Structural and Functional Properties of Human α-Thrombin, Phosphopyridoxylated α-Thrombin, and γT-Thrombin

IDENTIFICATION OF LYSYL RESIDUES IN α-THROMBIN THAT ARE CRITICAL FOR HEPARIN AND FIBRIN(0GEN) INTERACTIONS*

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Frank C. Church†, Charlotte W. Pratt, Claudia M. Noyes, Tul Kalayanamit, G. Bradley Sherrill, Rebecca B. Tobin, and James B. Meade

From the Department of Pathology and Medicine and the Center for Thrombosis and Hemostasis, the University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7035

α-Thrombin derivatives obtained either by site-specific modification at lysyl residues (phosphopyridoxylated) or by limited trypsinolysis (γT-thrombin) were compared to correlate structural modifications with the functional reactivity toward fibrin(ogen) and heparin. α-Thrombin phosphopyridoxylated in the absence of heparin (unprotected) showed approximately 2 mol of label incorporated/mol of thrombin, but only 1 mol of label incorporated/mol of proteinase when modified in the presence of added heparin (protected). In contrast to native α-thrombin, both phosphopyridoxylated α-thrombin derivatives failed to interact with a fibrin monomer-agarose column and had reduced fibrinogen clotting activity, which is very similar to γγ-thrombin. Heparin accelerated the rate of antithrombin III inhibition of α-thrombin. heparin-protected modified-α-thrombin, and γγ-thrombin in a manner consistent with a template mechanism but was without effect on unprotected modified α-thrombin. In a heparin-catalyzed antithrombin III inhibition assay of α-thrombin, we found that D-Phe-Pro-Arg chloromethyl ketone-active site-inactivated γγ-thrombin competed for heparin binding. It has been shown that limited proteolysis/autolysis of the B-chain of α-thrombin in the area around Arg-B73 (in βγ/βγ and γγ/γγ-thrombin), but not that around Lys-B154 (in γγ/γ-thrombin), diminishes specific interactions with fibrinogen (Hosfesteane, J., Braun, P. J., and Stone, S. R. (1988) Biochemistry 27, 2144–2151). In unprotected modified α-thrombin, lysyl residues B21, B65, B174, and B252 were phosphopyridoxylated. In heparin-protected modified α-thrombin, only lysyl residues B21 and B65 were phosphopyridoxylated. These observations suggest that lysyl residues 21/65 of the B-chain of α-thrombin are involved in fibrinogen interactions, and lysyl residues 174/252 of the B-chain are important in heparin interactions.

α-Thrombin (EC 3.4.21.5) is a trypsin-like serine proteinase that plays a key role both in blood coagulation and in other physiological processes that involve catalytic functions and nonenzymatic intermolecular interactions (1–3). Thrombin cleaves or interacts with many different blood components including fibrinogen, fibrin, platelets, coagulation factors V, VIII, and XIII, thrombomodulin, and protein C. One mechanism for regulating thrombin activity is inhibition by plasma proteinase inhibitors, including antithrombin III, heparin cofactor II, α1-proteinase inhibitor, and α2-macroglobulin (4). Antithrombin III and heparin cofactor II are distinguished from the other proteinase inhibitors by their ability to show greatly accelerated thrombin inhibition in the presence of heparin and other glycosaminoglycans (see Refs. 5 and 6 and references cited therein). The heparin-catalyzed thrombin inhibition reaction with antithrombin III (and heparin cofactor II) requires simultaneous binding of proteinase and proteinase inhibitor to heparin (7–10).

The physiological substrate specificity of α-thrombin is mediated through both the active site region and a separate binding site(s) associated with the active site (11). α-Thrombin autolysis or limited proteolysis with trypsin yields β- and γ-thrombin derivatives (12). βγ/γγ-Thrombin and βγ/γ-thrombin (obtained by trypsinolysis (βγ/γγ) or autolysis (β/γ), respectively) have virtually no fibrinogen clotting ability but are active toward synthetic substrates. Structural analysis of human γγ/γ-thrombin reveals that 5 basic amino acid residues in the B-chain, Arg-B62, Arg-B70, Arg-B73, Arg-B123, and Lys-B154, are highly susceptible to proteolysis (12–16). Recent work with βγ-thrombin (cleavage only at Arg-B73) suggests that the βγ-cleavage site in α-thrombin is directly involved in interactions with fibrinogen, protein C, thrombomodulin, and hirudin (17–19).

