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The covalent nature of the human antithrombin III–thrombin bond

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1. Cleavage of the human antithrombin III–thrombin complex with [^{14}C]methoxyamine hydrochloride results in inactive thrombin and ^{14}C-labelled antithrombin III. 2. Discontinuous polyacrylamide-gel electrophoresis of the reduced dissociation fragments of the complex in the presence of sodium dodecyl sulphate reveals two antithrombin III bands that do not resolve during electrophoresis without reduction. The heavy band has the electrophoretic mobility of the native protein. The light band has an apparent mol.wt. that is approx. 4000 less than the molecular weight of native antithrombin III. 3. Treatment of the cleavage products of the complex with carboxypeptidase B yields 1 μmol of arginine, a new *C*-terminal amino acid, per μmol of thrombin dissociated. The results indicate that during formation of the antithrombin III–thrombin complex, the inhibitor is cleaved at an arginine–X bond; this arginine residue forms a carboxylic ester with the enzyme, while the excised polypeptide remains bound through a disulphide bridge(s).

The reaction of thrombin, one of the serine proteinases of blood coagulation, with antithrombin III (also known as heparin cofactor), an α-2-glycoprotein composed of a single polypeptide chain, is involved in the maintenance of haemostasis in mammals (Harpel & Rosenberg, 1976). Studies performed with purified thrombin and antithrombin III indicate that the product of this reaction is a 1:1 inactive complex, whose rate of formation is greatly accelerated by heparin. This complex is stable to denaturing and reducing agents (Rosenberg & Damus, 1973) and requires pH > 8.5 or < 2.5 to be dissociated (Owen, 1975; Jesty, 1979; T. H. Finlay & M. O. Longas, unpublished work).

The thrombin–antithrombin III association in serum has also been investigated. Pepper *et al.* (1977) purified ^{125}I-labelled thrombin–antithrombin III complex formed after addition of ^{125}I-labelled thrombin to human serum *in vitro*. This complex had an apparent mol.wt. of 250000 estimated on SDS/polyacrylamide gels. By utilizing gel-filtration and immunological techniques, Binder (1973) isolated a high mol.wt. (190000) antithrombin III–thrombin complex from human serum. These observations suggest formation of polymeric antithrombin III–thrombin complexes *in vivo*. The antithrombin III–thrombin complex in human blood may be determined by the agglutination method of Collen *et al.* (1977).

Even though the thrombin–antithrombin III interaction is physiologically very significant (Harpel & Rosenberg, 1976), the mechanism by which these two proteins associate has not been elucidated, and the nature of the antithrombin III–thrombin bond has not been clearly established. In an attempt to identify the specific moieties of thrombin and antithrombin III that react during complex formation, Rosenberg & Damus (1973) found that the active-site serine residue of thrombin and the arginine residue(s) of antithrombin III were essential for effective thrombin–antithrombin III associations. Because of the stability of the antithrombin III–thrombin complex and its pH requirements for dissociation, a carboxylic ester between the two proteins has been postulated (Owen, 1975).

In the present paper we report a series of experiments that include quantitative formation of the antithrombin III–thrombin complex, and its cleavage with [^{14}C]methoxyamine hydrochloride (*O*-[^{14}C]methyl hydroxylamine hydrochloride), to elucidate the nature of the antithrombin III–thrombin bond in the human species.

Materials and methods

Human thrombin, 2500 NIH units (0.3 NIH unit = 0.16 nmol of substrate transformed/s =

Abbreviations used: SDS, sodium dodecyl sulphate.

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1 nkat)/mg of protein and 87.5% α-thrombin, was a gift from Dr. J. W. Fenton, II, Division of Laboratories and Research, New York State Department of Health, Albany, NY 12201, U.S.A. Phenylmethanesulphonyl fluoride-treated carboxypeptidase B, 79 units (1 unit = 1 μmol of substrate transformed/min)/ml was purchased from Worthington Biochemical Corp., Freehold, NJ 07728, U.S.A. Antithrombin III was purified essentially by the method of Miller-Andersson *et al.* (1974), with some modifications that were necessary to obtain a final product with high specific activity. The antithrombin III prepared by this technique is a single polypeptide chain. Its molecular weight determined on polyacrylamide gels in the presence of SDS is 64000 (Weber & Osborn, 1969), and its specific activity is 1200 ± 30 NIH units/mg of protein. Phosphorylase, bovine serum albumin, ovalbumin and trypsinogen were obtained from Sigma Chemical Co., St. Louis, MO 63178, U.S.A. Compound S2160 (Benzoylphenylalanylvalylarginine *p*-nitroanilide) was from Bofors, Nobel Division Peptide Research, Molndal, Sweden. [^{14}C]Methoxyamine hydrochloride was purchased from New England Nuclear, Boston, MA 02118, U.S.A. Heparin (158 USP units/mg from porcine gastric mucosa) was a product of Research Plus, Denville, NJ, U.S.A. All other chemicals were reagent grade and were used without further purification.

