Active Site-independent Recognition of Substrates and Product by Bovine Prothrombinase

A FLUORESCENCE RESONANCE ENERGY TRANSFER STUDY

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The conversion of prothrombin to thrombin is catalyzed by prothrombinase, an enzyme complex composed of the serine proteinase factor Xa and a cofactor protein, factor Va, assembled on membranes. Kinetic studies indicate that interactions with extended macromolecular recognition sites (exosites) rather than the active sites of prothrombinase are the principal determinants of binding affinity for substrate or product. We now provide a model-independent evaluation of such ideas by physical studies of the interaction of substrate derivatives and product with prothrombinase. The enzyme complex was assembled using Xa modified with a fluorescent peptidyl chloromethyl ketone to irreversibly occlude the active site. Binding was inferred by prethrombin 2-dependent perturbations in the fluorescence of Oregon Green at the active site of prothrombinase. Active site-independent binding was also unequivocally established by fluorescence resonance energy transfer between 2,6-dansyl tethered to the active site of Xa and eosin tethered to the active sites of either thrombin or meizothrombin des fragment 1. Comparable interprobe distances obtained from these measurements suggest that substrate and product interact equivalently with the enzyme. Competition established the ability of a range of substrate or product derivatives to bind in a mutually exclusive fashion to prothrombinase. Equilibrium dissociation constants obtained for the active site-independent binding of prothrombin, prethrombin 2, meizothrombin des fragment 1 and thrombin to prothrombinase were comparable with their affinities inferred from kinetic studies using active enzyme. Our findings directly establish that binding affinity is principally determined by the exosite-mediated interaction of either the substrate, both possible intermediates, or product with prothrombinase. A single type of exosite binding interaction evidently drives affinity and binding specificity through the stepwise reactions necessary for the two cleavage reactions of prothrombin activation and product release.

Several of the highly specific proteolytic activation steps of the blood coagulation cascade are catalyzed by enzyme complexes assembled through protein-protein and protein-membrane interactions between a trypsin-like serine proteinase and a cofactor protein (1–3). This architecture is typified by the prothrombinase complex that catalyzes the conversion of prothrombin to thrombin. Specific recognition and proteolysis of two peptide bonds in prothrombin is achieved by prothrombinase, which is composed of the serine protease, Factor Xa, assembled with the cofactor protein, Factor Va, in the presence of a suitable membrane surface and calcium ions (1).

The serine proteinases of coagulation belong to the chymotrypsin family and possess catalytic domains that are structurally homologous to each other (4, 5). Despite this homology, the coagulation enzymes display distinctive specificities in cleaving their protein substrates (1). Substrate affinity and enzyme specificity in this enzyme family has been considered to be dictated by the engagement of unique peptidyl sequences preceding the scissile bond in the substrate with complementary structures surrounding the active site of the proteinase (6, 7).

In contrast, a series of contributions now support a major role for extended macromolecular interactions at sites (exosites) removed from the active site in determining the action of prothrombinase on its protein substrate (8–11). Studies of the individual reactions of prothrombin activation catalyzed by prothrombinase have been assessed using prethrombin 2 (Pre-2) (11) and meizothrombin des fragment 1 (mIIaF1) as valid substrate analogs for the two half-reactions of prothrombin activation (8, 10). For each of these substrate analogs, the data are consistent with a kinetic model in which protein substrate recognition by prothrombinase is achieved through stepwise

