A fragment of antithrombin that binds both heparin and thrombin

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In order to identify the regions of antithrombin that interact with heparin and thrombin, it was degraded with CNBr and the activities of the isolated products were investigated. These fragments did not exhibit direct thrombin-neutralizing activity; however, one unique fragment was found to bind to heparin–Sephadex and also to interfere with the inhibition of thrombin by intact antithrombin. This fragment was identified as the one consisting of three disulphide-linked polypeptide chains containing residues 1–17, 104–251 and 424–432. At a concentration of 46 nM, this product decreased the heparin-enhanced thrombin-inhibitory activity of antithrombin by half, and completely abolished this inhibition when above 300 nM. In the absence of heparin, the action of antithrombin was not completely nullified by the fragment, even when present at relatively high concentrations. At a given fragment concentration, the extent of inhibition was independent of antithrombin concentration over the range tested. It was found that the fragment decreased the second-order rate constant for the antithrombin–thrombin reaction. Reduction and alkylation of the fragment showed that the above properties reside primarily in the peptide with residues 104–251. It is concluded that this peptide possesses portions of the antithrombin molecule that bind to heparin as well as to a site on thrombin.

INTRODUCTION

Antithrombin (AT) is a plasma glycoprotein that inhibits the enzymic activities of thrombin and various other activated blood coagulation factors (Travis & Salvesen, 1983). Neutralization of thrombin by this inhibitor is greatly accelerated by the glycosaminoglycan heparin (Rosenberg, 1985). This effect is correlated with the presence in heparin of a specific saccharide sequence (Atha *et al.*, 1984; Lindahl *et al.*, 1984) located in a section near the non-reducing terminus of the chain (Radoff & Danishefsky, 1984). The combination of heparin with AT produces a conformational change in the protein (Einarsson & Andersson, 1977; Villanueva & Danishefsky, 1977; Nordenman & Björk, 1978; Olson *et al.*, 1981) that can be correlated with its enhanced activity. Experiments on limited proteolysis of AT by proteinase II of *Crotalus* venom also suggest that heparin produces a conformational change in the protein leading to the exposure of the *N*-terminal region (Kress & Catanese, 1981).

Elucidation of the mechanism of action of AT requires that the regions and specific amino acid residues in the protein that interact with both thrombin and heparin be defined. Reports on effects of chemical modifications of amino acid residues have shown that arginine is involved in the reaction with thrombin and that lysine and arginine residues are required for the accelerating action of heparin (Rosenberg & Damus, 1973; Pecon & Blackburn, 1984; Jorgensen *et al.*, 1985). Theoretical predictions of the secondary structure of AT suggest that Lys-290, Lys-294 and Lys-297, located in a helical region of the molecule, may bind to the active saccharide sequence of heparin (Villanueva, 1984). [In the present paper the numbering of amino acid residues is according to Bock *et al.* (1982) and Chandra *et al.* (1983). Residue numbers above 210 have been altered to fit the complete

sequence of AT.] Also, tryptophan residues were found to be involved in the AT–heparin interaction (Blackburn & Sibley, 1980; Villanueva *et al.*, 1980; Karp *et al.*, 1984). It has been demonstrated that modification of Trp-49 in AT results in a loss of heparin binding (Blackburn *et al.*, 1984). Structural analysis of a naturally occurring mutation of human AT revealed that substitution of Arg-47 by a cysteine residue results in the loss of heparin binding (Koide *et al.*, 1984). With respect to the reaction of AT with thrombin, it has been demonstrated that the enzyme cleaves the inhibitor between Arg-393 and Ser-394 (Björk *et al.*, 1982). The oligosaccharide chains on AT are not required for heparin-binding or thrombin interactions since their removal did not alter these activities (Rosenfeld & Danishefsky, 1984).

The aim of the present study is to identify in a direct manner which regions of AT interact with heparin and with thrombin. In this paper we describe the isolation of defined sections of AT obtained after its degradation with CNBr and studies on their binding to heparin and on their effects on thrombin–AT interactions. One of these fragments was found to bind with heparin and to block the thrombin-inhibitory effect of AT.

EXPERIMENTAL

Materials

AT was prepared in this laboratory as described by Danishefsky *et al.* (1978). These preparations were greater than 95% pure as judged by SDS/polyacrylamide-gel electrophoresis and amino acid analysis. Thrombin was kindly provided by Dr. J. W. Fenton, Albany, NY, U.S.A. Bovine lung heparin (162 units/mg) was a gift from the Upjohn Co., courtesy of Dr. P. W. O'Connell.

