Demonstration of Exosite I-Dependent Interactions of Thrombin with Human Factor V and Factor Va Involving the Factor Va Heavy Chain: Analysis by Affinity Chromatography Employing A Novel Method for Active-Site-Selective Immobilization of Serine Proteinases†

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ABSTRACT: In an essential step of blood coagulation, factor V is proteolytically processed by thrombin to generate the activated protein cofactor, factor Va, and to release the activation fragments E and C1. For the identification and characterization of sites of thrombin binding to factor V and its activation products, a new method was developed for immobilizing thrombin and other serine proteinases specifically (≥92%) through their active sites and used in affinity chromatography studies of the interactions. Interactions of factor V with exosite I of thrombin were shown to regulate the factor V activation pathway from the 93% ± 12% inhibition of the rate of activation correlated with specific binding of hirudin$^{54-65}$ to this exosite. Chromatography of factor V on active-site-immobilized thrombin showed only a weak interaction, while the factor Va heterodimer bound specifically and with apparently higher affinity, in an interaction that was prevented by hirudin$^{54-65}$. The heavy chain of subunit-dissociated factor Va bound to immobilized thrombin, while the light-chain subunit and fragment E had no detectable affinity. These results demonstrate a previously undescribed, exosite I-dependent interaction of thrombin with factor Va that occurs through the factor Va heavy chain. They support the further conclusion that similar exosite I-dependent binding of thrombin to the heavy-chain region of factor V contributes to recognition of factor V as a specific thrombin substrate and thereby regulates proteolytic activation of the protein cofactor.

The blood clotting proteinase, thrombin, interacts with factor V as a physiological substrate, converting the inactive, precursor form of the protein into factor Va through a multistep activation process that is essential to the normal hemostatic response (*1*−*4*). Factor Va functions as a cofactor in prothrombin activation by directing the assembly of the factor Xa−factor Va−prothrombin complex on phospholipid membranes in the presence of calcium, which generates thrombin at a ∼300000-fold faster rate than factor Xa alone (*5*). Human factor V is a 330 000 molecular weight single-chain glycoprotein that is activated by thrombin cleavage at three sites: Arg^{709}, Arg^{1018}, and Arg^{1545} (*3, 4, 6*−*9*). This generates the heavy chain of factor Va from the amino-terminal part of the molecule, produces the light chain from the carboxy-terminal end, and releases the activation fragments, E and C1, from the central portion (*3*). Factor Va is a heterodimer of the heavy and light chains noncovalently associated in the presence of calcium (*3, 10*). In addition to the thrombin catalytic site, regulatory exosites I and II have been shown to be involved in activation of bovine factor V by human thrombin (*11*). This is consistent with the role of exosite I in contributing to the specificity of thrombin for a number of protein substrates by facilitating their specific binding (*12, 13*). Exosite II also mediates interactions of thrombin with heparin and the fragment 2 domain of prothrombin (*12, 13*). Activation of bovine and human factor V proceeds through different pathways (*3, 8, 9, 14, 15*), and the role of thrombin exosites in human factor V activation has not been established. Although it has been suggested on the basis of sequence homology that thrombin may bind to the heavy-chain and fragment C1 regions of factor V through exosite I (*16, 17*), the sites of nonenzymatic interactions of thrombin on factor V have not been defined in direct-binding studies. Whether thrombin binds to factor Va or to the other factor V activation products has not been previously investigated. However, it has been suggested from the effect of human factor Va on thrombin activation of protein C that thrombin may interact with factor Va through the factor Va light chain (*18, 19*).

The present studies were undertaken to characterize interactions of thrombin with human factor V and its activation products involved in the mechanism of factor V activation. The peptide, hirudin$^{54-65}$ (Hir$^{54-65}$),[1] which binds specifically to exosite I (*12, 13*), is shown to inhibit factor V activation, demonstrating a key role for this site in regulating factor V activation. A new method for specific immobilization of proteinases through their active sites, based on previous studies of proteinase labeling with thioester peptide chloromethyl ketones (*20*−*22*), was developed for

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affinity chromatography studies of the thrombin exosite interactions. The scheme enables immobilization of thrombin and other serine proteinases with comparable, ≥92% active site specificity, indicating that the approach is highly specific and broadly applicable. Affinity chromatography of factor V and its activation products on active-site-immobilized thrombin demonstrate a previously undescribed, exosite I-dependent interaction of thrombin with factor Va that occurs through the factor Va heavy chain. Similar, exosite I-dependent thrombin binding to the heavy-chain region of factor V is concluded to contribute to the recognition of factor V as a thrombin substrate, and to regulate the specificity of proteolytic cleavages in the factor V activation pathway.

EXPERIMENTAL PROCEDURES

Protein Purification and Characterization. Human prothrombin and α-thrombin were obtained as described previously (*21*). Preparations of thrombin were >95% active as determined by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate (*21*). Bovine pancreatic, ((*p*-toluenesulfonyl)-Phe-CH_2Cl)-treated trypsin (Sigma type XIII) was dialyzed against 1 mM HCl, pH 3.0, and was 85−89% active by active site titration as described for thrombin. Human α-factor Xa was purified as described previously and was 96% active as determined by initial rate assay with the kinetic constants previously determined (*23*). Human Glu-plasminogen form 1 was purified by published procedures (*24*, *25*). Plasmin was prepared by activation of plasminogen with streptokinase immobilized on AffiGel-10 (BioRad; 4 mg of streptokinase/mL of gel) and purified by soybean trypsin inhibitor−Sepharose chromatography (*26*). The chromatography was carried out as before, except at 4 °C, and the plasmin was eluted with 10 mM MES, 10 mM HEPES, 0.8 M benzamidine, 20 mM 6-aminohexanoic acid, 1 mg/mL PEG, pH 5.2. After concentration by ultrafiltration with a PM 30 membrane, plasmin was dialyzed exhaustively against 5 mM MES, 0.3 M NaCl, 10 mM 6-aminohexanoic acid, and 1 mg/mL PEG, pH 7.0, quick-frozen in a dry ice−2-propanol bath, and stored at −70 °C. Plasmin was 71% active by active site titration as described for thrombin. Preparations of bovine serum albumin (BSA) monomer (Sigma) contained 0.36−0.41 mol of thiol/mol of protein determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described below. The thiol concentration was taken to represent the concentration of monomeric BSA. Protein concentrations were determined from the 280-nm absorbance with the following absorption coefficients ($(mg/mL)^{-1}\ cm^{-1}$) and molecular weights: prothrombin, 1.47, 72 000; meizothrombin des-fragment 1, 1.78, 50 000 (*27*); thrombin, 1.74, 36 600 (*28*); trypsin, 1.6, 23 300; factor Xa, 1.16, 46 000 (*29*); plasminogen, 1.69, 92 000; plasmin, 1.70, 84 000 (*24*,*30*); BSA, 0.67, 66 000.

