Biochem. J. (1983) **213**, 345–353
Printed in Great Britain

Properties of antithrombin–thrombin complex formed in the presence and in the absence of heparin

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(*Received 14 February 1983/Accepted 13 April 1983*)

Purification of antithrombin–thrombin complex by ion-exchange chromatography on DEAE-agarose resulted in predominantly monomeric complex, whereas purification on matrix-linked heparin produced large amounts of aggregated complex. Monomeric antithrombin–thrombin complexes formed in the presence and in the absence of heparin had similar conformations and heparin affinities. Moreover, the first-order dissociation rate constants, measured by thrombin release, of these complexes were similar, 2.3×10^{-6}–3.4×10^{-6} s^{-1}, regardless of whether newly formed or purified complex was analysed. Similar dissociation rate constants were also obtained for purified complex formed with or without heparin, from analyses by dodecyl sulphate/polyacrylamide-gel electrophoresis of the release of modified antithrombin, cleaved at the reactive-site bond. No dissociation of intact antithrombin from the complex was detected by activity measurements or by gel electrophoresis. Aggregation of the complex was found to be accompanied by a decrease in apparent dissociation rate. The similar properties of antithrombin–thrombin complexes formed with or without heparin support the concept of a catalytic role for the polysaccharide in the antithrombin–thrombin reaction. Furthermore, the results indicate that the reaction between enzyme and inhibitor involves the rapid formation of an irreversible, kinetically stable, complex that dissociates into active thrombin and modified, inactive, antithrombin by a first-order process with a half-life of about 3 days. The inhibition thus resembles a normal proteolytic reaction, one intermediate step of which is very slow.

The plasma proteinase inhibitor antithrombin inactivates the serine proteinases of the intrinsic coagulation system by forming stable equimolar enzyme–inhibitor complexes (for reviews see Rosenberg, 1977*a*,*b*; Barrowcliffe *et al.*, 1978; Björk & Lindahl, 1982). These reactions involve interaction between the active-site serine residue of the enzyme and a specific Arg–Ser bond in the *C*-terminal portion of the inhibitor (Fish & Björk, 1979; Jörnvall *et al.*, 1979; Longas & Finlay, 1980; Björk *et al.*, 1981, 1982). A modified antithrombin, cleaved at this reactive-site bond, has been shown to be released by slow spontaneous dissociation of the antithrombin–thrombin complex (Danielsson & Björk, 1980), concurrently with active thrombin (Jesty, 1979*b*). Antithrombin may thus act essentially as a substrate for its target enzymes, forming a kinetically stable complex with the latter, in which normal proteolysis has been arrested at some intermediate stage. However, in apparent contradiction to this proposal it has been suggested that predominantly intact antithrombin dissociates from the antithrombin–thrombin complex at neutral pH and that the release of the modified inhibitor occurs by a secondary reaction that is not directly involved in thrombin inhibition (Griffith & Lundblad, 1981).

The rate of complex-formation between antithrombin and coagulation enzymes increases dramatically in the presence of heparin (Rosenberg & Damus, 1973; Jordan *et al.*, 1979). A certain fraction of the polysaccharide binds to antithrombin and induces a conformational change of the inhibitor that may be involved in the mechanism of the rate enhancement (Rosenberg & Damus, 1973; Villanueva & Danishefsky, 1977; Einarsson & Andersson, 1977; Nordenman & Björk, 1978*a*; Nordenman *et al.*, 1978; Olson & Shore, 1981; Olson *et al.*, 1981). In addition, an interaction between heparin and the enzyme also appears to be necessary for the accelerating effect of the polysaccharide, at least for certain enzymes (Laurent *et al.*, 1978; Jordan *et al.*, 1980; Holmer *et al.*, 1981;

Oosta *et al.*, 1981; Griffith, 1982). Several observations support the view that heparin acts as a catalyst in the antithrombin–thrombin reaction (Markwardt & Walsman, 1959; Biggs *et al.*, 1970; Gitel, 1975; Björk & Nordenman, 1976; Carlström *et al.*, 1977; Jordan *et al.*, 1979). However, so far no evidence has been presented that the reaction products in the presence and in the absence of heparin are identical.

The present study was undertaken to elucidate further the mechanism of the reaction between antithrombin and thrombin. The results show that enzyme–inhibitor complexes of similar, or identical, conformation and stability are formed in the presence and in the absence of heparin, consistent with a catalytic function for the polysaccharide. The results also demonstrate that the major dissociation pathway of the antithrombin–thrombin complex at neutral pH is by a slow first-order process into modified antithrombin and active thrombin, supporting the contention that the inhibition is akin to normal, slow, proteolysis.

