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

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

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α-Thrombin derivatives obtained either by site-specific modification at lysyl residues (phosphopyridoxylated) or by limited trypsinolysis (γ_T-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 γ_T-thrombin. Heparin accelerated the rate of antithrombin III inhibition of α-thrombin, heparin-protected modified-α-thrombin, and γ_T-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 γ_T-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 β_T/β- and γ_T/γ-thrombin), but not that around Lys-B154 (in γ_T/γ-thrombin), diminishes specific interactions with fibrinogen (Hofsteenge, 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 fibrin(ogen) 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, α_1-proteinase inhibitor, and α_2-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). β_T/γ_T-Thrombin and β/γ-thrombin (obtained by trypsinolysis (β_T/γ_T) or autolysis (β/γ), respectively) have virtually no fibrinogen clotting ability but are active toward synthetic substrates. Structural analysis of human γ_T/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 β_T-thrombin (cleavage only at Arg-B73) suggests that the β-cleavage site in α-thrombin is directly involved in interactions with fibrin(ogen), 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 fibrin(ogen). 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 γ_T-thrombin. We have also identified the

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position of the modified lysyl residues in the amino acid sequence of α-thrombin.

EXPERIMENTAL PROCEDURES AND RESULTS[1,2]

DISCUSSION

The purpose of this investigation was to further characterize the reaction properties of phosphopyridoxylated α-thrombin species compared to α-thrombin and γ_T-thrombin and to identify some of the lysyl residues that are involved in heparin and fibrin(ogen) 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 fibrin(ogen) 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 phosphopyridoxyl incorporation.[3,4] 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 γ_T-thrombin is more labile than α-thrombin and phosphopyridoxylated 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 phosphopyridoxylation resemble native α-thrombin, limited proteolysis in the B-chain of α-thrombin produces a protein structure more susceptible to denaturation.

The conformations of the phosphopyridoxylated α-thrombins and γ_T-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 fibrin(ogen)-specific epitopes on α-thrombin (EST2, -6, and -7). Unprotected modified α-thrombin and γ_T-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 γ_T-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 γ/γ_T-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 fibrin(ogen) interactions.[4]

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, γ_T-thrombin does not interact with immobilized fibrin monomer (15). In the present study using γ_T-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 phosphopyridoxylated α-thrombin (see below for the heparin binding properties of γ_T-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 phosphopyridoxylation or limited proteolysis of α-thrombin on the rate of inhibition by antithrombin III is small. The results with γ_T-thrombin are consistent with recently published observations of proteolyzed 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 phosphopyridoxylation 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 γ_T-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 γ_T-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 γ_T-thrombin is totally functional. We also showed that γ_T-

[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_2Cl, tosylphenylalanine chloromethyl ketone; TosGlyPro-ArgNA, N^{α}-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dansyl-Glu-Gly-Arg-CH_2Cl, dansyl-Glu-Gly-Arg chloromethyl ketone; D-Phe-Pro-Arg-CH_2Cl, D-Phe-Pro-Arg chloromethyl ketone; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GdnHCl, guanidine hydrochloride; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatograph(y).

[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 fibrin(ogen) interactions, Lys-B21 and Lys-B65, have an initial equal opportunity of being phosphopyridoxylated, 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 fibrin(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 fibrin(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.

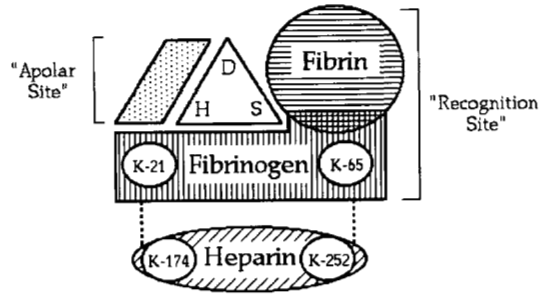

FIG. 6. **Schematic model of the active site region of α-thrombin illustrating the relationships between functional portions of the proteinase (B-chain) involved in interactions with fibrin(ogen) and heparin.** The catalytic active site triad residues (histidine (H-43), aspartic acid (D-99), and serine (S-205)) are shown within the *triangle*.