Factor V was purified from recovered normal human plasma by modifications of published procedures (*1*, *3*, *31*). Nine liters of plasma was thawed partially and made 25 μg/mL soybean trypsin inhibitor, 140 μM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 0.2 μM FPR-CH_2Cl and FFR-CH_2Cl. The cryoprecipitate was removed by centrifugation at 4000*g*, and the vitamin K-dependent clotting factors were collected by barium chloride precipitation (*32*). The 6−12% (w/v) PEG fraction of the plasma supernatant at 4 °C was collected by centrifugation at 4000*g* and dissolved in approximately one tenth of the initial plasma volume of 50 mM Tris-Cl, 0.075 M NH_4Cl, 10 mM $CaCl_2$, 10 mM benzamidine, 0.02% NaN_3, pH 8.0, containing 140 μM phenylmethylsulfonyl fluoride and 0.4−0.5 μM of the above peptide chloromethyl ketones, and chromatographed on DEAE−Sepharose fast flow (5 cm × 38 cm) at room temperature. The column was washed with ∼5 column volumes of the pH 8.0 equilibration buffer and eluted with a 3 L-gradient of NH_4Cl up to 0.25 M. FPR-CH_2Cl and FFR-CH_2Cl were added at 0.1 μM to all of the buffers for the chromatographic steps just before their use. The factor V-containing fractions were pooled, inhibitors were added as above, and factor V was precipitated by addition of ammonium sulfate to 70% saturation at 4 °C. The precipitate was dissolved in a minimum volume of 50 mM Tris-Cl, 0.1 M NH_4Cl, 10 mM $CaCl_2$, 10 mM benzamidine, and 1 mg/mL PEG, pH 7.5 containing phenylmethylsulfonyl fluoride as above and 4−5 μM of the peptide chloromethyl ketones, and chromatographed on Sephacryl-S300 HR (2.5 cm × 117 cm) equilibrated with the pH 7.5 buffer at 4 °C. Factor V, eluting from this column slightly before the main protein peak, was pooled, and inhibitors were added as above before dialysis against ≥60 volumes of 25 mM HEPES, 0.050 M NH_4Cl, 5 mM $CaCl_2$, and 10 mM benzamidine, pH 6.5 at 4 °C. The factor V was made 0.2 μM FPR-CH_2Cl and FFR-CH_2Cl and chromatographed on S-Sepharose (1.5 cm × 27 cm) equilibrated with the pH 6.5 buffer at 4 °C. The column was washed with ∼8 column volumes of equilibration buffer and eluted with a 400-mL gradient of NH_4Cl up to 1 M. Pooled fractions were made 1 μM in the peptide chloromethyl ketones and concentrated to ∼5 mg/mL by YM 100 ultrafiltration. The peptide chloromethyl ketones were added to 5 μM, and the factor V was dialyzed overnight at 4 °C in 50 000 molecular weight cutoff dialysis tubing (Spectrum) against two changes of 1000−2000 volumes of 50 mM HEPES, 0.11 M NaCl, 5 mM $CaCl_2$, and 1 mg/mL PEG, pH 7.4 to reduce the final inhibitor concentration to a negligible level. The purified factor V, averaging 28 mg, was centrifuged, quick-frozen, and stored at −70 °C. Factor V concentration was determined from the 280-nm absorbance with an absorption coefficient of 0.89 $(mg/mL)^{-1}\ cm^{-1}$ (*2*) and a molecular weight of 330 000 (*3*). The same absorption coefficient was used for the complete mixture of thrombin activation products, while a value of 1 was assumed for protein mixtures from the chromatography experiments.

Factor V activity was measured by coagulation assay, relative to normal human plasma as a 1 unit/mL standard. The time for clot formation was measured at 37 °C with a fibrometer, after addition of 100 μL of thromboplastin (Sigma) containing 25 mM $CaCl_2$ to a mixture of 100 μL

[1] Abbreviations: FPR-CH_2Cl, (D-Phe)-Pro-Arg-CH_2Cl; FFR-CH_2-Cl, (D-Phe)-Phe-Arg-CH_2Cl; ATA-FPR-CH_2Cl, N^{α}-[(acetylthio)acetyl]-(D-Phe)-Pro-Arg-CH_2Cl; ATA-FFR-CH_2Cl, N^{α}-[(acetylthio)acetyl]-(D-Phe)-Phe-Arg-CH_2Cl; Hir^{54-65}, $hirudin^{54-65}$, Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-Hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol) 8000; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); iodoacetyl−agarose, iodoacetyl-(3,3′-iminobispropylamine)−agarose; BSA, bovine serum albumin; Va-h, factor Va heavy chain; Va-l, factor Va light chain; Va-hl, isolated two-subunit form of factor Va; U, clotting activity units.

each of 20 mM HEPES, 0.15 M NaCl, 1 mg/mL PEG, pH 7.4, factor V-deficient plasma (*33*), and the sample containing factor V.

Factor V was activated at 1.7−6.0 mg/mL in the storage buffer given above by addition of 50 nM thrombin and incubation for 40 min at 37 °C. These conditions resulted in maximum activation and processing of activation intermediates. The thrombin was inactivated by addition of 1 μM FPR-CH_2Cl and incubation for 15 min at room temperature, and the factor Va was stored at −70 °C.

Factor V Activation Experiments. For monitoring thrombin activation of factor V by SDS gel electrophoresis, aliquots of reaction mixtures in 50 mM HEPES, 0.11 M NaCl, 5 mM $CaCl_2$, and 1 mg/mL PEG, pH 7.4 at 37 °C were removed at various times and 1 μM FPR-CH_2Cl was added to stop the reaction. Reduced samples were analyzed by electrophoresis on 4%−15% gradient gels. The effect of Hir^{54-65} on factor V activation was measured from its effect on the rate of increase in factor V clotting activity. Aliquots removed from mixtures of 0.1 μM factor V and 3 nM thrombin were made 1 μM FPR-CH_2Cl and diluted extensively before measurement of clotting activity with normal plasma as a standard, at the same final peptide concentrations. The rates of activation were obtained from the linear least-squares fits of the activity versus time data for ≤60% of the full reaction. The dependence of the rate of activation on Hir^{54-65} concentration was fit by the equation for a hyperbola to obtain the inhibition constant and maximum decrease in the activation rate.

Binding of Hir^{54-65} to thrombin was measured from the increase in tryptophan fluorescence (295 nm excitation, 360 nm emission) with an SLM 8100 spectrofluorometer, using acrylic cuvettes. Titrations of 0.4 μM thrombin with the peptide under the conditions of the activation experiments given above were fit by the quadratic equilibrium binding equation to obtain the dissociation constant and maximum fluorescence change, with one binding site assumed for the peptide on thrombin.

