Identification of the Cleavage Sites Resulting from Enzymatic Inactivation of Human Antithrombin III by *Crotalus adamanteus* Proteinase II in the Presence and Absence of Heparin†

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ABSTRACT: The effect of heparin on the enzymatic inactivation of human antithrombin III by *Crotalus adamanteus* proteinase II and the location of the inhibitor bonds cleaved were investigated. Incubation of antithrombin III with heparin followed by catalytic amounts of proteinase II resulted initially in the conversion of intact inhibitor (M_r 60 000) into an intermediate species (M_r 56 000) which retained inhibitory activity against thrombin. Further proteolysis resulted in the gradual appearance of a third band (M_r 50 000) and the corresponding loss of inhibitory activity against thrombin. Electrophoretic analysis of the reaction products under nonreducing conditions revealed only one band (M_r 60 000), indicating that the cleavages had occurred within disulfide loops in the terminal regions of the inhibitor molecule. Amino- and carboxy-terminal sequence analyses of the inactivated antithrombin III and amino-terminal analysis of the peptides released showed that cleavages had occurred at Glu_{37}–Gln_{38}, Ala_{375}–Ser_{376}, and Ala_{378}–Val_{379}. Characterization of the peptide initially released during conversion of the intact inhibitor to the intermediate species indicated that the Glu–Gln cleavage occurred first and did not affect inhibitory activity against thrombin. Reaction of antithrombin III with proteinase II in the absence of heparin resulted in a slow loss of inhibitory activity against thrombin and the appearance of a band (M_r 56 000) corresponding to inactive inhibitor. No further digestion products were detected. Carboxy-terminal analyses indicated that antithrombin III inactivated in the absence of heparin was cleaved at Ala_{375}–Ser_{376}. Dansylation of the inactivated antithrombin III revealed that no cleavage had occurred in the amino-terminal region in the absence of heparin. The reactive-site Arg_{384}–Ser_{385} was not cleaved either in the presence or in the absence of heparin. The data show that proteinase II enzymatically inactivated antithrombin III by cleavage of a site in the carboxy-terminal portion of the molecule nine residues from the reactive-site bond cleaved by thrombin during complex formation with the inhibitor. Also, the results in the presence of heparin support the view that heparin causes a conformational change in antithrombin III which allows proteinase II to inflict a noninactivating cleavage in the amino-terminal region of the molecule.

Venoms from various poisonous snakes contain metalloproteinases which enzymatically inactivate human plasma proteinase inhibitors by limited proteolysis (Kress & Paroski, 1978; Kress & Kurecki, 1979; Kress & Catanese, 1980). Proteinase II, purified from *Crotalus adamanteus* venom (Kurecki et al., 1978), inactivated α_1-proteinase inhibitor by cleavage of an X–Met bond (Kress et al., 1979) which was eight residues from the postulated reactive site of the inhibitor (Johnson & Travis, 1978; Morii et al., 1979; Carrell et al., 1980). More recent sequence data for α_1-proteinase inhibitor indicate that proteinase II cleaved the inhibitor at an Ala–Met bond (Petersen et al., 1979; Carrell et al., 1979).

The plasma proteinase inhibitor antithrombin III exhibits considerable homology to α_1-proteinase inhibitor, in both amino acid and carbohydrate sequences (Carrell et al., 1979; Hunt & Dayhoff, 1980; Hodges et al., 1979; Mega et al., 1980; Franzen et al., 1980). Antithrombin III forms stoichiometric enzyme–inhibitor complexes with virtually all the serine proteases involved in the coagulation–fibrinolysis system and is considered critical for regulation of the hemostatic mechanism (Stead et al., 1976). Complex formation between antithrombin III and thrombin is accompanied by proteolysis of the antithrombin III molecule (Fish et al., 1979; Jesty, 1979), and an Arg–Ser bond in the carboxy-terminal region of the inhibitor has been identified as the site of cleavage (Jornvall et al., 1979; Longas & Finlay, 1980). This corresponds to the unique Arg–X bond originally postulated as the reactive site of antithrombin III (Rosenberg & Damus, 1973).

A previous report indicated that inactivation of antithrombin III by metalloproteinases from crude venoms was enhanced by preincubation of the inhibitor with heparin (Kress & Catanese, 1980). The present investigation was undertaken to determine the effect of proteinase II on antithrombin III in the presence and absence of heparin and to identify the bond(s) in antithrombin III cleaved by proteinase II. Data are presented which indicate that prior exposure of antithrombin III to heparin allows a noninactivating cleavage by proteinase II in the amino-terminal region of the inhibitor molecule, followed by inactivating cleavages in the carboxy-terminal region. The results also show that in the absence of heparin an inactivating cleavage in the carboxy-terminal region occurs, and the bond cleaved is nine residues removed from that cleaved by thrombin during complex formation with antithrombin III.