thrombin (with its active site blocked by D-Phe-Pro-Arg-CH_2Cl) 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 (γ_T-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 fibrin(ogen) and heparin binding sites in α-thrombin. This is schematically shown in Fig. 6. As illustrated, the overall fibrin(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, γ_T-thrombin does not interact with fibrin(ogen) but can still effectively bind to D-Phe-Pro-Arg-CH_2Cl). Chemical modification of Lys-B21 and Lys-B65 also uncouples the fibrin(ogen) 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. Proteolyzed α-thrombin derivatives (such as γ_T-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*-dimethylaminoazobenzene-4′-isothiocyano-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 fibrin(ogen) interactions but also with a conformational change centered around Lys-B174 (including Lys-B252 as detected by phosphopyridoxylation) which is perturbed during α-thrombin-hirudin interactions and is protected during phosphopyridoxylation 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, 53), 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 fibrin(ogen) 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 fibrin(ogen) interactions.

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Supplementary Material
to
STRUCTURAL AND FUNCTIONAL PROPERTIES OF HUMAN α-THROMBIN, PHOSPHOPYRIDOXYLATED-α-THROMBIN AND γ_T-THROMBIN: Identification of Lysyl Residues in α-Thrombin That Are Critical for Heparin and Fibrin(ogen) Interactions
by
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EXPERIMENTAL PROCEDURES

Materials. Prothrombin and antithrombin III were isolated from human plasma obtained from the American Red Cross (Carolinas Region, Charlotte, NC) following previously described procedures (30). Human α-thrombin was prepared from prothrombin and purified as detailed previously (31). Human γ_T-thrombin was prepared by limited digestion with trypsin as described (16). Heparin was generously provided by Dr. G. van Dedem (Diosynth BV, Oss, The Netherlands). Heparin-agarose was prepared as described previously (30). Pyridoxal 5'-phosphate, BSA[2], TosPhe-CH_2Cl-treated trypsin, $NaBH_4$, DEAE-Sephacel, anti-mouse IgG-alkaline phosphatase conjugate and p-nitrophenyl phosphate were obtained from Sigma; bovine fibrinogen (75% clottable) was from Miles Laboratories. Affigel 15 was purchased from BioRad; Polybrene and iodoacetic acid were from Aldrich. Protein-sequencing reagents were products of Beckman; dansyl-Glu-Gly-Arg-CH_2Cl and D-Phe-Pro-Arg-CH_2Cl were from Calbiochem. TosGly-ProArgNA for the determination of amidolytic activity of thrombin was obtained from Boehringer-Mannheim; GdnHCl was from Pierce. Monoclonal antibodies (EST1, 2, 6, and 7) were obtained from American Diagnostica. DAPA was synthesized following the procedure of Nesheim et al. (32). With the exception of the buffers used to prepare fibrin monomer-agarose and the ELISA, all buffers used in this study contained 0.1% (w/v) polyethylene glycol (M_r = 8000) to limit protein adsorption to surfaces.

Phosphopyridoxylation of α-Thrombin. Pyridoxal 5'-phosphate modification of thrombin was carried out essentially as described previously (26): 100 μl of 25 mM pyridoxal 5'-phosphate was added to 10 ml of 26 μM thrombin in 100 mM triethanolamine-acetate at pH 8.0. This mixture was incubated in the dark at room temperature. After three hours, 500 μl of sodium borohydride (100 mM) was added and this mixture was incubated overnight at 4°C. Pyridoxyl 5'-phosphate was removed by gel filtration chromatography (Sephadex G-25). Heparin-protected modification was carried out in the presence of a tenfold molar excess of heparin to α-thrombin. Heparin was removed by passage of the reaction mixture over a DEAE-Sephacel column equilibrated in 50 mM Tris-HCl, 250 mM NaCl at pH 7.4.

Enzyme and Inhibitor Assays and ELISA. Fibrinogen clotting activity, amidolytic activity, dansyl-Glu-Gly-Arg-CH_2Cl, and antithrombin III reaction rates were determined for various forms of thrombin as described previously (30,31). The buffer for the heparin titration of antithrombin III-thrombin reaction rates, however, was 20 mM HEPES, 150 mM NaCl at pH 7.4.