* This work was supported by National Institutes of Health Grants HL-62523 and HL-74124 (to S. K.) and P41 RR-001348 (to T. T.). The abbreviations used are: Pre-2, prethrombin 2; 2,6-Dns-EGR-CH2Cl, 2-dimethyloxiranopenaphthalene-6-sulfonfyl-t-glutamyl-glycyl-t-arginine chloromethyl ketone; ATA-EGR-CH2Cl, acetothioacetyl adduct of EGR-CH2Cl; ATA-FPR-CH2Cl, acetothioacetyl adduct of FPR-CH2Cl; EGR-CH2Cl-t-glutamyl-glycyl-t-arginine chloromethyl ketone; FPR-CH2Cl, t-phenylalanilanyl-t-prolyl-t-arginine chloromethyl ketone; FRET, fluorescence resonance energy transfer; IIa,K, thrombin inactivated with ATA-FPR-CH2Cl and modified with 5-(iodoacetamido)eosin following thioester hydrolysis; IIa,F1, thrombin inactivated with FPR-CH2Cl; mIIaF1, meizothrombin des fragment 1; mIIaF1a, mIIaF1 inactivated with FPR-CH2Cl; mIIaF1b, mIIaF1 inactivated with ATA-FPR-CH2Cl and modified with 5-(iodoacetamido)eosin following thioester hydrolysis; FCPS, small unilamellar vesicles composed of 75% (w/w) L-α-phosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; Xa,K, factor Xa inactivated by 2,6-Dms-EGR-CH2Cl; Xa,F1, factor Xa inactivated by EGR-CH2Cl; Xa,F1a, factor Xa inactivated with ATA-EGR-CH2Cl and modified with Oregon Green after maleimide following thioester hydrolysis; F2, fragment 2.

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interactions at an exosite, followed by active site docking and catalysis (8, 10). Substrate affinity is determined in a predominant way by the initial exosite binding interaction (8). Active site docking, which occurs in a subsequent unimolecular binding step, contributes in an undetectable or minor way to substrate affinity and instead affects maximum catalytic rate (8, 11). A distinctive feature of this kinetic pathway is that small ligands that bind reversibly to the active site of Xa within prothrombinase do not affect protein substrate affinity and therefore act as noncompetitive inhibitors (8, 10). In contrast, thrombin, the product of the reaction, acts as a competitive inhibitor of protein substrate cleavage without engaging the active site of Xa within prothrombinase (8, 9). The data suggest that equivalent exosite tethering interactions drive affinity in the productive pathway for the cleavage of both half-reaction substrate analogs for prothrombin activation as well as product release (10).

Surprisingly, it follows that substrate derivatives and product should bind to prothrombinase, with affinities comparable with those inferred from steady state kinetic studies, even following modification of the enzyme by peptidyl chloromethyl ketones that are known to occlude structures necessary for substrate docking at the active site (12, 13). We have directly tested this prediction by the use of fluorescent strategies to investigate the binding of substrates and product to prothrombinase assembled using Xa covalently inactivated with a peptidyl chloromethyl ketone.

EXPERIMENTAL PROCEDURES

Materials—l-α-phosphatidylcholine (hen egg) and t-α-phosphatidyl-serine (porcine brain) were from Avanti Polar Lipids (Alabaster, AL). P-Phenylalanine-t-prolyl-t-arginyl chloromethyl ketone (FPR-CH2Cl), 2-dimethylaminophenyl-6-sulfonyl-t-glutamyl-t-arginyl chloromethyl ketone (2,6-Dns-EGR-CH2Cl), and L-glutamyl-glycyl-L-(NH4)2SO4 (80% saturation), collected by centrifugation (100,000 g, 45 min), dissolved in 50% glycerol, and stored at −20 °C.

Substrates and products interact with greatly decreased affinity for factor Xa in the absence of factor Va (22). Thus, the trivial and nonspecific interactions due to the inner filter effect (29) before corrected spectra were obtained using tabulated values provided with the instrument. The quantum yields were taken as 0.71 for fluorescein in 0.1 M NaOH with known absorbance at 330 nm. Quantum yields of the eosin derivatives were recorded using a solution of fluorescein in 0.1 M NaOH with known absorbance at 330 nm. Quantum yields for the eosin derivatives were determined with a solution of fluorescein in 0.1 M NaOH with known absorbance at 496 nm. Quantum yields were taken as 0.71 for fluorescein (30) and 0.95 for fluorescein (31).