α-Thrombin has a net positive surface charge of approximately 7 at physiological pH (20). As a polycation, α-thrombin binds not only to heparin (a highly negatively charged glycosaminoglycan) but also other polyanionic substances (21–24). The importance of positively charged lysyl residues of (pro)thrombin in polyanion binding and macromolecular protein interactions (such as with fibrinogen, antithrombin III, and factor V) has been examined by specific chemical modification (25–29). Griffith (26) showed that there are relatively few lysyl residues in α-thrombin that are essential for fibrinogen clotting activity and heparin binding.

This report further examines the importance of α-thrombin lysyl residues in binding to heparin and fibrinogen. For this study, we compared the structural and functional properties of α-thrombin chemically modified with pyridoxal 5'-phosphate (in the absence and presence of added heparin) to those of α-thrombin and γγ-thrombin. We have also identified the
position of the modified lysyl residues in the amino acid sequence of α-thrombin.

**EXPERIMENTAL PROCEDURES AND RESULTS**

The purpose of this investigation was to further characterize the reaction properties of phosphoryridoxylated α-thrombin species compared to α-thrombin and γT-thrombin and to identify some of the lysyl residues that are involved in heparin and fibrinogen interactions. The present study and previous work (26) have used pyridoxal 5'-phosphate modification of lysyl residues of thrombin to produce a proteinase with greatly reduced fibrinogen clotting and binding activities, but with a competent active site (catalytic triad). Analysis of α-thrombin treated with pyridoxal 5'-phosphate reveals that 4 out of a total of 22 lysyl residues are highly susceptible to modification. Selective modification of lysyl residues 21, 65, 174, and 252 in the B-chain of α-thrombin account for ~80% of the total phosphoryridoxyl incorporation.3 We feel that the contribution of other, minimally modified peptides to any decrease in α-thrombin activity would be minor.

Comparison of the thrombin derivatives to GdnHCl denaturation showed that γT-thrombin is more labile than α-thrombin and phosphoryridoxylated α-thrombins. These observations agree with urea denaturation studies of proteolyzed α-thrombin derivatives (2, 19). While the ε-amino group-labeled α-thrombin molecules produced by phosphoryridoxylation resemble native α-thrombin, limited proteolysis in the B-chain of α-thrombin produces a protein structure more susceptible to denaturation.

1 Portions of this paper (including “Experimental Procedures,” “Results,” Tables I and II, and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: BSA, bovine serum albumin; TosPhe-Ch, tosylphenylalanine chloromethyl ketone; TosGlyPro-ArgNA, Nα-p-tosyl-Gly-Pro-Arg-p-nitroanilide; HEFES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dansyl-Glu-Gly-Arc-CH2, dansyl-Glu-Gly-Arc-chloromethyl ketone; D-Phe-Pro-Arc-Arg-chloromethyl ketone; DAPA, dansylarginine N3-thyl-1,5-pentamethyylimidamide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GdnHCl, guanidine hydrochloride; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography).

3 Lysyl residues 21, 65, 174, and 252 in the B-chain of α-thrombin correspond to lysyl residues 341, 385, 494, and 572 in the zymogen, prothrombin, respectively (45).

4 Our data demonstrate that while 2 residues are modified, the total incorporation of pyridoxyl phosphate is 1 mol/mol of α-thrombin (in the presence of heparin). Our interpretation is that the residues found to be essential for fibrinogen(ogen) interactions, Lys-B21 and Lys-B65, have an initial equal opportunity of being phosphoryridoxylated, but following modification of either amino acid residue steric hindrance (or a localized conformational change) blocks or limits further modification (reactivity) of the other lysyl residue. Therefore, our observations suggest that modification of either reactive lysyl residue results in the loss of α-thrombin fibrinogen(ogen) activity. A similar explanation can be argued for the other 2 residues modified in α-thrombin, Lys-B174 and Lys-B252, which were determined to be important for heparin interactions. The individual contribution of α-thrombin B-chain lysyl residues 21 and 65 during fibrinogen(ogen) interaction and lysyl residues 174 and 252 during heparin binding is currently being evaluated using antipeptide antibodies prepared to synthetic peptides containing each respective lysyl residue. Noe et al. (46) recently demonstrated that an antipeptide polyclonal antibody prepared against residues 62-73 of the α-thrombin B-chain competitively inhibited not only fibrinogen clotting activity but also other enzymic functions without affecting tripeptidyl p-nitroanilide substrate hydrolysis by the proteinase.

