty (1979a) and Fish et al. (1979a) that proteolysis of enzyme–AT complexes by excess enzyme affected primarily the bound protease. Some proteolysis of a nonspecific nature must occur, however, and we feel that the presence of free trypsin in solution at long incubation times places a limitation on the study of the ultimate fate of the AT–trypsin complex.

Dissociation of Antithrombin–Trypsin Complexes. It is now firmly established that antithrombin inhibits proteases by forming a 1:1 complex involving the enzyme active site. The fate of the initial inhibited complex, however, has been harder to understand. Dissociation under a variety of conditions has been reported in the literature, and a modified inactive antithrombin is the usual product. In the trypsin reaction, the spontaneous breakdown of the complex is readily observable. This rapid regeneration of enzyme is, perhaps, the most striking difference between the trypsin–AT reaction and the reaction with the coagulation enzymes. Although speculative at this point, it is possible that the biologic specificity for an enzyme in the antithrombin reaction may reside in a reduced rate of dissociation from the inhibited complex. Our observation that the major antithrombin species that appears during dissociation does not, in fact, react with proteases is consistent with many reports in the literature involving other enzymes. Thus, Fish et al. (1979b), Fish & Bjork (1979), and Jesty (1979a) have shown that thrombin can be dissociated from its AT complex by a variety of conditions and in each case the predominant species is a modified antithrombin in which a small fragment (approximately 5K daltons) appears to have been removed from the inhibitor. Danielsson & Bjork (1980) have shown that thrombin can be dissociated by incubation at pH 7.4 in Tris as judged by $NaDodSO_4$ electrophoresis. Although Jesty (1979a) has found that complex dissociated at pH 9 appears to reassociate if dialyzed at pH 7, to our knowledge, only the report of Griffith & Lundblad (1981) has shown any substantial production of active AT species. By complexing dissociated enzyme with a tripeptide chloromethyl ketone and assaying active and inactive AT, these workers were able to show stoichiometric release of active AT at pH 7, and they found an increasing fraction of modified inhibitor as the pH is raised. The material dissociated from the trypsin complex in the presence of DipF or soybean trypsin inhibitor has little or no inhibitory activity. It is significant that this product of complex dissociation is able to bind to heparin–agarose. If this turns out to be generally true of modified AT isolated from protease reactions, it may provide a valuable structural tool for studying the heparin–AT interaction. This is also an interesting observation since it is known that heparin has a greatly reduced binding to the AT–protease complex compared to free inhibitor [see, e.g., Feinman (1979) and Jordan et al. (1979)].

Mechanism of the Antithrombin–Protease Reaction. The reaction of trypsin with antithrombin is consistent with the sequential formation of inhibited complex followed by conversion to modified inhibitor as shown in eq 2, using the

$$E + I \rightarrow C \rightarrow E + I^* \qquad (2)$$

symbols of the protease inhibitor mechanisms. As far as we have been able to determine, I* does not react with trypsin or thrombin. On the other hand, dissociation does not proceed to completion, and some stable complex exists, although it appears that this complex can be dissociated by hydroxylamine, soybean trypsin inhibitor, or DipF. This behavior is paradoxical, and we feel that this apparently metastable complex cannot be incorporated into a specific mechanism without further study. The major product is, in any case, the modified AT. We do detect, however, a small amount of active AT which may arise from the complex. In this regard, an additional stable intermediate (X in eq 1) has been proposed by Griffith & Lundblad (1981). They were able to show generation of both active and inactive AT species and proposed that each arose from a different stable intermediate. In our

view, it is not necessary to invoke a second species, and both forms could arise from one complex; the simplest mechanism would be that shown in eq 3:

$$E + I \rightleftarrows C \rightarrow E + I^* \quad (3)$$

Evidence for mechanisms of the type shown in eq 3 is obtained by trapping released enzyme, and it should be pointed out that, regardless of the number of sequential intermediates, the prediction of this mechanism is that ultimately there must be complete conversion to I*.

The existence of labile ES-type complexes (L) has been a subject of some study, and many groups have attempted to find such an intermediate without success (Feinman, 1979; Griffith & Lundblad, 1981; Jesty, 1979a). If such an intermediate exists, it must have a K_m greater than 10^{-4} M. In the work presented here with trypsin, we can see some saturation of the rate of association consistent with eq 4, but because the

$$E + I \rightleftharpoons L \rightarrow C \rightarrow I^* + E \quad (4)$$

measured constant (40 μM) is so close to the highest concentration used in the experiment, we feel more study is needed before such an intermediate is definitely established.

In summary, whereas a totally consistent model for antithrombin reactions is probably not possible at this time, the most comprehensive scheme that can be proposed is that shown in eq 5:

$$E + I \rightleftharpoons L \rightleftharpoons C \rightarrow I^* + E \quad (5)$$

For the reaction with trypsin, reversibility is minimal, the intermediate L is suggested, and the formation of the unreactive I* is relatively rapid.

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