CNBr was purchased from Aldrich Chemical Co., formic acid from J. T. Baker and iodoacetamide from

Abbreviations used: AT, antithrombin; S-2238, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide.

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Sigma Chemical Co. Dithiothreitol, Bio-Gel P-60, Bio-Gel P-150 and chemicals for electrophoresis were obtained from Bio-Rad Laboratories. The thrombin substrate S-2238 was the product of Kabi Diagnostica. Iodo[1-¹⁴C]acetamide (23.6 mCi/mmol) was purchased from New England Nuclear and diluted with non-radioactive alkylating reagent before use. Activated CH- (6-carboxyhexyl-)Sephacrose 4B was a product of Pharmacia.

Heparin-CH-Sephacrose

The reactive *N*-hydroxysuccinimide ester of CH-Sephacrose 4B was washed and coupled to the available free primary amino groups of heparin (unfractionated) in 0.5 M-NaCl/0.1 M-sodium bicarbonate buffer, pH 8.0, at 4 °C for 6.5 h. Residual unmodified groups on the gel were blocked by treating with 1 M-ethanolamine at pH 9.0 for 1 h at 4 °C. The heparin-CH-Sephacrose 4B gel was washed thoroughly with 0.5 M-NaCl/0.1 M-sodium formate buffer, pH 4.0, and with 0.5 M-NaCl/0.1 M-Tris/HCl buffer, pH 8.0, according to the manufacturer's specifications. The resulting gel contained 0.23 mg of heparin/ml of gel and bound 0.4 mg of AT/ml.

To investigate the retention of AT and polypeptide fragments by heparin-CH-Sephacrose 4B, samples were applied to a 10 ml column of this gel in 0.15 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.5, and washed with 2 column volumes of the same buffer. A linear gradient of 0.15–1.0 M-NaCl in 0.05 M-Tris/HCl buffer, pH 7.5, was then run through the column. Concentrations of NaCl in the fractions were determined by conductivity measurements.

Protein and peptide determination

Protein concentrations were determined by A_{280} measurements, which were corrected for light-scattering (Baughman & Waugh, 1967). Absorption coefficients ($A_{1\text{cm}}^{1\%}$) of 6.5 (Nordenman *et al.*, 1977) and 18.3 (Fenton *et al.*, 1977) were utilized for AT and thrombin respectively. M_r values of 58000 for AT (Nordenman *et al.*, 1977) and 36500 for thrombin (Fenton *et al.*, 1977) were employed.

Concentrations of CNBr-cleavage peptides of AT were measured at 230 nm, an absorption coefficient ($A_{1\text{cm}}^{1\%}$) of 48.4 being used. This value was calculated from the absorbance of a known concentration of intact AT at this wavelength. M_r values of the CNBr-cleavage peptides of AT were assigned on the basis of the number of amino acid residues of each fragment (Petersen *et al.*, 1979; Koide *et al.*, 1984). Thus fragment 2 was assigned an M_r of 19100, peak 3 M_r 9200 (average of two polypeptides) and peak 4 M_r 3400 (average of two polypeptides). Peak 1 was assigned an M_r value of 45000 on the basis of SDS/polyacrylamide-gel electrophoresis.

Gel electrophoresis

The procedure based on the method of Laemmli (1970) was used for SDS/polyacrylamide-slab-gel electrophoresis, employing a 7–20% (w/w) polyacrylamide gradient.

Amino acid analysis

The sample was freeze-dried and hydrolysed at 110 °C for 24 h with constant-boiling HCl under vacuum. The analysis was performed on a Beckman System 6300

high-performance amino acid analyser, with norleucine as internal standard.

Assays for AT activity

AT activity was measured by its ability to inhibit the hydrolysis of S-2238 by thrombin (Radoff & Danishefsky, 1981). All determinations were performed in a solution consisting of 0.15 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.5, at 42 °C. Thrombin solutions in this medium contained 0.825% poly(ethylene glycol)-6000. Stock solutions of substrate consisted of 0.75 mM-S-2238 in 0.01% Polybrene. Thrombin concentrations were adjusted for each experiment to give a change in absorbance of 0.20 ± 0.02 A unit/min in the final solution (1.3 ml) after all dilutions.