Preparation and Characterization of Enzyme Derivatives. ATA-FPR-CH_2Cl and ATA-FFR-CH_2Cl were prepared as described previously (*20*, *21*). Reactions of proteinases with the inhibitors were followed by the loss of chromogenic substrate activity, and the inactivated enzymes were dialyzed against 50 mM HEPES, 0.3 M NaCl, 1 mM EDTA, and 1 mg/mL PEG, pH 7.0 (coupling buffer). Incorporation of inhibitor−thioester groups was quantitated from the amplitudes of the NH_2OH-initiated thiol burst reactions measured with 500 μM DTNB in coupling buffer (*34*). Chromogenic substrate activity was measured from the initial rates of hydrolysis at 405 nm, with 100 μM H-D-Phe-Pip-Arg-*p*-nitroanilide for trypsin and thrombin in 50 mM HEPES, 0.125 M NaCl, 1 mM EDTA, and 1 mg/mL PEG, pH 7.4; 200 μM methoxycarbonyl-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide for factor Xa in 20 mM sodium phosphate buffer, 0.25 M NaCl, 0.1 mM EDTA, and 1 mg/mL PEG, pH 7.4; and 100−200 μM H-D-Val-Leu-Lys-*p*-nitroanilide for plasmin in 0.1 M HEPES, 0.1 M NaCl, 1 mM EDTA, 10 mM 6-aminohexanoic acid, and 1 mg/mL PEG, pH 7.4.

Thrombin was inactivated (<0.2% active) with ATA-FPR-CH_2Cl by incubation of 30−44 μM enzyme with a 2.0−2.5-fold excess of inhibitor for 1−2 h at 25 °C. ATA-FPR-thrombin contained 0.83−0.92 mol of thioester/mol of active sites. Active-site-blocked thrombin (<0.02% active) was prepared by incubation of 30 μM enzyme with a 2.4-fold excess of FPR-CH_2Cl for 1 h. Active-site-blocked thrombin and prothrombin were incubated subsequently with ATA-FPR-CH_2Cl under conditions similar to those employed for preparing ATA-FPR-thrombin. ATA-FPR-meizothrombin des-fragment 1 containing 1.0 mol of thioester/mol of protein was obtained as described previously (*22*). Derivatives of trypsin, factor Xa, and plasmin were prepared similarly, by inactivation (<0.2% active) with a 2.1−2.7-fold excess of ATA-FPR-CH_2Cl (trypsin and factor Xa) or ATA-FFR-CH_2-Cl (plasmin) for 2 h in pH 7 buffers. Buffers for plasmin contained 10 mM 6-aminohexanoic acid and for factor Xa, 10 mM $CaCl_2$. Active-site-blocked enzymes were prepared similarly, and the blocked enzymes and zymogens were incubated with the thioester peptide chloromethyl ketones under the same conditions used for the active enzymes. Stoichiometries of inhibitor incorporation (mol of thioester/mol of active sites) were 0.89−1.03 (trypsin), 0.95 (factor Xa), and 1.01 (plasmin).

Immobilization Reactions. Cross-linked, beaded 6% agarose containing iodoacetyl groups linked by 3,3′-iminobispropylamine spacer arms (iodoacetyl−agarose) was obtained from Pierce Chemical Co. (SulfoLink Coupling Gel). The gel (0.5−1.0 mL) was washed rapidly with ∼40 volumes of N_2-purged coupling buffer in a 1-mL plastic column at room temperature and protected from light. The settled gel was diluted with an equal volume of coupling buffer, and coupling reactions were carried out by incubating 200 μL of the 1:1 gel slurry with an equal volume of protein. Incubations were mixed continuously with an Eppendorf Thermomixer at room temperature.

All proteins were in coupling buffer, except ATA-FPR-meizothrombin des-fragment 1 and plasmin. ATA-FPR-meizothrombin des-fragment 1 in 5 mM MES, 0.3 M NaCl, pH 6.0 was mixed with 0.1 volume of 1 M HEPES, 10 mM EDTA, pH 7.2 before reactions with the gel. Coupling buffer containing 15 mM 6-aminohexanoic acid (Fluka) was used for plasmin. Reactions at 4.7−6.4 μM enzyme derivative were initiated by addition of 0.1 M NH_2OH. The time courses were followed by centrifugation of the gel for 1 min at 14 000 rpm at various times and removal of a 2-μL aliquot of the supernatant. Protein concentrations were determined from the protein fluorescence (280 nm excitation, 350 nm emission) of the aliquots diluted in coupling buffer, with the purified proteins as standards. Immobilization reactions at higher protein concentrations were measured by 280-nm absorbance. Concentrations of proteins coupled were calculated from the difference between the coupling reaction and control incubations in which buffer was substituted for the gel. Reaction of 2-mercaptoethanol with iodoacetyl−agarose was measured from the change in thiol concentration determined with DTNB. The maximum percentage of immobilization for the proteinases was calculated from the concentration of protein bound following completion of the reaction (typically from 15 to 240 min) as a fraction of the initial active enzyme concentration.

The high-substitution thrombin−agarose matrixes used for the affinity chromatography studies were prepared by reacting 30 μM ATA-FPR-thrombin with 1.5−2.0 mL of gel, initiated with 0.1 M NH_2OH and incubated for 3−4 h. The gel was washed with coupling buffer, and BSA monomer

was added to 20−25 mg/mL of gel and incubated ∼12 h. Thrombin−agarose contained 3.5 or 4.2 mg of thrombin and 6.4 or 7.2 mg of BSA coupled/mL of gel. The low-substitution thrombin−agarose, containing 6 μM thrombin in the gel volume, was prepared by reaction of 4 μM ATA-FPR-thrombin with 3 mL of gel, followed by blocking with BSA monomer. BSA monomer−agarose was prepared by overnight reaction of 18 mg of BSA monomer/mL of gel and contained 11 mg of BSA coupled/mL of gel. 2-Mercaptoethanol-blocked iodoacetyl−agarose was prepared by reaction with 8−14 mM 2-mercaptoethanol for ∼18 h. The gels were stored at 4 °C in 5 mM MES, 0.3 M NaCl, 10 mM benzamidine, 0.02% NaN_3, pH 6.0 buffer containing 0.1 μM FPR-CH_2Cl.

Affinity Chromatography Experiments. Factor V/Va species (0.8−2.0 A_{280nm} units in 0.5−2.0 mL) were chromatographed on 1.5-mL columns (0.7 cm × 4 cm) at 3−4 mL/h in 50 mM HEPES, 0.11 M NaCl, 5 mM $CaCl_2$, 1 mg/mL PEG, 0.1 μM FPR-CH_2Cl, pH 7.4 buffer, at 22 °C. For the experiments with the low-substitution thrombin−agarose, 200 μg of factor Va in 300 μL was chromatographed on 3-mL columns at 1.5 mL/h. Fractions of 0.6 or 0.76 mL were collected, and elution of protein was measured by absorbance at 280 nm and factor V/Va activity by clotting assay. Protein peaks were concentrated by ultrafiltration with YM 10 membranes before SDS gel electrophoresis.