Experimental Procedures

Materials. Heparin (lot no. 736-0160, 158 USP units/mg), α-*N*-benzoyl-L-arginine ethyl ester hydrochloride, and dithioerythritol were from Sigma; bovine trypsin and carboxypeptidases A and B were from Worthington; dansyl chloride and polyamide thin-layer sheets were from Pierce; human thrombin (Fibrindex) was from Ortho, D-Phe-Pip-Arg-*p*-nitroanilide·2HCl (S-2238) was from Kabi, USA; Sephacryl S-200 was from Pharmacia, and fluorescamine was from Roche. Iodoacetic acid from Fisher was recrystallized from hexane–ether immediately before use (Noltmann et al., 1962). Spectrapor dialysis tubing from Fisher was used according to the manufacturer's instructions. *C. adamanteus* proteinase II (Kurecki et al., 1978) was prepared as described previously; an $E^{1\%,1\ cm}_{280\ nm} = 8.8$ was used to determine concentration. Purified, nonpasteurized human antithrombin III (Wickerhauser

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& Williams, 1979) was kindly provided by the American Red Cross Blood Services Laboratory with the partial support of NIH Grant HL 13881. The material had a biological activity of 37.2 plasma equivalents per mL (Wickerhauser et al., 1979) and showed a single band on immunoelectrophoresis (Scheidigger, 1955) against anti-whole human serum and anti-human antithrombin III. Disc electrophoresis (Davis, 1964) showed a major band and a trace amount of a less anodically migrating band. The preparation showed only a single band on electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$)[1] (Weber et al., 1972) under both reducing and nonreducing conditions. The concentration of antithrombin III was determined by using $E^{1\%,1\ cm}_{280\ nm} = 6.1$. Heparin concentration was determined by weight.

Inactivation of Antithrombin III and Electrophoretic Analysis of Reaction Products. Antithrombin III (4.0 mg) was incubated with heparin (0.6 mg) for 3 min in 0.02 M sodium phosphate, pH 7.5, at 23 °C, and then proteinase II (either 13 or 40 μg) was added. Controls consisted of inhibitor incubated with heparin only. The final reaction volume was 0.6 mL. The molar ratio of inhibitor to proteinase was 120:1 or 40:1. Reaction aliquots (45 μL) were withdrawn at various times after addition of enzyme; a portion of this (30 μL) was added to an equal amount of 0.05 M Tris–0.01 M EDTA, pH 8.0, to end the reaction. These samples were assayed for inhibitory activity against thrombin by using S-2238 substrate (Abildgaard et al., 1977). The remainder of each aliquot (15 μL) was added to 1.5 μL of 10% $NaDodSO_4$ and 1.5 μL of 10% metcaptoethanol and heated at 100 °C for 3 min. Separate aliquots (15 μL) were also taken at various times during the reaction and treated with $NaDodSO_4$ only. Samples for electrophoresis were reacted with fluorescamine and then adjusted to pH 6.8 for running in the system described by Laemmli (1970) using a 4.5% stacking gel and a 7.5% running gel. Visualization of the fluorescent bands on the vertical slab gels was as previously described (Kurecki et al., 1978). Molecular weight determinations of reaction aliquots, not treated with fluorescamine, were run in the same system and stained for 4 h with 0.125% Coomassie brilliant blue R250, and diffusion destained for 4 h in water–methanol–acetic acid (5:4:1). The standard proteins used were β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin. An identical protocol was followed for an inactivation reaction in which buffer was substituted for heparin; controls consisted of inhibitor alone.

The optimal antithrombin III/heparin ratio was determined by incubating 350-μg amounts of the inhibitor with 10–525 μg of heparin and then adding 3.5 μg of proteinase II to give an inhibitor:enzyme molar ratio of 40:1 in a final volume of 100 μL of 0.02 M sodium phosphate, pH 7.5. Reaction aliquots were withdrawn, assayed, and analyzed electrophoretically as described above.

Isolation and Characterization of Antithrombin III Inactivation Products. Antithrombin III (12.2 mg) was incubated with heparin (1.8 mg), and proteinase II (0.12 mg) was added in a final reaction volume of 4.2 mL. Residual inhibitory activity was monitored periodically as described above. After 8 h, the reaction was terminated by addition of 0.1 M EDTA (adjusted to pH 8.0) to a final concentration of 0.01 M. Isolation of reaction products was based upon slight modifications of methods used by Jornvall et al. (1979). The reaction mixture was dialyzed against 0.1 M Tris-HCl–0.05% $NaDodSO_4$, pH 8.4, and charged on a 1.5 × 90 cm column of Sephacryl S-200 equilibrated and eluted at 23 °C with the same buffer. Essentially all material eluted in one peak near the void volume, and this was pooled, dialyzed against distilled H_2O, and lyophilized. The lyophilized material (10 mg) was dissolved in 0.5 mL of 0.1 M Tris-HCl, pH 8.4, containing 30 mg of $NaDodSO_4$ and reduced overnight with dithioerythritol (10 mM final concentration) under an N_2 atmosphere. The reduced inhibitor was reacted for 1 h with iodoacetic acid (25 mM final concentration) after which an excess of dithioerythritol was added, and the material was dialyzed overnight against 0.05 M Tris-HCl–0.1 M NaCl–0.05% $NaDodSO_4$–0.005 M mercaptoethanol, pH 7.5. Spectrapor 6 (molecular weight cutoff = 1000) dialysis tubing was used. The dialyzed material containing RCM-inactivated antithrombin III and RCM peptides from antithrombin III was separated on a second Sephacryl S-200 column eluted with 0.05 M Tris-HCl–0.1 M NaCl–0.05% $NaDodSO_4$, pH 7.5. The protein material eluted in one sharp peak (inactivated antithrombin III) and one broad peak (peptides). The peaks were pooled separately, dialyzed for 24 h against distilled H_2O, and lyophilized. An identical protocol was followed for an inactivation reaction in which buffer was substituted for heparin.