Protein assay

Protein concentration was determined by alkaline hydrolysis followed by reaction with ninhydrin (Moore, 1968) or by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Antithrombin III assay

The heparin catalysed inactivation of thrombin by antithrombin III was determined at 37°C by a modification of the two-stage assay of Ødegård *et al.* (1975). Heparin (0.5 unit), human thrombin (0.1–0.5 unit) and antithrombin III (0.1–0.5 unit) were incubated in 0.5 ml of 0.025 M-Tris/0.075 M-NaCl buffer, pH 8.3, for 30 s. The substrate (compound S2160; 0.1 ml of a 0.5 mM solution containing 0.33 mg of polybrene/ml) was then added, and residual thrombin activity was monitored at 405 nm in a model 240 Gilford spectrophotometer equipped with a Gilford 6051 recorder. Polybrene was added to neutralize heparin, which is an inhibitor of thrombin (Longas *et al.*, 1980). The slow inactivation of thrombin by antithrombin III, which occurs in the absence of heparin (Rosenberg & Damus, 1973), was assayed in the same conditions without heparin and polybrene, and the incubation time was 30 min. One antithrombin III unit inactivated one NIH unit of thrombin.

Thrombin assay

The amidolytic activity of thrombin on the synthetic tripeptide compound S2160 was determined as described in the antithrombin III assay, but in the absence of heparin, antithrombin III and polybrene.

Preparation of the antithrombin III–thrombin complex

Thrombin (4.3 μM) and methoxyamine hydrochloride (1 mM) in 0.05 M-Tris/0.15 M-NaCl buffer, pH 7.5, were incubated at 0°C for 1 min. Antithrombin III (8.1 μM) was added and incubation was continued for 5 min. The reaction mixture was then adjusted to 0.1 M in methoxyamine hydrochloride and allowed to stand at 37°C for 1 h followed by dialysis at 4°C against 1 litre of 0.02 M-Tris/0.4 M-NaCl buffer, pH 7.5, with four changes.

Reagents for the Sakaguchi reaction

Reagent A. This was 0.01 g of α-naphthol dissolved in 100 ml of 5% (w/v) urea prepared in 95% (v/v) ethanol.

Reagent B. This was 5% sodium hypobromite [0.7 ml of bromine diluted to 100 ml with 5% (w/v) NaOH].

Reagent C. This was prepared by adding five NaOH pellets to 10.0 ml of reagent A (Acher & Crocker, 1952).

Determination of the absorption coefficient of arginine

The Sakaguchi reaction of guanidino and monosubstituted guanidino groups adapted to t.l.c. by Acher & Crocker (1952) was modified to determine the absorption coefficient (A_{1cm}) of arginine. Arginine (0.5 ml, 1 μM in water) and Sakaguchi reagent C (0.4 ml) were mixed thoroughly and combined with Sakaguchi reagent B (0.1 ml). After 5 min at standard temperature, the mixture was scanned for absorbance between 400 and 800 nm against a blank containing all reagents in the same proportion except arginine. The calculated $A_{1\,cm,505}$ is $1.35 \times 10^5\,M^{-1}\cdot cm^{-1}$.

Results

Cleavage of the antithrombin III–thrombin complex with [^{14}C]methoxyamine hydrochloride

The antithrombin III–thrombin complex (0.67 mg/ml) in 0.02 M-Tris/0.4 M-NaCl buffer, pH 7.5, was adjusted to pH 8.4 ± 0.05 with 1 M-NaOH. A portion was removed and made 0.01 M with respect to methoxyamine hydrochloride, with [^{14}C]methoxyamine hydrochloride (1.62×10^5 c.p.m./μmol in Tris/NaCl buffer, pH 8.4). Thrombin and antithrombin III (0.67 mg/ml) were treated identically and all the samples were incu-

Table 1. *Cleavage of the antithrombin III–thrombin complex with [^{14}C]methoxyamine hydrochloride*
The complex was treated with [^{14}C]methoxyamine hydrochloride as described in the text. To establish changes in the biological activities of the proteins due to reaction with [^{14}C]methoxyamine hydrochloride, portions of native thrombin and antithrombin III in 0.05 M-Tris/0.15 M-NaCl buffer, pH 7.5, were subjected to the steps of the cleavage experiment, but in the absence of methoxyamine hydrochloride.

Reaction	Antithrombin III activity (fast) (%)*	Antithrombin III activity (slow) (%)†	Thrombin activity (%)‡	[^{14}C]methoxyamine hydrochloride incorporated (μmol/μmol of protein)
Antithrombin III–thrombin complex	0	0	0	
Antithrombin III–thrombin complex + [^{14}C]methoxyamine hydrochloride	0	22	0	1.371
Antithrombin III + [^{14}C]methoxyamine hydrochloride	0	15		0.318
Thrombin + [^{14}C]methoxyamine hydrochloride			0	0.184
Native antithrombin III	90.0	80.0		
Native thrombin			82	

* Assayed in the presence of heparin as described in the text.
† Determined in the absence of heparin after 30 min incubation at 37°C with fresh thrombin in a 1 : 1 molar ratio.
‡ Amidolytic activity on the synthetic tripeptide compound S2160.