For preparative separation of fragment C1 from thrombin-activated factor V, up to 5 mg of factor Va was chromatographed on a 5.5-mL column of (2-mercaptoethanol)−agarose. This separation was performed at 4 °C to minimize degradation of factor Va. To dissociate the factor Va subunits, a sample of the isolated two-subunit form of factor Va (1−2 A_{280nm} units) was mixed with 0.1 volume of 50 mM HEPES, 0.125 M NaCl, 100 mM EDTA, pH 8.2 buffer to give 10 mM EDTA and pH 7.4. Factor Va activity was monitored over 2−3 h of incubation at room temperature until it was ≤4% of the initial value. The inactivated factor Va was dialyzed against 50 mM HEPES, 0.125 M NaCl, 2 mM EDTA, 0.1 μM FPR-CH_2Cl, pH 7.4.

RESULTS

Effect of Hirudin$^{54-65}$ on Factor V Activation. The involvement of exosite I of thrombin in factor V activation was investigated from the effect of the nonsulfated form of the carboxy-terminal hirudin dodecapeptide (Hir$^{54-65}$) on proteolytic processing of factor V, as observed by SDS gel electrophoresis (Figure 1). The peptide decreased the rate of disappearance of factor V, slowed the rate of appearance and disappearance of activation intermediates, and inhibited the rate of formation of the final factor Va light-chain and heavy-chain products. The main intermediates accumulating in the presence of 98 μM peptide were (Va-h)-E and C1-(Va-l) resulting from cleavage at Arg^{1018} (Figure 1). Inhibition of factor V activation by the peptide was characterized further from its effect on the thrombin-catalyzed rate of increase in factor Va activity measured by coagulation assays. Analysis of the decrease in the rate of factor V activation as a function of Hir$^{54-65}$ concentration gave an inhibition constant of 3.9 ± 1.4 μM and a maximum decrease in the activation rate of 93% ± 12% (Figure 2). This ∼14-fold inhibition agreed with the ∼10-fold decrease in the rate of

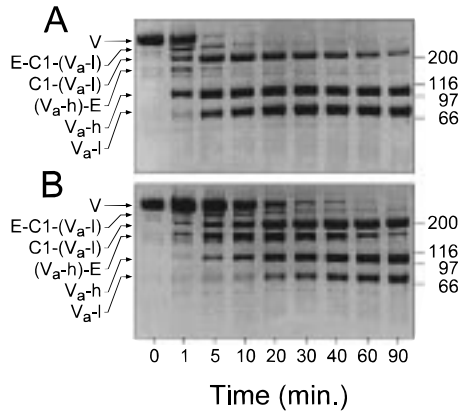


FIGURE 1: Effect of Hir$^{54-65}$ on thrombin cleavage of factor V. Coomassie blue-stained SDS gels are shown of reduced samples (∼12 μg) from reaction mixtures of 2.9 μM factor V and 3 nM thrombin. Samples were taken at the indicated times from reactions in the absence (A) and presence (B) of 98 μM Hir$^{54-65}$. The identities of the reaction intermediates and products are indicated, as well as the migration positions of molecular weight markers, with the molecular weights in thousands.

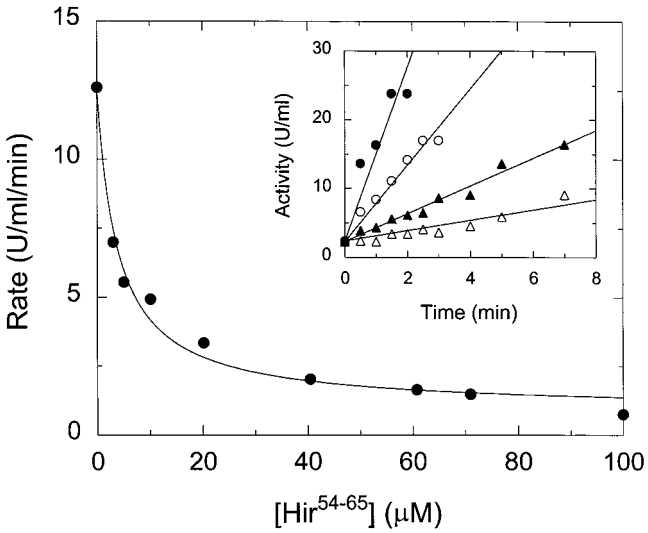
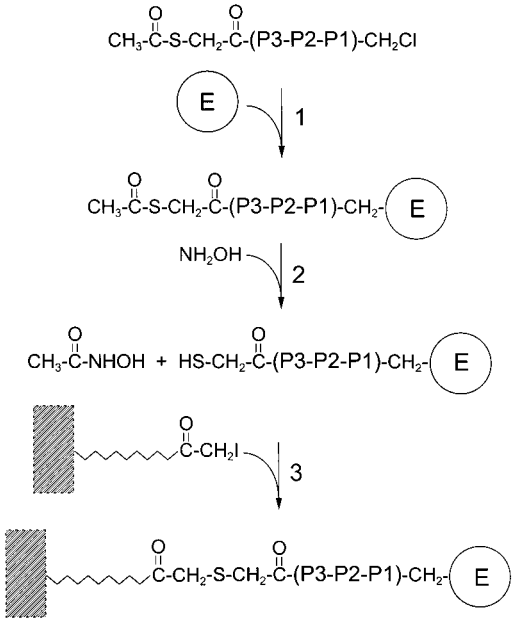


FIGURE 2: Effect of Hir$^{54-65}$ on the rate of factor V activation. The rate of activation (Rate) of 0.1 μM factor V by 3 nM thrombin is shown as a function of the concentration of Hir$^{54-65}$ ([Hir$^{54-65}$]). The solid line represents the least-squares fit of the data by a hyperbolic dependence with the parameters given in the text. Inset: Representative progress curves for the increase in factor V activity (Activity) are shown for reactions at Hir$^{54-65}$ concentrations of 0 μM (●), 5 μM (○), 40 μM (▲), and 100 μM (△). The lines represent the linear least-squares fits. Reactions were performed and analyzed as described under Experimental Procedures.

the proteolytic reactions estimated from the difference in time required to deplete factor V or generate equivalent levels of the final products in the SDS gel experiments (Figure 1). The inhibition constant for the peptide was in good agreement with the dissociation constant of 2.9 ± 1.5 μM determined from titration of the tryptophan fluorescence enhancement of 8.4% ± 1.7% accompanying binding of the peptide to thrombin (data not shown). This correlation indicated that binding of the peptide to exosite I of thrombin was responsible for the observed inhibition of factor V activation.