Another reaction similar to the reaction described above (with heparin present) was performed, with the exception that the reaction was terminated after 30 min by addition of EDTA. The peptide material from this reaction which was isolated after reduction and carboxymethylation was pooled, lyophilized, and subjected to amino acid and manual sequence analysis (Gray, 1972).

Amino Acid Analyses. Samples containing 0.5–1.5 nmol of protein were hydrolyzed in triplicate for 21 h and analyzed for amino acid composition (Moore & Stein, 1963) with a Dionex D-400 analyzer. Half-cysteine was determined as carboxymethylcysteine.

Automated Sequence Analyses. Automated amino-terminal sequence analyses were performed on a Beckman 890 sequenator as previously described (Seon & Pressman, 1979). Polybrene carrier and 0.25 M quadrol coupling buffer were used. A 12-nmol sample of inactivated antithrombin III (50 000-dalton material from the second Sephacryl column) and 36 nmol of the peptide mixture (5000-dalton material from the second Sephacryl column) were sequenced. Residues obtained after each cycle were identified as the PTH derivatives by high-performance liquid chromatography (Bhown et al., 1978). Amounts of amino acid recovered were calculated and corrected against an internal standard of PTH-norleucine. The repetitive yield was 94% for inactivated antithrombin III and 91% for the peptide mixture.

Carboxypeptidase Analyses. Carboxypeptidase A was washed 3 times with cold distilled H_2O and centrifuged; the enzyme pellet was dissolved in 10% LiCl. Carboxypeptidase B was dialyzed overnight against 0.01 M sodium borate–0.1 M NaCl, pH 8.5. Analyses using carboxypeptidases A and B were performed on samples containing approximately 3 nmol of protein according to published procedures (Ambler, 1972). Unless otherwise indicated, incubations were at 37 °C in 0.4 M sodium borate, pH 8.5, for 1 and 4 h at enzyme:substrate ratios of 1:100 (w/w). Residues released were identified on the amino acid analyzer, and all values were corrected by using controls consisting of enzyme only and substrate only.

Results

Enzymatic Inactivation of Antithrombin III. When catalytic amounts of proteinase II were reacted with antithrombin

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; RCM, reduced and carboxymethylated; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

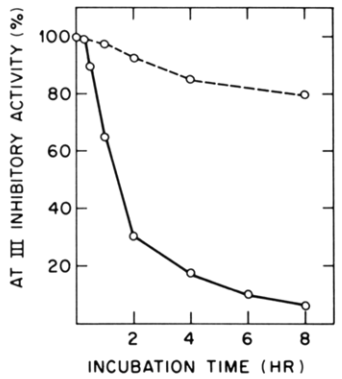

FIGURE 1: Inactivation of antithrombin III by proteinase II in the presence and absence of heparin. Antithrombin III was incubated with proteinase II at an inhibitor:enzyme molar ratio of 40:1 (see Experimental Procedures). Aliquots were assayed for residual inhibitory activity toward thrombin. The zero time activity is that of a comparable aliquot prior to proteinase addition and is normalized to 100%. The curves show loss of antithrombin III activity in the absence of heparin (O--O) and in the presence of heparin (O—O). Electrophoretic analyses of these incubation mixtures are shown in Figure 2.

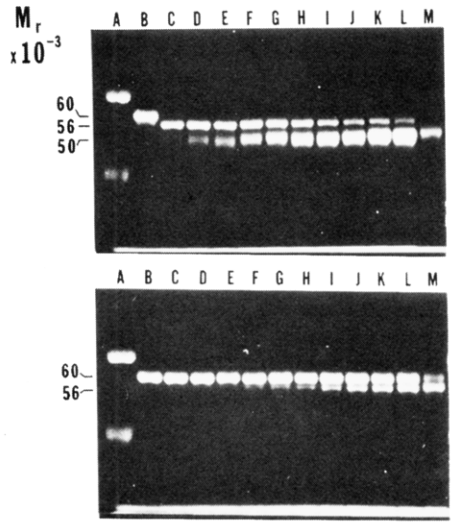

FIGURE 2: Electrophoretic analysis of the products resulting from inactivation of antithrombin III by proteinase II. Aliquots from the reaction mixtures containing antithrombin III and proteinase II (see Figure 1) were treated with $NaDodSO_4$–mercaptoethanol, reacted with fluorescamine, and subjected to electrophoresis in the system described by Laemmli (see Experimental Procedures). (Upper panel) Heparin present; (lower panel) heparin absent. Gel channels contained aliquots of the incubation mixtures taken after the following times of reaction: B, 15 s; C, 15 min; D, 30 min; E, 45 min; F, 1 h; G, 1.5 h; H, 2 h; I, 3 h; J, 4 h; K, 6 h; L, 8 h; M, 24 h. Channel A contained the following standards: bovine serum albumin and ovalbumin. The molecular weight values were calculated from separate gels (not shown) by using four standard proteins and reaction aliquots not treated with fluorescamine (see Experimental Procedures).