D-Phe-Pro-Arg-CH_2Cl-γ_T-thrombin was prepared by sequential addition, at 10-min intervals, of 10 μL aliquots of 10 mM of the active site-directed reagent D-Phe-Pro-Arg-CH_2Cl (in 1 mM HCl) to 8.4 μM γ_T-thrombin in 20 mM HEPES, 300 mM NaCl at pH 6.5. The extent of modification was followed by the loss of amidolytic substrate hydrolysis activity and was terminated when no activity remained. Residual D-Phe-Pro-Arg-CH_2Cl in the modified-γ_T-thrombin was removed by dialysis versus the HEPES buffer (4 x 4L at 4°C).

Monoclonal antibody reactivity was determined with an ELISA. Microtiter plate wells were coated overnight at ambient temperature with the antigen (various thrombin derivatives) at a concentration of 3 μg/mL in the coating buffer (10 mM Tris-HCl, 10 mM NaCl, 0.1% NaN_3, pH 8.0). The plates were washed three times with the wash buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2% BSA, 0.05% Tween 20, 0.1% NaN_3, pH 8.0). Stock anti-α-thrombin solutions of the monoclonal antibodies (EST1, 2, 6, and 7) were diluted 1:1000 in the coating buffer, added to the microtiter 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 1:1000 in the coating buffer). After washing, bound anti-IgG-alkaline phosphatase conjugate was then quantified by reaction with p-nitrophenyl phosphate (7.6 mM) in 100 μM glycine, 0.05% Tween 20, 0.1% NaN_3, pH 9.0. Absorbances were read with a Molecular Devices Vmax microplate reader at 405 nm.

Stability Studies. GdnHCl denaturation studies of the thrombin derivatives (each at 10 nM) were performed by incubation in 0.1 mL of 50 mM Tris-HCl at pH 7.4, and 0-2.2 M GdnHCl for 1 hr at ambient temperature. Residual thrombin amidolytic activity was measured and compared to control α-thrombin derivatives which were incubated in NaCl solutions.

Chromatography on Fibrin Monomer-Agarose. Bovine fibrinogen was coupled to Affigel 15 as described in the package insert provided by BioRad. Affigel slurry (15 ml) was added to 30 ml of fibrinogen (5 mg/ml) dissolved in 100 mM HEPES at pH 7.5. Fibrin monomer-agarose was prepared from the resulting fibrinogen-agarose by a method patterned after that of Heene and Matthias (33) which included incubation with α-thrombin followed by washing the matrix with acetic acid and urea solutions.

Fibrin chromatography of thrombin derivatives was carried out on a 1.3 x 4-cm fibrin monomer-agarose column equilibrated with 50 mM 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/h, 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/h. The eluate was collected in 1 ml fractions and thrombin was identified by amidolytic activity.

Determination of Rate Constants for the Antithrombin III-Thrombin Inhibition Reaction by DAPA Fluorescence. Thrombin inhibition assays with antithrombin III-heparin were performed by including the active site-specific thrombin inhibitor DAPA (9,32,33). Enhanced fluorescence occurs during DAPA binding to thrombin's active site (K_d = 43 nM). Thus, in a continuous assay, a decrease in fluorescence occurs when DAPA is displaced from thrombin during antithrombin III inhibition. Fluorescence measurements were performed using a Perkin-Elmer Model LS-5 spectrofluorometer. Emission was monitored at 540 nm with a 20 nm band-pass 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 (35). Thrombin and DAPA were mixed to final concentrations 50 and 450 nM, respectively, in 20 mM HEPES, 150 mM NaCl buffer at pH 7.4 and ambient temperature (34). Concentrated solutions of antithrombin III-heparin (ranging from 8-60 μM and 4-2000 μg/mL for antithrombin III and heparin, respectively) were then added to the DAPA-thrombin solution, which yielded ≥10-fold molar 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 monitored until fluorescence levels equal to those of DAPA alone were achieved.