The conformations of the phosphoryridoxylated α-thrombins and γT-thrombin were compared to α-thrombin using monoclonal antibodies prepared against α-thrombin (41). Interestingly, α-thrombin and heparin-protected modified α-thrombin reacted essentially the same with the monoclonal antibodies directed to fibrinogen-specific epitopes on α-thrombin (EST2, -6, and -7). Unprotected modified α-thrombin and γT-thrombin were similar to each other and showed reduced ability to interact with EST2, -6, and -7. This differential reactivity suggests that a specific protein conformation is conserved in heparin-protected modified α-thrombin. In contrast, the lowered reactivity of unprotected modified α-thrombin and γT-thrombin suggests an altered protein conformation (either by chemical modification of lysyl residues B-21, B-65, B-174, and B-252 or by limited proteolysis near Arg-B73 and/or Lys-B154) that is poorly recognized by these monoclonal antibodies (EST2, -6, and -7).

Lysyl residues 21 and 65 of the B-chain of α-thrombin are modified both in the absence and presence of added heparin. Peptide bond cleavage in γT-thrombin occurs within the regions containing Arg-B62 to Arg-B73 and Arg-B123 to Lys-B154 (12-16). Loss of clotting activity is correlated solely with proteolysis in the region centered around Arg-73 in the B-chain of α-thrombin (13, 18, 19). Whether or not the activity loss demonstrated in α-thrombin following limited proteolysis/chemical modification is the result of a conformation change, these observations suggest that the areas around Lys-B21, Lys-B65, and Lys-B73 are important for fibrinogen(ogen) interactions.

α-Thrombin fibrinogen clotting and fibrin monomer binding activities are inhibited by negatively charged nucleotides and other anionic compounds (22, 23). It appears that fibrin binding to α-thrombin involves an "anionic binding site" (or "recognition site") in the proteinase (14, 15, 24). Unlike α-thrombin, γT-thrombin does not interact with immobilized fibrin monomer (15). In the present study using γT-thrombin and lysine-modified α-thrombin species, we investigated whether both fibrin and heparin binding occur at the same site in α-thrombin. We found that the interaction with immobilized fibrin monomer was greatly reduced for both heparin- and non-heparin-binding species of phosphoryridoxylated α-thrombin (see below for the heparin binding properties of γT-thrombin). For these reasons, we conclude either that the heparin and fibrin binding sites in α-thrombin are different or that if they are the same site, then the requirements for fibrin binding are more extensive than for heparin binding.

The effect of either phosphoryridoxylation or limited proteolysis of α-thrombin on the rate of inhibition by antithrombin III is small. The results with γT-thrombin are consistent with recently published observations of proteolized thrombin derivatives and indicate that the region of α-thrombin centered around Arg-B73 and Lys-B154 is not significantly involved in interaction with antithrombin III (19, 47). Likewise, the lysine residues modified by phosphoryridoxylation are not required for the interaction with antithrombin III (in the absence of heparin).

Elution of the thrombin derivatives from immobilized heparin revealed that only unprotected modified α-thrombin eluted abnormally, while heparin-protected modified α- and γT-thrombin eluted essentially at the same ionic strength as native α-thrombin. A comparison of α-thrombin, unprotected and heparin-protected modified α-thrombins, and γT-thrombin inhibition by antithrombin III over a broad range of heparin concentrations (44) indicates that the heparin binding site in heparin-protected modified α-thrombin and γT-thrombin is totally functional. We also showed that γT-
thrombin (with its active site blocked by D-Phe-Pro-Arg-CH₂Cl) can compete for heparin binding to α-thrombin during a heparin-catalyzed antithrombin III inhibition reaction. These results suggest that neither proteolysis of α-thrombin near the area of Arg-B73 and Lys-B154 (γγ-thrombin) nor site-selective chemical modification at Lys-B21 and Lys-B65 (heparin-protected modified α-thrombin) compromises heparin binding. Our results also indicate that modification of Lys-B174 and Lys-B252 of α-thrombin greatly reduces proteinase-heparin binding; thus, these residues are required for the interaction with heparin.