III in the absence of heparin, a slow inactivation of the inhibitor occurred (Figure 1). After 8 h, 80% of the initial antithrombin III activity against thrombin still remained. However, when antithrombin III was preincubated with heparin and then reacted with proteinase II, inhibitor inactivation was greatly accelerated. After a 15-min incubation with proteinase II, antithrombin III activity against thrombin began to decrease noticeably, and after an 8-h incubation, only 6% of the initial inhibitory activity remained. The antithrombin III:proteinase II molar ratio was 40:1, indicating that the inhibitor was being enzymatically inactivated in both cases. Experiments in which the amount of heparin was varied indicated that optimal rates of antithrombin III inactivation occurred at an inhibitor:heparin ratio of 6.7:1 (w/w), as judged by the loss of antithrombin III activity against thrombin and by the disappearance of the band corresponding to intact antithrombin III on the electrophoretic patterns (not shown). When heparin was present in higher amounts, antithrombin III inactivation was retarded. Therefore, the 6.7:1 ratio was employed in all experiments described in the present report.

The electrophoretic analyses of antithrombin III inactivation by proteinase II in the presence and absence of heparin are shown in Figure 2. (The data in Figures 1 and 2 are from the same experiment and are directly comparable). In the presence of heparin (Figure 2, upper panel), intact antithrombin III (60 000 daltons) was rapidly converted to an intermediate species (56 000 daltons). After 30 min, essentially all the intact antithrombin III had disappeared. However, only 10% of the inhibitory activity had been lost at this time. As the reaction proceeded, further proteolysis of the intermediate species resulted in the appearance of a third band (50 000 daltons). The amount of the third band detected corresponded to the observed loss of antithrombin III activity. After an 8-h incubation, a trace of the 56 000-dalton band and 6% of the original antithrombin III activity remained. Electrophoretic analyses of the reaction products using gradient slab gels showed that peptide material of approximately 5000 daltons was released during the proteolysis of antithrombin III. However, the system used did not resolve the peptides (data not shown). No bands with molecular weights greater than the 60 000-dalton intact inhibitor band were detected on any of the gel patterns, indicating that antithrombin III had been enzymatically inactivated without formation of an enzyme–inhibitor complex. The data indicate that reaction of antithrombin III with proteinase II in the presence of heparin results in the rapid conversion of the 60 000-dalton intact inhibitor to an active intermediate species of 56 000 daltons which is subsequently digested to an inactivated inhibitor of 50 000 daltons.

It should be noted that some preliminary experiments were run in the presence of 0.02 M phosphate buffer containing 0.5 M NaCl. There was no apparent alteration of the results. That is, the optimal antithrombin III/heparin ratio remained the same, as did the inhibitor inactivation rate, and the electrophoretic pattern was the same as that in the experiments described above (Figure 2, upper panel). This apparently excludes effects due to nonspecific binding of heparin to antithrombin III or to proteinase II. Since the high salt concentration did cause smearing of bands on electrophoresis, all experiments described in the present report used samples dialyzed against 0.02 M phosphate.

The electrophoretic analyses of inhibitor inactivation in the absence of heparin are shown in the lower panel of Figure 2. The 60 000-dalton antithrombin III band remained intact for approximately 1 h of incubation, and during that time, only 3% of antithrombin III activity was lost. From 90 min on, a second band (56 000 daltons) became apparent and gradually increased during the remainder of the 8-h incubation. Loss of antithrombin III activity corresponded to the amount of second band which was detected electrophoretically, and no further proteolysis of the 56 000-dalton band was observed. Peptide material of approximately 5000 daltons was detected by gradient slab gel electrophoresis (data not shown). The 56 000-dalton antithrombin III species formed in the presence of heparin retained its activity, whereas the 56 000-dalton band formed in the absence of heparin was inactive. Since electrophoretic analyses of the reaction products under nonreducing conditions (not shown) revealed only a single band of 60 000 daltons, it was tentatively concluded that all cleavages

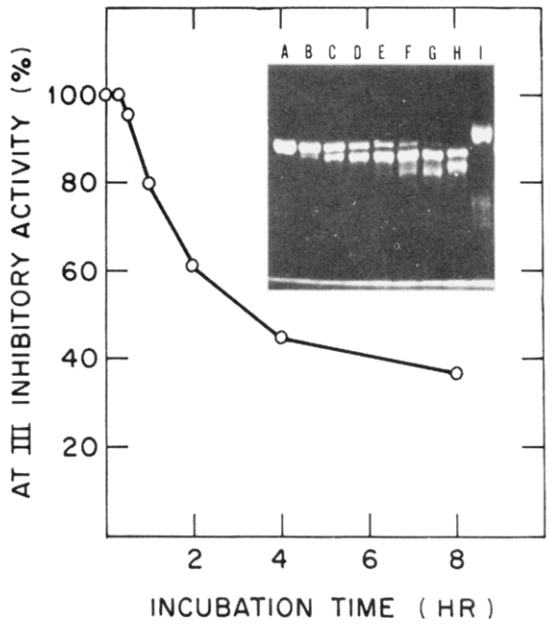

FIGURE 3: Partial inactivation of antithrombin III in the presence of heparin by using lowered amounts of proteinase II. Experimental details are as described in Figure 1 (heparin present) except that the antithrombin III:proteinase II molar ratio was 120:1. The insert shows electrophoretic analyses (see Figure 2 for details). Gel channels contained aliquots taken after the following times of reaction: A, 15 s; B, 5 min; C, 15 min; D, 30 min; E, 1 h; F, 2 h; G, 4 h; H, 8 h. Channel I contains the following standards: bovine serum albumin and ovalbumin.

by proteinase II had occurred within disulfide loop(s) in the terminal region of the antithrombin III molecule (Petersen et al., 1978).