Active-Site-Selective Immobilization of Thrombin and Other Proteinases. A new method for active-site-specific immobilization of thrombin was developed to investigate binding of factor V and its activation products to exosites

Scheme 1



on thrombin by affinity chromatography. The approach consists of the three reactions shown in Scheme 1: (1) active-site-specific inactivation of a proteinase with a thioester tripeptide chloromethyl ketone, where the peptide is represented as P3-P2-P1, according to the nomenclature for proteinase substrates (*35*); (2) conversion of the thioester group of the covalently incorporated inhibitor to a free thiol with NH_2OH; and (3) reaction of the thioacetyl-P3-P2-P1-enzyme with iodoacetyl-(3,3′-iminobispropylamine)−agarose (iodoacetyl−agarose). To evaluate the specificity of the immobilization scheme, the reactions were quantitated for thrombin, the thioester peptide chloromethyl ketone, N^{α}-[(acetylthio)acetyl]-(D-Phe)-Pro-Arg-CH_2Cl (ATA-FPR-CH_2-Cl), and iodoacetyl−agarose. Irreversible inactivation of thrombin with ATA-FPR-CH_2Cl was accompanied by stoichiometric incorporation (0.83−0.92 mol/mol of active sites) of the inhibitor, in agreement with previous studies (*20, 21*). The immobilization reactions (reactions 2 and 3, Scheme 1) were measured by the NH_2OH-initiated disappearance of protein from the solution phase in mixtures of 5 μM covalent thrombin−inhibitor complex and an excess (100 μL) of iodoacetyl−agarose. As shown in Figure 3, 95% ± 3% of ATA-FPR-thrombin was immobilized within about 10 min. Immobilization of ATA-FPR-thrombin was almost completely dependent on generation of the inhibitor thiol group with NH_2OH, as shown by the absence of significant immobilization over 3−4 h in reactions without NH_2OH (Figure 3, Table 1). Thrombin that had been active-site-blocked with FPR-CH_2Cl prior to incubation with ATA-FPR-CH_2Cl, or prothrombin treated similarly with the thioester peptide chloromethyl ketone did not react significantly with the gel (Figure 3, Table 1). An estimate of 103% ± 7% for the active site specificity of the immobilization reactions was obtained from the fraction of enzyme immobilization that was dependent on blocking the active site (Table 1).

To determine whether the immobilization scheme could be applied to other proteinases, the reactions of Scheme 1 were quantitated for four other proteinases in time-course experiments similar to those done for thrombin. Inactivation of meizothrombin des-fragment 1, factor Xa, and trypsin with

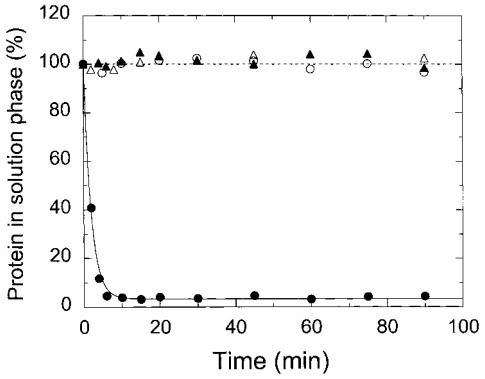


FIGURE 3: Time courses of thrombin immobilization reactions. The percent of the initial protein concentration remaining in the solution phase is shown as a function of time after initiating reactions of 5 μM protein with 100 μL of iodoacetyl−agarose. Results are shown for ATA-FPR-thrombin initiated with 0.1 M NH_2OH (●), ATA-FPR-thrombin in the absence of NH_2OH (○), FPR-thrombin in the presence of NH_2OH (▲), and prothrombin in the presence of NH_2OH (△). Reactions were performed as described under Experimental Procedures.

ATA-FPR-CH_2Cl, and plasmin with ATA-FFR-CH_2Cl resulted in stoichiometric incorporation (0.89−1.03 mol/mol of active sites) of the inhibitors. The enzyme derivatives reacted similarly with iodoacetyl−agarose, reaching levels of ≥92% immobilized within 10−15 min (Table 1). Estimation of the active site specificity of the reaction scheme from the dependence on blocking the active site for all of the enzymes studied indicated that ≥97% of the coupled enzymes were linked through the active site (Table 1).

The capacity of iodoacetyl−agarose for reaction with ATA-FPR-trypsin and BSA monomer was determined from the amount of protein coupled at saturating levels, which gave maximum levels of 318 and 166 μM in the gel volume, respectively (Table 1). This was a small fraction of the total concentration of thiol-reactive groups on iodoacetyl−agarose of 8800 μM determined with 2-mercaptoethanol. Reactions of 6−9 mg of ATA-FPR-thrombin with 1.5−2.0 mL of gel for preparation of matrixes for chromatographic experiments resulted in immobilization of 94−117 μM thrombin in the gel volume.

Chromatography of Factor V on Thrombin−Agarose. Interactions of factor V and its activation products with active-site-immobilized thrombin were studied in small-scale chromatographic experiments. Results are presented for thrombin−agarose in which residual iodoacetyl groups on the gel were blocked with BSA monomer, although similar results were obtained without the additional coupling of BSA. Results for thrombin−agarose were compared to those for control matrixes of (2-mercaptoethanol)−agarose or BSA monomer−agarose to assess the specificity of the interactions.

Factor V did not interact with the (2-mercaptoethanol)−agarose control matrix, as shown by its elution with the *I* 0.15 M, 5 mM Ca^{2+}, pH 7.4 equilibration buffer as a relatively sharp peak of protein and activity, and the absence of detectable protein eluted subsequently with buffer containing 1 M NaCl (Figure 4A). The majority of factor V eluted from thrombin−agarose in the equilibration buffer as a broad peak (Figure 4B). A small amount of protein was subsequently eluted with high-salt buffer, which contained factor V, as observed by SDS gel electrophoresis (Figure 4B). The

Table 1: Active Site Specificity of Proteinase Immobilization with Thioester Peptide Chloromethyl Ketones[a]

protein/ligand	gel capacity (μM in gel volume)	immobilization (%)				active site specificity (%)
		+NH_2OH	−NH_2OH	active-site-blocked	zymogen	
ATA-FPR-thrombin	≥117	95 ± 3	1 ± 5	−3 ± 6	2 ± 5	103 ± 7
ATA-FPR-meizothrombin des-fragment 1		95 ± 1	3 ± 3			97 ± 4
ATA-FPR-factor Xa	≥88	95 ± 4	2 ± 5	−2 ± 8	−3 ± 5	103 ± 10
ATA-FFR-plasmin	≥37	104 ± 4	5 ± 7	2 ± 5	−1 ± 5	98 ± 8
ATA-FPR-trypsin	318 ± 4	92 ± 12	6 ± 9	1 ± 13		99 ± 24
		100 ± 5[b]	6 ± 17[b,c]	2 ± 5[b,c]		98 ± 9
BSA monomer	166 ± 36					
2-mercaptoethanol	8800 ± 630					