These observations and previous reports (see references cited below) can be used to construct a model relating the fibrinogen and heparin binding sites in α-thrombin. This is schematically shown in Fig. 6. As illustrated, the overall fibrinogen (ogen) binding area is composed of the "recognition site" and the "apolar site" (11, 14, 15). The rationale for showing the recognition site for fibrinogen and fibrin as overlapping is based on limited proteolysis studies of α-thrombin in which both fibrinogen and fibrin interactions are greatly reduced without affecting binding to the apolar site (that is, γγ-thrombin does not interact with fibrin(ogen) but can still effectively bind to D-Phe-Pro-Arg-CH₂Cl). Chemical modification of Lys-B21 and Lys-B65 also uncouples the fibrinogen interactions of the recognition site. The model also depicts the heparin binding site as a distinct region but with a conformation-dependent linkage to the recognition site. Proteolysed α-thrombin derivatives (such as γγ-thrombin) bind heparin essentially like the native molecule. Heparin-protected modified α-thrombin, which resembles native α-thrombin in its ability to react with both heparin and the monoclonal antibodies, has lost its fibrin binding properties yet retains some fibrinogen clotting activity. Unprotected modified α-thrombin is greatly reduced in all of these properties. Chang (48) has recently reported that Lys-B21, Lys-B65, and 4 other lysyl residues of α-thrombin are protected from modification by 4-N,N-dimethylaminobenzene-4'-isothiocyanato-2'-sulfonic acid when the proteinase is complexed with hirudin. Additionally, he showed that Lys-B174 becomes available for modification in α-thrombin following complex formation with hirudin (48). These observations are compatible not only with the essential role of Lys-B21 and Lys-B65 during fibrinogen heparin interactions but also with a conformational change centered around Lys-B174 (including Lys-B252 as detected by phosphopyridoxylization) which is perturbed during α-thrombin-hirudin interactions and is protected during phosphopyridoxylization by heparin. Evidence for significant α-thrombin conformational changes following binding to hirudin has been described (49, 50). This model is also consistent with α-thrombin-hirudin complexes having reduced affinity for immobilized heparin (51) as a consequence of an altered protein conformation centered around Lys-B174 (and presumably including Lys-B252).

In conclusion, amino acid residues within α-thrombin that are critical for substrate/cofactor binding have been characterized using chemical modification (25–29, 48, 52, 55), limited proteolysis (1, 2, 12–19, 47, 51, 54), comparative structural modeling (55, 56), and naturally occurring genetic variants (57–60). Although not all the determinants for heparin or fibrinogen interactions with α-thrombin have been identified, our results demonstrate the importance of B-chain lysyl residues 174 and 252 in heparin binding and lysyl residues 21 and 65 in fibrinogen(ogen) interactions.

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REFERENCES

![Fig. 6. Schematic model of the active site region of α-thrombin illustrating the relationships between functional portions of the proteinase (B-chain) involved in interactions with fibrinogen (ogen) and heparin. The catalytic active site triad residues (histidine (H-43), aspartic acid (D-99), and serine (S-205)) are shown within the triangle.](attachment:image)
Supplementary Material


Monoclonal antibody reactivity was determined with an ELISA. Microtitre plates were coated overnight at ambient temperature with the antigen (various thrombin derivatives) at a concentration of 1.5 µg/ml in the carbonate buffer (pH 9.6) (see table 1). Plates were washed three times with Tween (PBS) (150 µM NaCl, 0.05% BSA, 0.05% Tween 20, 0.1% NaCl, pH 8.3). A stock anti-thrombin solution of the monoclonal antibodies (2 T, 2 T, and 2 T) were diluted 1:000 in the coating buffer, added to the monolayer plates, and incubated for 4 hr at ambient temperature. The plates were washed as described above and incubated with anti-mouse IgG alkaline phosphatase conjugate for 4 hr (diluted 1:000) in the coating buffer. After washing, bound anti-mouse alkaline phosphatase conjugate was then quantitated by reaction with 4-nitrophenylphosphate (76 µM) in a 100 µM glycine, 0.05% Tween 20, 0.1% NaCl, pH 9.6. Absorbances were read with a Molecular Devices microplate reader at 405 nm.

