The Contribution of Factor Xa to Exosite-dependent Substrate Recognition by Prothrombinase*

Received for publication, November 12, 2001, and in revised form, December 25, 2001
Published, JBC Papers in Press, January 8, 2002, DOI 10.1074/jbc.M110848200

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Kinetic studies support the concept that protein substrate recognition by the prothrombinase complex of coagulation is achieved by interactions at extended macromolecular recognition sites (exosites), distinct from the active site of factor Xa within the complex. We have used this formal kinetic model and a monoclonal antibody directed against Xa (αBFX-2b) to investigate the contributions of surfaces on the proteinase to exosite-mediated protein substrate recognition by prothrombinase. αBFX-2b bound reversibly to a fluorescent derivative of factor Xa (Kd = 17.1 ± 5.6 nM) but had no effect on active site function of factor Xa or factor Xa saturably assembled into prothrombinase. In contrast, αBFX-2b was a slow, tight binding inhibitor of the cleavage of either prethrombin 2 or meizothrombin des-fragment 1 by prothrombinase (Kd = 0.55 ± 0.05 nM). Thus, αBFX-2b binding to factor Xa within prothrombinase selectively leads to the inhibition of protein substrate cleavage without interfering with active site function. Inhibition kinetics could adequately be accounted for by a kinetic model in which prethrombin 2 and αBFX-2b bind in a mutually exclusive way to prothrombinase. These are properties expected of an exosite-directed inhibitor. The site(s) on factor Xa responsible for antibody binding were evaluated by identification of immunoreactive fragments following chemical digestion of human and bovine Xa and were further confirmed with a series of recombinantly expressed fragments. These approaches suggest that residues 82–91 and 102–116 in the proteinase domain contribute to αBFX-2b binding. The data establish this antibody as a prototypic exosite-directed inhibitor of prothrombinase and suggest that the occlusion of a surface on factor Xa, spatially removed from the active site, is sufficient to block exosite-dependent recognition of the protein substrate by prothrombinase.

Although factor Xa can activate prothrombin by itself, the catalytic efficiency for prothrombin activation is increased by a factor of ~300,000 upon the incorporation of the proteinase into the prothrombinase complex (2, 3, 5). Factor Va is considered to contribute to a major way to the enhanced catalytic efficiency for prothrombin activation by prothrombinase (2, 6). However, it appears increasingly improbable that the role of the cofactor is primarily realized by perturbing the catalytic site of factor Xa, as has been tacitly assumed (2, 6, 7). Instead, the data are more consistent with the idea that factor Va either contributes additional binding sites for the protein substrate and/or perturbs sites on factor Xa removed from the catalytic site, leading to enhanced extended interactions between the protein substrate and prothrombinase (8, 9). It therefore follows that ground state rather than transition state effects likely play a significant role in the enhanced catalytic efficiency of prothrombinase toward its protein substrate.

Support for the latter concept has accumulated from mechanistic studies of the individual half-reactions of prothrombin activation (10, 11). In these approaches, the kinetics of substrate recognition and cleavage at the two sites has been discretely assessed using proteolytic derivatives of prothrombin containing one of the two cleavage sites as substrate analogs. In either case, the bimolecular reaction between the protein substrate and prothrombinase results from interactions between extended macromolecular interaction sites (exosites) on the enzyme removed from the catalytic site and substrate sites distinct from structures surrounding the scissile bond (10, 11). Docking of substrate structures at the active site of the enzyme occurs in a second unimolecular binding step preceding scissile bond cleavage. The exosite-binding step dominates the perceived affinity of the enzyme for the protein substrate (10–12).

