The Reaction of Bovine α-Thrombin with Tetranitromethane

CHARACTERIZATION OF THE MODIFIED PROTEIN*

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Previous studies from several laboratories have shown that thrombin is inactivated by tetranitromethane with the formation of nitrotyrosine. The inactivation is characterized by an apparently greater loss of fibrinogen-clotting activity than activity toward synthetic ester substrates, suggesting that the residues modified by tetranitromethane are involved in the interaction of thrombin with fibrinogen. This study was designed 1) to determine the effect of solvent conditions on the rate of modification and the stoichiometry of the reaction of tetranitromethane with bovine α-thrombin; 2) to identify the residue(s) modified; and 3) to characterize the modified enzyme with respect to its interaction with peptide nitroanilide substrates and fibrinogen.

The inactivation of thrombin by tetranitromethane proceeded more rapidly in 50 mM Tris, pH 8.0, than in 50 mM sodium phosphate, 100 mM NaCl, pH 8.0. Approximately 10% fibrinogen-clotting activity remained at maximal inactivation. A study of the effect of tetranitromethane concentration on the rate of inactivation suggested that the loss of activity was the result of the modification of 1 mol of tyrosine/mol of thrombin. A similar result was obtained from the analysis of the extent of inactivation as a function of the extent of protein modification. Structural analysis of the modified protein showed substantial modification at both Tyr[71] and Tyr[85]. Enzyme kinetic studies were performed with the modified protein and a control thrombin with N^2-tosylglycylprolylarginine p-nitroanilide, H-D-phenylalanylpipecolylarginine p-nitronailide, and purified bovine fibrinogen. With all three substrates, a substantial decrease in k_{cat} was observed, whereas there was essentially no change in K_m. These results suggest that, contrary to previous suggestions, the modification of Tyr[71] and Tyr[85] in thrombin does not influence the binding of substrates, but rather influences active site reactivity.

Thrombin is a trypsin-like regulatory serine protease with diverse biological activities (1) primarily recognized as the enzyme responsible for conversion of fibrinogen to fibrin in

the process of blood coagulation. The elucidation of the structural factors in thrombin responsible for the specific interaction with fibrinogen has been the subject of study in a number of laboratories. Some approaches have used limited proteolysis of α-thrombin to obtain derivative forms such as β-thrombin (2, 3) and γ-thrombin (4–6). Although there has been a significant amount of work on these proteins, information regarding specific regions of thrombin critical for interaction with fibrinogen is still lacking. Other studies (7, 8) have attempted to use specific chemical modification to explore structural features of thrombin outside of the active site region.

The reaction of thrombin with tetranitromethane (TNM)[1] is an example of such a modification. The partial inactivation of thrombin by TNM was first reported by Astrup in 1947 (9). Subsequent studies by Sokolovsky and Riordan (10) showed that fibrinogen-clotting activity was lost more rapidly than esterase activity. Our laboratory (11) extended these earlier studies and demonstrated that the reaction of thrombin with TNM resulted in the formation of nitrotyrosine residues. None of these studies established stoichiometry for the reaction, nor were there efforts to identify the site(s) of modification. However, the above studies on the reaction of TNM with thrombin are consistent with the hypothesis that tyrosyl residues are involved in the processing of fibrinogen as a substrate but may not be involved with thrombin catalysis of smaller, synthetic substrates. These studies were designed both to test this hypothesis as well as to characterize the modified protein as obtained under well-defined reaction conditions.

MATERIALS AND METHODS

Bovine α-thrombin was prepared as described by Workman and Lundblad (12). Bovine fibrinogen (60% clottable) was obtained from Miles. For the assay of thrombin using fibrinopeptide release, the fibrinogen was purified by ammonium sulfate fractionation as described by Hageman and Scheraga (13). p-Chlorobenzylamido-aminocaproyl-agarose was prepared as described by Thompson (14). 4-Chlorobenzylamine and tetranitromethane were obtained from Aldrich. Tosylglycylprolylarginine p-nitroanilide (Tos-Gly-Pro-Arg-pNA) was obtained from Sigma, whereas H-D-phenylalanylpipecolylarginine p-nitroanilide (H-D-Phe-Pip-Arg-pNA) was a product of AB Kabi and was obtained from Helena Laboratories. p-Nitrophenyl-p'-guanidinobenzoate (NPGB) was obtained from Research Organics. The C_{18} reverse-phase column (C_{18} type TP silica, 4.6 mm, inner diameter, × 25 cm) was obtained from The Separations Group. All