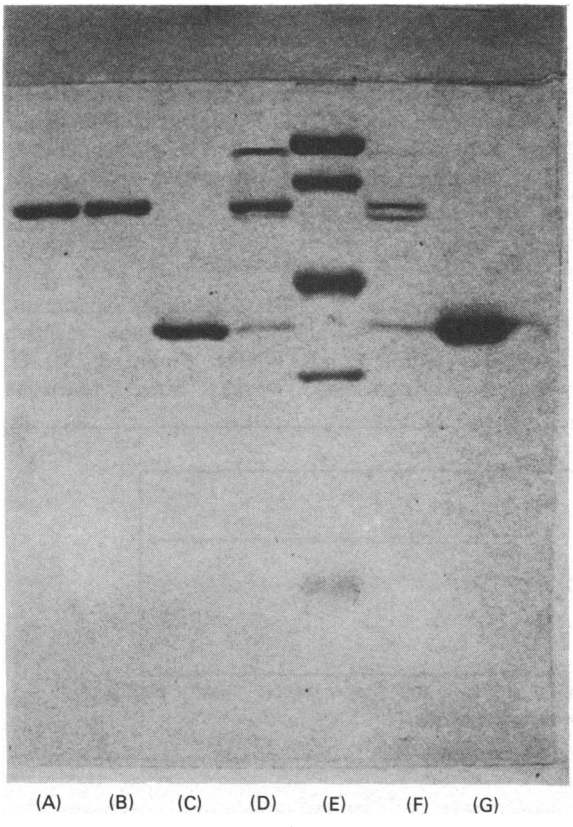


Fig. 1. *Discontinuous SDS/polyacrylamide-gel electrophoresis of the cleavage fragments of the antithrombin III–thrombin complex*
All samples were dialysed against 500 ml of 67 mM-Tris, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) Bromophenol Blue. After incubation in a boiling-water bath for 1½ min, portions containing 3 μg of protein/band were subjected to electrophoresis by a modification of the method of Laemmli (1970). The stacking and separating gels were 5 and 12.5% (w/v) in acrylamide respectively; the cathode buffer was pH 8.4. Electrophoresis was carried out with a voltage gradient from 45 to 110 V and 15 mA until the blue dye reached the bottom of the gel or the anode. (A) Native antithrombin III; (B) antithrombin III treated with [^{14}C]methoxyamine hydrochloride under the conditions of the cleavage experiment; (C) thrombin treated with [^{14}C]methyoxyamine hydrochloride under the same conditions; (D) the antithrombin III–thrombin complex incubated at pH 8.4 ± 0.05 during the cleavage reaction; (E) proteins of known molecular weight, 4 μg each [phosphorylase *b* (mol.wt. 95000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), trypsinogen (mol.wt. 24000)]; (F) dissociation products of the antithrombin III–thrombin complex obtained as described in the text; (G) native thrombin.

bated at 37°C for 16 h. The reaction mixtures were then dialysed against 1 litre of 0.05 M-Tris/0.15 M-NaCl buffer, pH 7.5, with several changes, until no ^{14}C was detectable in the diffusate. Samples were counted for radioactivity in 10 ml of Aquasol with a model 3320 Packard Tri-Carb liquid-scintillation spectrometer. The results of this experiment indicate that some [^{14}C]methoxyamine hydrochloride bound to the individual proteins, and as a consequence, they lost their biological activities (Table 1). The incorporation of this reagent in thrombin and

antithrombin III might be due to reaction with the carbonyl carbon atoms of the side chains of acidic amino acids or any electrophilic moiety other than peptide bonds (T. H. Finlay & M. O. Longas, unpublished work). Table 1 also shows that cleavage of the antithrombin III–thrombin complex took place with concomitant incorporation of 1.371 μmol of [^{14}C]methoxyamine hydrochloride per μmol of complex, assuming its mol.wt. to be 90000. Deduction of the [^{14}C]methoxyamine hydrochloride in the individual proteins used as controls (0.318 μmol/μmol of antithrombin III and 0.184 μmol/μmol of thrombin) from the μmol of this reagent in the dissociation products yields 0.869 μmol of [^{14}C]methoxyamine hydrochloride incorporated at the cleavage site/μmol of complex. Fig. 1 shows formation of a new antithrombin III band that we term cleaved antithrombin III and is lighter than the native protein by approx. 4000-mol.wt. units (Fig. 2). These results agree with the data of other investigators (Fish & Björk, 1979; Jesty, 1979) who reported modified antithrombin III released on cleavage of the bovine antithrombin III–thrombin complex. Fig. 3 shows that most of the ^{14}C is in the antithrombin III band of the cleavage fragments of the complex.