Stability Studies: GlucCIC functional studies of thrombin derivatives (structure shown in table 1) were performed by incubating thrombin 100 µM in 1/50 mM Tris·HCl pH 8.0 at 37°C, for 1 hr at ambient temperature. Residual thrombin amidolytic activity was measured and compared to control a-thrombin derivatives which were included in NaCl solutions.

Chromatography on Fiber Membrane Agarose: bovine thrombin was applied to Affigel 15 as described in the package insert provided by Bio-Rad. Affigel slurry (15 ml) was added to 30 ml of (10 mg/ml) dissolved in 100 mM HEPS at pH 7.5. Thrombin-immune agarose was prepared from the resulting fibrinogen-agarose by a method patterned after that of Heine and Matthews (1983) which included incubation with a-thrombin followed by washing with the matrix with acetic acid and water solutions. Fibrin chromatography of thrombin derivatives was carried out on a 1.3 x 4.0 cm fibrin monomer-agarose column equilibrated with 0.1 M Tris·HCl, 50 mM NaCl, pH 7.4. Approximately 15 µg of protein in 1 ml of buffer was added to the column at a flow rate of 3 ml/min, followed by washing the column with the buffer. The protein was eluted using a linear gradient with a total volume of 40 ml from 50 to 200 mM NaCl in 50 mM Tris·HCl, pH 7.4, at a flow rate of 25 ml/min. The eluate was collected in 1 ml fractions and thrombin was identified by amidolytic activity.

Determination of Rate Constants for the Antithrombin III-Thrombin Interaction by DAPA Fluorochromatography: Thrombin inhibition assays with antithrombin III-heparin were performed by including the active amidolytic thrombin inhibitor DAPA (9,20,29). Eluted fluorescence occurs during DAPA binding to thrombin's active site (Kg = 43 M). Thus, in a continuous assay, a decrease in fluorescence occurs when DAPA is displaced from thrombin. Determination of the rate constants for the antithrombin III-thrombin interaction was performed using a Perkin-Elmer Model LS-5 spectrophotometer. Emission was monitored at 460 nm with a 20 nm bandwidth and a 450 nm cut-off filter in the emission beam. Thrombin-DAPA complexes were excited at 280 nm with a 3 nm band-pass, providing excitation of the dansyl group by energy transfer from thrombin, thus eliminating background fluorescence due to unbound DAPA. Thrombin and DAPA were used to final concentrations 30 and 450 µM, respectively. Concentrated solution of antithrombin III-heparin (ranging from 400 µM to 4000 µM for antithrombin III and heparin, respectively) were then added to the DAPA/thrombin solution, which yielded 2 fold lower excess of antithrombin III to thrombin in the assay system (this permitted measurement of pseudo first order rate constants of inhibition). The loss of fluorescence was measured until fluorescence levels equal those of DAPA alone were achieved.
Essential Lysyl Residues of α-Thrombin

Active site titration of the thrombin derivatives (except unprotected-modified α-thrombin) was performed by the DAPA fluorescence assay with 15 α-DAP and 1 µM cleavage in 20 mM HEPPS, 150 mM NaCl at pH 7.4. Small portions (usually 1 µl) of α-thrombin IIIa were added and the DAPA fluorescence measured. The amount of α-thrombin IIIa required to displace all of the DAPA from the active site is equal to the active enzyme concentration.