Materials and methods

Bovine antithrombin was prepared as described by Miller-Andersson *et al.* (1974). The rates of inactivation of thrombin by the inhibitor in the absence and in the presence of Polybrene were identical, indicating that the antithrombin preparation was not contaminated with heparin (Jesty, 1979*b*).

Bovine α-thrombin was prepared from prethrombin 1 by published procedures (Owen *et al.*, 1974; Lundblad *et al.*, 1975; Carlström *et al.*, 1977). The purified enzyme was 90% active, as determined by active-site titration with 4-nitrophenyl 4-guanidinobenzoate (Chase & Shaw, 1970), and its specific activity was 2900 N.I.H. units/mg. Thrombin was radiolabelled with NaB^3H_4 in terminal sialic acid residues after periodate oxidation (Van Lenten & Ashwell, 1971). The labelled material had a specific radioactivity of 2700 d.p.m./μg and was fully active towards D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide (compound S-2238; AB Kabi, Stockholm, Sweden).

Heparin from pig intestinal mucosa (stage 14; Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A.) was purified (Lindahl *et al.*, 1965), fractionated by gel chromatography and then separated into species with high and low affinities for antithrombin (Höök *et al.*, 1976). High-affinity heparin with an average M_r of about 13000 (Danielsson & Björk, 1981) was used in most experiments. Purified (but unfractionated) commercial heparin was also coupled to CNBr-activated Sepharose with a resulting heparin content of 2.2 mg/ml of wet gel. Affinity chromatography on heparin–Sepharose was done as described previously (Carlström *et al.*, 1977; Nordenman & Björk, 1978*b*).

Nascent antithrombin–thrombin complex formed in the absence of heparin was prepared by addition at 37°C of thrombin to antithrombin to a final molar ratio of 1:6. Analyses were started immediately after the reaction was complete (i.e. 10 min), without purification of the complex; hence the term 'nascent complex'. In experiments with nascent complex formed in the presence of heparin, the inhibitor was first incubated with high-affinity heparin for 5 min at a molar ratio of about 1:5. The inhibitor/heparin mixture was then allowed to react for 60 s at 37°C with thrombin at an enzyme/inhibitor molar ratio of 1:6. Polybrene (final concn. 0.3 g/l) was then added in order to neutralize the heparin and prevent it from interfering with further analyses.

Most experiments with purified antithrombin–thrombin complex were done with complex isolated by ion-exchange chromatography. Antithrombin (18 mg) and thrombin (3 mg) were mixed, either for 10 min at 4°C in the presence of 2 mg of unfractionated heparin, or for 30 min at room temperature in the absence of the polysaccharide. The mixture was then applied at 4°C to a column (2.6 cm × 13 cm) of DEAE-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) in 50 mM-sodium phosphate buffer, pH 6.5. The complex was eluted with a linear gradient to 0.25 M-NaCl in the same buffer, whereas non-complexed antithrombin remained bound to the column at this salt concentration. The complex was further purified on a Sephacryl S-200 column (1.6 cm × 90 cm) in 0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4. In some analyses antithrombin–thrombin complex formed in the presence of heparin and purified by chromatography on heparin–agarose, followed by gel chromatography (Danielsson & Björk, 1980), was also used.

Sedimentation-equilibrium experiments were conducted in a Beckman model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). The rapid long-column meniscus-depletion method (Chervenka, 1970), in conjunction with interference optics, was used. The partial specific volume of the antithrombin–thrombin complex was calculated to be 0.72 ml/g from the values for the two moieties of the complex (Magnusson *et al.*, 1975; Nordenman *et al.*, 1977).

C.d. measurements were made at 22 ± 2°C with a Jasco J41 A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Analyses in the near-u.v. range (250–310 nm) were done in cells with 1 cm path-length at a protein concentration of 1 g/l, and analyses in the far-u.v. range (200–250 nm) were done in cells with 1 mm path-length at a protein concentration of 0.2 g/l.