Binding of Prethrombin 2 to Prothrombinase Measured by Fluorescence Resonance Energy Transfer (FRET)—Spectral measurements were performed in assay buffer using a scanning fluorescence plate reader (SpectraMax Gemini; Molecular Devices, Inc., Sunnyvale, CA) maintained at 25 °C. Emission spectra were acquired using λex = 330 nm and scanning the emission monochromator between 420 and 600 nm. Reaction mixtures (200 μl) containing a fixed concentration of prethrombinase and increasing concentrations of IIa or mIIaF1 were prepared in wells of nonbinding, black, 96-well plates (catalog no. 3650; Corning Glass). Four different reaction mixtures to protein solutions were exchanged into this buffer either by dialysis or centrifugal gel filtration before use.

Binding of Prethrombin 2 to Prothrombinase—Reaction mixtures (200 μl) in 1×1-cm stirred quartz cuvettes containing 20 mM XaOG, 50 mM NaCl, and 0.4 μM PCPS in assay buffer were titrated with increasing concentrations of Pre-2. Steady state fluorescence intensity was measured following each addition at 25 °C using an SLM 8000 instrument with modifications by On-Line Instrument Systems (OLIS, Bogaert, GA) as described (14), with λex = 490 nm, λem = 515 nm with a long pass filter (K5-500, Schott, Duruyes, PA) in the emission beam.
cific component of the FRET signal was extracted by repeating the same experiment in the absence of factor Va followed by integration of spectra to yield $F_{D*} - A^*$, $F_{D^*} - A^*$, $F_{D^*} - A$ and $F_{D^*} - A'$. The fractional change in donor fluorescence as a function of acceptor concentration, corrected for trivial effects, was calculated from the integrated areas as follows.

$$
\frac{F - F_o}{F_o} = \left( \frac{F_{D^*} - A'}{F_{D^*} - A'} - \frac{F_{D^*} - A''}{F_{D^*} - A''} \right)
= \frac{F_{D^*} - A'}{F_{D^*} - A'} - \frac{F_{D^*} - A''}{F_{D^*} - A''}
\quad (Eq. 1)
$$

**Competitive Binding Measurements**—Exactly the same experimental strategy described above was used to assess the ability of various nonfluorescent derivatives to displace IIaE or mIIaE from prothrombinase. For measurements with IIa, FRET was measured with the same concentrations of prothrombinase constituents described above, IIaE (and unlabeled controls) fixed at 1.0 $\mu M$ and increasing concentrations of IIaE or prothrombin. Competition measurements with mIIaE were similarly performed using mIIaF1 (and unlabeled controls) fixed at 6.0 $\mu M$ and increasing concentrations of IIaE, Pre-2, or F2.

**Dynamic Fluorescence Measurements**—Fluorescence excited state lifetimes were measured by time-correlated, single photon counting using instrumentation described previously (33). The laser system consisted of a mode-locked Nd:YAG laser (Coherent Antares, Santa Paula, CA) used to synchronously pump a home-built single-jet cavity dumped dye laser (4-dicyanomethylene-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran in methanol/ethyleneglycol). Frequency doubling with a $\beta$-barium borate crystal yielded 330-nm excitation light. Emission from a 10 cm long pathlength cell was collected and analyzed using a two-exponential decay to extract the intensity-weighted average of the observed donor lifetimes in the presence of increasing concentrations of IIaE ($\tau_o$) or in the presence of increasing concentrations of IIaE ($\tau_r$).

**Data Analysis**—Based on established equilibrium constants and stoichiometries of the individual component interactions for prothrombinase assembly (34), concentrations of Va and PCPS were chosen to be saturating relative to factor Xa. The concentration of prothrombinase (E) was therefore considered equal to the limiting concentration of the Xa derivative present in the reaction mixture. When Va was absent, all of the factor Xa was considered to be present as the Xa-PCPS binary complex (34). Constants were extracted by nonlinear least squares analysis according to the indicated equations using the Levenberg-Marquardt algorithm (35). Uncertainties in the fitted parameters represent 95% confidence limits.