[1] The abbreviations used are: TNM, tetranitromethane; NPGB, p-nitrophenyl-p'-guanidinobenzoate; HPLC, high-performance liquid chromatography; Tos-Gly-Pro-Arg-pNA; N^α-tosylglycylprolylarginine p-nitroanilide; H-D-Phe-Pip-Arg-pNA, H-D-phenylalanylpipecolylarginine p-nitroanilide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

other chemicals were of reagent-grade and used without further purification.

α-Thrombin was dialyzed into either 50 mM Tris, pH 8.0, or 50 mM sodium phosphate, 100 mM NaCl, pH 8.0, for 4 h at ambient temperature prior to modification with TNM. There was no change in the specific activity of the α-thrombin preparation as a result of the dialysis procedure. The preparations of α-thrombin used in these experiments were typical of previous preparations of α-thrombin (12) with a specific activity of 2000–2500 NIH units/mg of protein without correction for active site concentration. The reaction of TNM was performed essentially as described by Riordan and Vallee (15) except that the reactions were terminated by the addition of a 1.3 M excess of cysteine to TNM, and, unless otherwise indicated, the modified proteins were dialyzed into 50 mM Tris, pH 8.0, prior to further study. Spectra (230–600 nm) were obtained for all modified proteins both to establish nitrotyrosine formation and to evaluate possible modifications of tryptophan residues. The extent of nitrotyrosine formation was determined by spectroscopy of the modified protein in 0.1 M NaOH using the extinction coefficient at 428 nm reported in the literature (15). Protein concentration of native thrombin was determined by spectroscopy at 280 nm using a milligram/milliliter extinction coefficient of 1.7 (16). The protein concentration of modified proteins was determined by the biuret reaction (17, 18). Samples for structural analysis were reduced and carboxymethylated basically as described by Canfield and Anfinsen (19). Tryptic hydrolysis was performed on 250–500-µg samples in 100 mM sodium bicarbonate, pH 8.0, with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin which had been previously allowed to stand in 1 mM HCl (20). The tryptic hydrolysates were taken directly to a C₁₈ reverse-phase column. The columns were developed as described in the text,

and effluent was monitored at 210, 280, and 360 nm. Selected effluent peaks were subjected to amino-terminal sequence analysis using a Beckman 890 automated sequenator (21).