Active site titration of the thrombin derivatives (except unprotected modified-α-thrombin) was performed by the DAPA fluorescence assay with 10 μM DAPA and 1 μg/mL heparin in 20 mM HEPES, 150 mM NaCl at pH 7.4. Small portions (usually 10 μL) of antithrombin III were added and thrombin-DAPA fluorescence measured. The amount of antithrombin III required to displace all of the DAPA from the active site is equal to the active enzyme concentration.

HPLC and Primary Structure Analyses. Samples of protein for primary structural analysis were reductively-denatured, S-carboxymethylated with iodoacetic acid and then digested with TosPheCH$_2$Cl-treated trypsin 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 332 gradient liquid chromatograph and a Hewlett-Packard 1040A diode array detector. A column temperature of 45°C and a flow rate of 1 ml/min were maintained. Solvent A was 50 mM sodium phosphate (pH 6.5); solvent B was acetonitrile. Solvent strength was increased linearly from 1 to 28% solvent B in 100 min and then to 62% solvent B in 55 min. Under these chromatographic conditions, no peptides were eluted after 120 min. The elution was monitored at 210, 280, and 325 nm (36). Peptides chosen for sequencing were rechromatographed on the same column in a trifluoroacetic acid solvent system (37). 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 (38). Phosphopyridoxylated lysine was identified by the absence of the usual lysine peak (36).

Other Determinations. Protein concentrations were determined spectro-photometrically using specific extinction coefficients at 280 nm of 1.75 ml mg^{-1}cm^{-1} for human α-thrombin (M_r = 36,600) and γ-thrombin (M_r = 31,100) (12), 0.65 ml mg^{-1} cm^{-1} for antithrombin III (M_r = 58,000) (39), and 1.47 ml mg^{-1} cm^{-1} for prothrombin (M_r = 71,600) (40). Protein-bound pyridoxyl 5'-phosphate concentrations were determined spectrophotometrically using an extinction coefficient at 322 nm of 9000 M^{-1} cm^{-1} (26).

RESULTS

Thrombin Derivatives. To assess the involvement of thrombin lysyl residues during both heparin and fibrinogen interactions, Griffith (26) used pyridoxal 5'-phosphate as a specific reagent for ε-amino group modification. Under identical experimental conditions, we found incorporation of approximately 2 mole of pyridoxyl phosphate/mole of thrombin in the absence of heparin. Modification of thrombin in the presence of added heparin resulted in incorporation of only 1 mole of reagent/mole of proteinase. Reaction characteristics of these phosphopyridoxylated-α-thrombin species, modified in the absence and presence of added heparin, toward fibrinogen clotting (~5 and ~30-40% for unprotected and heparin-protected modified derivatives, respectively) and amidolytic substrate hydrolysis (100% of the activity compared to α-thrombin) were essentially the same as those described previously by Griffith and associates (26,29). γT-Thrombin, prepared as described by Braun et al. (16), had lost more than 99% of its fibrinogen cleaving activity, had essentially the same amidolytic activity as α-thrombin, and it appeared to contain three non-covalently linked components during SDS-PAGE under non-reducing conditions [and did not contain detectable amounts of α- or β-thrombin) (data not included)]. Inactivation rate constants for the thrombin derivatives by dansyl-Glu-Gly-Arg-CH$_2$Cl (1.5 μM) were determined and found to be 2.4 ± 0.1, 2.1 ± .05, 1.9 ± 0.1 and 1.3 ± 0.1 x 10^5 M^{-1} min^{-1} for α-thrombin, heparin-protected modified-α-thrombin, unprotected modified-α-thrombin, and γT-thrombin, respectively.

Structural Stability and Monoclonal Antibody Reactivity. The structural stability of the thrombin derivatives was examined by incubation with increasing amounts of GdnHCl. Compared to γT-thrombin, α-thrombin and the phosphopyridoxylated-α-thrombins were substantially more stable to denaturation (data not shown). The GdnHCl concentration that resulted in 50% loss of amidolytic activity was 0.6, 1.0, 1.3 and 1.3 M for γT-thrombin, unprotected modified-α-thrombin, heparin-protected modified-α-thrombin and α-thrombin, respectively.