[a] Percentage immobilization was calculated from the concentration of protein bound as a fraction of the initial active enzyme concentration for reactions of 4.7−6.4 μM enzyme with 100 μL of gel, monitored by protein fluorescence, as described under Experimental Procedures. Results are means ±2 SD from 2−4 reaction time courses, determined from data representing complete reaction. Active site specificity was calculated as described in the text. Gel capacity was determined from the amount bound in reactions of 100 μL of gel with an excess of ATA-FPR-trypsin (1 mg), BSA monomer (1.4−2.1 mg), or 2-mercaptoethanol. The capacities listed for other proteins represent the minimum levels based on the highest amount observed in larger-scale reactions at less than saturating protein concentrations. [b] Results from absorbance-monitored reactions. [c] Results of single reactions.

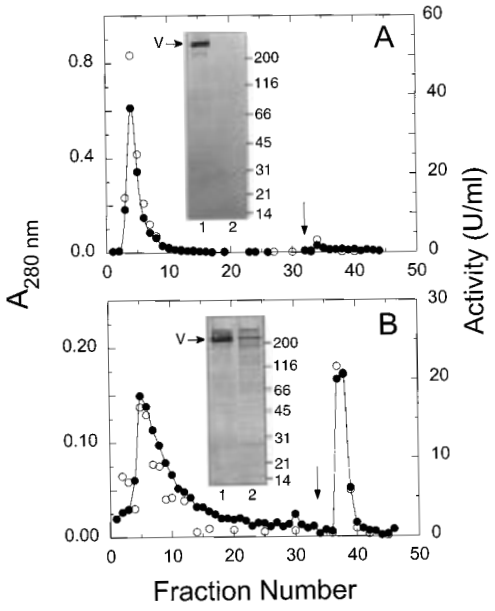


FIGURE 4: Chromatography of factor V on thrombin−agarose and (2-mercaptoethanol)−agarose. (A) Elution profile from chromatography of 1.3 mg of factor V on a 1.5-mL column of (2-mercaptoethanol)−agarose, monitored by absorbance at 280 nm (A_{280nm}, ●) and clotting activity (○) in 0.6-mL fractions. Elution with buffer containing 1 M NaCl was started at the point indicated by the arrow. Inset: SDS gel electrophoresis of samples (~3 μg) of fractions 3−16 (lane 1) and fractions 34−40 (lane 2). The factor V band is indicated (V), and the migration positions of molecular weight markers are indicated with the molecular weights in thousands. (B) Chromatography of 1.5 mg of factor V on thrombin−agarose as described in A. Inset: SDS gel electrophoresis of samples (~4 μg) of fractions 2−20 (lane 1) and fractions 35−42 (lane 2). Chromatography and electrophoresis were performed as described in Experimental Procedures.

protein from both of the peaks appeared functionally similar, in that the factor V was converted to factor Va by incubation with thrombin, as determined by SDS gel electrophoresis. Additional experiments produced similar results, but the amount of protein eluted with 1 M NaCl was variable, depending on the volume of equilibration buffer used to wash the column prior to high-salt elution. The elution of factor V in a broad peak indicated an interaction of factor V with thrombin−agarose but with a low affinity for the immobilized enzyme. The small amount of factor V eluted with salt represented residual factor V from the trailing edge of the broad peak, and as shown below, this fraction may also have contained low levels of factor V activation intermediates that bind more tightly to the matrix. Attempts to increase the affinity of factor V for the matrix by decreasing the ionic strength were not successful and resulted in large losses of protein.

Chromatography of Factor V Activation Products on Thrombin−Agarose. Chromatography of thrombin-activated factor V, consisting of a mixture of factor Va and fragments E and C1 on (2-mercaptoethanol)−agarose showed that fragment C1 interacted nonspecifically with the control matrix. Factor Va eluted as a noninteracting protein and activity peak, separated from an inactive protein peak that was eluted with buffer containing 1 M NaCl (Figure 5A). SDS gel electrophoresis showed that the nonbinding fraction contained factor Va and fragment E, while the bound protein was fragment C1, which was detected by periodic acid Schiff staining (Figure 5A). Similar results were obtained with BSA monomer−agarose, although fragment C1 was bound somewhat less efficiently to this matrix (results not shown). This property of fragment C1 was used to remove it from factor V activation products prior to further studies. Results of several such separations with different preparations of activated factor V, some of which contained residual levels of the major 220 000 molecular weight activation intermediate, showed that this species also bound to the control column.

Chromatography of factor V activation products, from which fragment C1 was removed, on thrombin−agarose resulted in elution with the equilibration buffer of a small, inactive protein peak that was identified as fragment E (Figure 5B). In contrast to the low affinity of factor V for immobilized thrombin, the two-subunit form of factor Va was bound quantitatively to the matrix under the same conditions and was eluted with buffer containing 1 M NaCl (Figure 5B). The capacity of thrombin−agarose for factor Va was high enough to bind at least 2 mg to 1.5 mL of gel.

Chromatography of Subunit-Dissociated Factor Va on Thrombin−Agarose. To determine whether one of the factor Va subunits was responsible for binding of factor Va to thrombin, the isolated two-subunit form of factor Va (Va-hl) was dissociated by incubation in EDTA and chromatographed on thrombin−agarose in the presence of EDTA. Factor Va light chain eluted with the equilibration buffer,