The rate of dissociation of thrombin from both the nascent and the purified antithrombin–thrombin complexes was monitored at 37°C by a continuous assay, based on the release of active enzyme in the presence of a chromogenic substrate. The procedure essentially followed that described by Jesty (1979*b*); the major modification was that the final reaction volume was 2.1 ml. The analyses of the nascent complexes were started 10 min after antithrombin and thrombin were mixed; at this time essentially all thrombin was inactivated. The analyses of the purified complexes were started immediately after transfer of the complex to 37°C. First-order dissociation rate constants were obtained by computer-fitting the spectrophotometer tracings to a theoretical equation (Jesty, 1979*b*).

The rate of dissociation of modified antithrombin from the purified antithrombin–thrombin complex was studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Danielsson & Björk, 1980). The complex (2 g/l in 0.18 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4) was incubated at 37°C in the presence of chloroform to prevent microbial growth. In some experiments the irreversible thrombin inhibitor D-Phe-Pro-Arg-CH_2Cl ('D-phenylalanyl-prolyl-arginine chloromethyl ketone') (Kettner & Shaw, 1979; kindly given by Dr. E. Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A.) was also added in a 5-fold molar ratio to the complex. Samples were removed at different times, and were analysed by gel electrophoresis under reducing conditions (Weber & Osborn, 1969; Danielsson & Björk, 1980). The gels were stained with Coomassie R-250, and were scanned at 570 nm. The relative amounts of complex, native antithrombin, the large chain of modified antithrombin, and thrombin were determined by planimetry.

Attempts were made to measure the release of native antithrombin from the nascent antithrombin–thrombin complex. The method used by Griffith & Lundblad (1981) was employed, with a few modifications. Antithrombin (2.0 g/l) and thrombin (1.3 g/l) in 0.1 M-NaCl/50 mM-sodium phosphate buffer, pH 7.5, were mixed and incubated for 10 min at 37°C to form the complex. A 160 μl amount of D-Phe-Pro-Arg-CH_2Cl (1 g/l) was then added to inactivate remaining free thrombin. After different additional incubation times at 37°C, 300 μl samples were applied to a column (0.9 cm × 27 cm) of Sephadex G-25 in order to remove excess free synthetic inhibitor. The centre fraction of the protein peak was then assayed for antithrombin activity in the presence of heparin, essentially as described by Fish & Björk (1979), although a lower thrombin concentration (0.22 mg/l) was used.

Protein concentrations were measured spectrophotometrically at 280 nm. Specific absorption coefficients for antithrombin, thrombin and antithrombin–thrombin complex of 0.67 (Nordenman *et al.*, 1977), 1.75 (Fish *et al.*, 1979) and 1.10 (Fish & Björk, 1979) $l \cdot g^{-1} \cdot cm^{-1}$ respectively were used.

Results

Purification and relative molecular mass

Most studies were done with complex purified by ion-exchange chromatography, followed by gel chromatography. In this procedure, both complex formed in the presence and that formed in the absence of heparin were eluted from the gel-chromatography column as a single symmetrical peak at a position corresponding to that of a globular protein with an M_r of about 90000. The purified complexes were >95% homogeneous in dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions; an apparent M_r of about 75000 was obtained for both complexes by this method. Sedimentation equilibrium showed the preparations of the two complex forms to contain mainly molecules with an M_r value (i.e. the M_r estimated for the smallest component in the solution) of 93000–108000. However, a small amount of material with higher M_r was apparent, presumably chiefly aggregated complex formed during the sedimentation-equilibrium analyses (see further below). These results thus show that both complex preparations contained predominantly monomeric antithrombin–thrombin complex, which has theoretical M_r values of 92000 when native and 81000 when denatured and reduced (Magnusson *et al.*, 1975; Nordenman *et al.*, 1977; Petersen *et al.*, 1979; Jörnvall *et al.*, 1979).

A few analyses (namely comparison of the dissociation rates of monomeric and aggregated complex) were done with complex formed in the presence of heparin and purified by affinity chromatography on heparin–agarose, followed by gel chromatography. This procedure gave two peaks, containing approximately equal amounts of material, in the final gel-chromatography step. Both peaks contained >95% homogeneous complex, as judged by dodecyl sulphate/polyacrylamide-gel electrophoresis. The second peak was eluted at a position corresponding to monomeric complex, and was also shown to contain preponderantly this form of the complex by sedimentation equilibrium. In contrast, the first peak, which was eluted close to the void volume of the gel-chromatography column, was grossly heterogeneous in sedimentation-equilibrium analyses and was concluded to contain aggregates of different sizes. For unknown reasons, purification on heparin–agarose thus promotes aggregation of the antithrombin–thrombin complex.