Structural similarities of the thrombin derivatives were assessed by determining their binding to four monoclonal antibodies specific for α-thrombin (41). Reactivity of the thrombin derivatives to EST2, 6 and 7 (these monoclonal antibodies recognize areas within the fibrinogen binding site of α-thrombin, see Ref. 41) was varied in that the binding characteristics of α-thrombin and heparin-protected modified-α-thrombin were identical while unprotected modified-α-thrombin and γT-thrombin exhibited essentially the same reduced binding properties to these monoclonal antibodies (Table I). The differential binding properties of the thrombin derivatives with these monoclonal antibodies (EST2, 6 and 7) suggest altered reactivity rather than reduced binding (of proteinase) to the microtiter plate well surface because all of the thrombin derivatives had an equal reactivity with EST1 (Table I).

Interaction with Fibrin. Fibrin monomer-agarose chromatography was used to assess the interaction of α-thrombin and phosphopyridoxylated-α-thrombin species with fibrin (Fig. 1). Native (or NaBH$_4$-reduced) α-thrombin reversibly bound to immobilized fibrin monomer (Fig. 1A) in agreement with previously reported results (24,42). Unlike native α-thrombin, both unprotected and heparin-protected modified-α-thrombins showed greatly reduced interactions with the fibrin monomer column and eluted essentially in the void volume (shown for unprotected modified-α-thrombin in Fig. 1A). Our data are in excellent agreement with those recently reported by Kaminski and McDonagh (43) who measured fibrin monomer interactions with various α-thrombin derivatives. These results imply that fibrin interaction is greatly reduced in the phosphopyridoxylated-α-thrombin species due to modification either at the fibrinogen binding site or at another site specific for fibrin. Further, the interaction of the phosphopyridoxylated-α-thrombins is essentially the same as that reported previously for γT-thrombin (15).

To additionally assess the interaction of lysine-modified-α-thrombin (unprotected) with fibrin, we used heparin-agarose affinity chromatography to obtain heparin- and non-heparin-binding fractions of phosphopyridoxylated-α-thrombin. Griffith (26) determined that incorporation of the first mole of pyridoxyl phosphate into α-thrombin is associated with the loss of clotting activity and that this modification rate had a $t_{1/2}$ =1 min. Therefore, to prepare modified α-thrombin species that would possess heparin- and non-heparin-binding fractions, the modification time was reduced from 3 h to ≤3 min (data not included). Under the detection limits used, both the heparin- and the non-heparin binding fractions of lysine-modified thrombin had greatly lowered affinity for immobilized fibrin monomer (both thrombin species had ~1 5 mol of pyridoxyl phosphate incorpo-rated/mol of proteinase) (Fig. 1B). These results indicate that phosphopyridoxylated-α-thrombin species do not interact with immobilized fibrin monomer regardless 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. 2A, the thrombin derivatives exhibited slightly different inhibition rates with antithrombin III, and second order rate constants of 4.10, 3.21, 3.19 and 0.97 x 10^5 M^{-1} min^{-1} were calculated for α-thrombin, heparin-protected modified-α-thrombin, unprotected modified-α-thrombin, and γT-thrombin, respectively. Therefore, compared to α-thrombin, the phosphopyridoxylated-α-thrombin species were inhibited at about the same rate (~1.3-fold less) while the inhibition rate of γT-thrombin was reduced by 4.2-fold.

We also examined the inhibition rates of the thrombin derivatives by antithrombin III in the presence of varying amounts of heparin. As shown in Fig. 2B, heparin did not enhance the rate of antithrombin III inhibition of unprotected modified-α-thrombin. In contrast, α-thrombin, heparin-protected modified-α-thrombin and γT-thrombin were rapidly inhibited by antithrombin III as the heparin concentration increased from 0.03 to 1 μg/ml; the inhibition rate (k_2) reached a maximum between 1 and 2 μg/ml heparin, and subsequently decreased as the heparin concentration increased above 5 μg/ml (Fig. 2B). The shape of these reaction rate curves is indicative of formation of a complex containing inhibitor, heparin and proteinase (8-10). Furthermore, heparin binding by these proteinases is similar since the heparin-dependency of the antithrombin III reaction for α-thrombin, heparin-protected modified-α-thrombin and γT-thrombin is essentially the same (43). The differences found in maximum rate enhancement of inhibition between α-thrombin, heparin-protected modified-α-thrombin and γT-thrombin by antithrombin III and heparin is consistent with those values calculated for inhibition in the absence of added heparin. These results suggest that the heparin binding site in both heparin-protected modified-α-thrombin and γT-thrombin is intact although the fibrin(ogen) binding site is not fully functional.

