A Disulfide Bond in Antithrombin Is Required for Heparin-accelerated Thrombin Inactivation*

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Heparin accelerates the rate of reaction of antithrombin with thrombin, an effect which is abolished by mild reduction of the antithrombin with dithiothreitol. Reduced antithrombin incorporates 1.7 mol of [14C]acetamide/mol of protein, with cysteine as the only amino acid modified. Tryptic digestion of the reduced and alkylated antithrombin results in the formation of only two labeled peptides. In the absence of heparin, the second order rate constant for the reaction of thrombin with both reduced and native antithrombin is 5.9 to 9.8 $\times 10^8$ M$^{-1}$ min$^{-1}$. In the presence of heparin, the rate constant for the reaction between reduced antithrombin and thrombin is 8.3 to 12.2 $\times 10^8$ M$^{-1}$ min$^{-1}$, while the rate of reaction between native antithrombin and thrombin is too fast to follow under the conditions used. Reduced antithrombin elutes from a heparin-Sepharose column at 0.5 M NaCl, in contrast to 1.0 M NaCl required for elution of the native protein. The intrinsic tryptophan fluorescence enhancement caused by heparin binding to native antithrombin is not observed with reduced antithrombin. These data indicate that cleavage of one of the three antithrombin disulfide bonds results in reduced affinity for heparin and the loss of heparin-accelerated antithrombin activity and imply that heparin and thrombin bind at different sites on the antithrombin molecule.

Antithrombin, a single-chain α-glycoprotein found in mammalian plasma, inhibits a variety of trypsin-like serine proteases by forming a stable, inactive, 1:1 enzyme-inhibitor complex (1).

Antithrombin is unique among the plasma and non-plasma protease inhibitors in that its rate of reaction with proteases is markedly increased in the presence of heparin (2–5). It has been suggested (6) that neutralization of thrombin by antithrombin involves the active site serine of the thrombin and arginine residues of the inhibitor and that heparin binds to lysyl residue(s) at a separate site on the antithrombin molecule. The acceleration in reaction rate could be due to a heparin-dependent conformational change in the antithrombin, rendering the susceptible arginine bond more accessible to attack by thrombin. Conformational changes in antithrombin resulting from the presence of heparin have been observed by various physical techniques (7–9).

Kurachi et al. (10) purified and compared the properties of antithrombin isolated from human, bovine, and equine plasma by a procedure that included chromatography on heparinagarose. The proteins are similar in molecular weight and amino acid and carbohydrate composition. Each has 6 half-cysteine residues and no free sulfhydryl groups.

This communication reports the existence of a disulfide bond in human antithrombin which must remain intact for the binding and rate-accelerating effects of heparin to occur. Selective reduction of this bond does not interfere with the slow neutralization of thrombin observed in the absence of heparin.

EXPERIMENTAL PROCEDURES

Materials—Peptide substrates for thrombin, Cbz-Phe-Val-Arg-p-nitroanilide (S-2160) and Cbz-Gly-Pro-Arg-p-nitroanilide (Chromozym TH) were obtained from Bofors and Boehringer Mannheim, respectively. Foetive gastric mucosal heparin (from Research Plus) was purified by chromatography on Sephadex G-75 in 0.2 M NaCl, fractions from the central portion of the heparin peak (assayed with azure A (11)) were combined and the polysaccharide was precipitated with ethanol. The molecular weight of this preparation was determined to be 8900 ± 1000 by viscometry (12). The range was estimated from the width of the heparin peak, assuming a molecular weight fractionation range of 1,000 to 50,000 for dextrans on Sephadex G-75. Specific activity was 159 units/mg. Iodo[1-14C]acetamide was from New England Nuclear. Iodo[1-14C]acetate was from Amersham. Polyethylene glycol 4000 was a product of Union Carbide. All other chemicals were purchased as reagent grade. Antithrombin was purified from human plasma essentially by the method of Miller-Andersson et al. (13), except that the immobilized heparin was made using triazine-activated Sepharose (14). The purified antithrombin inactivated 200 NIH units of thrombin/mg and demonstrated only a single protein-staining band following reduction and electrophoresis on 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate (15). Human thrombin (2440 NIH units/mg, 84% active enzyme, 99% α-thrombin) was a gift from Dr. J. W. Fenton, II. Human fibrinogen was prepared by differential polyethylene glycol precipitation of aluminum hydroxide-adsorbed, cryoprecipitated human plasma. The fibrinogen was over 95% clottable, had only NH2-terminal alanine and tyrosine residues, and showed only three protein-staining bands, corresponding to the Aα, Bβ, and γ subunits (16), following reduction and electrophoresis (15).