HPLC and Edman-Sequence Analyses

Samples of protons for primary structural analysis were reduced-thioesterified S-carboxyamidomethyl and then digested with TFA-solubilized cyanogen bromide (CNBr) as described previously (30). Peptide maps of the unprotected and heparin-protected-modified α-thrombin samples were prepared using reverse-phase HPLC as detailed previously (30). A Vydac 218TP column was used with a Beckman 3200 gradient liquid chromatograph and a Beckman P-480A UV detector. A column temperature of 45°C and a flow rate of 0.5 ml/min were maintained. Solvent A (5% acetonitrile, 0.05 M sodium phosphate buffer, pH 6.5) solvent B was 95% acetonitrile. Solvent strength was increased linearly from 1 to 5% solvent B in 10 min and then to 100% solvent B in 50 min. Under these chromatographic conditions, no peptides were eluted after 130 min. The eluate was monitored at 210, 280, and 285 nm. Peptides chosen for sequencing were rechromatographed on the same column in a reverse-phase solvent and system (31). Acidic amino acid sequences were determined by automated Edman degradation in a Beckman Model 890C sequencer with identification of phenylthiohydantoin amino acids by HPLC as previously described (32). Phosphoprenylated tyrosine was identified by the absence of the usual tyrosine peak (18).

Ober Determinations

Protein concentrations were determined spectro-photometrically using the method of Bradford (33), with BSA as standard (10 mg/ml in 0.1 M NaCl, 0.01 M sodium phosphate buffer, pH 4.6), and 34,690 and tyrosine (M, 190), 123, 166 µg/ml in 0.1 M NaCl for bovine serum albumin IIIa (11) and 90, 183 and 187 mg/ml for prothrombin (M, 71,680). (19). Protein-bound pyridylphosphatic 3'-phosphate structural similarities of the thrombin derivatives were assessed by determining their binding to four monoclonal antibodies specific for α-thrombin (14). Reactivity of the thrombin derivatives to FESI, 6 and 7 (three monoclonal antibodies recognize regions in the thrombin binding site of α-thrombin, 14) was reversed. The binding characteristics of heparin and heparin-protected-modified α-thrombin were identical with unmodified modified α-thrombin and 73% thrombin exhibited essentially the same reduced binding properties to these monoclonal antibodies. These results show that the thrombin derivatives with monoclonal antibodies (6, 7, and 9) suggest altered reactivity (19). The interaction of the thrombin derivatives with phospholipid microsomes and surface bivalency of the α-thrombin derivatives and phospholipase C-α-thrombin is essentially the same as that reported previously for 73- thrombin (15).

To further assess the interaction of (lysine-modified α-thrombin) with fibrin, we used heparin-Sepharose chromatography to affinity-heparin and non-heparin-binding fractions of phospholipid-modified α-thrombin. Griffith (32) determined that incorporation of the first mole of pentameric phase into α-thrombin is associated with the loss of clotting activity and that this modification rate had a 1:1:1 stoichiometry. Therefore, to assess modified α-thrombin species that would possess heparin- and non-heparin-binding fractions, the modification was reduced from 5 to 0.5 µM (19). These results suggest that the thrombin derivatives with monoclonal antibodies of their ability to bind to heparin.

Inhibition by Antithrombin III and Interaction with Heparin: Inhibition of the thrombin derivatives by the primary physiologic thrombin inhibitor, antithrombin III, was determined both in the absence and presence of added heparin. As shown in Fig. 23, the thrombin derivatives exhibited slight inhibition with antithrombin III, and second order rate constants for 3, 3.5, 3.9 and 5.0 × 10^(-5) M·M-1·sec-1 were calculated for α-thrombin, heparin-protected-modified α-thrombin, unprotected-modified α-thrombin and heparin-thrombin, respectively. Therefore, compared to antithrombin III, an increase in the ratio of heparin concentration increased above 0.1 µg/ml (the inhibition rate of 0.53 M·M-1·sec-1) more than the inhibition rate of heparin thrombin increased above 0.1 µg/ml (the inhibition rate 0.42 M·M-1·sec-1). These results suggest that the heparin binding site in both heparin-protected-modified α-thrombin and 73% thrombin is intact although the thrombin binding site in the heparin thrombin is not.

The thrombin derivatives were further analyzed by reactivity with immobilized heparin as a function of their inhibition. With the exception of unprotected-modified α-thrombin, the map of proteins eluted in the void volume was the same at an inhibition strength of >17 M NaCl, the other thrombin derivatives were eluted from heparin-Sepharose at about the same 10. The ability of the various thrombin derivatives to elicit both immobilized heparin as a function of the thrombin-thrombin interaction with a heparin-binding site in the absence of added heparin. These results suggest that the heparin binding site in both heparin-protected-modified α-thrombin and heparin thrombin is intact although the thrombin binding site in the heparin thrombin is not intact.