The thrombin derivatives were further analyzed for reactivity with immobilized heparin as a function of ionic strength. With the exception of unprotected modified-α-thrombin (the majority of protein eluted in the void volume fraction and the remainder eluted at an ionic strength of ~0.17 M NaCl), the other thrombin derivatives were eluted from heparin-agarose at about the same ionic strength (~0.4 M NaCl) (Fig. 3). Thus, the ability of the various thrombin derivatives to elute from immobilized heparin as a function of the ionic strength is consistent with a functional heparin binding site in the proteinase.

We further investigated the function of the heparin binding properties of γT-thrombin by competing with α-thrombin for heparin binding in an antithrombin III-heparin assay. This was performed by adding varying amounts of D-Phe-Pro-Arg-CH$_2$Cl-active site inactivated-γT-thrombin to a solution containing a fixed amount of heparin, antithrombin III and α-thrombin. As the concentration of D-Phe-Pro-Arg-CH$_2$Cl-γT-thrombin increased from 4.8- to 19-fold greater than that of α-thrombin, the rate of α-thrombin inhibition by heparin-antithrombin III was substantially decreased (Fig. 4). Control experiments verified that D-Phe-Pro-Arg-CH$_2$Cl-γT-thrombin had no effect in the α-thrombin-antithrombin III inhibition assay performed in the absence of heparin (pseudo first order rate constants were 0.10 ± .01 and 0.12 ± .01 min^{-1}, in the absence and presence of the D-Phe-Pro-Arg-CH$_2$Cl-γT-thrombin (19:1 to α-thrombin), respectively]. These results and those above collectively demonstrate that the mechanism of heparin binding in α-thrombin, heparin-protected modified-α-thrombin and γT-thrombin is essentially the same.

Identification of Lysyl Residues in α-Thrombin that are Involved in Heparin and Fibrin(ogen) Interactions. To identify the essential lysyl residues in α-thrombin, the unprotected and heparin-protected phosphopyridoxylated-α-thrombin species were trypsin hydrolyzed to produce HPLC peptide maps (Fig. 5). Although the overall extent of labeling was in the range of 1-2 mole of reagent incorporated/mole of thrombin (depending upon whether the sample was modified in the absence or presence of added heparin), inspection of the HPLC profiles revealed that more than 1-2 lysyl residues were susceptible to modification. The phosphopyridoxyl label was detected at 325 nm, and the peptide map of the unprotected modified-α-thrombin sample showed that a majority of the label (~80%) was contained in four peptides (number 1-4 in Fig. 5, middle panel). The peptide map of heparin-protected modified-α-thrombin clearly showed that two of these four peptides were totally protected (>97%) from phosphopyridoxylation (Fig. 5, top panel).

The six HPLC peptides highlighted in Fig. 5 (number 1-6) were additionally processed in order to determine the position of the phosphopyridoxyl label in both the unprotected and heparin-protected thrombin species. These six peptides were chosen for sequence analysis because they were clearly labeled in the absence of heparin and/or they were totally absent when modified in the presence of added heparin. The peptides were collected, rechromatographed in a trifluoroacetic acid system to insure their purity, and then subjected to automated Edman degradation (Table II). The lysyl residues modified in the absence of heparin were residues B21, B65, B174 and B252 of the B-chain of α-thrombin (using the thrombin numbering system).[3] The lysyl residues protected from modification in the presence of added heparin were residues B174 and B252. Also listed in Table II is the relative percentage of phosphopyridoxyl label found in each peptide and the amount recovered of phenylthiohydantoin derivatives. The six peptides range individually from 8-56% of the overall phosphopyridoxyl label. Each of the other labeled peptides individually account for <7% of the total phosphopyridoxyl label.