Fibrinogen-clotting activity was determined as previously described (22). All thrombin preparations were evaluated for active site integrity using NPGB (23), and kinetic data were corrected for percent active sites. All preparations had at least 80% active sites. Affinity chromatography of native and modified thrombin preparations on p-chlorobenzylamido-aminohexyl-agarose was performed at ambient temperature. The sample, containing 500–1000 µg of protein in 50 mM Tris-HCl, pH 8.0, was applied to a 0.9 × 15-cm column previously equilibrated with 30 mM sodium phosphate, 0.75 M NaCl, pH 8.0. After emergence of unabsorbed material, thrombin was eluted with 25% 1,4-dioxane in 30 mM sodium phosphate, 0.75 M NaCl, pH 8.0, essentially as described by Thompson (14). Native and modified thrombin preparations were subjected to high-performance size-exclusion chromatography as described by Tarvers (24) using a TSK-3000SW column in 50 mM HEPES, 150 mM NaCl, pH 6.8. An ISCO high-performance liquid chromatography system was used in these studies. Amidase activity with the peptide nitroanilide substrates was determined in 50 mM Tris, pH 8.0, and the release of nitroaniline was followed at 405 nm (25) using a Kontron 810/820 UV-VIS spectrophotometer. The absorbance data were converted directly to rate data using linear regression analysis using the data analysis module of the spectrophotometer. Kinetic constants were determined using nonlinear regression analysis (26, 27). The hydrolysis of fibrinogen by thrombin resulting in the release of fibrinopeptide A was performed in 0.15 M ammonium acetate, pH 8.0 (28), in the presence of 100 mM 6-aminohexanoic acid (29). Portions of the reaction mixture were removed at selected time points and added to perchloric
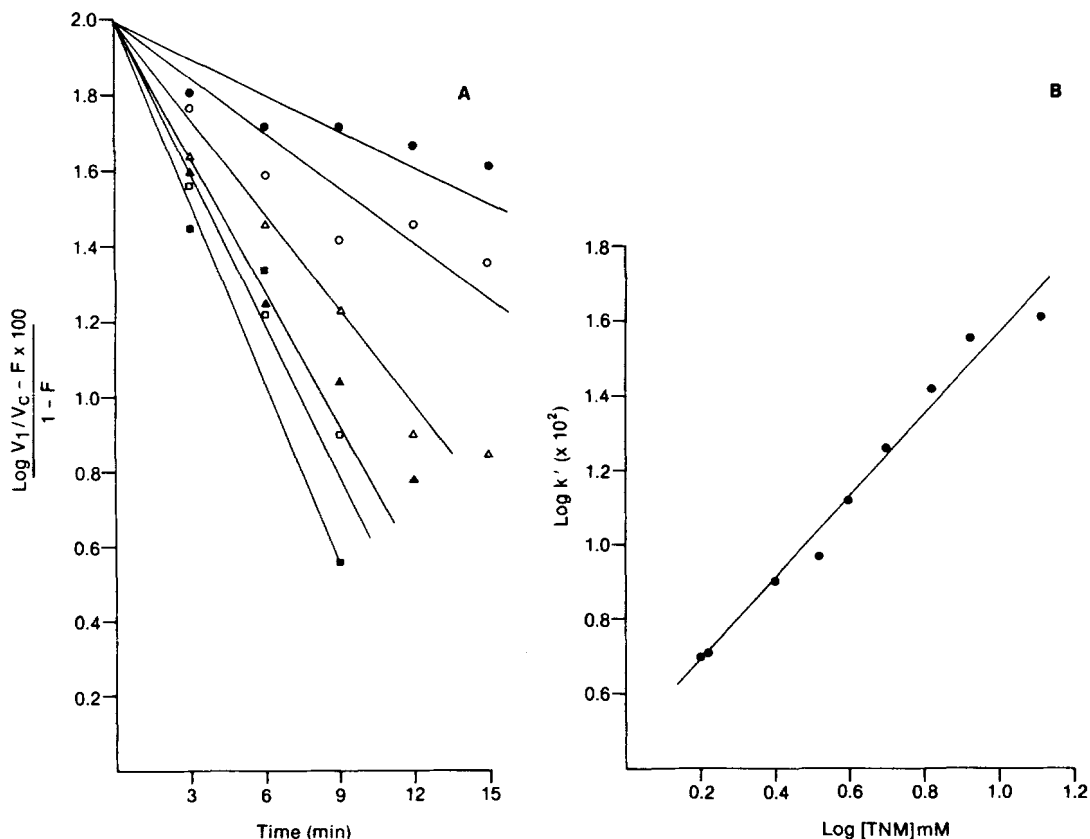