Inactivation of antithrombin III in the presence of heparin with an antithrombin III:proteinase II ratio of 120:1 is shown in Figure 3. Under these conditions of lowered enzyme concentration, the reaction did not go to completion in 8 h, but the early stages of the inactivation process were more readily visualized. Conversion of intact antithrombin III (60 000 daltons) to the 56 000-dalton intermediate species occurred gradually, and loss of inhibitory activity against thrombin was correlated with the appearance of the 50 000-dalton band.

Characterization of Antithrombin III Inactivation Products. The amino acid compositions of the pooled peaks from the Sephacryl columns are shown in Table I. The single peak from the first Sephacryl column migrated electrophoretically under nonreducing conditions as a single 60 000-dalton band on gradient gels, and no peptide band was detected. Its composition is shown as antithrombin III in Table I. Reduction and carboxymethylation of this material, followed by gel filtration on a second Sephacryl S-200, resulted in the separation of two protein peaks. The first migrated electrophoretically as a single 50 000-dalton band, the second as a 5000-dalton band. Total separation of the 50 000-dalton material from traces of peptide was achieved only when NaDodSO$_4$ was employed in the elution buffers. The composition of these two peaks is shown in Table I as inactivated antithrombin III and peptides, respectively.

The sum of the compositions of inactivated antithrombin III plus peptides approximates the composition of the antithrombin III material from the first Sephacryl column for each amino acid residue except glycine. Unhydrolyzed antithrombin III contained only trace amounts of glycine, and a hydrolysate of undialyzed antithrombin III had the expected amount of glycine. The excess amounts of glycine were possibly due to

Table I: Amino Acid Composition of Reaction Products Resulting from Inactivation of Antithrombin III by Proteinase II in the Presence of Heparin[a]

	inactivated antithrombin III[b]	peptides[c]	antithrombin III[d]	N-terminal peptide[e]
Asp	36.0 (36)	9.0 (9)	43.0 (45)	4.0 (4)
Thr	18.0 (17)	4.9 (5)	22.4 (22)	2.1 (2)
Ser	25.5 (27)	5.2 (5)	35.0 (32)	2.7 (3)
Glu	45.7 (46)	6.1 (5)	51.0 (51)	3.9 (4)
Pro	11.3 (12)	7.7 (9)	19.6 (21)	5.3 (5)
Gly	17.1 (14)	7.6 (4)	29.4 (18)	5.7 (2)
$^1/_2$-Cys	3.1 (3)	2.6 (3)	6.0 (6)	1.9 (2)
Ala	26.2 (25)	6.5 (6)	32.0 (31)	2.4 (2)
Val	15.8 (18)	8.2 (8)	24.7 (26)	1.6 (1)
Met	7.6 (8)	2.8 (3)	10.8 (11)	1.8 (2)
Ile	15.0 (14)	5.6 (7)	19.9 (21)	2.9 (3)
Leu	33.4 (36)	3.4 (3)	38.5 (39)	0.5 (0)
Tyr	7.8 (8)	1.3 (1)	10.2 (9)	0.7 (1)
Phe	19.9 (21)	4.8 (4)	24.8 (25)	0.4 (0)
Lys	31.8 (30)	5.0 (5)	32.7 (35)	3.3 (3)
His	4.4 (4)	1.3 (1)	5.1 (5)	0.6 (1)
Arg	16.1 (15)	5.8 (7)	22.6 (22)	2.2 (2)

[a] Values in parentheses are the amounts expected for the various reaction products as calculated from the composition given by Petersen et al. (1978). [b] 50 000-dalton material from second Sephacryl column. [c] 5000-dalton peptide mixture from second Sephacryl column. [d] 50 000-dalton material (under nonreducing conditions) from first Sephacryl column. [e] 5000-dalton peptide from antithrombin III inactivation reaction terminated after 30 min. See text for details.

Table II: Amino-Terminal Sequence Analysis of the Products Resulting from Inactivation of Antithrombin III by Proteinase II in the Presence of Heparin

cycle	inactivated anthrombin III	peptide 1	peptide 2	peptide 3
1	Gln_{38} (6.6)[a]	His_1 (4.8)	Ser_{376} (4.2)	Val_{379} (6.5)
2	(Lys)[b]	Gly (7.3)	Thr (4.6)	Val (5.9)
3	Ile (4.3)	Ser (0.3)	Ala (6.7)	Ile (5.3)
4	Pro (6.9)	Pro (3.6)	Val (4.1)	Ala (4.5)
5	Glu (6.2)	Val (4.8)[c]	Val (4.8)	Gly (3.7)
6	Ala (7.9)		Ile (1.0)	
7	Thr (2.1)		Ala (3.7)	
8	Asn (1.1)		Gly (1.1)	

[a] Subscripts after residues detected in the first cycle refer to respective positions in the published antithrombin III sequence (Petersen et al., 1978); values in parentheses indicate nmoles recovered. [b] Lys overlaps the internal standard, and the amount recovered was not determined. [c] In cycle 5 of the peptide mixture, the amount of Val was allocated equally between peptides 1 and 2.

use of untreated dialysis tubing (Brown & Howard, 1980). Furthermore, the antithrombin III composition itself compares favorably with published literature values. The close correspondence between the composition of the antithrombin III material and the expected values for intact inhibitor indicates that no significant portions of the antithrombin III molecule have been deleted due to random proteolysis during the inactivation reaction.