TABLE I

α-Thrombin, Phosphopyridoxylated-α-Thrombin and γT-Thrombin Reactivity with α-Thrombin Specific Monoclonal Antibodies

Thrombin Derivative	% Reactivity with Monoclonal Antibodies[a] EST1	EST2	EST6	EST7
α-Thrombin	100	100	100	100
Heparin-protected modified-α-thrombin	100	100	100	100
Unprotected modified-α-thrombin	100	60	50	75
γT-Thrombin	100	60	30	70

[a]Reactivity of the thrombin derivatives was assessed by ELISA as described under "Experimental Procedures" and the values given are the means of at least three separate determinations performed in triplicate.

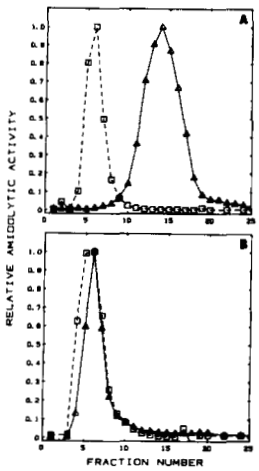

Fig. 1. Fibrin monomer-agarose chromatography of α-thrombin derivatives. **(A)** Native α- and phosphopyridoxylated-α-thrombin (unprotected) were chromatographed on immobilized fibrin monomer as described under "Experimental Procedures". The void volume fraction was determined using ovalbumin and was contained within fraction number 5. A linear NaCl gradient was started at fraction number 7 and amidolytic activity measurements were used to identify elution positions of native α-thrombin (△) and phosphopyridoxylated-α-thrombin (□). **(B)** Fibrin monomer-agarose chromatography of the non-heparin-binding (□) and heparin-binding (△) phosphopyridoxlyated-α-thrombin species (modified in the absence of added heparin).

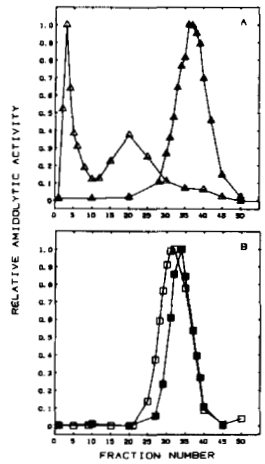

Fig. 3. Heparin agarose elution of various thrombin derivatives. Portions of the thrombin derivatives (about 250 nmol in a total volume of 1.0 mL) were loaded onto a 1.0-mL heparin agarose column equilibrated in 20 mM HEPES at pH 7.4, washed for 5 mL with the buffer, and then eluted with a 25 x 25 mL linear gradient of NaCl from 0-0.75 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 γγ-thrombin (□).

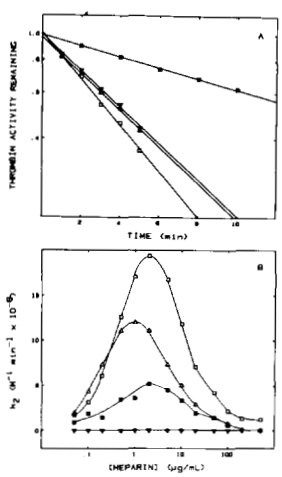

Fig. 2. Inhibition of thrombin derivatives by antithrombin III in the absence and presence of added heparin. **(A)** Thrombin derivatives (at 5 or 10 nM) were incubated with antithrombin III (500 nM) at 25°C in 20 mM HEPES, 150 mM NaCl, 2.5 or 5.0 mg/mL BSA, and 0.1 mg/mL Polybrene. Portions were withdrawn at intervals and assayed for remaining thrombin amidolytic activity. α-Thrombin (□), unprotected modified-α-thrombin (▼), heparin-protected modified-α-thrombin (△) and γγ-thrombin (■). **(B)** Thrombin derivatives (0.5 nM) were incubated with antithrombin III (5 nM) in the HEPES buffer in the presence of various concentrations of heparin. Second order rate constant (k_2) for thrombin inhibition were determined. α-Thrombin (□), unprotected modified-α-thrombin (▼), heparin-protected modified-α-thrombin (△) and γγ-thrombin (■).