Assay of Antithrombin and Thrombin—Rapid neutralization of thrombin by antithrombin in the presence of heparin was determined at 37°C by a modification of the two-stage assay described by Odegard et al. (17). Heparin (0.6 unit), human thrombin (0.3 to 0.5 unit), and antithrombin were incubated for 30 s in 0.45 ml of 0.067 M Tris-Cl, 0.2 M NaCl pH 8.3. At the end of the incubation, 0.15 ml of substrate (0.5 M) containing 0.25 mg/ml of Polybrene was added and residual thrombin activity was determined from the change in adsorbance at 405 nm as a function of time. One unit of antithrombin neutralized 1

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1 T. H. Finlay, V. Troll, and L. T. Hodgins, manuscript in preparation.

2 M. O. Longas and T. H. Finlay, unpublished experiments.
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NIH unit of thrombin under the conditions of the assay. To measure the slow neutralization of thrombin occurring in the absence of heparin, conditions were the same as above except that heparin and Polybrene were omitted and the first incubation was increased to 30 min.

In some experiments, thrombin activity was determined using fibrinogen as a substrate. These assays were conducted by the method of Lundblad et al. (18) except for the addition of the highly purified fibrinogen described above (1.8 mg/ml) and determination of clotting times with a Fibrometer (BioQuest). A thrombin concentration of 0.4 unit/ml gave a clotting time of 18.8 s. Plots of thrombin concentration against the reciprocal of the clotting times were linear for thrombin concentrations between 0.05 and 1 unit/ml. Activity of the thrombin preparation as determined with the synthetic peptide substrate was routinely compared to its activity as determined with fibrinogen.

Protein Assay—The method of Lowry et al. (20) was used with bovine serum albumin as a standard.

RESULTS

Effect of Dithiothreitol on the Activity of Antithrombin—When antithrombin was incubated with dithiothreitol at pH 8.3 and 37°C for 2 min, its ability to neutralize thrombin rapidly in the presence of heparin was decreased (Fig. 1A). In the absence of 1 mM dithiothreitol, heparin-mediated antithrombin activity was completely lost. This concentration of dithiothreitol had no effect on thrombin amidolytic activity, whether thrombin was incubated alone or with heparin or antithrombin. The effect of the reducing agent on the inactivation of thrombin by antithrombin in the absence of heparin was also examined (Fig. 1B). Dithiothreitol concentrations as high as 1 mM had no apparent effect on the slow neutralization of thrombin by antithrombin which occurs in the absence of heparin. No loss in thrombin amidolytic activity during the course of these experiments was observed.

Effect of Dithiothreitol on Thrombin Clotting Activity—Although no obvious effect of dithiothreitol on thrombin amidolytic activity was found (Fig. 1), it is possible that reduction might have caused some minor structural change affecting binding of antithrombin but not hydrolysis of small substrates. Clotting activity, which is a better measure of thrombin structure, was examined to assess such minor structural alterations. Thrombin was incubated at 37°C for either 0.5 or 30 min with various concentrations of dithiothreitol and assayed for clotting activity with fibrinogen (Table I). When the reduced thrombin was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (15) without additional reduction, some dissociation of the A and B chains was observed following incubation with 10 mM dithiothreitol for either 0.15 or 30 min or with 1 mM dithiothreitol for 30 min. Treatment of thrombin with 0.1 mM dithiothreitol had no apparent effect on clotting activity and did not result in discernable dissociation.