FIG. 1. **Inactivation of thrombin by tetranitromethane in sodium phosphate buffer.** *A*, semilogarithmic plot of the rate of loss of fibrinogen-clotting activity as a function of time at different concentrations of TNM. The concentration of thrombin was 30 µM, and the TNM concentrations were 1.66 (●), 3.31 (○), 4.98 (△), 6.64 (▲), 8.30 (□), and 12.45 (■) mM in 50 mM sodium phosphate, 100 mM NaCl, pH 8.0. Reactions were initiated by the addition of TNM (diluted in absolute EtOH), and portions were removed at the indicated times, diluted in cold 60 mM Tris-HCl, 90 mM NaCl, pH 7.5, and assayed for residual fibrinogen-clotting activity. The amount of residual activity remaining after 30 min of reaction at the highest concentration of TNM was 10% of initial activity and was taken equal to F for the calculations. *B*, plot of the log first-order rate constants (k') as determined from the data in *A* versus log TNM concentration. The slope of the line obtained in *B* determined by linear regression analysis was 1.10 with $r = 0.98$.
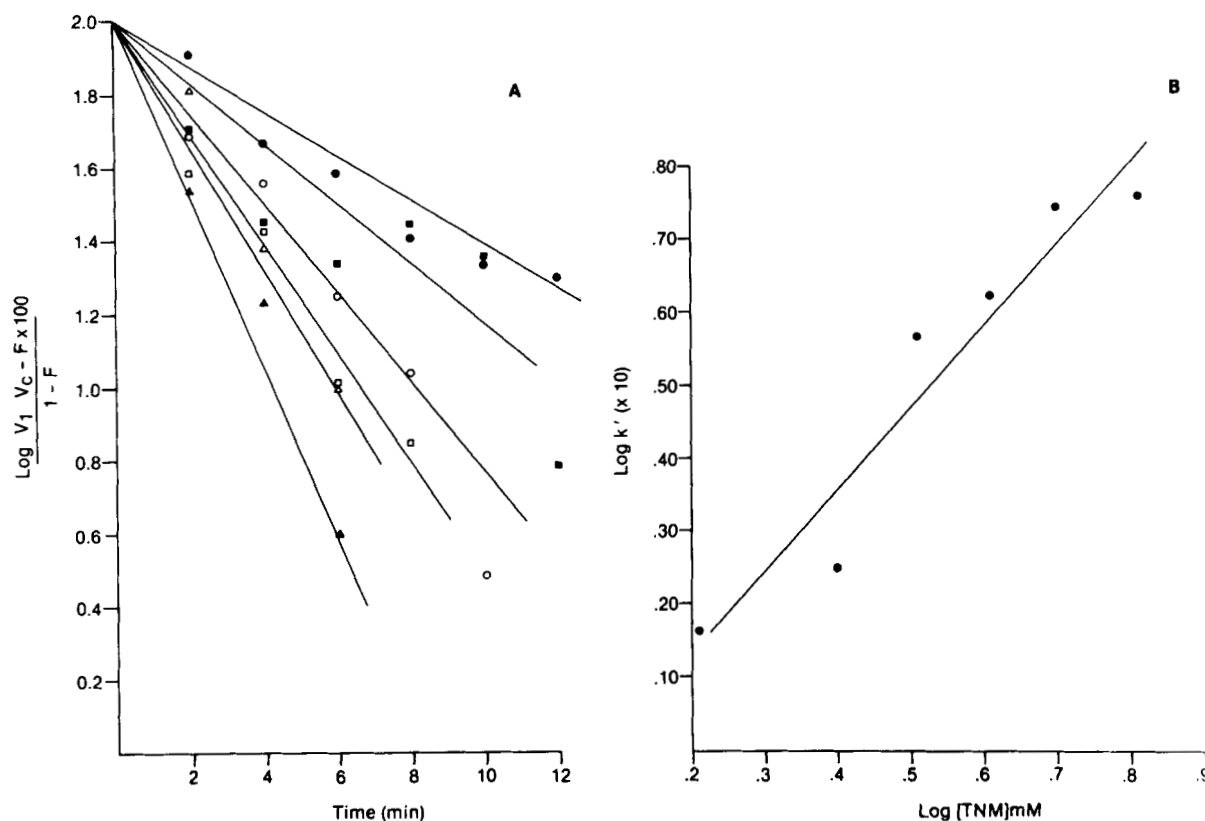
FIG. 2. **Inactivation of thrombin by tetranitromethane in Tris-Clo buffer.** *A*, semilogarithmic plot of the rate of loss of fibrinogen-clotting activity as function of time at different concentrations of TNM. The concentration of thrombin was 30 μM, and the TNM concentrations were 1.66 (●), 3.31 (O), 4.98 (■), 6.64 (□), 8.30 (△), and 12.45 (▲) mM in 50 mM sodium phosphate, 100 mM NaCl, pH 8.0. Reactions were initiated by the addition of TNM (diluted in absolute EtOH), and portions were removed at the indicated times, diluted in cold 60 mM Tris-HCl, 90 mM NaCl, pH 7.5, and assayed for residual fibrinogen-clotting activity. The amount of residual activity remaining after 30 min of reaction at the highest concentration of TNM was 10% of initial activity and was taken equal to *F* for the calculations. *B*, plot of the log first-order rate constants (*k'*) as determined from the data in *A* versus log TNM concentration. The slope of the line obtained in *B* determined by linear regression analysis was 1.13 with *r* = 0.96.

acid to terminate the reaction. After centrifugation, a portion of the supernatant fraction was analyzed by high-performance liquid chromatography using a C_{18} reverse-phase column with an isocratic eluent of 0.05 M sodium phosphate, pH 3.1, acetonitrile (81:19). The kinetic constants for fibrinopeptide A release were determined by nonlinear regression analysis as cited above.