Conformation and heparin binding

Monomeric antithrombin–thrombin complex, formed in the absence or in the presence of heparin, was analysed by c.d. (Fig. 1). The spectra for the two forms of the complex were identical, within experimental error, in both the far-u.v. and near-u.v. wavelength regions. This indicates that the antithrombin–thrombin complex has a similar or identical secondary structure and conformation around aromatic amino acid residues, regardless of whether or not heparin has been present to accelerate its formation.

The binding of heparin to the complex was analysed semi-quantitatively by affinity chromatography on heparin–agarose. Complex formed in the absence or in the presence of heparin was eluted by a salt gradient at ionic strengths of 0.47 and 0.49 respectively. The two complex forms thus bind heparin with similar affinity.

Dissociation

The dissociation rate of the nascent antithrombin–thrombin complex, formed by mixing thrombin with an excess of antithrombin but not purified, was monitored by measurements of the release of active thrombin (Jesty, 1979*b*). This release followed apparent first-order kinetics during the time of the spectrophotometric analyses (which, however, lasted only about 15 min), both for complex formed in the presence and for that formed in the absence of heparin. The apparent first-order rate constants obtained from successive analyses, however, decreased slowly with time for both complexes, as noted earlier for the complex formed without heparin (Jesty, 1979*b*). This behaviour indicates that a secondary reaction is superimposed on the simple first-order dissociation of the complex; as discussed further below, this reaction probably is an aggregation of the complex. The rate constants for the dissociation of the nascent complexes therefore were obtained by extrapolation to zero time (Jesty, 1979*b*). The apparent first-order rate constants obtained by this procedure were independent of complex concentration and were identical for the two complex forms, i.e. 3.4 (S.D. $\pm 0.3) \times 10^{-6} s^{-1}$ ($n = 8$) and 3.4 (S.D. $\pm 0.4) \times 10^{-6} s^{-1}$ ($n = 8$) for complex formed in the presence and in the absence of heparin respectively (Fig. 2). Jesty (1979*b*) reported a somewhat lower first-order rate constant for nascent complex formed without heparin, $1.43 \times 10^{-6} s^{-1}$. The reason for this discrepancy is unknown.

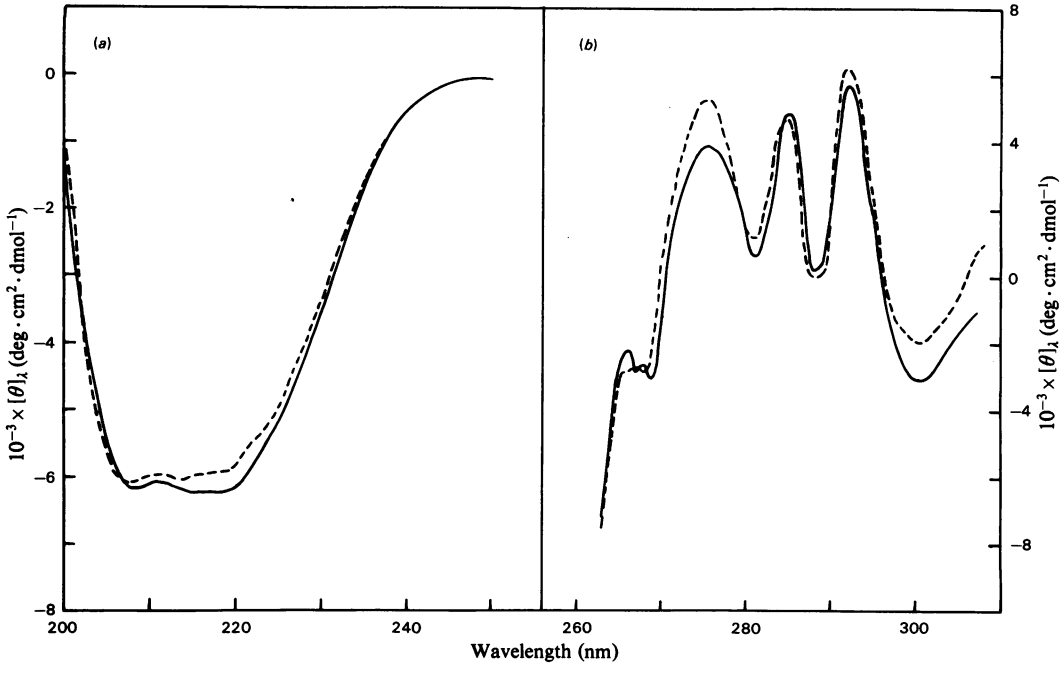

Fig. 1. *C.d. spectra of purified antithrombin–thrombin complex formed in the presence and in the absence of heparin*
(*a*) Far-u.v. wavelength region. The unit on the ordinate is mean residue ellipticity. (*b*) Near-u.v. wavelength region. The unit on the ordinate is molar ellipticity. ——, Complex formed in the absence of heparin; – – – –, complex formed in the presence of heparin. The solvent was 0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4.