Sequence Analyses of the Antithrombin III Inactivation Products. The inactivated antithrombin III (50 000 daltons) and the peptide mixture were subjected to eight cycles of automated sequence analysis. The results are summarized in Table II. The amino-terminal sequence of inactivated antithrombin III corresponded to residues Gln_{38} through Asn_{45} in the antithrombin III sequence. Traces of contaminating amino acids were detected only during the first sequenator

Table III: Effect of Carboxypeptidase Treatment on Reaction Products Resulting from Proteinase II Inactivation of Antithrombin III

substrate	reaction time (h)	amino acid residue released (nmol) by carboxypeptidase A	amino acid residue released (nmol) by carboxypeptidase B
intact antithrombin III	1	ND[a]	Lys (0.9)[b]
	4	ND	Lys (0.9)
inactivated antithrombin III (heparin present)	1	Ala (2.4)[b]	ND
	4	Ala (2.7)	ND
inactivated antithrombin III (heparin absent)	1	Ala (2.2)[c]	Lys (0.7)[d]
	4	Ala (2.5)	Lys (0.8)

[a] No amino acid residues detected in excess of the trace amounts present in enzyme and substrate controls. [b] Nanomoles released per nanomole of substrate. [c] Nanomoles released per nanomole of inactivated anthrombin III. [d] Nanomoles released per nanomole of active antithrombin III.

cycle, but no significant impurities were noted in the remaining cycles. It was concluded that inactivated antithrombin III was essentially homogeneous and that proteinase II had cleaved a single bond in the amino-terminal region of the antithrombin III molecule at residues Glu_{37}–Gln.

The action of proteinase II on antithrombin III in the presence of heparin (Figure 2, upper panel) had resulted in the rapid conversion of the intact inhibitor (60 000 daltons) to an active intermediate species (56 000 daltons). For trapping of the peptide released initially, the reaction was terminated by the addition of EDTA after a 30-min incubation. The peptide material was isolated as described previously and characterized. Its amino acid composition is shown in Table I as an N-terminal peptide. The composition of the peptide corresponded closely with that expected for residues 1 through 37 of the antithrombin III molecule. Again, Gly content was unexpectedly high as had previously been noted. No correlation existed between the composition of the initial peptide released and that of the carboxy-terminal region of the molecule. Amino-terminal analysis of this peptide using manual methods revealed the sequence His-Gly-Ser-Pro, which corresponds to the first four residues of intact antithrombin III. Characterization of this peptide confirmed that antithrombin III in the presence of heparin is initially cleaved by proteinase II at Glu_{37}–Gln and that this cleavage does not result in loss of antithrombin III inhibitory activity against thrombin.

The electrophoretic data (Figures 2 and 3) had shown that loss of antithrombin III activity in the presence of heparin is correlated with the appearance of a 50 000-dalton band, indicating that a further cleavage must have occurred in the carboxy-terminal region. In order to determine the site of this cleavage, the inactivated antithrombin III was subjected to carboxypeptidase analysis. The results are summarized in Table III. Incubation of inactivated antithrombin III with carboxypeptidase A for 4 h resulted in the release of 2.7 nmol of Ala per nmol of inactivated antithrombin III. No other amino acids were released. This indicated that cleavage of the carboxy-terminal region of antithrombin III by proteinase II had occurred at Ala_{375}, since this is the only section of the molecule which contains three consecutive Ala residues. Adjacent Ala residues occur at only one other site in antithrombin III, positions 184 and 185. The possibility of cleavage in this region near the middle of the molecule was excluded since it would have been readily apparent on the gel electrophoresis patterns (Figures 2 and 3). Release of greater than two residues of Ala could, therefore, result only from cleavage by proteinase II at Ala_{375}. No Arg was released by carboxypeptidase B, indicating that proteinase II did not cleave antithrombin III at the Arg_{384}–Ser site cleaved by thrombin.

The carboxypeptidase data showed that proteinase II had inactivated antithrombin III by cleavage of at least one bond in the carboxy-terminal region of the molecule. Therefore, an amino-terminal peptide and a carboxy-terminal peptide were expected as digest products. The amino-terminal peptide was characterized as described above. However, dansylation of the unresolved peptide mixture suggested the presence of a third component. Attempts to satisfactorily resolve the small amounts of peptide material were unsuccessful. Automated sequence analysis was, therefore, performed on the peptide mixture.[2]

As expected, the peptide material was heterogeneous, and several amino acid residues were detected with each cycle (Table II). Despite the heterogeneity of the peptide material, it was possible to unambiguously assign the residues detected to known regions of the published antithrombin III sequence. One sequence corresponded to His_1 through Val_5 and is the amino-terminal peptide resulting from the Glu_{37}–Gln cleavage mentioned previously. A second sequence corresponded to Ser_{376} through Gly_{383} and confirmed cleavage of the Ala_{375}–Ser bond. The third peptide sequence corresponded to Val_{379} through Gly_{383} and indicated that another cleavage had occurred in the carboxy-terminal region of antithrombin III at the Ala_{378}–Val bond.