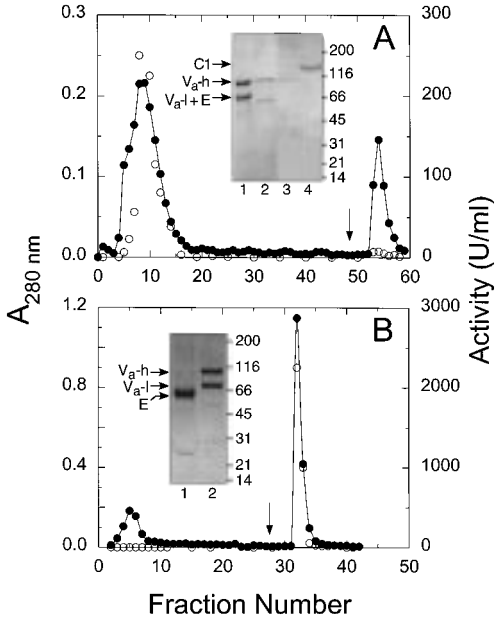


FIGURE 5: Chromatography of factor V activation products on thrombin−agarose and (2-mercaptoethanol)−agarose. (A) Chromatography of 1.6 mg of thrombin-activated factor V on a 1.5-mL column of (2-mercaptoethanol)−agarose, monitored by absorbance at 280 nm (A_{280nm}, ●), and clotting activity (○) in 0.6-mL fractions. Elution with buffer containing 1 M NaCl was started at the point indicated by the arrow. Inset: SDS gel electrophoresis of ∼6-μg samples of fractions 4−17 (lanes 1 and 2) and fractions 53−58 (lanes 3 and 4). Lanes 1 and 3 were stained with Coomassie blue and lanes 2 and 4 with periodic acid Schiff stain. Bands identified as fragments C1 (C1), factor Va heavy chain (Va-h), and factor Va light chain plus fragment E (Va-l+E) are identified, along with migration positions of molecular weight markers with the molecular weights in thousands. (B) Chromatography of 2.2 A_{280nm} units of fragment C1-depleted factor V activation products on thrombin−agarose as described in A. Inset: SDS gel electrophoresis of 6−10 μg of concentrated fractions 2−11 (lane 1) and fractions 31−38 (lane 2) stained with Coomassie blue. Bands corresponding to fragment E (E) and the factor Va heavy (Va-h) and light (Va-l) chains are indicated. Chromatography and electrophoresis were performed as described in Experimental Procedures.

indicating little or no affinity for immobilized thrombin, while the heavy chain was bound specifically and eluted with high-salt buffer (Figure 6). The isolated subunits were functionally active, as shown by the return of factor Va clotting activity when they were incubated together in the presence of calcium. Chromatography of EDTA-dissociated factor V activation products on BSA monomer−agarose showed no nonspecific binding of the dissociated subunits (results not shown).

Effect of Hir$^{54-65}$ on Factor Va Binding to Thrombin−Agarose. To determine if exosite I was involved in factor Va binding to thrombin−agarose, the effect of Hir$^{54-65}$ on the chromatography of factor Va was examined. For these experiments, a matrix containing a lower level of immobilized thrombin (6 μM) was used to reduce the affinity of factor Va for the column and to allow its elution under isocratic conditions. Under these conditions, factor Va eluted as a broad peak at 3.6 column volumes of equilibration buffer (Figure 7). This was significantly retarded compared to the elution of factor Va at 1.5 column volumes from a control column of BSA monomer−agarose, which represented no interaction. Equilibration of the thrombin−agarose column with Y^{63}-sulfated Hir$^{54-65}$ shifted the elution volume of factor

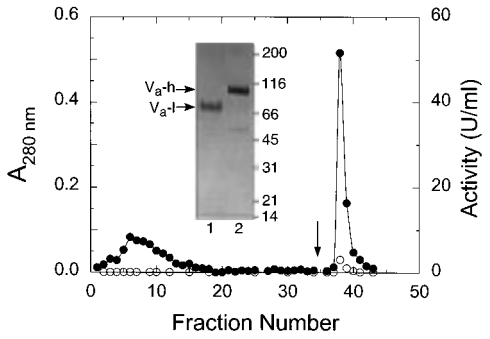


FIGURE 6: Chromatography of factor Va subunits on thrombin−agarose. Chromatography of factor Va-hl (1.0 A_{280nm} units) that was dissociated into subunits by incubation with EDTA as described under Experimental Procedures on a 1.5-mL column of thrombin−agarose in buffer containing 1 mM EDTA. Absorbance at 280 nm (A_{280nm}, ●) and clotting activity (○) measured in 0.6-mL fractions. Elution with buffer containing 1 M NaCl was started at the point indicated by the arrow. Inset: SDS gel electrophoresis of 4−8-μg samples of fractions 2−20 (lane 1) and fractions 37−43 (lane 2). Bands identified as the factor Va heavy chain (Va-h) and light chain (Va-l) are indicated. Migration positions of molecular weight standards are indicated with the molecular weights in thousands. Chromatography and electrophoresis were performed as described in Experimental Procedures.

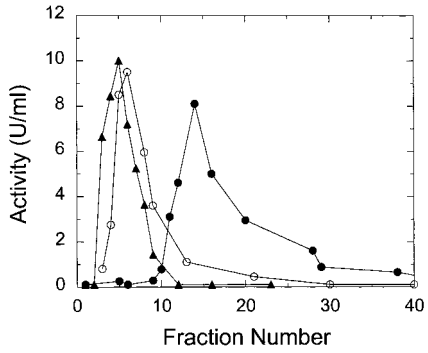


FIGURE 7: Effect of Hir$^{54-65}$ on elution of factor Va from thrombin−agarose. Chromatography of 200 μg of factor Va on 3-mL columns of thrombin−agarose containing 6 μM thrombin in the gel volume and BSA monomer−agarose was monitored by clotting activity (Activity) in 0.76-mL fractions. Results are shown for thrombin−agarose in the absence of Hir$^{54-65}$ (●) and after equilibration of the column with buffer containing 4 μM Y^{63}-sulfated Hir$^{54-65}$ (▲), and for BSA monomer−agarose in the absence of Hir$^{54-65}$ (○). Chromatography was performed as described in Experimental Procedures.

Va to 1.3 column volumes, indistinguishable from the result for the control matrix. This demonstrated that binding of Hir$^{54-65}$ to exosite I of immobilized thrombin prevented binding of factor Va.

DISCUSSION

The results of these studies demonstrate that exosite I of thrombin plays a key role in recognition of human factor V as a specific substrate and in regulation of factor V activation. A ∼14-fold inhibition of the rate of factor V activation by binding of Hir$^{54-65}$ to exosite I was correlated with inhibition of the initial cleavage reactions in factor V as well as those involved in processing of the activation intermediates to the final products. These observations extend the results of previous studies of a mixed system of bovine factor V activation by human thrombin which provided evidence of a role for exosite I (*11*). The products of human and bovine

factor V activation are very similar, but the activation pathways are different. Human factor V activation follows a kinetically preferred pathway in which cleavage at Arg^{709} occurs rapidly to generate the factor Va heavy chain, followed closely by cleavage at Arg^{1018}, and the slowest reaction at Arg^{1545} to generate the light chain (*3, 8, 9*). The first step produces an activation intermediate with little or no activity (*8*). This reaction is thought to accelerate the subsequent cleavages, with the ultimate generation of the light chain required for expression of full factor Va activity (*8*). Activation of bovine factor V by bovine thrombin proceeds instead with an initial preferential cleavage at the site corresponding to Arg^{1018} in the human protein (*14, 15, 36*). The present results showed increased accumulation of the intermediates resulting from cleavage at Arg^{1018} when exosite I-dependent reactions were inhibited by Hir^{54-65}, resulting in initial products similar to those described for bovine factor V. These results indicate that cleavages at Arg^{709} and Arg^{1545} are facilitated by exosite I, and suggest that a relatively smaller effect on cleavage at Arg^{1018} may account for greater accumulation of early intermediates resulting from cleavage at this site. Interestingly, the affinity of human thrombin for exosite I ligands, such as the hirudin peptide, is ~10-fold greater than that of bovine thrombin (*37*), suggesting that differences observed in the accumulation of factor V activation intermediates may be due to differences in the affinities of exosite I-mediated interactions.