**Binding Studies Using XaOG**—The change in fluorescence intensity observed after the addition of increasing concentrations of Pro-2 to prothrombinase assembled using XaOG was analyzed according to Equation 2,

$$
1 - \frac{F}{F_o} = \left( 1 - \frac{F}{F_o} \right) \frac{[\text{Pre-2}]}{K_e + [\text{Pre-2}]} \quad (Eq. 2)
$$

where $F$ and $F_o$ denote fluorescence intensities observed in the presence and absence of Pre-2, $F_o$ is the fluorescence intensity at infinite concentrations of Pre-2, and $K_e$ is the equilibrium dissociation constant for the binding of Pre-2 to active site occluded prothrombinase. This equation is based on the assumptions that $K_{D} > [E]$ and that 1 mol of Pre-2 is bound per mol of E at saturation. Analysis according to Equation 2 yielded fitted values for $K_e$ and $1 - (F/F_o)$.

**Binding Measurements by FRET**—Binding was inferred from changes in donor fluorescence ($F$) at a fixed concentration ([D]$_0$) and increasing concentrations of acceptor ([A]$_0$). In the absence of acceptor, donor fluorescence intensity ($F_{D*}$) is given as follows,

$$
F_{D*} = \frac{\alpha_D [D]}{\tau_D}
\quad (Eq. 3)
$$

where $\tau_D$ is the excited state lifetime of the donor and $\alpha_D$ incorporates a series of constants related to the optical properties of the donor. In the presence of acceptor, total donor fluorescence results from the individual contributions of free ([D]$_0$) and acceptor-bound donor ([DA]$)_0$.

$$
F = \frac{\alpha_D [D]}{\tau_D} + \frac{\alpha_{DA} [DA]}{\tau_{DA}}
\quad (Eq. 4)
$$

where $\tau_{DA}$ is the excited state lifetime of the donor-acceptor complex and $\alpha_{DA}$ encompasses constants related to the optical properties of DA. Assuming that 1 mol of A binds per mol of D, substitution of the resulting conservation expression $[D] = [D]_0 - [DA]$ into Equation 4 yields the following.

$$
F = \frac{\alpha_D [D]}{\tau_D} + \frac{\alpha_{DA} [DA]}{\tau_{DA}}
\quad (Eq. 5)
$$

Dividing Equation 5 by Equation 3 results in the following.

$$
\frac{F}{F_o} = 1 + \frac{\alpha_{DA} [DA]}{\alpha_D [D]} \quad (Eq. 6)
$$

In the general case, fractional saturation of D is given by the following.

$$
\frac{[D]}{[D]_0} = \frac{[DA]}{[D]_0} - \frac{[A]}{[A]_0} - 2 \left( \frac{[D]}{[D]_0} - \frac{[A]}{[A]_0} - 2 \right)
\quad (Eq. 7)
$$

Combining Equations 6 and 7 with rearrangement yields the following.

$$
1 - \frac{F}{F_o} = \left( 1 - \frac{\alpha_{DA} \tau_D}{\alpha_D \tau_{DA}} \right) \frac{[DA]}{[D]_0}
\quad (Eq. 8)
$$

Analysis according to Equation 8 was used to derive fitted values for $K_e$ and a maximal signal at saturation corresponding to energy transfer efficiency, $T_{sat} = (1 - (F/F_o))$, as described by Lakowicz (36).

**Competition Studies**—Changes in donor quenching at one fixed concentration of acceptor and increasing concentrations of unlabeled substrate or product were analyzed as described previously (9, 37). Analysis according to these implicit functions was aided by assuming a stoichiometry of 1 mol of acceptor or competitor bound/mol of prothrombinase at saturation and by fixing maximal energy transfer to values directly determined from the FRET studies. This approach was used to derive fitted values for $K_j$ and $K_{Comp}$ reflecting the equilibrium dissociation constants for the interaction of acceptor and unlabeled competitor with prothrombinase, respectively.