Antithrombin III Inactivation in the Absence of Heparin. Antithrombin III was incubated with proteinase II without heparin and the reaction terminated after 8 h. Assays indicated that 70% of initial antithrombin III activity remained at this time. Approximately 30% of the starting material, therefore, was converted to inactivated inhibitor and peptide. The products were separated and characterized as described previously, and the site of bond cleavage was identified by carboxypeptidase analysis of the inactivated inhibitor, and reference to the published sequence (Petersen et al., 1978). The large molecular mass material from the second Sephacryl column consisted of a mixture of proteins with electrophoretic migrations corresponding to 60 000 and 56 000 daltons. Amino-terminal analysis of the mixture showed only didansylhistidine, indicating that the amino terminus of antithrombin III had not been cleaved in the absence of heparin. Reaction with carboxypeptidase B (Table III) released 0.8 nmol of Lys per nmol of active antithrombin III. No Arg was released either from the large molecular mass mixture (Table III) or from the peptide (not shown). This excluded cleavage at the Arg_{384}–Ser reactive site. Reaction with carboxypeptidase A for 4 h released 2.5 nmol of Ala per nmol of inactivated antithrombin III. Incubation with higher amounts of carboxypeptidase A (enzyme:substrate = 1:40 w/w), longer time periods (8 and 14 h), or at pH values of 5.5 also resulted in the release only of Ala and always in amounts exceeding 2 nmol per nmol of inactivated inhibitor. For the same reasons stated above, the carboxypeptidase A data indicated cleavage of inactivated antithrombin III at Ala_{375}. Although no detectable amounts of Ser or Thr (residues 376 and 377) were

[2] Similar sequencing approaches in which several residues were detected per sequenator cycle have been utilized by Homandberg & Laskowski (1979) in investigating peptide bond resynthesis in ribonuclease A and also by Jornvall & Philipson (1980) for determining the sites of limited proteolysis of adenovirus hexon protein. In both instances, assignments of residue positions were possible because the sequence of the proteins being studied was known.

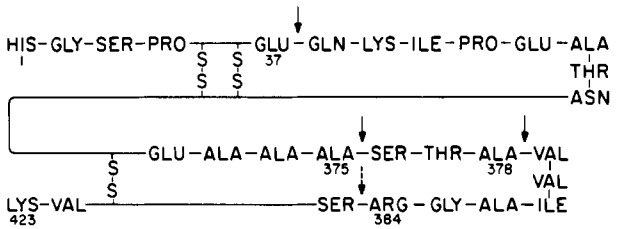

FIGURE 4: Schematic representation of the inactivation of antithrombin III by proteinase II in the presence and absence of heparin. The structure, residue numbering, and disulfide linkages are as reported by Petersen et al. (1978). The solid arrows indicate bonds cleaved by proteinase II in the presence of heparin. The Ala_{375}–Ser bond is also cleaved in the absence of heparin, and no cleavage occurs at Glu_{37}–Gln. The dashed arrow indicates the reactive-site bond cleaved by thrombin during complex formation (Rosenberg & Damus; 1973; Jornvall et al., 1979; Longas & Finlay, 1980).

released, the possibility of a slight amount of secondary cleavage in the carboxy-terminal region (as had occurred at Ala_{378}–Val in the presence of heparin) was not excluded by the present data. Simultaneous incubation with both carboxypeptidases A and B (not shown) resulted in the release of lysine, valine, and alanine. The lysine and valine confirm that the 60 000-dalton protein is intact antithrombin III. Release of 2.5 nmol of Ala per nmol of inactivated antithrombin III indicates that the 56 000-dalton form is antithrombin III which has been inactivated by proteinase II in the absence of heparin via cleavage of the Ala_{375}–Ser bond in the carboxy-terminal region of the inhibitor.

Discussion

The effects of proteinase II on antithrombin III in the presence and absence of heparin are summarized schematically in Figure 4.

In the presence of heparin, an initial and rapid cleavage of antithrombin III by proteinase II occurred at Glu_{37}–Gln (Figure 2; Table II). However, no loss of inhibitory activity against thrombin was noted. Proteolysis in this region of the inhibitor, at Lys_{29}–Ala, has previously been observed during complex formation with trypsin (Mahoney et al., 1980). However, several other tryptic cleavages also occurred, and no conclusions regarding the necessity of the Lys–Ala bond for inhibitory activity could be made. In the present study, the observed amino acid composition of the isolated peptide (Table I, N-terminal peptide) corresponded closely to that expected for residues 1 through 37, and no contaminating sequences were noted in the analysis of inactivated antithrombin III (Table II). This indicated that Glu_{37}–Gln was the only bond in the amino-terminal region of antithrombin III susceptible to proteinase II when heparin was present.