RESULTS

Previous studies (10, 11) on the reaction of thrombin with TNM have shown that there is residual fibrinogen-clotting activity remaining after apparently complete reaction. Similar results were obtained in this study (data not shown) where, under our assay conditions, 90% inactivation of thrombin fibrinogen-clotting activity was obtained upon maximum reaction with TNM. As a result, the residual activity must be considered in calculation of rate constants for the inactivation reaction and for studying the stoichiometry of the inactivation reaction (30–32). The following equation was used in the calculation of the data described below: $A/A_0 = (1 - F)e^{-kt}$ (32).

Initial experiments were performed in 50 mM sodium phosphates, 100 mM NaCl, pH 8.0, as in previous studies from this laboratory (11). First-order rate plots for the inactivation of thrombin in this solvent over a range of TNM concentration are shown in Fig. 1*A*. A double logarithmic plot of TNM concentration *versus* the observed first-order rate constant is shown in Fig. 1*B*. The slope of the line obtained is 1.10,

suggesting that inactivation is the result of reaction at a single site on the protein. Since we had observed that sodium ions influence the activity of purified bovine thrombin (33), the reaction of thrombin with TNM was also performed in 50 mM Tris-HCl, pH 8.0. A first-order rate plot of the inactivation of thrombin by TNM in this solvent is shown in Fig. 2*A*. Comparison of these data with those shown in Fig. 1*A* reveals that the inactivation reaction proceeds approximately 3 times more rapidly in Tris than in the sodium-containing solvent. A double logarithmic plot of TNM concentration *versus* the observed first-order rate constant for the reaction in Tris is shown in Fig. 2*B*. The slope of this line is 1.13, suggesting that, as with the sodium phosphate solvent, the inactivation of thrombin by TNM in Tris is the result of reaction at single site on the protein. The decreased rate of inactivation observed in the phosphate buffer is not solely the result of increased ionic strength since the inclusion of 100 mM NaCl in the Tris buffer had little effect on the rate of reaction of TNM with thrombin.

The above results suggest that the inactivation of thrombin by TNM is the result of reaction at a single site in the protein. Previous studies (11) had shown that the reaction of TNM with thrombin resulted in the modification of only tyrosine. Similar results were observed in this study. In an attempt to establish stoichiometry for the inactivation reaction, the modification of thrombin by TNM was performed under a variety

of solvent conditions, and the extent of modification was determined by spectral analysis. A plot of the extent of inactivation (after correction for residual activity) *versus* nitrotyrosine formation is shown in Fig. 3. The results are consistent with the inactivation of thrombin by TNM resulting from modification of a single tyrosine residue.

The modified protein was then subjected to structural analysis. Fig. 4 represents the HPLC analysis of a tryptic digest of the modified protein after reduction and carboxymethylation. The effluent was monitored at 210, 280, and 360 nm. Only the 360 nm profile showing the presence of nitrotyrosine (15) is shown. The peaks absorbing at 360 nm were collected and subjected to amino-terminal sequence analysis. Two major sites of modification were observed, Tyr[71] and Tyr[85], in the B chain with minor modification at Tyr[215]. A similar analysis of the human protein showed modification at Tyr[71] and Tyr[85] and lesser modification at Tyr[134]. There was also apparent modification at Trp[148] in the human protein. The modification reactions for these proteins were performed in the sodium phosphate buffer. In an attempt to identify a single residue, the modification reaction was performed in both Tris buffer and phosphate buffer, and the two modified proteins were subjected to structural analysis. The results of this experiment are shown in Fig. 5. There was no significant difference in the extent of modification at Tyr[71] and Tyr[85].