Interaction of Heparin with Reduced Antithrombin—Two mechanisms for the loss of the heparin-accelerated thrombin neutralizing activity of antithrombin following reduction were considered: 1) reduction of antithrombin results in a decreased affinity for heparin or 2) reduction does not effect heparin binding but interferes in some way with the conformational change in the antithrombin presumed to occur subsequent to binding. To distinguish between these two mechanisms, antithrombin was chromatographed on columns of heparin-Sepharose in the presence and absence of 1 mM dithiothreitol using a gradient of NaCl to elute antithrombin activity (Fig. 2). In 1 mM dithiothreitol, antithrombin eluted at approximately 0.5 M NaCl, while in the absence of reducing agent it eluted at 1 M NaCl. To rule out the possibility that the dithiothreitol caused a breakdown of the heparin-Sepharose, the same affin-

### Table I

<table>
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<th>Dithiothreitol</th>
<th>Incubation time</th>
<th>Thrombin activity (NIH units/ml)</th>
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<tr>
<td>10^{-4} M</td>
<td>30 s</td>
<td>0.120</td>
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**FIG. 1. Effect of dithiothreitol on the inactivation of thrombin by antithrombin.** A, inactivation of thrombin in the presence of heparin. Initial incubation [if present]: antithrombin (1.6 units/ml), heparin (1 unit/ml), and dithiothreitol at 0.15 M NaCl, 0.05 M Tris-Cl, pH 8.3, containing the dithiothreitol concentrations indicated. After either 0.5 or 30 min, an aliquot was withdrawn and added to 300 ml of assay mixture (1.8 mg/ml of fibrinogen, 3.3% gum acacia, 0.11 M NaCl, 6 mM CaCl$_2$, 0.017 M imidazole, pH 7.2) at 37°C and the clotting time was recorded.
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Second Order Rate Constants for the Reaction of Antithrombin with Thrombin—Conceivably, antithrombin activity determined in the absence of heparin (Fig. 1B) might not reflect small but significant alterations in antithrombin structure. To demonstrate unequivocally that mild reduction resulted only in the loss of heparin-accelerated activity, second order rate constants for the reaction between reduced and native antithrombin with thrombin in the presence and absence of heparin were determined (Fig. 5). The dithiothreitol concentration during the reaction between antithrombin and thrombin was 0.1 mM; this level was found to have no effect on thrombin amidolytic or clotting activity (Fig. 1 and Table II). The second order rate constant for the reaction of both native and reduced antithrombin with thrombin, calculated from a plot of the reciprocal of thrombin activity against time (Fig. 5, inset), was $6.8 \times 10^{-4} \text{M}^{-1} \text{min}^{-1}$. In the presence of $1.1 \times 10^{-5} \text{M}$ heparin, the rate constant for the reaction of reduced antithrombin with thrombin was increased slightly to $10.1 \times 10^{-4} \text{M}^{-1} \text{min}^{-1}$. These values are in good agreement with those reported for the reaction between human thrombin and antithrombin by Downing et al. (22). In contrast, the rate of reaction between native antithrombin and thrombin in the presence of $1.1 \times 10^{-5} \text{M}$ heparin was at least 100-fold faster (Fig. 5) and the rate constant was impossible to measure under these conditions. Similar results were obtained at other heparin and protein concentrations (Table III).