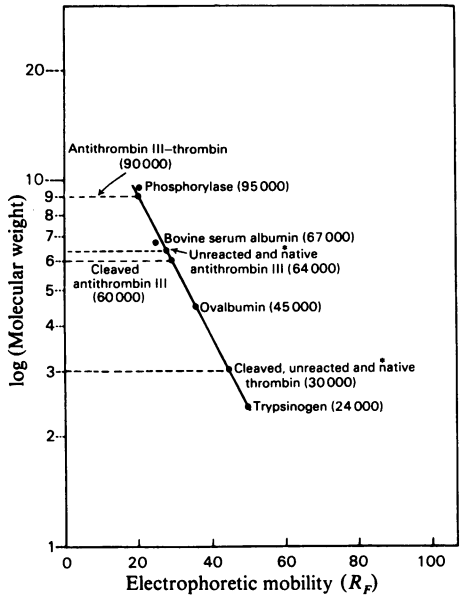


Fig. 2. *Determination of the apparent molecular weights of the dissociation fragments of the antithrombin III–thrombin complex*

The logarithms of the molecular weights of the proteins used as standards were plotted against the electrophoretic mobility of the reduced proteins on discontinuous SDS/polyacrylamide gels prepared as indicated in the legend to Fig. 1. The molecular weights of the unknown polypeptides were obtained by extrapolation. *Differentiates thrombin and antithrombin III from the proteins obtained after cleavage of the antithrombin III–thrombin complex.

Discontinuous SDS/polyacrylamide-gel electrophoresis of the dissociation products of the antithrombin III–thrombin complex without reduction failed to resolve the heavy and light antithrombin III bands shown in gel (F) of Fig. 1. In contrast, they migrated as a sharp single band of electrophoretic mobility different from that of native antithrombin III (gel B, Fig. 4).

Hydrolysis of [^{14}C]*methoxamate(s)*

In the conditions of the cleavage experiment, methoxyamine hydrochloride was not reactive towards peptide bonds (T. H. Finlay & M. O. Longas, unpublished work). Since reactions

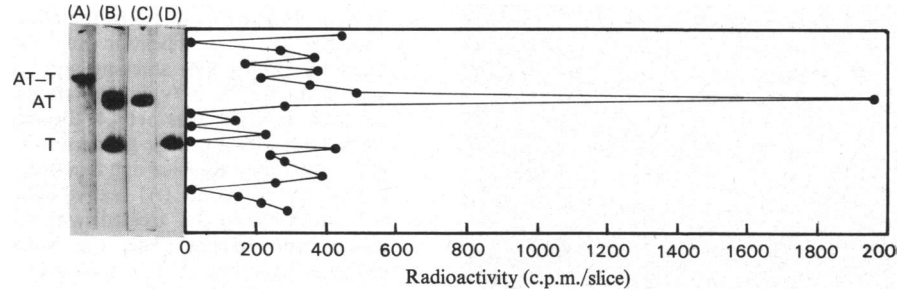


Fig. 3. *Determination of ^{14}C in SDS/polyacrylamide gels of the dissociation products of the antithrombin III–thrombin complex*

Cylindrical gels were prepared by the method of Weber & Osborn (1969), with 100 μg of protein/band. Duplicates of the gels shown were divided into 2 mm slices. Each slice was incubated with 30% (v/v) H_2O_2 (0.5 ml) at 37°C overnight and counted in 10.0 ml of Aquasol. (A) Antithrombin III–thrombin complex prepared in the presence of 1 mM-methoxyamine hydrochloride, using a 1:1 molar ratio of proteins; (B) the dissociation products of the antithrombin III–thrombin complex after treatment with [^{14}C]methoxyamine hydrochloride as described in the text; (C) native antithrombin III; (D) native thrombin. AT-T, antithrombin III–thrombin complex; AT, antithrombin III; T, thrombin.

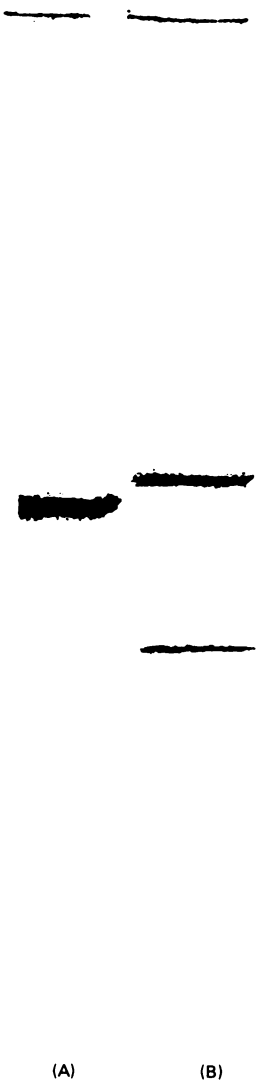


Fig. 4. *Discontinuous SDS/polyacrylamide-gel electrophoresis without reduction*
(A) Native antithrombin III; (B) dissociation products of the antithrombin III–thrombin complex, which also appear in Fig. 1 (gel F). All other conditions are described in the legend to Fig. 1.