Since these results suggested possible heterogeneity of the products derived from the modification of α-thrombin by TNM, we sought to demonstrate that there were multiple products resulting from the reaction of thrombin with TNM. We subjected the product of the modification reaction to chromatographic fractionation on SP-Sephadex C-50 under conditions demonstrated by our laboratory to separate multiple forms of thrombin (2, 3). The modified protein chromatographed in an manner identical to native α-thrombin. Furthermore, the modified protein bound to *p*-chlorobenzylamido-ε-aminocaproylagarose (14) as effectively as the native
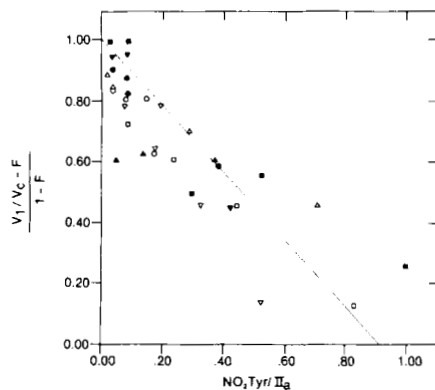

FIG. 4. **Comparison of tryptic maps of TNM-modified thrombin.** Thrombin was allowed to react with a 50-fold (*trace A*) or 200-fold (*trace B*) molar excess of TNM for 30 min in 50 mM sodium phosphate, 100 mM NaCl, pH 8.0. The reactions were terminated by dialysis against 100 mM ammonium bicarbonate. The content of nitrotyrosine was determined by spectroscopy after adjustment of the pH to 12.0. The preparations were hydrolyzed with trypsin, and the hydrolysates were chromatographed on a C_{18} column with an acetonitrile gradient. Effluent absorbance was monitored at 210, 280, and 360 nm. The absorbance at 360 is shown. Discrete peaks absorbing at 360 nm were collected and subjected to primary structure analysis. Sequence information was compared with existing primary structure information to determine placement in the amino acid sequence of thrombin.

FIG. 3. **Relationship between loss of fibrinogen-clotting activity on thrombin and extent of formation of nitrotyrosine.** Thrombin (290 nmol in 10 ml to reaction mixture (50 mM Tris-Cl, pH 8.0)) was incubated with a 25-fold molar excess of TNM (○), a 50-fold molar excess of TNM (●), a 50-fold molar excess of TNM in the presence of 200 mM NaCl (▲), a 50-fold molar excess of TNM in the presence of 10 mM benzamidine (△), a 50-fold molar excess of TNM in the presence of 200 mM NaCl and 10 mM benzamidine (▼), a 100-fold molar excess of TNM (□), a 100-fold molar excess of TNM in the presence of 10 mM benzamidine (■), or a 100-fold molar excess of TNM in the presence of 200 mM NaCl (▽). The reactions were initiated by the addition of TNM (diluted in absolute EtOH), and portions were removed at various times and added to a 1.3 M excess of cysteine (to TNM) to terminate the reaction. After dialysis against 0.1 M NH₄CO₃, the samples were assayed for fibrinogen-clotting activity and content of nitrotyrosine (after adjustment of pH to 12.0). The V_c value is corrected for a residual activity (F) of 10% of a control preparation.
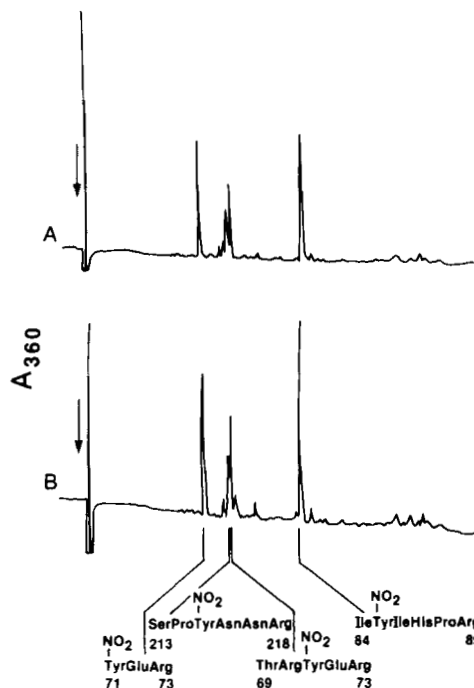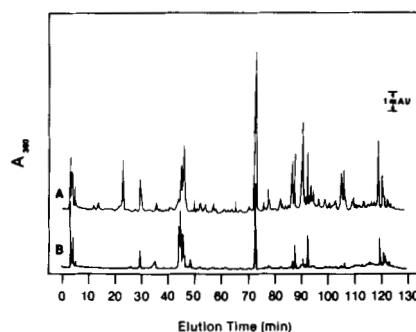