Heparin cofactor activity was measured by the amount of thrombin inhibited after a 30 s incubation with AT and heparin (0.091 $\mu\text{g/ml}$). Residual thrombin was assayed by its action on S-2238 according to the procedure of Radoff & Danishefsky (1981). In each experiment a standard curve was constructed relating residual thrombin activity to AT concentration in the presence of a fixed heparin concentration (0.091 $\mu\text{g/ml}$). The effects of CNBr-cleavage fragments on heparin cofactor activity were determined with incubation mixtures containing AT concentrations that, in the absence of fragment, produced 80–90% thrombin inhibition. The observed residual AT activity was determined from the respective standard curve. The equation used to calculate percentage inhibition of heparin cofactor activity was:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Observed AT}}{\text{Actual AT}}\right) \times 100$$

For scanning the effects of different column fractions (see Tables), a single large excess of the test fragment was employed, whereas titration experiments employed a wide range of concentrations (see Figures). Details of specific experiments are described in the Figure and Table legends. Final thrombin concentrations for different heparin-dependent inhibition experiments ranged from 3.5 to 5.0 nM before addition of 0.20 ml of substrate. The final concentration of AT, before addition of substrate, varied from 30 to 61 nM.

Progressive AT inhibition of thrombin activity was determined in the absence of heparin by two methods. One method was employed to scan fractions for AT inhibitory activity. This involved preincubation of thrombin with an excess of the specific fraction followed by addition of AT. Thrombin activity that remained 10 min after the last addition was measured by appropriate dilution with buffer and reaction with S-2238. Percentage inhibition of AT activity was calculated in a manner analogous to that used to calculate percentage inhibition of heparin cofactor activity. Preliminary experiments showed that under these conditions the loss in thrombin activity was directly related to incubation time for at least 10 min. Controls containing thrombin alone showed there was no loss in its activity within the time span of the incubation. In the other procedure, employed for titration studies, solutions containing thrombin, fragment and AT were incubated at 42 °C, and samples, removed after 1.0, 5.0 and 10.0 min, were measured for remaining thrombin activity with S-2238. The final thrombin concentrations in the incubation mixture, before transfer of the samples to the diluted

substrate solution, ranged from 8.3 to 19 nM. AT concentrations in these incubation mixtures were sufficient to inhibit thrombin by 80–90% in the absence of fragment. To calculate percentage inhibition, pseudo-first-order rate constants obtained in these experiments were compared with those in which fragment was absent. For a fixed fragment concentration, percentage inhibition of AT activity was shown to be independent of AT concentration under these conditions (see Fig. 8).

CNBr degradation of AT

A modification of the procedures of Petersen *et al.* (1979), Blackburn *et al.* (1984) and Koide *et al.* (1984) for CNBr digestion of AT was followed. A solution of AT (4.6 mg/ml) in 70% (v/v) formic acid was flushed with N₂, and CNBr was added (16 mg/ml). The mixture was stirred for 24 h in the dark, in a fume hood, then diluted with at least 9 vol. of water and freeze-dried. The residue was resuspended in 0.1 M-formic acid, and insoluble material was removed by centrifugation.

Reduction and alkylation of peptide fragments

Reduction of the disulphide bonds of fragment 2 with dithiothreitol and alkylation with iodo[1-¹⁴C]acetamide were performed according to the procedure of Waxdal *et al.* (1968). The alkylation reaction was stopped after 30 min by addition of excess dithiothreitol.

RESULTS AND DISCUSSION

Degradation of AT and isolation of fragments

AT was treated with CNBr as described in the Experimental section and fractionated on a column of Bio-Gel P-60. This resulted in the separation of at least four polypeptide peaks (Fig. 1). The fractions included in peak 2 were combined and rechromatographed on Bio-Gel P-150. This provided a purification of the principal fragment of peak 2 (subsequently denoted as fragment 2) from small amounts of higher-*M_r* and lower-*M_r* materials. The contents of peak 1 were repurified in a similar manner. SDS/polyacrylamide-gel electrophoresis showed that the material in peak 1 consisted of a product that was partially degraded by CNBr. On the basis of SDS/polyacrylamide-gel electrophoresis and previously reported separations of CNBr-

Table 1. Assignment of CNBr-digest peaks from the Bio-Gel P-60 column

Peak no.	Description
1*	Partially degraded products
2*	Residues 104–251 disulphide-linked to residues 1–17 and to residues 424–432
3	Mixture of residues 21–103 and residues 339–423
4	Mixture of residues 253–281 and 282–314