involving this reagent produce oximes (March, 1968), and the specific incorporation of [^{14}C]methoxyamine hydrochloride during cleavage of the antithrombin III–thrombin complex took place in antithrombin III (Fig. 3), cleaved antithrombin III might be an oxime formed by nucleophilic attack of [^{14}C]methoxyamine hydrochloride at the carbonyl carbon atom of its arginine residue(s) that associates with the side chain of the active-site serine residue of thrombin (Rosenberg & Damus, 1973). Therefore, a modification of the method of Reeves (1962) was used to hydrolyse the methoxamate(s). The dissociation fragments of the complex (0.6mg in 1.2ml of 0.05M-Tris/0.15M-NaCl buffer, pH7.5) were dialysed at standard temperature against 500ml of 0.2M-sodium phosphate buffer, pH7.5, containing 6.9mM-SDS. The diffusate was changed every day until it contained no detectable ^{14}C. SDS was included in the buffer to avoid possible precipitation of thrombin, whose isoelectric points range from 6.0 to 7.6 (Fenton *et al.*, 1977). Complete removal of ^{14}C was achieved in 5 days.

C-terminal arginine

The cleavage products of the antithrombin III–thrombin complex after hydrolysis of the methoxamate(s) (0.6mg in 1.3ml of 0.2M-sodium phosphate, pH7.5, containing 6.9mM-SDS) and carboxypeptidase B (0.196ml, 79units/ml) were mixed and allowed to interact at 37°C for 1h (Guidotti, 1960). The reaction was stopped by the addition of an equal volume of 20% (w/v) chilled trichloroacetic acid followed by incubation at 0°C for 1h. The precipitated protein was removed by centrifugation at 2000*g* for 5min, and the pH of the supernatant was adjusted to 7.0 with 10M-NaOH. Arginine was identified from its chromogenesis in the Sakaguchi reaction, after mixing 0.5ml of this supernatant with 0.4ml of Sakaguchi reagent C and 0.1ml of Sakaguchi reagent B prepared as described in the Materials and methods section. It was quantified by absorbance at 505nm, and the nmol obtained were read on a standard curve of known concentrations of arginine treated under the same conditions.

As shown in Table 2, *C*-terminal arginine in the dissociation fragments of the antithrombin III–thrombin complex exceeded *C*-terminal arginine in the complex or in the starting thrombin by 56%. The amount of thrombin incorporated in the complex was 56 ± 2%, and cleavage efficiency was >96%. Therefore, approx. 1μmol of new *C*-terminal arginine was formed/μmol of thrombin dissociated.

Discussion

The human antithrombin III–thrombin complex may be cleaved at pH > 8.5 in a reaction that is time- and temperature-dependent and requires no added nucleophiles (T. H. Finlay & M. O. Longas, unpublished work). Dissociation of the bovine antithrombin III–thrombin complex in the course of electrophoresis at pH9.0 was reported by Jesty (1979). Hence, in the present study, the cathode

Table 2. *C-terminal arginine of the dissociation fragments of the antithrombin III–thrombin complex*
The cleavage fragments of the complex were treated with carboxypeptidase B as indicated in the text.

Sample	Area (cm^2)*	Proteins incorporated in the complex (%)	Arginine (nmol)†	Complex cleavage (%)‡
Antithrombin III–thrombin complex	0.72		25.5	
Cleavage products of the antithrombin III–thrombin complex			39.3	>96
Thrombin	1.25	56.0 ± 2	25.0	
Antithrombin III	2.40	30.0 ± 2		

* Areas of the protein-stained bands after scanning of gel D shown in Fig. 1. The results obtained with gels in which the antithrombin III–thrombin complex was maintained at neutral pH were approximately the same.

† Determined on a standard curve of known concentrations of arginine versus A_{505} after Sakaguchi reaction. See the Materials and methods section.

‡ Calculated from the yield of *C*-terminal arginine.