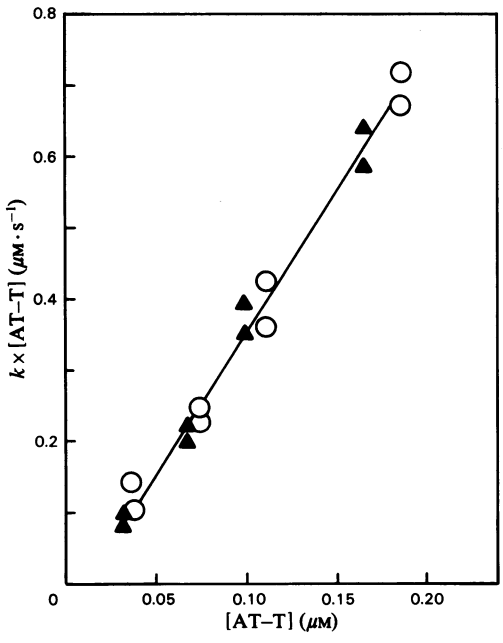

Fig. 2. *Dissociation, monitored by thrombin assay, of nascent antithrombin–thrombin complex formed in the presence and in the absence of heparin*

The rate of thrombin release from the complex was analysed by a continuous spectrophotometric assay with a chromogenic thrombin substrate (Jesty, 1979*b*). The kinetic parameter obtained by computer-fitting the resulting dissociation curves is the product between the apparent first-order dissociation rate constant (k') and the concentration of the complex ([AT–T]). This parameter was extrapolated to zero time (i.e. the time at which the reactants were mixed) by plotting $\ln(k' \times [AT-T])$ versus time for four or five analyses done at 10–15 min intervals. The extrapolated values ($k \times [AT-T]$) are plotted versus complex concentration. O, Complex formed in the absence of heparin; ▲, complex formed in the presence of heparin.

The release of thrombin from the purified monomeric antithrombin–thrombin complexes also followed apparent first-order kinetics during the time of the measurements. The rate constants were independent of complex concentration, and were determined to be 2.3 (S.D. $\pm 0.1) \times 10^{-6} s^{-1}$ ($n = 4$) and 2.6 (S.D. $\pm 0.5) \times 10^{-6} s^{-1}$ ($n = 3$) for complex formed in the presence and in the absence of heparin respectively. Also, these two values are identical with each other within the errors of the measurements. However, they are slightly lower than the rate constants obtained for the nascent complexes by extrapolation to zero time. This difference probably is due to the formation of some aggregated complex, with lower apparent dissociation rate constant (see below), during preparation or analysis of the purified complexes.

The rate of dissociation of the purified monomeric complexes was also monitored by analyses by dodecyl sulphate/polyacrylamide-gel electrophoresis of the amounts of modified antithrombin liberated from the complexes and of remaining intact complex. The dissociation was monitored for a considerably longer time than the release of thrombin activity from the complexes. First-order kinetics were obtained in all experiments (Fig. 3). No differences between experiments with or without the thrombin inhibitor D-Phe-Pro-Arg-CH_2Cl were observed. The rate constants for release of modified antithrombin were $1.8 \times 10^{-6} s^{-1}$ and $1.6 \times 10^{-6} s^{-1}$ for complex formed in the presence and in the absence of heparin respectively, and the corresponding rate constants calculated from the disappearance of the complex were $1.9 \times 10^{-6} s^{-1}$ and $1.4 \times 10^{-6} s^{-1}$. The differences between these four values are within the experimental errors of the method; the disappearance of the complex thus correlates well with the appearance of modified antithrombin. However, the rate constants obtained in the gel-electrophoretic analyses are slightly lower than those obtained by thrombin release from the purified complexes. Again, this difference can be ascribed to aggregation, which is unavoidable during the long incubation times inherent in the former experiments (see below).