Reduction and Alkylation of Antithrombin—Presumably, mild reduction of antithrombin with dithiothreitol results in the cleavage of disulfide bonds. To prove this and to determine

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**Fig. 2.** Effect of diethiothreitol on the binding of antithrombin to heparin-Sepharose. Antithrombin (660 units in 1.0 ml of 0.15 M NaCl, 0.05 M Tris-Cl, pH 8.3) was applied to a column (0.9 x 10 cm) of heparin-Sepharose (see "Materials and Methods"). The column was eluted with 10 ml of 0.15 M NaCl, 0.05 M Tris/citrate, pH 8.3, and then with a linear gradient of 10 ml of this buffer and 10 ml of 1.5 M NaCl, 0.05 M Tris/citrate, pH 8.3. Fractions of 1.0 ml were collected and antithrombin activity was determined using the synthetic substrate assay in the absence of heparin. In a second experiment, conditions were the same except that the antithrombin was incubated in buffer containing 10^-3 M diethiothreitol for 30 min prior to chromatography and all buffers contained 10^-3 M diethiothreitol.

- no heparin; 
- 10^-3 M diethiothreitol

Assay columns used for the experiment with the reducing agent was washed with several column volumes of buffer containing 0.15 M NaCl and reloaded with antithrombin. Again, approximately 1 M NaCl was required to elute the antithrombin activity. In a second experiment, 0.35 mg of human thrombin was chromatographed on heparin-Sepharose under essentially identical conditions except that the diethiothreitol concentration was 0.1 mM and the incubation time 10 min. In this experiment thrombin required approximately 0.4 M NaCl for elution whether or not diethiothreitol was present.

**Effect of Reduction on the Binding of Heparin to Antithrombin as Measured by Fluorescence Enhancement**—The uncorrected fluorescence emission spectra of both reduced and native antithrombin in the presence and absence of heparin are shown in Fig. 3. An emission maximum at 335 nm was found for both the native and reduced proteins. However, the intensity of the emission peak was reduced by approximately 17% for the reduced protein, although its concentration was slightly higher. In the presence of 25 µg/ml of heparin, native antithrombin exhibited a fluorescence enhancement of 25%; increasing the heparin concentration beyond this value did not increase the fluorescence enhancement. Reduced antithrombin showed no fluorescence enhancement even at a heparin concentration of 50 µg/ml.

**Effect of Heparin and Reduction of Antithrombin on the Formation of the Antithrombin-Thrombin Complex**—These experiments were performed in order to determine whether the antithrombin-thrombin complex formed with reduced antithrombin was the same as that formed with the native protein. Antithrombin (0.52 mg/ml) in 0.05 M Tris-Cl, 0.10 M NaCl, pH 8.3) was incubated with and without heparin (10^-6 M) and with and without dithiothreitol (1 mM) at 37°C. After 30 min the reactions were cooled to 0°C, an equal volume of thrombin (0.32 mg/ml in the same buffer) was added, and the reactions were continued for either 10 or 30 s at 0°C. The reactions were then brought to 0.1 M in diisopropylphosphofluoridate and diluted into phosphate buffer containing 2% sodium dodecyl sulfate and 2% mercaptoethanol. Prior reduction of the antithrombin or incubation of reduced or native antithrombin with heparin had no apparent effect on the nature of the antithrombin-thrombin complex (Fig. 4) as determined by gel electrophoresis.