The noninactivating cleavage in the amino-terminal region was followed by cleavages at Ala_{375}–Ser and Ala_{378}–Val (Figure 4; Tables II and III), and a gradual loss of inhibitory activity occurred. Recently, evidence was presented that formation of the stoichiometric complex with thrombin results in proteolysis of an X–Ser bond in bovine antithrombin III (Jornvall et al., 1979) and an Arg–X bond in human antithrombin III (Longas & Finlay, 1980). In both instances, the cleavage occurred in the carboxy-terminal region of the molecule and resulted in inactivated antithrombin III. Because of the homology between bovine and human antithrombin III (Jornvall et al., 1979; Kurachi et al., 1976), cleavage at Arg_{384}–Ser (Figure 4) was postulated (Jornvall et al., 1979), and this bond is now considered the reactive site of antithrombin III for thrombin (Danielsson & Bjork, 1980). Proteinase II, therefore, cleaves the antithrombin III molecule at bonds six and nine residues removed from the reactive-site bond cleaved by thrombin (Figure 4). This is similar to the effect of proteinase II on α_1-proteinase inhibitor (Kress et al., 1979) in which case inactivation occurred by cleavage of an Ala–Met bond eight residues from the postulated reactive site (Johnson & Travis, 1978). The result with antithrombin III is not unexpected in view of the considerable homology between α_1-proteinase inhibitor and antithrombin III (Petersen et al., 1979; Carrell et al., 1979; Hunt & Dayhoff, 1980). One major difference between the inhibitors is that α_1-proteinase inhibitor contains no disulfide loops (Morii et al., 1978), and limited proteolysis by proteinase II resulted in release of a 4000-dalton peptide from the molecule. However, antithrombin III contains three disulfide bonds, and all cleavages by proteinase II occurred within disulfide loops at either end of the molecule (Figure 4). Therefore, no large peptides were released from antithrombin III as a result of inactivation by proteinase II, and the reactive-site bond remained intact. Limited proteolysis at bonds near the reactive site was sufficient to cause loss of inhibitory activity. Since peptides beginning with Ser_{376} and Val_{379} were both detected in the sequenator analyses but only Ala (not Thr or Ser) was released by carboxypeptidase A, it is apparent that only partial cleavage occurred at Ala_{378}–Val. On the other hand, almost all inhibitor activity was lost (Figure 1). It therefore seems likely that loss of activity was due to cleavage of the Ala_{375}–Ser bond rather than to deletion of the tripeptide Ser_{376}-Thr-Ala from the molecule. It should be noted that the antithrombin III sequence is complete except for the possible omission of several residues (Petersen et al., 1978). Since the omissions occur at residues 210 and 306, it is unlikely that this will affect the interpretation of the bond cleavages in the present paper.

Several important differences were noted during the inactivation of antithrombin III by proteinase II when heparin was absent from the incubation mixture. The rate of proteolysis of the antithrombin III molecule (Figure 2) and the corresponding loss of inhibitory activity (Figure 1) were both considerably lower in the absence of heparin. Also, no cleavage was observed in the amino-terminal region of the antithrombin III molecule (Figure 4) even after 8 h of incubation. Lack of cleavage of any bond in this part of the inhibitor when heparin was absent contrasts sharply with the rapid cleavage of the Glu_{37}–Gln bond when heparin was present. Thus, both the rate of proteolysis of antithrombin III by proteinase II and also the accessibility of the amino-terminal region of the inhibitor to the enzyme are affected by the presence of heparin.

Previous studies have indicated that heparin greatly accelerates enzyme–antithrombin III interactions (Rosenberg & Rosenberg, 1975). This effect on complex formation between thrombin and antithrombin III has been attributed to direct binding of heparin causing a conformational change in the inhibitor (Rosenberg & Damus, 1973; Nordenman & Bjork, 1978; Villanueva & Danishevsky, 1979; Jordan et al., 1980) or to binding of heparin by the enzyme (Li et al., 1974; Machovich et al., 1975; Sturzebecher & Markwardt, 1977; Griffith, 1979). The results in the present study do not appear to be caused by binding of heparin to proteinase II. Inhibitor inactivation was optimal when the antithrombin:heparin ratio was approximately 6.7:1 (w/w). Additional amounts of heparin relative to inhibitor retarded the reaction, presumably due to electrostatic interaction between the excess heparin and proteinase II which is a basic protein (Kurecki et al., 1978). The data do, however, support the view that exposure to heparin caused a conformational change in antithrombin III which allowed cleavage by proteinase II of a bond in the

inhibitor which was not accessible to the enzyme in the absence of heparin.

Finally, in the absence of heparin, proteinase II cleaved the Ala_{375}–Ser bond near the reactive-site region of antithrombin III (Figure 4), and proteolysis of this bond was correlated with loss of inhibitory activity (Figures 1 and 2). The assignment of the Ala_{375}–Ser bond cleavage in the absence of heparin is based on carboxypeptidase analyses of inactivated antithrombin III; no amino-terminal analyses of the peptide were performed. However, this is the only region of the inhibitor molecule containing three adjacent Ala residues. The fact that only Ala (in the amounts expected) and no other residues were released tends to preclude the ambiguity of primary bond cleavage assignment which ordinarily would accompany carboxypeptidase analysis if multiple residues were involved. In conclusion, it seems likely that the primary cleavage in the reactive-site region in the presence of heparin also occurred at Ala_{375}–Ser and that proteolysis of this bond accounts for the inactivation of antithrombin III by proteinase II.

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