It has been suggested previously that intact antithrombin is released from the human antithrombin–thrombin complex (Griffith & Lundblad, 1981). However, in our earlier experiments (Danielsson & Björk, 1980), and in the analyses presented above, only inactive modified antithrombin and no intact inhibitor was found to dissociate from the purified bovine complexes. The possibility that intact active antithrombin is released from the nascent antithrombin–thrombin complex was also tested. Two mixtures of thrombin and antithrombin, with apparent molar ratios of 1:1 and 1.05:1, were prepared in order to form the nascent complex. A larger excess of thrombin, to diminish further the background activity of antithrombin and thus possibly to increase the sensitivity of the analyses, was avoided, since that would have resulted in extensive proteolytic degradation of the complex (Jesty, 1979*a*; Fish *et al.*, 1979). Only complex prepared in the absence of heparin was studied. The antithrombin activity of the samples was measured after increasing incubation times at 37°C (Fig. 4). In contrast with the study by Griffith & Lundblad (1981), the nascent bovine antithrombin–thrombin complex did not release active antithrombin in detectable amounts. Such release could only have corresponded to, at most, 10% of the concomitant release of thrombin under our experimental conditions.

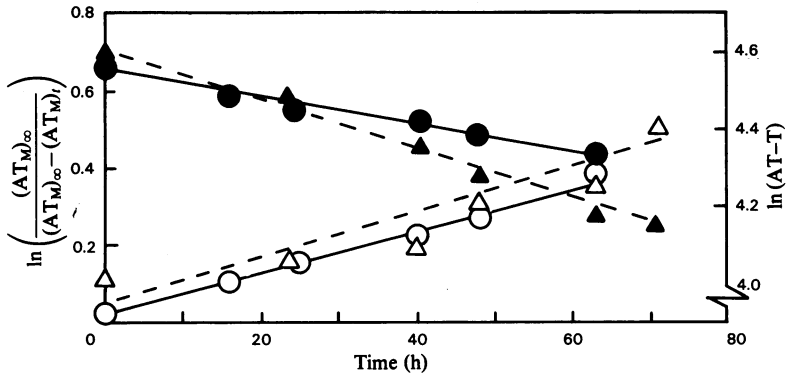

Fig. 3. *Dissociation, monitored by dodecyl sulphate/polyacrylamide-gel electrophoresis, of purified antithrombin–thrombin complex formed in the presence and in the absence of heparin*

The gel electrophoresis was run under reducing conditions. First-order dissociation rate constants were calculated from the slopes of plots of either the rate of appearance of modified antithrombin or the rate of disappearance of the complex. (AT_M) and (AT–T) are the amounts of modified antithrombin and antithrombin–thrombin complex respectively, expressed as per cent of the total amount of stained material on the gels. The small fragment of modified antithrombin and the A-chain of thrombin, which were liberated by the reduction but not clearly visible on the gels, were not included in these calculations. $(AT_M)_\infty$ was taken as the weight per cent of modified antithrombin in the reduced complex (i.e. complex without the small fragment of the modified inhibitor and the thrombin A-chain). ○, Complex formed in the absence of heparin, appearance of AT_M; ●, complex formed in the absence of heparin, disappearance of complex; △, complex formed in the presence of heparin, appearance of AT_M; ▲, complex formed in the presence of heparin, disappearance of complex.

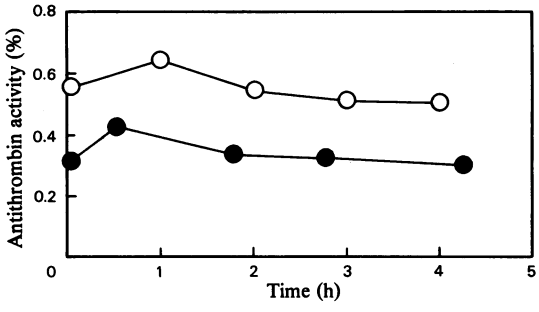

Fig. 4. *Analysis of antithrombin activity in preparations of nascent antithrombin–thrombin complex after different incubation times at 37°C*

The nascent complex was formed at two different apparent molar ratios of antithrombin to thrombin in the absence of heparin. Antithrombin activity was assayed after different incubation times at 37°C, as described in the Materials and methods section. The assay was calibrated with known amounts of antithrombin. Antithrombin activity was expressed as per cent of the total amount of antithrombin in the sample, estimated from the measured concentration of the complex and the weight fraction of antithrombin in the complex. Each curve is the mean of two different experiments. ○, Thrombin/antithrombin apparent molar ratio 1.0:1.0; ●, thrombin/antithrombin apparent molar ratio 1.05:1.0.