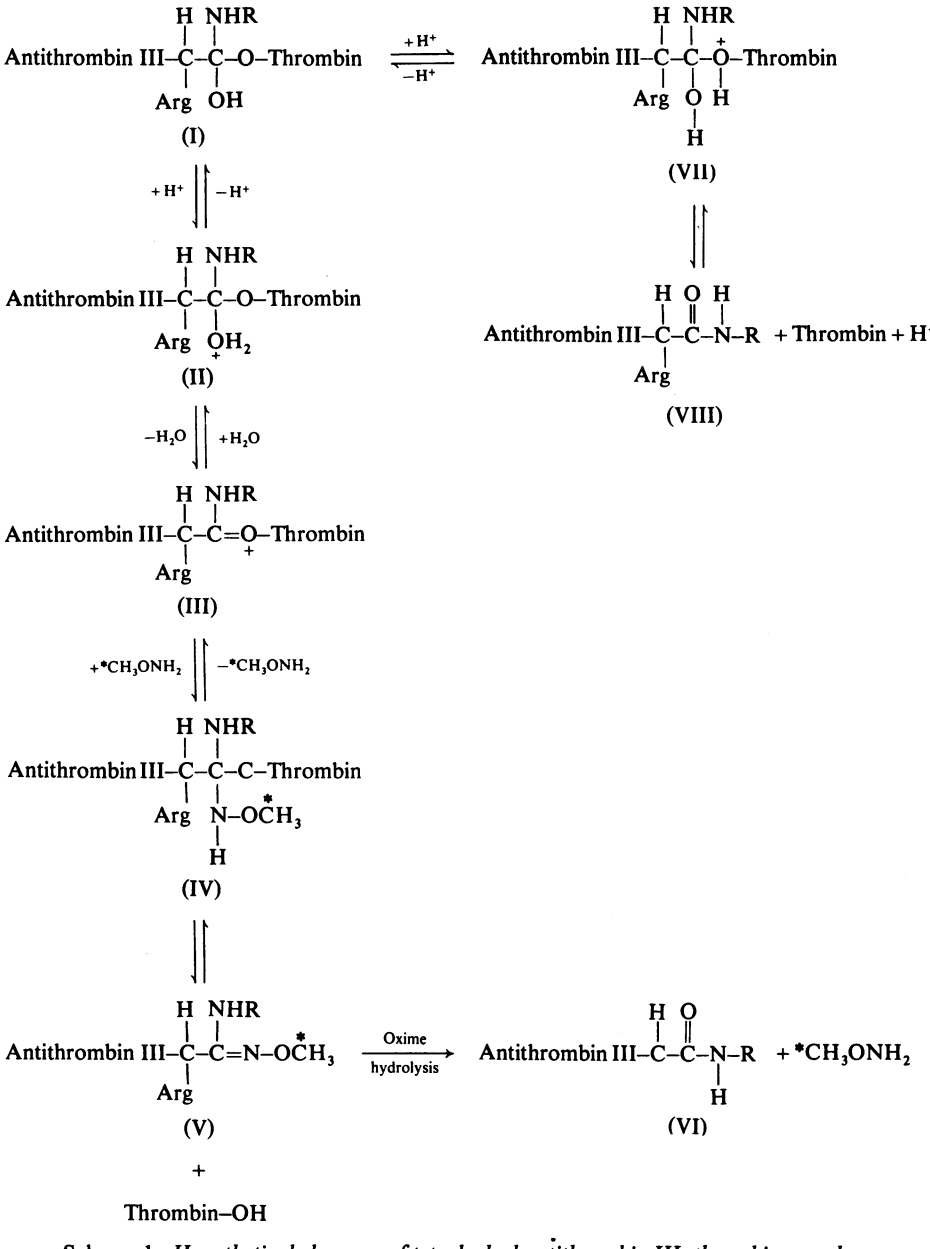
buffer used for electrophoresis of the complex and its cleavage products was pH 8.4. Fig. 1 (gel D) and Table 2 indicate that no significant dissociation of the complex took place at pH 8.4 during the cleavage experiment or electrophoresis. Consequently, the products shown in gel (F) of Fig. 1 are due to reaction of the complex with [^{14}C]methoxyamine hydrochloride.

In the reaction of thrombin with antithrombin III, the complex formed is cleaved by free thrombin present in solution (Rosenberg & Damus, 1973; Jesty, 1979; Fish *et al.*, 1979). Therefore, we prepared the complex in the presence of 1 mM-methoxyamine hydrochloride, a good competitive inhibitor of this enzyme (Longas & Finlay, 1980). Whether a 1 : 1 molar ratio of proteins (Fig. 3, gel A) or a 2-fold molar excess of antithrombin III was used (Fig. 1, gel D), the resulting complex was apparently clean.

Heavy and light antithrombin III obtained on cleavage of the complex did not resolve during electrophoresis without reduction (Fig. 4, gel B). This indicates that the molecular weights of the unreduced proteins were the same. Furthermore, under reduced conditions, the heavy band had the electrophoretic mobility of native antithrombin III [Figs. 1 (gel F) and 2]. The complex displayed only a trace of modified antithrombin III, and the amount of this inhibitor that was not incorporated in the complex had the apparent molecular weight of native antithrombin III [Figs. 1 (gel D) and 2]. These findings are significant; they demonstrate that not all the antithrombin III was altered during formation or cleavage of the complex, and suggest that light antithrombin III might be inhibitor-cleaved in the course of complex formation, whose molecular weight was affected only after reaction with reducing agents. Modified antithrombin III was previously identified after cleavage of the bovine antithrombin III–thrombin complex (Fish & Björk, 1979; Jesty, 1979).

No free arginine was released by the action of carboxypeptidase B on ^{14}C-labelled fragments of the antithrombin III–thrombin complex. The specific incorporation of 0.869 μmol of [^{14}C]methoxyamine hydrochloride/μmol of complex cleaved took place in antithrombin III (Table 1 and Fig. 3). Moreover, preliminary *C*-terminal amino-acid analysis of the dissociation products of the complex after its treatment with carboxypeptidases A and B displayed arginine as the only new *C*-terminal residue. (These results are not included here because complex cleavage was performed with unlabelled methoxyamine hydrochloride). Therefore, the ^{14}C-labelled antithrombin III moiety might be the precursor of the new *C*-terminal arginine residue in the cleavage products (Table 2). Thus, the formation of 1 μmol of new *C*-terminal arginine/μmol of thrombin removed from the complex indicates that the antithrombin III–thrombin bond involves 1 mol of a specific antithrombin III arginine/mol of thrombin.