A new method was developed for active-site-specific immobilization of proteinases to identify and characterize the sites of thrombin binding interactions involved in factor V activation by affinity chromatography. Results of quantitating the reactions of the immobilization scheme support the conclusion that this approach enables immobilization of thrombin and several other serine proteinases with equivalent, ≥92% active site specificity. This estimate is based on the fraction of active enzyme that is coupled to iodoacetyl−agarose and is similar to the estimate of ≥97% from the fraction of active enzyme coupled that is not eliminated by initial blocking of the active site. Both estimates are comparable to the specificity observed for labeling with fluorescence probes by the use of thioester peptide chloromethyl ketones (*21, 22*). The experiments designed to test the specificity of the reactions were done under conditions of a large excess of iodoacetyl−agarose, to enable quantitation of specific and nonspecific reactions. In larger-scale reactions for preparation of gels for affinity chromatography studies, the fraction of thrombin immobilized was typically lower (80−85%). This is thought to be due to partial saturation of reactive groups on the gel and to less efficient mixing of the gel in larger-scale reactions and the competing rate of thiol oxidation (*21, 34*). While this may decrease the efficiency of coupling slightly, the specific linkage of the enzyme to the matrix should not be affected.

The high specificity of enzyme immobilization is due largely to the selective labeling of only the catalytically active proteinases by peptide chloromethyl ketones (*38, 39*). Although the rates of inhibition of different proteinases show a very large dependence on inhibitor structure, these rates are typically much faster than the rate of nonspecific protein alkylation by peptide chloromethyl ketones and their derivatives, even for slow reactions (*21, 22, 38, 40−42*). Unlike the inhibition reactions, the reactivity of the thiol group exposed on the incorporated thioester peptide chloromethyl ketones does not depend greatly on the substrate specificity of the proteinase or the sequence of the inhibitor (*22*). These properties account for the absence of significant nonspecific immobilization observed in the present studies, and the similar reactivity with iodoacetyl−agarose of the thioester−inhibitor derivatives of five different proteinases. On this basis, the same approach should enable specific immobilization of any of the enzymes previously shown to be labeled by these inhibitors (*22, 43−45*), and likely a wide variety of other serine proteinases. The results support the structure for the immobilized proteinases shown in Scheme 1, in which the enzymes are linked to the gel by a single covalent bond. The resulting homogeneity of the coupled product leaves the surface of the enzyme unmodified and accessible for macromolecular binding, unlike conventional nonspecific coupling methods. Estimates of the capacity of iodoacetyl−agarose indicate that levels of ~100 μM enzyme in the gel volume can be readily achieved. These properties should be advantageous for preparative and analytical affinity chromatography studies of regulatory interactions of many different proteinases.

The active site immobilization approach enabled the characterization of binding interactions of thrombin with human factor V activation products for the first time. In view of the evidence for the role of exosite I in factor V activation, it was surprising that the affinity of factor V for thrombin−agarose was too low for the protein to be substantially retained by the matrix, while factor Va was more tightly bound under the same conditions. Experiments with control matrixes showed that factor Va binding was not due to nonspecific interactions with the gel, the spacer arm, or BSA used to block residual iodoacetyl groups. This observation may reflect an intrinsically higher affinity of factor Va for active-site-blocked thrombin compared to factor V, or only apparent differences in affinity due to differences in accessibility of the proteins to immobilized thrombin. Binding of factor Va to thrombin−agarose confirmed the expectation that exosites on the active-site-immobilized enzyme would be accessible to large macromolecules. However, in addition to the ~2-fold higher molecular weight of factor V compared to factor Va, the factor V molecule has a disproportionately larger hydrodynamic volume because of its asymmetric shape, which results in its greater exclusion from gel-filtration columns, such as the 6% agarose from which iodoacetyl−agarose is prepared (*10, 46−48*). Thus, the low affinity of factor V for thrombin−agarose is probably due partly to a small accessible chromatographic volume.[2]

Comparison of the interactions of factor V activation products with thrombin−agarose supports the conclusion that factor Va binds to thrombin through sites on the factor Va heavy chain. Activation fragment E and the factor Va light chain had no detectable affinity for thrombin. The results did not provide evidence for the previous suggestion that thrombin may bind with relatively high affinity to the factor Va light chain, which was based on kinetic studies of the rate-enhancing effect of the light chain on protein C

[2] Preliminary results of fluorescence studies indicate that factor V has a ~3-fold lower affinity for active-site-labeled thrombin compared to factor Va.

activation (*18*, *19*). This interaction could possibly be affected by blocking of the thrombin catalytic site by the peptide inhibitor in the immobilized enzyme, or more complex interactions may contribute to the effect on protein C activation. Unexpectedly, fragment C1 bound nonspecifically to control matrixes of iodoacetyl−agarose blocked with 2-mercaptoethanol or BSA. This interaction appears to involve the 3,3′-iminobispropylamine spacer arm and was observed only for fragment C1 and the longest-lived factor V activation intermediate that contains this fragment. This precluded an assessment of the interaction of fragment C1 with thrombin. A practical benefit of this effect, however, is that sequential chromatography of factor V activation products on (2-mercaptoethanol)−agarose and thrombin−agarose provides an economical means of isolating each of the factor V activation products.

The observation that binding of factor Va to thrombin is prevented by Hir^{54-65} demonstrates that this interaction is exosite I-dependent and indicates that similar interactions of comparable affinity do not occur with the other activation products, with the possible exception of fragment C1. This interaction is likely due to direct binding of factor Va to exosite I. However, it should be noted that the results do not exclude the possibility that binding of Hir^{54-65} to exosite I causes a conformational change in thrombin that weakens the affinity of factor Va binding at a different site on the enzyme. On the basis of the evidence for the role of exosite I in activation of factor V, binding of thrombin to the factor Va heavy chain is thought to reflect an interaction involved in recognition of factor V as a specific thrombin substrate. These results provide experimental evidence for previous suggestions that thrombin may interact with the heavy-chain and fragment C1 regions of factor V based on the proximity of the thrombin cleavage sites to sequences homologous to those known to bind exosite I (*16*, *17*). Thus, exosite I-dependent binding of thrombin to a site on the heavy-chain region of factor V may direct the initial recognition of factor V as a substrate and regulate the specificity of the cleavage reactions in the proteolytic processing pathway.

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