Aggregation

As described above, and also reported earlier (Jesty, 1979*b*), the apparent dissociation rate of the nascent antithrombin–thrombin complexes decreases initially with time. An explanation for this decrease was suggested by analyses of antithrombin–thrombin complex purified by affinity chromatography on heparin–agarose, followed by gel chromatography. The second, monomeric, gel-chromatography peak obtained by this procedure showed the dissociation rate constant (monitored by the rate of thrombin release) typical of monomeric complex, i.e. about $2.5 \times 10^{-6} s^{-1}$. In contrast, the rate constant for the release of thrombin from the first peak, containing mainly aggregated complex, was only about $0.8 \times 10^{-6} s^{-1}$. Thus aggregation of the antithrombin–thrombin complex is accompanied by a decrease of the apparent dissociation rate constant of the complex. Conversely, the decrease in the dissociation rate of the nascent complex may be due to aggregation of the complex.

As a further verification of this suggestion, the formation of aggregates of the nascent antithrombin–thrombin complex at 37°C was shown by gel chromatography. Two mixtures of antithrombin and thrombin were prepared, identical with those used in measurements of the rate of thrombin release, but with tritiated thrombin. The samples were incu-

bated at 37°C for 10min and 8h respectively. Both mixtures were then analysed by gel chromatography at 4°C (Fig. 5). The 10min sample gave one radioactive complex peak, eluted at a position corresponding to that of monomeric complex. In contrast, the sample incubated for 8h contained a large amount of radioactive material which was eluted close to the void volume of the column (Fig. 5), indicating that aggregates of the complex had been formed.

Aggregation of the purified, initially monomeric, antithrombin–thrombin complexes also occurred. Thus, storage for several days at +4°C, or for shorter times at higher temperatures, produced appreciable amounts of aggregated complex, as evident by gel chromatography. Moreover, the rapid formation of aggregates at 37°C was indicated by the fact that the dissociation rate for purified monomeric complex (which had been kept at 4°C) decreased in manner similar to that for the nascent complex when the temperature was increased to 37°C.

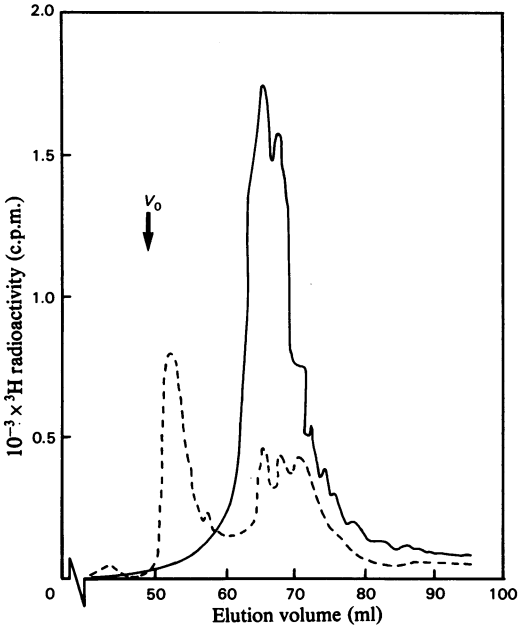

Fig. 5. *Gel chromatography of antithrombin–[^3H]thrombin mixtures after different incubation times at 37°C*
Equal volumes of antithrombin (0.36g/l) and [^{3}H]-thrombin (0.04g/l) in 50mM-sodium phosphate buffer, pH7.5, containing 0.13% bovine serum albumin were mixed and incubated for 10min (——) or 8h (– – – –) at 37°C. An amount of 1ml was then applied to a Sephacryl S-200 column (1.6cm × 90cm), equilibrated with 0.2M-NaCl/50mM-Tris/HCl buffer, pH7.4, at 4°C. The elution rate was 5ml/h. Samples (600μl) of the collected fractions were analysed for radioactivity. The void volume (V_0) and the total volume (which was 120ml) of the column were determined with high-M_r dextran and tritiated water respectively.