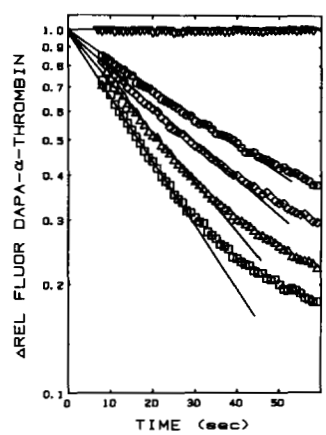

Fig. 4. Inhibition of α-thrombin by antithrombin III-heparin in the presence of varying amounts of D-Pro-Phe-Arg-CH_2Cl-γγ-thrombin. Displacement of the DAPA fluorophore from the active site of α-thrombin during the heparin-catalyzed antithrombin III inhibition reaction was performed as described under "Experimental Procedures". DAPA fluorescence was monitored continuously upon addition of 100 μL of a antithrombin III (8 μM)-heparin (8 μg/mL) solution to a final volume of 1.5 mL containing α-thrombin (50 nM) in the presence of DAPA (450 nM). Varying amounts of D-Phe-Pro-Arg-CH_2Cl-γγ-thrombin (dilutions of a stock solution of 8.4 μM were added in a total volume of 100 μL) were added to the α-thrombin-DAPA solution prior to the addition of antithrombin III-heparin: 0 (□), 240 (△), 480 (◇) and 960 (○) nM D-Phe-Pro-Arg-CH_2Cl-γγ-thrombin. Control experiments verified that D-Phe-Pro-Arg-CH_2Cl-γγ-thrombin had no effect on the fluorescence of α-thrombin-DAPA (▽) and did not itself react with DAPA.

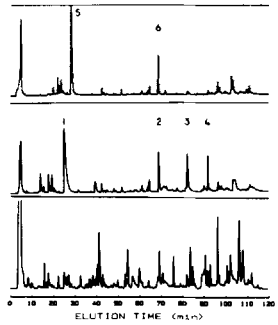

Fig. 5. Peptide maps of phosphopyridoxylated-α-thrombin species. Tryptic digests of unprotected and heparin-protected phosphopyridoxylated-α-thrombin species were chromatographed by reverse-phase HPLC as described under "Experimental Procedures". The lower panel shows the detection of unprotected peptides at 210 nm. Phosphopyridoxyl-containing peptides were monitored at 325 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 0.04 for the middle and top panels.

Table II
Amino Acid Sequences of α-Thrombin Peptides Containing Phosphopyridoxylated Lysyl Residues

Peptide[a]	Peptide Sequence[b]	% Label Incorporated[c]	Heparin Protected
1,5	Ile 63 (5500)-Gly (4100)-Lys (0)-His (NQ)-Ser (NQ)-Arg (ND)	45, 56	–
2,6	Lys 21 (0)-Ser (NQ)-Pro (240)-Gln (220)-Leu (410)-Leu (460)-Cys(NQ)-(Gly-Ala-Ser-Leu-Ile-Ser-Asp-Arg)	11, 15	–
3	Trp 249 (NQ)-Ile (780)-Gln (NQ)-Lys (0)-Val (580)-Ile (580)-Asp (370)-Gln (NQ)-Phe (340)-Gly (230)-Glu (90)	15	+
4	Gly 155 (350)-Gln (NQ)-Pro (150)-Ser (NQ)-Val (210)-Leu (170)-Gln (NQ)-Val (NQ)-Val (150)-Asn (90)-Leu (120)-Pro (32)-Ile (120)-Val (91)-Glu (54)-Arg (ND)-Pro (24)-Val (30)-Cys (NQ)-Lys (0)-Asp (23)-(Ser-Thr-Arg)	8	+

[a]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).

[b]The underlined residue represents phosphopyridoxylated lysyl residues. All of the labeled peptides sequenced were from the B-chain of thrombin. Quantitative yields of amino acid residues in pmol are given in parentheses following the identified amino acid residue; sequence data for amino acid residues within parentheses was not obtained; NQ, not quantitated; ND, not detected.

[c]The total amount of phosphopyridoxyl label in the HPLC chromatograms present in the absence (Fig. 5, middle panel) and presence of added heparin (Fig. 5, top panel) were normalized to 100%, and the amount of label incorporated in each peptide is expressed relative to this normalized value.