Identification of Lysyl Residues in α-Thrombin That are Involved in Heparin and Heparinlike Interactions. To identify the essential lysine residues in α-thrombin, the thrombin derivatives with monoclonal antibodies specific for α-thrombin (14). Reactivity of the thrombin derivatives to FESI, 6 and 7 (three monoclonal antibodies recognize regions in the thrombin binding site of α-thrombin, 14) was reversed. The binding characteristics of heparin and heparin-protected-modified α-thrombin were identical with unmodified modified α-thrombin and 73% thrombin exhibited essentially the same reduced binding properties to these monoclonal antibodies. These results show that the thrombin derivatives with monoclonal antibodies (6, 7, and 9) suggest altered reactivity (19). The interaction of the thrombin derivatives with phospholipid microsomes and surface bivalency of the α-thrombin derivatives and phospholipase C-α-thrombin is essentially the same as that reported previously for 73- thrombin (15).

Interaction with Fibrin. Fibrin inhibitor-aggregate chromatography was used to assess the interaction of α-thrombin and phospholipid-modified α-thrombin with fibrin (19). Native (S-Alc) modified α-thrombin reversibly bound to immobilized fibrin monomer (Fig. 1A) in agreement with previous results (26, 27). Unlike native α-thrombin, both unprotected and heparin-protected-modified α-thrombin showed greatly reduced interactions with the form monomer, but the thrombin essentially retained all the vesicle volume (shown for unprotected modified α-thrombin in Fig. 1A). Our data are in excellent agreement with those recently reported by Kaminski and McEntee (34) who measured fibrin monomer interactions with various α-thrombin derivatives. These results imply that fibrin interaction is greatly reduced in the phospholipid-modified α-thrombin species due to modification either at the thrombin binding site or at another site specific for fibrin. Further, the interaction of the phospholipid-modified α-thrombin is essentially the same as that reported previously for 73% thrombin (15).

To further assess the interaction of (lysine-modified α-thrombin) with fibrin, we used heparin-Sepharose chromatography to affinity-heparin and non-heparin-binding fractions of phospholipid-modified α-thrombin. Griffith (32) determined that incorporation of the first mole of pentameric phase into α-thrombin is associated with the loss of clotting activity and that this modification rate had a 1:1:1 stoichiometry. Therefore, to assess modified α-thrombin species that would possess heparin- and non-heparin-binding fractions, the modification was reduced from 5 to 0.5 µM (19). These results suggest that the thrombin derivatives with monoclonal antibodies of their ability to bind to heparin.

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*Some reactivity of the thrombin derivatives was assessed by ELISA as described under Experimental Procedures.*

\*The values given are the means of at least three separate determinations performed in triplicate.
Fig. 1. Fibron monomer-aggregate chromatography of α-thrombin derivatives. (A) Native α- and phosphopentadecanoylated-α-thrombin (unprotected) were chromatographed on immobilized fibron monomer as described under 'Experimental Procedures'. The void volume fraction was determined using ovalbumin and was contained within fraction number 3. A linear NaCl gradient was started at fraction number 7 and anti-aggregic activity measurements were used to identify elution positions of native α-thrombin (•) and phosphopentadecanoylated-α-thrombin (○) (B) Fibre monomer-aggregate chromatography of the non-heparin-binding (□) and heparin-binding (●) phosphopentadecanoylated-α-thrombin species (modified in the absence of added heparin).

Fig. 2. Inhibition of thrombin derivatives by antithrombin III in the absence and presence of added heparin. (A) Thrombin derivatives (5 or 10 μM) were incubated with antithrombin III (500 nM) at 25°C in 20 mM HEPES, 150 mM NaCl, 2.5 mM MgCl₂, 0.1 g/mL BSA, and 0.1 mg/mL Polystyrene. Portions were withdrawn at intervals and assayed for remaining thrombin amidolytic activity. α-Thrombin (●), unprotected modified-α-thrombin (○), heparin-protected modified-α-thrombin (□) and γ-thrombin (■). (B) Thrombin derivatives (5 μM) were incubated with antithrombin III (5 nM) in the HEPES buffer in the presence of various concentrations of heparin. Half order rate constant (k₅) for thrombin inhibition were determined. α-Thrombin (●), unprotected modified-α-thrombin (○), heparin-protected modified-α-thrombin (□) and γ-thrombin (■).