FIG. 5. **Effect of solvent composition on sites of modification of thrombin with tetranitromethane.** Preparations of thrombin were modified with a 100-fold molar excess of TNM either in 50 mM Tris-Cl, pH 8.0 (*trace A*), or in 50 mM sodium phosphate, 100 mM NaCl, pH 8.0 (*trace B*). The analyses were performed as described for Fig. 4. Absorbance was monitored at 210, 280, and 360 nm. Only the absorbance at 360 nm is shown. The profiles shown were normalized to permit direct comparison as a function of protein concentration in hydrolysates (determined from total absorbance at 210 and 280 nm. *MAU*, milli-absorbance unit.

protein and was eluted by the same solvent conditions. As with our previous study (11), the modification of α-thrombin under these reaction conditions did not result in the formation of dimer or higher aggregate forms of thrombin as assessed by high-performance size-exclusion chromatography.

The final portion of this work concerns the study of the

TABLE I

Effect of nitration of thrombin on hydrolysis of two peptide nitroanilide substrates

The assays were performed over a 50-fold range of substrate concentration in 50 mM Tris, 50 mM HEPES, 0.01% polyethylene glycol, pH 7.8. Kinetic constants were obtained by nonlinear regression analysis of initial rate data as described in the text.

Substrate	K_m	k_{cat}
	μM	s^{-1}
Tos-Gly-Pro-Arg-pNA		
Control	12.6	63.4
Nitrated	16.7	44.8
H-D-Phe-Pip-Arg-pNA		
Control	20.9	90.4
Nitrated	21.3	60.0

TABLE II

Effect of nitration of thrombin on kinetic constants for release of fibrinopeptide A from fibrinogen

The release of fibrinopeptide A from reaction mixtures containing varying concentrations of fibrinogen in 0.15 M ammonium acetate, 0.1 M 6-aminohexanoic acid, pH 8.0, was determined by high-performance liquid chromatography as described in the text.

Enzyme source	K_m	k_{cat}
	μM	s^{-1}
Control	9.6	299.0
Nitrated	9.6	86.0

enzymatic properties of the modified protein. The activity of nitrated thrombin in the hydrolysis of Tos-Gly-Pro-Arg-pNA and H-D-Phe-Pip-Arg-pNA was compared with that of a control thrombin preparation. Table I shows the results of this study. With either substrate, there is only a minor change in K_m, whereas there is a decrease in k_{cat}. The calculations were made after correction for active site concentration in the two enzyme preparations. The data are inconsistent with the hypothesis that modification of thrombin with TNM influences substrate binding. However, it could be argued that these substrates do not accurately reflect the activity of thrombin toward fibrinogen (34). Therefore, kinetic constants for the release of fibrinopeptide A from bovine fibrinogen were obtained. The rate of fibrinopeptide release was obtained by HPLC analysis. The results of this experiment are shown in Table II. K_m values for nitrated thrombin and the control preparations were essentially identical, whereas there was a marked decrease in k_{cat} for the nitrated enzyme. The kinetic constants obtained for the control thrombin preparation are consistent with those reported by other investigators (29).

DISCUSSION

The initial bias of this study was that the apparently selective inactivation of the fibrinogen-clotting activity of thrombin by TNM resulted from the modification of a specific fibrinogen-binding site on the surface of thrombin. Results obtained show that modification of active site reactivity, not fibrinogen binding, is responsible for the change in catalytic activity. Therefore, the initial hypothesis is essentially incorrect.

The data obtained are equivocal in regard to the change in catalytic activity being due to modification of a single, specific tyrosine residue modification by TNM. Results obtained from experiments examining the effect of TNM concentration on inactivation rate and from experiments evaluating the correlation of nitrotyrosine formation with the loss of fibrinogen-clotting activity suggest that the loss of biological activity is the result of the modification of a single tyrosine residue. It is acknowledged that the data on the effect of TNM concentration on the rate of inactivation are also consistent with two sites having essentially equal reactivity. However, our laboratory (35) has previously shown that reaction of N-butyrylimidazole with thrombin results in the modification on only 1 mol of tyrosine/mol of protein at maximum inactivation.