* Further fractionated on Bio-Gel P-150 column (predominant peak material).

cleavage fragments of AT (Petersen *et al.*, 1979; Blackburn *et al.*, 1984; Koide *et al.*, 1984), the peaks were assigned to positions of the AT sequence shown in Table 1. The smallest fragments, including peptides with residues 321–338, 18–20 and 316–320, and the homoserine residues from positions 252 and 315, described by workers in other laboratories, were not detected by this method. In the present studies, the disulphide linkages were not reduced, since it has been reported that disruption of these bonds in intact AT results in the loss of heparin binding (Longas *et al.*, 1980).

Activities of the peaks

Assays for thrombin-inhibitory activity in the absence of intact AT show that none of the fragments has any measurable effects either in the absence or in the presence of heparin, even when they were added in large excess. Similarly, when mixed together they did not neutralize the

Fig. 1. Separation of CNBr-cleavage fragments on Bio-Gel P-60

The dimensions of the column were 1.7 cm × 116 cm and the fragments were eluted with 0.1 M-formic acid. Each fraction contained 2.6 ml. The individual fractions included in peak 2 are shown by the horizontal bar. Other fractions were pooled in a similar fashion.

Fig. 2. Heparin-Sepharose elution patterns

The column was run as described in the Experimental section and samples were eluted with a NaCl gradient (----) in 0.05 M-Tris/HCl buffer, pH 7.5. (a) Pattern for fragment 2, measured by absorbance at 230 nm. (b) Elution of AT monitored by absorbance at 280 nm.

action of thrombin. Fragment 2 and the contents of peaks 3 and 4 were screened with respect to their binding to heparin-Sepharose. Only fragment 2 showed an affinity for the column (Fig. 2) and was eluted at only a slightly lower NaCl concentration than was intact AT. The polypeptides of peaks 3 and 4 were totally eluted from the column in the loading buffer.

These fragments were also studied with regard to their ability to inhibit the neutralization of thrombin by AT. Concentrations of the fractions in excess of AT were used in these initial experiments to screen for their effect on AT activity. As seen from the data in Tables 2 and 3, fragment 2 produced the greatest degree of interference with the action of the inhibitor, both in the presence and in the absence of heparin. The fact that only fragment 2 inhibited heparin cofactor activity confirms that it alone binds to heparin. The contents of peak 3 showed a relatively small effect on the progressive inhibitory activity of AT. Additionally, Table 3 shows that fractions 2, 3 and 4 did not affect thrombin activity in the absence of AT.

Characteristics of fragment 2

Since the material in purified fragment 2 bound to heparin-Sepharose, and exhibited the highest AT inhibition, it was studied in detail. The origin of this fragment is shown schematically in the top of Fig. 3. Fragment 2 is the result of CNBr cleavages at the methionine residues at positions 17, 103, 251 and 423. Table 4 shows the results of amino acid analysis, which agrees with the known sequence of the fragment (Bock

Fig. 3. Formation of fragment 2 from AT and its reduction and alkylation

The thick horizontal line in the top diagram depicts the primary structure of intact AT. The positions of disulphide bonds are shown (S-S) as well as the carbohydrate chains (▼). Cleavage at the methionine residues indicated by the arrows (top) yields fragment 2, shown in the middle of the Figure. Reduction of the disulphide bonds with dithiothreitol and alkylation of the resultant cysteine residues with iodo[14 C]acetamide produces the three peptides shown at the bottom of the Figure. SAA represents the thiol unit linked to acetamide.

Table 2. Effect of CNBr-cleavage products on the inhibition of heparin cofactor activity

The solution contained heparin (0.091 μ g/ml, final concentration) and fragment preincubated with buffer for 5.0 min. AT was added to a final concentration of 31 nM, and after 1.0 min thrombin (3.8 nM final concentration) was added. Substrate was added after another 30 s. This concentration of AT was enough to inhibit the thrombin by 78% in the absence of fragment.