Fig. 3. Heparin aggregate elution of various thrombin derivatives. Portions of the thrombin derivatives (about 250 μL in a total volume of 1.0 mL) were loaded onto a 1.0 mL heparin agarose column equilibrated at 20 mM HEPES in pH 7.4, washed for 3 mL with the buffer, and then eluted with a 25 x 25 mL linear gradient of NaCl from 0.05  M NaCl (from fraction number 6-65). Elution was monitored by amidolytic activity. (A) Unprotected modified-α-thrombin (●) and heparin-protected modified-α-thrombin (□). (B) α-Thrombin (○) and γ-thrombin (■).

Fig. 4. Inhibition of α-thrombin by antithrombin III-heparin in the presence of varying amounts of L-Pro-Pro-Arg-Cys-Glu-Cys-thrombin. Displacement of the DAPA fluorophore from the active site of α-thrombin during the heparin-catalyzed antithrombin III inhibition reaction was performed as described under 'Experimental Procedures'. DAPA fluorescence was monitored continuously upon addition of 100 μL of a antithrombin III (5 μM) heparin (8 μg/mL) solution to a final volume of 1.5 mL containing α-thrombin (50 μM) and varying amounts of L-Pro-Pro-Arg-Cys-Glu-Cys-thrombin. Varying concentrations of L-Pro-Pro-Arg-Cys-Glu-Cys-thrombin (0.1, 0.5, 1.0, 5.0 and 10.0 μM) and L-Pro-Pro-Arg-Cys-thrombin and L-Pro-Pro-Arg-Cys-thrombin had no effect on the fluorescence of α-thrombin-DAPA (●) and DAPA not itself react with DAPA.
Essential Lysyl Residues of α-Thrombin

Fig. 3. Peptide maps of phospho-lysylated α-thrombin species. Trypsin digests of unprotected and heparin-protected phospho-lysylated α-thrombin species were chromatographed by reverse-phase HPLC as described under "Experimental Procedures". The lower panel shows the detection of unprotected peptide at 210 nm. Phospho-lysyl-containing peptides were monitored at 225 nm in the unprotected thrombin species (middle panel) and the heparin-protected species (top panel). About 1 mg each was injected in the middle and top panels. Absorbance units full scale for detection was 1.0 for the middle and top panels.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide Sequence</th>
<th>% Label Incorporated</th>
<th>Hepatic Protected</th>
</tr>
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<tbody>
<tr>
<td>1.5</td>
<td>Lys Leu-Ser-Arg-NP-Arg (NQ)</td>
<td>45, 56</td>
<td>–</td>
</tr>
<tr>
<td>2.6</td>
<td>Lys Leu-Ser-Arg-NP-Arg (NQ)</td>
<td>45, 56</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Try Leu-Ser-Arg-NP-Arg (NQ)</td>
<td>45, 56</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Gly Leu-Ser-Arg-NP-Arg (NQ)</td>
<td>45, 56</td>
<td>–</td>
</tr>
</tbody>
</table>

Peptides 1-4 were from the peptide map of the unprotected sample, while peptides 5 and 6 were from the heparin-protected sample (see Fig. 5).

The underlined residue represents phospho-lysylated lysyl residue. All of the labeled peptides sequenced were from the B-chain of thrombin. Quantitative yields of amino acid residues in peptide are given in parentheses following the identified amino acid residues; sequence data for amino acid residues within parentheses was not obtained; (NQ), not quantitated; (NO), not detected.

The total amount of phospho-lysyl label in the HPLC chromatogram present in the absence (Fig. 3, middle panel) and presence of added heparin (Fig. 3, top panel) were normalized to 100%, and the amount of label incorporated in each peptide is expressed relative to this normalized value.