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**Fig. 3.** Uncorrected fluorescence emission spectra of native and reduced antithrombin in the presence of: — no heparin; — 5 µg/ml of heparin; — 25 µg/ml of heparin. Excitation wavelength was 295 nm. Cell path length was 1.0 cm. Native antithrombin was 0.186 mg/ml (0.83 µM) and reduced antithrombin was 0.192 mg/ml (0.86 µM). Solvent was 0.10 M NaCl, 0.05 M Tris-Cl, pH 7.4. Antithrombin (1200 units/mg) was reduced with 1 mM dithiothreitol in the above solvent at 37°C for 30 min and then exhaustively dialyzed. Native antithrombin treated in a like manner, except for the absence of dithiothreitol, lost no activity. Spectra were taken as described under "Materials and Methods."
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the number of disulfide bonds cleaved to produce the loss of heparin-mediated antithrombin activity, mildly reduced antithrombin was alkylated with iodo\['"C\]acetamide (Table II). Antithrombin (3.3 mg/ml) was incubated in 0.15 M NaCl, 0.05 M Tris-Cl, pH 8.3, at 37°C with and without 1 mM dithiothreitol for 135 min. An excess (220% of the dithiothreitol on a molar basis) of iodo\['"C\]acetamide (6.3 × 10^4 cpm/mol) was added to the reaction containing the reduced antithrombin and the incubation was continued 15 min longer. The reactions were exhaustively dialyzed and antithrombin activity, protein concentration, and radioactivity were measured. Antithrombin was assayed in the presence of heparin using the tripeptide nitroanilide assay for thrombin activity. Reduction of antithrombin followed by alkylation with iodoacetamide resulted in the loss of 85% of the initial antithrombin activity and incorporation of 1.7 mol of acetamide/mol of protein, assuming a molecular weight of 56,000 (10). In a control experiment, incubation of antithrombin with 2 mM iodoacetamide for 135 min without prior reduction did not result in an appreciable loss of antithrombin activity or incorporation of radioactivity into the protein. Reduced and alkylated antithrombin also was without thrombin inhibitory activity using the slow neutralization assay in the absence of heparin.

To show conclusively that only a single disulfide bond was cleaved by mild reduction with dithiothreitol, 3 mg of antithrombin was reduced and alkylated by the procedure described above except that iodo\[14C\]acetate (1.14 × 10^4 cpm/μmol) was used in place of the iodo\[14C\]acetamide. Hydrolases (6 N HCl, 110°C, 6 h) of the labeled protein were examined by thin layer chromatography on Silica Gel G with two solvent systems (n-butyl alcoholacetic acid:water, 4:1:1, and n-propyl alcohol:water, 7:1) and by paper electrophoresis at pH 8.9. In each case, the sole or major radioactive spot migrated with authentic S-carboxymethyl-L-cysteine. A portion of the S-[14C]carboxymethylantithrombin then was re-
duced a second time in 6 m guanidinium hydrochloride, 10 mM dithiothreitol, 0.5 M NaCl, 0.25 M Tris, pH 8.3, at 37°C for 120 min. This strongly reduced protein was treated with a 2-fold molar excess of unlabeled iodoacetamide at room temperature for 30 min and desalted by chromatography on Sephadex G-10. The doubly reduced and alkylated antithrombin was digested with 10% by weight of tosyl L-phenylalanyn chloromethylketone-treated trypsin (two equal additions of trypsin, 0.2% NH_4HCO_3, pH 8.2, 37°C, 12 h). Thin layer chromato-

![Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the antithrombin-thrombin complex prepared with reduced and native antithrombin in the presence and absence of heparin. Samples were reduced with mercaptoethanol (1%, 100°C, 2 min) and aliquots containing 5 μg of protein were electrophoresed on 7.5% gels (15). A, antithrombin; B, antithrombin + thrombin at 0 time; C, antithrombin + thrombin, 10 s; D, antithrombin + thrombin, 30 s; E, reduced antithrombin + thrombin, 10 s; F, reduced antithrombin + thrombin, 30 s; G, antithrombin + thrombin + heparin, 10 s; H, antithrombin + thrombin + heparin, 30 s; I, reduced antithrombin + thrombin + heparin, 10 s; J, reduced antithrombin + thrombin + heparin, 30 s; K, thrombin. The uppermost band on the gels is the antithrombin-thrombin complex; the band just below it has been attributed to a partially degraded complex (21).](image)