**Interfluorophore Distances**—Forster distances ($r_j$) between the 2.6-dansyl moiety incorporated into the active site of Xa within prothrombinase and eosin incorporated into either IIaE or mIIaE were calculated using donor quantum yield and spectral overlap integrals as described (32, 36). For these calculations, the refractive index was taken as 1.4, and donor and acceptor dipoles were assumed to be randomly oriented, yielding $r^2 = 2/3$ (36). Interprobe distance was calculated from the Förster distance, and the efficiency of energy transfer was obtained for each of the two acceptor species from analysis according to Equation 8. Uncertainty of 10% was assumed for all values for which the S.D. was unknown. Uncertainties in the calculated distance were determined by propagating errors in the component terms (35).

**RESULTS**

**Binding of Prothrombin 2 to Prothrombinase**—Initial studies established that Pre-2 modestly quenched the fluorescence intensity of Oregon Green488 covalently tethered via a peptideyl chloromethylketone to the active site of factor Xa within prothrombinase. Increasing concentrations of Pre-2 produced a saturable change in fluorescence intensity of prothrombinase assembled with XaOG (Fig. 1), consistent with the ability of this substrate analog to bind to the enzyme complex despite an occluded active site. Analysis of the data, using this fluorescence change to infer binding, yielded a modest maximal quenching of $8.6 \pm 0.3\%$ and $K_j = 1.3 \pm 0.2 \mu M$ (Fig. 1). The data imply that Pre-2 can bind to prothrombinase even when the active site of factor Xa within the enzyme complex is irreversibly blocked. Taken along with the value of $K_j = 2.3 \pm 0.1 \mu M$ determined from initial velocity studies (8), the data suggest that the active site-independent binding of Pre-2 to prothrombinase can adequately account for the affinity of this half-reaction substrate analog for catalytically competent enzyme.

**Substrate and Product Interactions with Prothrombinase Measured by FRET**—There are a series of experimental limi-
tations to the single probe binding approach described above. Further work was pursued by the development of a FRET strategy to provide a signal unequivocally related to the binding of substrates and products to active site-blocked prothrombinase (36). The FRET approach exploited the ability to specifically incorporate fluorescent probes into the active site of Xa as well as the active sites of IIa and mIIa or mIIaΔF1 tethered with a peptidyl chloromethyl ketone (9, 10).

Screening of a series of fluorescent probes in this system yielded an adequate FRET signal with the 2,6-dansyl moiety incorporated into the active site of Xa as donor and eosin incorporated into the active sites of either IIa or mIIa or mIIaΔF1 as acceptor fluorophores. Absorbance and corrected fluorescence emission spectra for prothrombinase assembled with XaD, and IIaE illustrate a modest degree of spectral overlap between these probes (Fig. 2A). This feature proved advantageous in minimizing inner filter effects on donor fluorescence expected at μM concentrations of IIaE or mIIaΔF1 needed to measure binding to prothrombinase (below). Trivial contributions of the inner filter effect to donor quenching were further minimized by performing fluorescence measurements in front face format (29), with a scanning fluorescence plate reader.

Emission spectra obtained by excitation of the donor fluorophore in reaction mixtures containing prothrombinase assembled with XaD and IIaE are presented in Fig. 2B. In comparison with control spectra, the experimental spectrum obtained in the presence of both donor-labeled prothrombinase and acceptor-labeled thrombin was characterized by significant donor quenching (Fig. 2B). Acceptor fluorescence was only modestly enhanced because IIaE was present in excess of the concentration of prothrombinase (Fig. 2B). Minor effects on donor quenching were observed in the absence of Va (not shown), consistent with the greatly reduced affinity for the interaction of substrates or product with Xa in the absence of the cofactor (22). Further studies were therefore conducted by focusing on acceptor-dependent changes in donor fluorescence of prothrombinase, measured by integration of the signal between 420 and 450 nm, and by correcting for the small changes in donor fluorescence observed in the absence of Va (Equation 1).