Sample	Concn. (μ M)	Inhibition of AT activity (%)
Fragment 2	0.09	72
Peak 3 peptides	0.18	0
Peak 4 peptides	0.18	0

et al., 1982; Chandra *et al.*, 1983). It should be noted that fragment 2 does not contain the residues that have been reported previously to be involved in heparin binding, such as Arg-47 (Koide *et al.*, 1984) and Trp-49 (Blackburn *et al.*, 1984), and it does not include the residue cleaved by thrombin, Arg-393 (Björk *et al.*, 1982). The conclusion that residues 47 and 49 are important in AT-heparin interactions is based on experiments in which AT is modified in these positions. However, these residues may not be involved directly; rather, the loss in activity upon their alteration may be due to resultant conformational or other changes at remote sites.

The possibility that the fragment 2 preparation is contaminated by a longer incompletely digested fragment containing residues 47 and 49 is highly unlikely, since at

Table 3. Effect of CNBr-cleavage products on the inhibition of AT activity in the absence of heparin

Thrombin (final concentration 9.7 nM before dilution) and fragment were preincubated for 10 min, then AT was added to a final concentration before dilution of 87 nM. This concentration inhibited thrombin by 78% in the absence of peptide. After 10.0 min, the sample was diluted with prewarmed buffer and substrate, and the change in A_{405} per min was followed.

Sample	Concn. (μ M)	Inhibition of AT activity (%)	Thrombin activity (% of that in absence of AT)
Peak 1 peptides	0.4	57	—
Fragment 2	0.3	62	106
Peak 3 peptides	0.3	19	109
Peak 4 peptides	0.3	-1	105

Table 4. Amino acid composition of fragment 2

Uncorrected values are averages of four determinations. Expected values are based on the sum of the residues in the peptides 1–17, 104–251 and 424–432 from the sequence reported by Chandra *et al.* (1983).

Amino acid	Composition	
	(residues/173 residues)	(expected residues/molecule)
Asp	19.8	20
Thr	9.6	10
Ser	13.4	16
Glu	17.6	19†
Pro	7.6	7
Gly	6.8	7
Ala	12.7	12
Cys	3.6	4
Val	11.0	11
Ile	8.5	10
Leu	14.1	13
Tyr	7.3*	5
Phe	12.3*	11
His	2.1	2
Lys	18.3	18
Arg	8.3	8

* Residual glucosamine does not separate from tyrosine and phenylalanine on this analyser system.

† Includes three residues of homoserine, which does not separate from glutamic acid, per molecule.

least two methionine residues would have to remain intact and such a higher- M_r component would easily separate from fragment 2 during gel-filtration chromatography. No methionine residues were found in the analysis of fragment 2 (Table 4). The fact that the fragment inhibits AT half-maximally at molar concentrations in the same order of magnitude as the AT concentration (see below) also demonstrates that the observed activities cannot be due to a minor contaminant.

Inhibition of heparin cofactor activity by fragment 2

The effect of concentration of fragment 2 on the inhibition of heparin-enhanced AT activity is described in Fig. 4. The neutralization of this activity in the presence of 0.091 μg of heparin/ml (6.3 nM) increases with the concentration of fragment 2 and is essentially complete at a concentration of greater than 300 nM. The concentration of fragment 2 that results in 50% inhibition of this heparin cofactor activity is at about 46 nM. For comparison, AT concentration was 61 nM in this experiment. When heparin concentration was increased, the degree of inhibition of heparin cofactor activity by fragment 2 decreased; thus more AT is activated by additional heparin. In the assay with heparin, it appears that the inhibition of AT activity by the fragment is due to sequestering of the heparin by excess fragment, thus preventing AT from being activated by the glycosaminoglycan.

Inhibition of progressive AT activity by fragment 2

In the absence of heparin, the inhibition of AT by fragment 2 was incomplete, and reached a maximum in one experiment of about 36% (Fig. 5). In other

Fig. 4. Inhibition of heparin cofactor activity by fragment 2

The indicated concentrations represent those immediately before addition of substrate. Solutions of fragment 2 and heparin (0.091 $\mu\text{g}/\text{ml}$) were mixed with the buffer, and after 5 min AT (61 nM) was added. After exactly 60 s, this was mixed with thrombin (4.5 nM) and incubated for 30 s. S-2238 solution (200 μl) was then added to measure residual thrombin activity. Percentage inhibition was calculated as described in the Experimental section.