Alternatively, results of structural analysis of the modified protein indicate that modification at both Tyr[71] and Tyr[85] with TNM occurred concomitant with the change in catalytic activity. It is possible that modification at either residue might result in loss of activity or that, although both are modified, only one of the sites is responsible for the changes in catalytic activity assessed.

The effect of solvent conditions on the rate of inactivation of thrombin by TNM is of interest considering other results in the literature. Our laboratory (33, 34) has shown that NaCl has a significant effect on the catalytic properties of α-thrombin. However, the decreased rate in phosphate buffer is not solely a result of the presence of sodium ions. Although the effect of solvent on the rate of reaction was not pursued in greater detail, there appears to be a specific effect of phosphate. Berliner *et al.* (36) have studied the effect of inorganic phosphate and organic phosphates on the interaction of thrombin with nonpolymerized fibrin. Inorganic phosphate decreased the affinity of thrombin for fibrin at concentrations similar to those used in this study.

A major problem with this study resides in providing an adequate explanation for the observation that the modification of thrombin with TNM results in an enzyme with a decreased k_{cat} and unchanged K_m. With all three substrates used in this study, Tos-Gly-Pro-Arg-pNA, H-D-Phe-Pip-Arg-pNA, and fibrinogen, a marked decrease was observed in k_{cat} with little, if any, change in K_m. As assessed by active site titration, the reaction of TNM with thrombin under our experimental conditions does not affect reaction with NPGB, an ester substrate which is used as an active site titrant for thrombin (23). Thus, the observed decrease in k_{cat} is not the result of total loss of active site integrity. The lack of effect of reaction with TNM on active site reactivity of thrombin differs from the report of Glenn *et al.* (37) who reported a greater than 60% decrease in reactivity with NPGB after the modification of approximately 4 tyrosine residues in human thrombin with TNM.

An accurate three-dimensional structure for thrombin has not yet been developed. Furie *et al.* (38) have developed a structure for thrombin based on homology with the pancreatic serine proteases. The structure developed by these investigators is divided into two general regions: constant regions where there is extensive homology to the pancreatic serine proteases and variable regions where there is much less structural homology. According to this structure, both Tyr[71] and Tyr[85] are located in a variable region which is suggested to have an important role in fibrinogen binding. These residues are also located in the region of the protein where proteolysis occurs during the conversion of α-thrombin to β-thrombin (2). We have previously shown that the loss of the fibrinogen-clotting activity of β-thrombin is the result of a K_{cat} change and not the loss of a fibrinogen-binding site (39). Cook *et al.* (40), using a series of peptide thioesters as substrates, demonstrated that nitrated human thrombin has a decreased k_{cat} and an increased K_m with all substrates tested. Similar behavior was shown by γ-thrombin. These investigators suggested that the modification of tyrosine residues with TNM results in a conformational change similar to that seen in the limited proteolysis of human α-thrombin to produce γ-thrombin.

On the basis of the above discussion, we suggest that the

initial reaction of TNM with thrombin results in the disruption of the relationship between Asp^{99} and His^{43}, reducing efficiency of concerted proton transfer during the catalysis of the hydrolysis of specific substrates by thrombin. The importance of concerted proton transfer has been suggested to be critical in the efficiency of catalysis by regulatory proteases with their specific substrates (41, 42).

More extensive nitration may result in additional changes in the properties of thrombin. Nitrated thrombin prepared by our earlier procedure, having approximately 4 tyrosine residues modified, did not demonstrate high affinity binding to platelets (43). Alexander et al. (44), using nitrated human thrombin with a similar extent of modification, have also reported decreased reactivity with platelets. Other studies (37, 45) on the interaction of highly nitrated human thrombin with fibroblasts have shown decreased binding compared to control preparations. These studies suggest that there may be tyrosine residues in thrombin which are critical for binding with macromolecular substrates, but which are less reactive with TNM.

In conclusion, the reaction of TNM with bovine α-thrombin results in the formation of a modified protein with decreased catalytic activity. The loss of activity appears to result from the modification of active site reactivity rather than from modification of a residue actually involved directly in either catalysis or substrate binding.

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