Binding of Thrombin to Prothrombinase—Donor quenching increased saturaingly with increasing concentrations of IIaE (Fig. 3). The presence of IIaE shortened the excited state lifetime of the donor (Fig. 3, inset). Fractional changes in donor-excited state lifetime with increasing concentrations of acceptor parallelled changes in donor quenching (Fig. 3). These observations confirm resonance energy transfer between the donor fluorophore tethered to the active site of Xa within prothrombinase and the acceptor tethered to the active site of thrombin. Analysis of the donor quenching data yielded maximum quenching of 27.6 ± 1.3% at saturation and an equilibrium dissociation constant in agreement with the kinetically determined affinity for the binding of thrombin to prothrombinase (Table I). These data provide independent support for the conclusion derived from initial velocity studies that thrombin can bind to prothrombinase without engaging the active site of Xa within prothrombinase (8, 9).

The reversibility of the interaction measured by FRET was assessed by examining the ability of a nonfluorescent derivative of thrombin (IIaΔ) to displace IIaE bound to prothrombinase. Increasing concentrations of IIaΔ systematically decreased donor quenching to nearly zero in reaction mixtures containing XaD, incorporated into prothrombinase and a fixed

Fig. 1. Prethrombin 2 binding to prothrombinase. Reaction mixtures contained 20 nM XaD, 50 nM Factor Va, 50 μM PCPS, and increasing concentrations of Pre-2. Fluorescence intensity was measured using 0.82 μM XaD, 50 μM Factor Va, 50 μM PCPS, and increasing concentrations of Pre-2. Fluorescence intensity was monitored using λα = 490 nm and λβ = 515 nm. The line is drawn following analysis of the data according to Equation 2 using the following fitted parameters: K = 1.3 ± 0.2 μM, and 1 - (F/F0) = 0.006 ± 0.003.

![Fig. 1](image1)

Fig. 2. FRET between probes incorporated into the active site of thrombin and the active site of Xa within prothrombinase. A, absorbance spectra were measured using 0.52 μM XaD (D ABS) and 1.33 μM IIaE (A ABS). Corrected fluorescence emission spectra were obtained using 156 nM XaD, with 330 nm (D EM) and using 160 nM IIaE with 496 nm (A EM). All measurements were performed in assay buffer. B, fluorescence emission spectra obtained using λα = 330 nm are illustrated for reaction mixtures in assay buffer containing 0.4 μM Va, 50 μM PCPS, and either 0.2 μM XaD plus 1.7 μM IIaΔ (for D), 0.2 μM XaD plus 1.7 μM IIaΔ (for A), or 0.2 μM XaD plus 1.7 μM IIaΔ (for D plus A).
concentration of IIaE (Fig. 4). Analysis of these data according to the expressions describing mutually exclusive interactions between IIaE or IIa, and prothrombinase yielded equilibrium dissociation constants in tolerable agreement with each other and the value directly determined using IIa (Table I). These data establish that binding interactions inferred by FRET are freely reversible and that both IIaE and IIa bind indistinguishably to prothrombinase independent of the active site of Xa within the enzyme complex. The data also establish that the acceptor fluorophore tethered to the active site of thrombin does not detectably alter the interaction of product with prothrombinase.

**Binding of Meizothrombin-des Fragment 1 to Prothrombinase**—Previous studies have established meizothrombin, which contains an intact Arg274—Prothrombinase—does not detectably alter the interaction of product with acceptor fluorophore tethered to the active site of thrombin within the enzyme complex. The data also establish that the ability to prothrombinase independent of the active site of Xa mixes containing 0.2 μM factor Va, 50 μM PCPS, and 8 μM IIaE (upper trace) or 8 μM IIa (lower trace).

![Image](https://example.com/image.png)

**Table 1**

| Method | Acceptor | Titrant | $K_d$ ± S.D. $^a$ | $K_d$ or $K_s$ ± S.D. $^a$ |
|--------|----------|---------|------------------|--------------------------|
| Xa OG  | NA       | Pre-2   | $1.4 \pm 0.2$    | $2.3 \pm 0.1$             |
| FRET   | IIaE     | IIaE    | $0.9 \pm 0.2$    | $2.6 \pm 0.2$             |
| FRET competition | IIaE | IIaE | $0.5 \pm 0.2$ | $2.6 \pm 0.2$ |
| FRET   | mIIaΔF1E | mIIaΔF1E | $3.0 \pm 0.6$ | $11.7 \pm 1.4$ |
| FRET competition | IIaE | IIaE | $1.2 \pm 0.1$ | $2.6 \pm 0.2$ |

$^a$ Equilibrium dissociation constants presented ± 95% confidence limits correspond to those inferred for the binding of the substrate or product species listed as either acceptor or titrant to prothrombinase.