Discussion

Heparin accelerates the antithrombin–thrombin reaction in sub-stoicheiometric amounts (Markwardt & Walsman, 1959; Biggs *et al.*, 1970; Björk & Nordenman, 1976) and does not affect the amount of thrombin that can be neutralized (Quick, 1938; Seegers *et al.*, 1942). Moreover, heparin can be released for renewed binding to antithrombin once the inhibitor–thrombin complex is formed (Carlström *et al.*, 1977; Jordan *et al.*, 1979). These findings have led to the suggestion that heparin acts as a catalyst in the reaction between antithrombin and thrombin. The results presented here show that purified antithrombin–thrombin complexes, formed in the presence or in the absence of heparin, have similar conformations, as measured by c.d., and heparin affinities. Furthermore, the first-order dissociation rate constants of the two types of complex were found to be identical, regardless of the method used for the analyses and whether nascent or purified complex was analysed. The results thus provide evidence that the acceleration of the reaction between antithrombin and thrombin by heparin does not affect the properties of the complex formed, and thereby strongly support the proposal that heparin functions as a catalyst in this reaction (Markwardt & Walsman, 1959; Gitel, 1975; Björk & Nordenman, 1976).

Our previous studies have indicated that the antithrombin–thrombin complex irreversibly dissociates at neutral pH to active thrombin and a proteolytically cleaved antithrombin (Danielsson & Björk, 1980). In contrast, others have proposed that the antithrombin–thrombin reaction is basically reversible at neutral pH (Jesty, 1979*b*; Griffith & Lundblad, 1981) and only under certain conditions proceeds to the formation of an acyl-intermediate complex, which then may decompose to free enzyme and cleaved inhibitor (Griffith & Lundblad, 1981). The slow conversion observed by Jesty (1979*b*) of the initially formed antithrombin–thrombin complex into a form with a lower apparent dissociation rate constant seemingly supports this mechanism. Such a decrease in dissociation rate was observed also in the present work. However, several findings indicate that this decrease cannot be due to a slow stabilization of the complex, involving a conversion of an initial, reversible, 'enzyme–substrate' complex into a more stable, possibly covalently linked, form (Griffith & Lundblad, 1981). Thus the dissociation rate constants, determined from the rate of thrombin release, of nascent and mature (i.e. purified)

monomeric complexes were similar. These rate constants were also similar to those determined for the purified complex from the rates of release of modified antithrombin or of disappearance of the complex. Moreover, no dissociation of intact antithrombin from either the nascent or the purified complex was observed. Instead, the results indicate that complex-formation rapidly proceeds to the formation of a kinetically stable enzyme–inhibitor complex that dissociates by a simple first-order process with a half-life of about 3 days to active thrombin and modified antithrombin. This conclusion does not exclude the possibility that the antithrombin–thrombin reaction involves an initial, less stable, reversible form of the complex, as demonstrated by Olson & Shore (1982), but only shows that the decay of such an intermediate must occur on a much faster time-scale than previously suggested (Griffith & Lundblad, 1981). Together, the results strongly corroborate the suggestion that the inhibition of proteinases by antithrombin in essence is a normal proteolytic reaction, one intermediate step of which is very slow, most probably owing to the non-covalent interactions between enzyme and inhibitor (Fish & Björk, 1979; Danielsson & Björk, 1980; Longas & Finlay, 1980; Wallgren *et al.*, 1981). This rate-limiting step may be the hydrolysis of the intermediate acyl bond between the active serine residue of the enzyme and the arginine residue of the reactive bond of the inhibitor (Owen, 1975; Fish & Björk, 1979; Longas & Finlay, 1980; Wallgren *et al.*, 1981; Björk *et al.*, 1982).

An alternative reason for the slow initial decrease of the apparent dissociation rate of the nascent antithrombin–thrombin complex is suggested by the results. Aggregated complex thus was shown to dissociate with a markedly lower rate constant than monomeric complex; this is presumably due to the protein–protein interactions introduced by aggregate formation. Moreover, formation of aggregates in nascent complex preparations identical with those used in the analyses of dissociation rates was shown by gel chromatography. Slow aggregation of the antithrombin–thrombin complex after its formation thus adequately explains the decrease of the apparent dissociation rate. The rate of this aggregation was found to increase with increasing temperature. In keeping with this observation, polymeric antithrombin–thrombin complex appears to be the predominant form of the complex *in vivo* (Pepper *et al.*, 1977).

This investigation was supported by research grants from the Swedish Medical Research Council (Project no. 4212) and the Swedish Council for Forestry and Agricultural Research (Project no. A5861). We thank Ms. Elke Raub for excellent technical assistance.

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