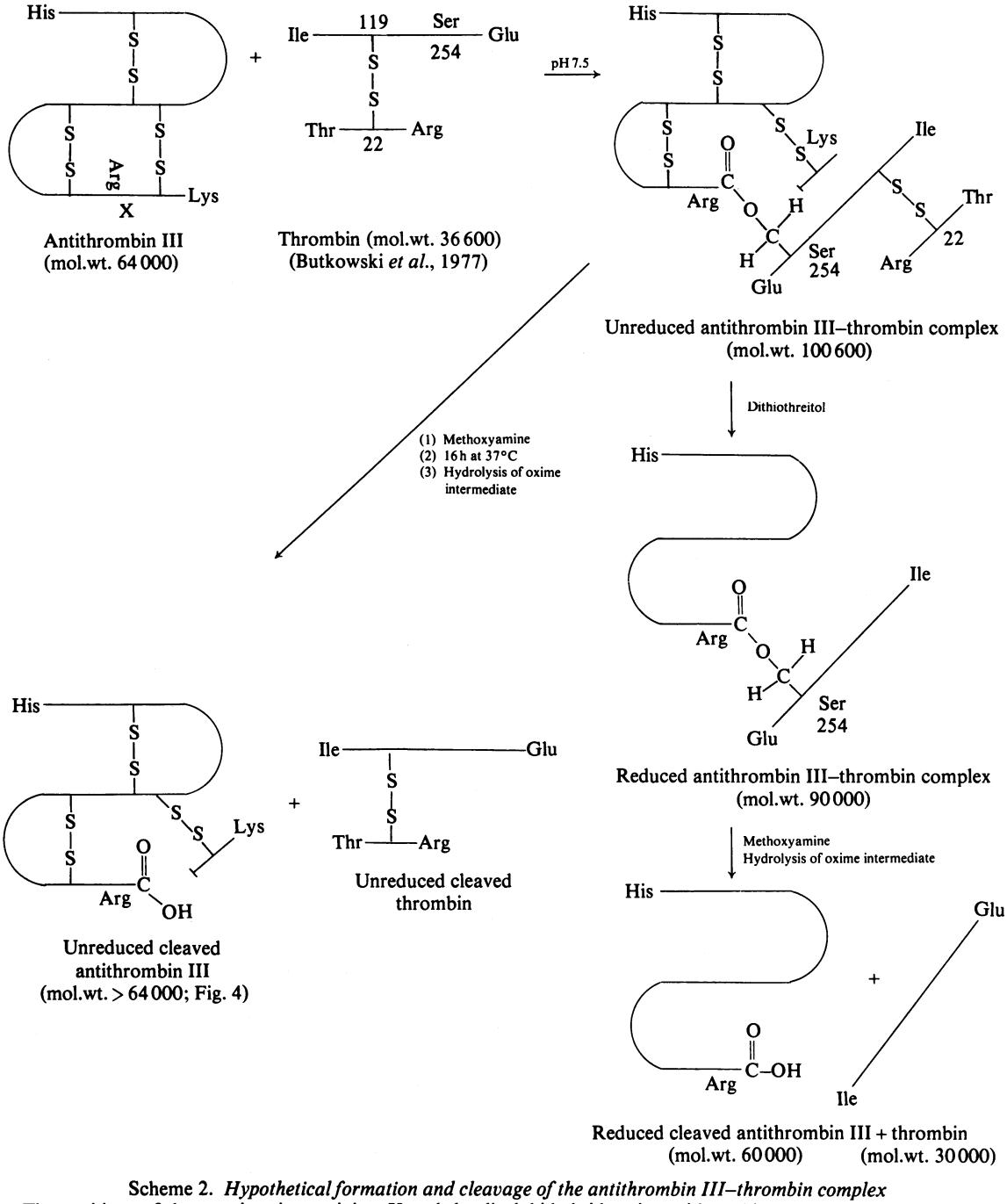
The possibility of a tetrahedral antithrombin III–thrombin complex and its dissociation with [^{14}C]methoxyamine hydrochloride was also considered. Hypothetical cleavage of a tetrahedral complex is shown in Scheme 1. To initiate splitting of compound I, tetrahedral antithrombin III–thrombin complex, an acidic medium is required. This compound should be stable to base unless distant substituents or a reaction medium have an electron-withdrawing effect on the tetrahedral centre that weakens its stability towards base (Morrison & Boyd, 1966). Compound III, an electrophile susceptible to attack by methoxyamine hydrochloride, does not form unless acidic conditions precede (March, 1968). Compounds VI and VIII, antithrombin III dissociated from a tetrahedral complex, should have the molecular weight of the native protein, since it was not cleaved during formation of the tetrahedral adduct. As a consequence, no new *C*-terminal amino acids should form on dissociation



Scheme 1. *Hypothetical cleavage of tetrahedral antithrombin III–thrombin complex*
* = ^{14}C; R = *C*-terminal region of antithrombin III.

of tetrahedral antithrombin III–thrombin complex, if it is free of fragments produced by the action of thrombin during its formation.