![Fig. 5. Reaction of reduced and native antithrombin with thrombin in the presence and absence of heparin. Antithrombin, 0.106 nmol in 0.10 ml of 0.05 M Tris, 0.15 M NaCl, pH 7.4 (with or without 1 mM dithiothreitol as indicated), was incubated at 37°C. After 30 min, the reaction was diluted approximately 10-fold with buffer (with or without 1.1 nmol of heparin) and incubated 1 min longer. Thrombin (0.107 nmol in 50 μl) then was added to bring the total volume to 1.0 ml and the incubation was continued at 37°C. At intervals, 25-μl aliquots from the reaction mixture were added to 0.675 ml of 0.15 M Cbz-Gly-Pro-Arg-p-nitroanilide in the same buffer and the change in A_405 as a function of time was followed. Thrombin activity was determined from the initial slopes of the recorder tracings. □, antithrombin + heparin; ■, reduced antithrombin + heparin; ▲, antithrombin; ●, reduced antithrombin.](image)

<table>
<thead>
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<th>Table II</th>
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<td>Reduction and alkylation of antithrombin</td>
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<tr>
<td>Additions</td>
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<td>Iodoacetamide (2 × 10^4 μM)</td>
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<td>Dithiothreitol (1 × 10^4 μM)</td>
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<td>Dithiothreitol + iodoacetamide</td>
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* Determined by the synthetic substrate assay in the presence of heparin after incubation at pH 8.3 for 135 min followed by dialysis.

* Assuming M, = 56,000 for antithrombin.
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In a study of the interaction of native and reduced antithrombin with immobilized heparin, it was found that, in the presence of 1 mM diithiothreitol, antithrombin eluted from heparin-Sepharose at approximately half the NaCl concentration required for elution in the absence of reducing agent (Fig. 2). Since the interaction of antithrombin with heparin is at least partially ionic (25), these data indicate that mild reduction of antithrombin results in reduced affinity for heparin. These experiments do not rule out the possibility that mild reduction in some way prevents a heparin-dependent conformational change in antithrombin. The observation that heparin did not cause a fluorescence enhancement of reduced antithrombin is consistent with both hypotheses. Heparin also binds to thrombin (26) and it has been proposed that this interaction plays a role in the heparin-mediated reaction of thrombin with antithrombin (27). However, the interaction of thrombin with heparin does not appear to be of significance in the experiments which we report on in this communication, as we have shown that 0.1 mM diithiothreitol has no effect on thrombin amidolytic and clotting activity or on the binding of thrombin to immobilized heparin.

Our results with native antithrombin are in agreement with those of Nordenman et al. (9) in that we also observe no shift in emission maxima on heparin binding. The observation that the intrinsic fluorescence of reduced antithrombin is lower than that for the native protein suggests that cleavage of one disulfide bond alters the conformation of the antithrombin molecule.

Alkylation of antithrombin with iodoacetic acid after reduction with 1 mM diithiothreitol resulted in the modification of slightly less than 2 mol of cysteine/mol of protein. As only two labeled peptides were found after tryptic digestion of the reduced and alkylated protein the effects noted here, namely loss of affinity for heparin and loss of heparin-accelerated activity, result from the reductive cleavage of only one of the three antithrombin disulfide bonds. Our findings confirm and extend those of Nordenman and Andersson (7) who report that completely reduced and S-carboxymethylated antithrombin does not bind heparin, as determined by fluorescence enhancement measurements. Although mild reduction of antithrombin resulted in the loss of only heparin-accelerated thrombin inhibitory activity, alkylation of the reduced antithrombin with iodoacetamide resulted in the loss of both heparin-accelerated and slow neutralization activity. We are unable to explain this. However, we recently found that S-cyanation of reduced antithrombin with 2-nitro-5-thiocyanato benzoic acid does not cause a loss of slow neutralization activity. This suggests that the size of the substituent on the modified cysteine may be important.

The experiments we report on here are consistent with separate heparin and thrombin binding sites on the antithrombin molecule. Our results, along with other kinetic data (28) and various physical measurements (7, 9), support the hypothesis that one way in which heparin can act as an anticoagulant is by binding to antithrombin and altering its conformation so as to increase its rate of reaction with serine proteases (6).

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REFERENCES


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