$^b$ Substrate and product affinities for prothrombinase inferred from initial velocity and product inhibition studies previously reported (8–10, 60).

$^c$ NA, not applicable.
both Pre-2 and mIIαΔF1 by prothrombinase (10). Our observations also imply limited effects, if any, of direct interactions between the F2 domain of the substrate and prothrombinase in mediating substrate recognition.

**Distance Calculations**—Relevant constants measured for the two donor-acceptor pairs used in the present study yielded Förster distances of 40.5 ± 0.8 or 41.7 ± 0.9 Å between the probes tethered at the active sites of Hα or mIIαΔF1g and the active site of prothrombinase (Table II). Based on the energy transfer efficiencies inferred for acceptor-saturated donor (Table II), these values yield interprobe distances of 47.7 ± 1.3 for Hα and 53.6 ± 1.9 Å for mIIαΔF1 bound to prothrombinase. These findings suggest comparable overall geometries for Hα and mIIαΔF1 bound to prothrombinase.

There are a series of established caveats associated with assuming that the donor and acceptor dipoles are randomly oriented (36). The peptidyl chloromethyl ketone tether can add significantly to extend the distance of the fluorophore from each of the active sites (38, 39). Recognizing these caveats, the molecular dimensions of the proteinase domains of Xa, Hα, and mIIαΔF1, determined by x-ray crystallography (12, 40, 41), raise the possibility that the active sites of Hα and mIIαΔF1 are comparably oriented and probably facing away from the active site of Xa within prothrombinase in the enzyme-substrate or enzyme-product complex.

**Discussion**

Multiple lines of evidence now point to a predominant contribution of exosite interactions rather than active site engagement in driving substrate affinity and binding specificity for each of the two half-reactions of prothrombin activation catalyzed by prothrombinase (8–10). These newer concepts contrast with the assumed basis for substrate affinity and proteinase specificity resulting from the engagement of unique sequences flanking the cleavage sites in the substrate with complementary sites in the active site of the proteinase (6, 7). However, evidence for a major contribution of exosite binding rather than active site engagement in protein substrate recognition by prothrombinase has largely been derived from steady state kinetic studies wherein interpretations can be highly model-specific. Results of the equilibrium binding studies described in the present work now provide unambiguous support, in a model-independent way, for the idea that substrate derivatives and product can indeed bind to prothrombinase even when the active site of the catalyst is covalently occluded. The fact that affinities determined in this way are not greatly weaker than those inferred from kinetic studies lends further support to the conclusion that it is exosite binding rather than active site docking by the substrate that largely determines
substrate affinity and binding specificity for prothrombinase.

A primary role for exosite-mediated interactions in the action of thrombin on several of its substrates has been established by extensive studies and confirmed by x-ray crystallography (42, 43). The collective findings with prothrombinase establish that this strategy also serves to enforce binding specificity in coagulation reactions where there is only one known biological substrate. Signature features associated with exosite-dependent substrate recognition are also evident in the action of the VIIa-TP complex on factor X (44, 45), factor X binding to IXa-VIIIa (46), Va cleavage by activated protein C (47), and IXa formation catalyzed by Xla (48). Thus, exosite-mediated function increasingly appears to represent a generalized mechanism underlying substrate specificity in the coagulation enzymes.