Fig. 5. Inhibition of progressive AT activity by fragment 2

Concentrations are final concentrations before addition of samples to buffer and substrate. Fragment 2 was incubated with thrombin (19 nM) for 15 min, followed by addition of AT (100 nM). After exactly 1.0, 5.0 and 10.0 min, portions were transferred to a cuvette containing a prewarmed mixture of buffer and S-2238. Percentage inhibition was calculated as described in the Experimental section.

experiments this maximum approached 50% inhibition. Half-maximal inhibition of AT activity was exhibited with 28 nM-fragment 2, with 100 nM-AT (Fig. 5). Thus the fragment appears to be a more effective competitor for thrombin than was AT in this experiment. This

Fig. 6. Comparison of effects of peak 1 material and fragment 2 on the inhibition of AT activity

The assay procedure was the same as described for Table 3, except that the final AT and thrombin concentrations were 220 nM and 8.3 nM respectively. The concentration for half-maximal inhibition for the material in peak 1 was estimated to be about 64 nM and that for Fragment 2 was about 21 nM. A logarithmic scale is used for the abscissa in order to show all the data. ○, Fragment 2; ●, peak 1.

inhibition is specific for fragment 2, since the contents of peaks 3 and 4 had no effect, and even Bio-Gel-P-150-purified peak 1 material required about 3 times as much material as fragment 2 to reach 50% of maximal inhibition (Fig. 6). SDS/polyacrylamide-gel electrophoresis of the material in peak 1 shows it to be homogeneous with an apparent M_r of 45000. Upon reduction with dithiothreitol, over half of the resulting material was changed to a faster-moving component with the same electrophoretic mobility as the large subunit from reduced fragment 2. The rest maintained the same mobility as the non-reduced material. Peak 1 material therefore is an incompletely digested CNBr-cleavage fragment that includes residues in common with fragment 2 and would be expected to have at least some of the properties of that fragment.

To determine whether the results depended on the order of addition, thrombin and AT were first mixed together, followed after 10 min by a large excess of fragment 2. Under these conditions the fragment had no effect on the inhibition of thrombin by AT (Fig. 7). This result is attributed to the relatively tight binding of the components in the AT-thrombin complex (Jesty, 1979). Only when fragment 2 was added first to either AT or thrombin, as described above (for example, Table 3 or Fig. 4), was interference with AT activity observed. The degree of inhibition of AT by fragment 2 was the same in experiments in which the thrombin or the AT was first incubated with the fragment.

AT inhibition of thrombin can be defined by second-order kinetics (Downing *et al.*, 1978). The effect of fragment 2 on the second-order rate constant for the inhibition of thrombin by AT was determined (Fig. 8). A concentration of fragment was used (25 nM) that would give about half-maximal inhibition of AT activity, as predicted from Fig. 5. The concentration of AT was varied. In this experiment the rate constant was reduced from $9.7 \times 10^5 \pm 0.1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ in the absence of fragment 2 to $7.7 \times 10^5 \pm 0.1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ in its

Fig. 7. Effect of incubation of AT with thrombin before addition of fragment

Buffer, thrombin (19 nM, final concentration) and AT (200 nM) were mixed together. Residual thrombin activity was measured in samples taken after exactly 1.0 min and 8.0 min after mixing. After another 2 min (arrow) either buffer (○) or an equal volume of fragment 2 (251 nM) (●) was added, and at 25 min further samples were taken for assay from both incubations. The 25 min-sample volumes were adjusted for dilution.

Fig. 8. Effect of fragment 2 on the kinetic constant for the thrombin-AT reaction

A solution of 17 nM-thrombin (final concentration, before dilution) and 25 nM of fragment 2 were preincubated for 15 min, and different concentrations of AT were then added. Samples of the mixture were withdrawn at exactly 60 s, 5 min and 10 min after the last dilution and assayed for activity as described for Fig. 3. ○, Fragment present; ●, buffer substituted for fragment solution. The pseudo-first-order rate constants ($k_{\text{obs.}}$) are plotted against the AT concentration. The slopes of the linear-regression lines going through the origin are the second-order rate constants (Jencks, 1969). The rates measured at the lowest concentration of AT shown were too low to measure accurately, although they are included in the graph. Analysis of co-variance, which compares the values from these two sets of data (Dixon & Massey, 1957), shows that the slopes of these lines are statistically significantly different (> 95th percentile confidence level).

presence, i.e. about 57% of maximal inhibition of AT activity was actually achieved. This experiment also shows that the percentage inhibition of AT by a fixed concentration of fragment 2 is independent of AT concentration. Using the method of partial fractions to evaluate the rate constant (Downing *et al.*, 1978) instead of the method described in Fig. 8 resulted in the same values. Previous studies have determined the second-order rate constant to range from 3.4×10^5 to $9.6 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Downing *et al.*, 1978; Jesty, 1979; Longas *et al.*, 1980; Griffith & Lundblad, 1981; Hoylaerts *et al.*, 1984; Karp *et al.*, 1984; Rosenberg, 1985). Our value in the absence of fragment is probably the result of the somewhat higher temperature employed. Thus fragment 2 inhibited this AT-thrombin reaction by causing a depression in the second-order rate constant.