In the present work, the conditions used in the cleavage experiment did not favour attack of peptide bonds by methoxyamine hydrochloride (T. H. Finlay & M. O. Longas, unpublished work). The antithrombin III–thrombin complex was not exposed to pH < 7.5; it contained only traces of a proteolytic fragment (Fig. 1, gel D); cleavage was performed in a basic medium (pH 8.4); cleaved antithrombin III appeared to have a lower molecular weight than the native protein (Fig. 2); and arginine was the new *C*-terminal residue in the dissociation fragments of the complex (Table 2). These observations imply that it would be very



Scheme 2. *Hypothetical formation and cleavage of the antithrombin III–thrombin complex*
The positions of the reactive site, arginine–X and the disulphide bridges in antithrombin III are tentative, since the exact locations are not known. The molecular weight of the unreduced complex is the sum of the molecular weights of enzyme and inhibitor. The molecular weights of the reduced proteins are taken from Fig. 2.

difficult to cleave the antithrombin III–thrombin complex under the conditions described in the present paper, if it had a tetrahedral structure. In contrast, Scheme 2 shows the antithrombin III–thrombin complex in which the enzyme is liberated by the action of methoxyamine hydrochloride, in the conditions of the present work. The cleavage products obtained appeared to be derived from this

type of complex. Besides, this structure has been proposed for another serine proteinase–proteinase inhibitor complex (Laskowski & Sealock, 1971).

The results of the present study confirm the findings of Rosenberg & Damus (1973) with regard to involvement of arginine residue(s) of antithrombin III in its interaction with thrombin. They also indicate that during formation of the human antithrombin III–thrombin complex the inhibitor is cleaved at an arginine–X bond; this arginine residue forms a carboxylic ester with the enzyme as postulated by Owen (1975), whereas the excised polypeptide, which has an apparent mol.wt. of approx. 4000, remains bound through disulphide linkage(s). Although the results presented here favour association of thrombin with antithrombin III through an ester, the structure of the antithrombin III–thrombin complex requires elucidation by other methods.

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References

Acher, R. & Crocker, C. (1952) *Biochim. Biophys. Acta* **9**, 704–707

Binder, B. (1973) *Thromb. Diath. Haemorrh.* **30**, 280–283

Butkowski, F. J., Elion, J. Downing, M. R. & Mann, K. G. (1977) *J. Biol. Chem.* **252**, 4941–4957

Collen, D., DeCock, F. & Verstraete, M. (1977) *Eur. J. Clin. Invest.* **7**, 407–411

Fenton, J. W., Fasco, M. J. & Stackrow, A. B. (1977) *J. Biol. Chem.* **252**, 3587–3598

Fish, W. W. & Björk, I. (1979) *Eur. J. Biochem.* **101**, 31–38

Fish, W. W., Orre, K. & Björk, I. (1979) *Eur. J. Biochem.* **101**, 39–44

Guidotti, G. (1960) *Biochim. Biophys. Acta* **42**, 177–179

Harpel, P. C. & Rosenberg, R. D. (1976) in *Progress in Hemostasis and Thrombosis* (Spaet, T. H., ed.), vol. 3, pp. 145–190, Grune and Stratton, New York

Jesty, J. (1979) *J. Biol. Chem.* **254**, 1044–1049

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Laskowski, M., Jr. & Sealock, R. W. (1971) *Enzymes 3rd Ed.* **3**, 375–473

Longas, M. O. & Finlay, T. H. (1980) *Int. J. Biochem.* in the press

Longas, M. O., Ferguson, W. & Finlay, T. H. (1980) *Arch. Biochem. Biophys.* **200**, 595–602

Lowry, O. D., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

March, J. (1968) in *Advanced Organic Chemistry: Reactions, Mechanisms and Structure*, pp. 675–839, McGraw-Hill Book Company, New York

Miller-Andersson, M., Borg, H. & Andersson, L. O. (1974) *Thromb. Res.* **5**, 439–452

Moore, S. (1968) *J. Biol. Chem.* **243**, 6281–6283

Morrison, R. T. & Boyd, R. N. (1966) in *Organic Chemistry*, pp. 642, 8785, Allyn and Bacon, Boston

Ødegård, O. R., Lie, M. & Abildgaard, U. (1975) *Thromb. Res.* **6**, 287–294

Owen, W. G. (1975) *Biochim. Biophys. Acta* **405**, 380–387

Pepper, D. S., Banhegyi, D. & Cash, J. D. (1977) *Thromb. Haemostas.* (Stuttg.) **38**, 494–503

Reeves, R. L. (1962) *J. Am. Chem. Soc.* **84**, 3332–3337

Rosenberg, R. D. & Damus, P. S. (1973) *J. Biol. Chem.* **248**, 6490–6505

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412