Equilibrium dissociation constants determined for active-site-independent interaction of substrates (prothrombin, Pre-2, and mIIaF1) and product (IIa) with prothrombinase were, if site-independent interaction of substrates (prothrombin, Pre-2, enzymes. A suggested mechanism underlying substrate specificity in the coagulation reactions where there is only one known biological substrate. Signature features associated with exosite-dependent substrate recognition are also evident in the action of the VIIa-TP complex on factor X (44, 45), factor X binding to IXa-VIIIa (46), Va cleavage by activated protein C (47), and IXa formation catalyzed by Xla (48). Thus, exosite-mediated function increasingly appears to represent a generalized mechanism underlying substrate specificity in the coagulation enzymes.

Table II shows the distances and energy transfer efficiencies for various pairs of probes, including the eosin probe at the active site of IIa or mIIa and the syl probe tethered at the active site of Xa within prothrombinase. The distances were determined by fluorescence resonance energy transfer (FRET) and are presented as mean values ± standard deviations (S.D.).

The ability of prothrombin derivatives to interact with factor Va through the F2 domain was previously considered essential for the enhanced action of prothrombinase on its protein substrate (52). More recent studies have established equivalent enhancing effects of Va on prothrombinase function regardless of the presence of the F2 domain in the substrate (22). The inability of F2 to block the binding of mIIaF1 to prothrombinase supports the results of kinetic studies (10) and indicates that interactions between the F2 domain and Va within prothrombinase probably contribute in a minor way, if at all, to substrate affinity for the enzyme complex.

Comprehensive work has established the ability of (pro)exosite I in thrombin and prothrombin derivatives to mediate interactions with factor Va (53–55). Inhibition of protein substrate cleavage by prothrombinase by (pro)exosite I ligands (9, 55), including peptidyl sequences from Va (56), has led to the suggestion that this interaction may play a key (55) or even singular (56) role in driving substrate affinity. These suggestions are in line with previous conclusions derived from kinetic studies of prothrombin activation by factors Va and Va in the absence of membranes (57). However, ligands that target extended surfaces on the proteinase domain of Xa have been shown to compete with exosite-dependent substrate binding to prothrombinase (51, 58). Competitive inhibition of protein substrate cleavage by prothrombinase has been documented with proteolytic derivatives of thrombin lacking exosite I (9). Finally, affinities of the various prothrombin derivatives for prothrombinase measured in the present work do not parallel differences in affinity observed for (pro)exosite I ligands for the various prothrombin derivatives (59). Overall, the observations suggest that whereas the interaction between Va and the (pro)exosite I region of the substrate probably contributes to substrate binding, this represents only one facet of a more complex mechanism underlying exosite-dependent recognition of the protein substrate by prothrombinase.

Interfluorophore distances of 48–54 Å between the 2,6-dansyl probe tethered at the active site of Xa within prothrombinase and the eosin probe at the active site of IIa or mIIaF1 further indicate that both product and substrate are bound in...
a comparable way to the enzyme complex. There are a series of well established caveats associated with the interpretation of distances calculated in these systems and the impact of the assumption yielding $\rho^2 = 2/3$ (36). Even considering the realistic limits of $\rho^2$, expected to yield deviations in calculated distance of ~20% (36), the data suggest that interprobe distances are comparable to the diameter of the proteinase domain of IIa (40). Thus, the active sites of prothrombinase and enzyme-bound IIa are relatively far from each other and possibly pointing away from one another. Although the significance of this observation is presently unclear, these distances may provide important constraints in future structure-function and molecular modeling studies of the interactions between prothrombinase and its substrate derivatives.

In summary, we have used fluorescence approaches to test a surprising and key prediction that arises from studies assessing the kinetic mechanism of protein substrate recognition by prothrombinase (8–10). Equilibrium binding studies now provide direct verification that prothrombin, its derivatives, and coagulation factors of coagulation.

The narrow and distinctive specificities of the serine proteinases do not contribute in an obvious way to affinity and binding specificity. These findings challenge existing paradigms explaining functional diversity in the chymotrypsin family and the narrow and distinctive specificities of the serine proteinases of coagulation.

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Active Site-independent Recognition of Substrates and Product by Bovine Prothrombinase: A FLUORESCENCE RESONANCE ENERGY TRANSFER STUDY

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