The mechanism for the actions of fragment 2 is consistent with the proposal that it contains a section of AT that normally binds to thrombin at a site other than the active serine residue. The latter is evidenced by the fact that the fragment, even at high concentrations, does not inhibit thrombin by itself. The existence of such a site has been demonstrated by experiments showing that active-site-blocked thrombin interferes with the AT-thrombin interaction (Griffith, 1982; Pletcher & Nelsestuen, 1983; Hoylaerts *et al.*, 1984). The approach of AT to this secondary site is blocked by fragment 2 when it is bound to thrombin, and thus it competes with AT for this site. Since AT activity is incompletely inhibited by excessive concentrations of the fragment, AT must still interact with the active serine residue of thrombin, although with a lesser effect, in the presence of fragment 2 bound to the secondary site.

Fig. 9. Separation of components after reduction and alkylation of fragment 2

Fragment 2 was reduced with dithiothreitol and alkylated with iodo[^{14}C]acetamide as described in the Experimental section. The mixture was applied to a column (1.0 cm $\times$ 54.0 cm) of Bio-Gel P-60 equilibrated and eluted with 0.1 M-formic acid. Radioactivities in 50 μl samples of each 1 ml fraction were determined. The large peak centred at fraction 39 represents the products from unused iodo[^{14}C]acetamide.

Reduction and alkylation of fragment 2

Fragment 2 was reduced with dithiothreitol and alkylated with iodo[^{14}C]acetamide. Separation of the large peptide (I) from the two small peptides (II and III) was effected by chromatography on a Bio-Gel P-60 column (Fig. 9). Peptide I had about twice the amount of radioactivity as either peptide II or peptide III, which would be expected from the structure of Fragment 2 (Fig. 3). The effects of large excesses of these peptides in inhibiting AT activity, in the absence and in the presence of heparin, is shown in Table 5. Most of the AT inhibitory activity resides in peptide I, although a small amount of interference is observed with peptides II plus III. Gel electrophoresis revealed that peptide I was greater than 95% pure, with only a trace of a component that had twice the molecular mass. Additionally, amino acid analysis

Table 5. Inhibition of AT activity by reduced and alkylated peptides

Activity assays are the same as indicated for Tables 2 and 3, except AT concentration was $0.22 \mu\text{M}$ without heparin and $0.061 \mu\text{M}$ with heparin. Thrombin concentration was 13 nM without heparin (before dilution) and 3.5 nM with heparin. Final peptide concentration is based on the specific radioactivity of iodo[^{14}C]acetamide.

Peptide	Concn. (μM)	Concn. of heparin ($\mu\text{g/ml}$)	Inhibition of AT (%)
I	3.8	0	46
II plus III	4.5	0	21
I	1.0	0.091	96
II plus III	1.2	0.091	15

Table 6. Amino acid analysis of peptide I

Uncorrected values are averages of two determinations. Expected values are based on the sum of the residues in peptide 104-251 from the sequence reported by Chandra *et al.* (1983).

Amino acid	Composition	
	(residues/144 residues)	(expected residues/molecule)
Asp	16.6	17
Thr	8.5	9
Ser	13.5	15
Glu	16.5	17†
Pro	3.2	3
Gly	5.5	5
Ala	10.1	10
Val	7.3	8
Ile	6.7	8
Leu	12.9	13
Tyr	7.2*	5
Phe	12.7*	11
His	1.0	1
Lys	15.4	16
Arg	6.0	6

* See footnote* in Table 4.

† Includes one residue of homoserine, which does not separate from glutamic acid, per molecule.

shows good agreement with the expected values (Table 6). Peptide I bound to a heparin-Sephadex column, whereas the other peptides did not (results not shown). Reduction and alkylation of fragment 2 narrows its active region to the peptide with residues 104–251. Further studies on this peptide should reveal new locations for the heparin- and thrombin-binding regions of AT.

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