The contributions of extended surfaces in factors Xa and Va within prothrombinase toward exosite interactions with the protein substrate remain uncertain. An important direct role for factor Va in binding the substrate is implied by the established ability of the cofactor to bind the fragment 2 domain of prothrombin (13–15), and by evidence documenting an interaction between the fibrinogen-binding site in the proteinase domain of thrombin and factor Va (16–18). However, a dominant role for such interactions in exosite-dependent protein substrate recognition is neither supported by kinetic studies using substrate derivatives lacking the fragment 2 domain nor by inhibition studies with proteolytic derivatives of thrombin lacking part or all of the fibrinogen-binding site (12).

Selective inhibition of protein substrate cleavage without restricting access of small ligands to the active site of factor Xa within prothrombinase or interfering with the assembly of the enzyme complex is the hallmark of an exosite-directed inhibitor (10, 11, 19). A monoclonal antibody directed against the proteinase domain of factor Xa that exhibits some of these properties has been described in previous work (20). We have fur-
ther characterized the properties of this antibody to probe the contributions of extended surfaces in factor Xa toward exosite-dependent macromolecular substrate recognition by prothrombinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—The IMPACT-CN protein expression kit, the pTY2B vector, *Escherichia coli* strain ER 2566, and restriction enzymes were from New England Biolabs (Beverly, MA). Immobilization membranes were from Millipore (Bedford, MA). The Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, CA). The peptideyl substrate methoxy carbonyl-o-cyclohexylglycyl-glycyl-γ-arginine-p-nitroanilide (Spectrozyme Xa, SpXa)1 was from American Diagnostica (Greenwich, CT). Stock solutions (4 × 10^−3 M) were prepared in water, and concentrations were determined using ε_{280} = 8270 M^−1 cm^−1 (21). Lyophilized vesicles from Sonicated PCPS were purchased from Venom Supplies (Tanunda, Australia). Cyano gen bromide, hen egg t-α-phosphatidylycholine (PC), and bovine brain t-α-phosphatidyl-t-serine (PS) were from Sigma. Oregon Green_350 maleimide was from Molecular Probes (Eugene, OR). The inhibitor 4-aminobenzamide (PAB) was from Aldrich, and concentrations of PAB in water were determined using ε_{280} = 15,000 M^−1 cm^−1 (22). Polyethylene glycol M_20,000 (PEG) was from J. T. Baker Inc. Phospholipid vesicles composed of 75% (w/w) PC and 25% (w/w) PS (PCPS) were prepared by sonication and differential centrifugation and quality-controlled by quasielastic light scattering as described (23). Phospholipid concentrations are expressed in terms of head group concentration determined by a colorimetric phosphate assay (24). Unless otherwise noted, all kinetic measurements were conducted in 20 mM Hepes, 0.15 M NaCl, 2 mM CaCl_2, 0.1% (w/v) PEG, pH 7.5 (assay buffer), at 25 °C.

**Proteins**—All measurements of Xa and prothrombinase function were performed using proteins of bovine origin. Instances in which human Xa was used to probe antibody reactivity either by fragmentation or by expression of recombinant derivatives are explicitly identified in the text. Bovine factor Va, factor X, prothrombin, prethrombin 1, and prethrombin 2 were isolated from bovine plasma using procedures described previously (12, 25). Human factor X was isolated from plasma by affinity chromatography on benzamidine-Sepharose (27, 28). Active site titration of several preparations using P-nitrophenoI-p'-guanidinobenzoate (29) yielded 1.08–1.15 mol of active sites per mol of factor Xa. Bovine factor Xa was inactivated with acetothioacetyl-L-Glu-Gly-L-Arg–CH_3Cl and labeled with Oregon Green_350 maleimide to yield OG_350-Xa as described (12). The factor Xa-like enzyme (trocacin) from the venom of *T. carinatus* was purified by barium citrate adsorption of the crude venom. Barium citrate was eluted with 0.2 M EDTA, dialyzed into 20 mM HEPES, 0.15 M NaCl, pH 7.5, and applied to a 1 × 10-cm HMG column (Pierce Separation Products, Framingham, MA) at 6 ml/min. Bound protein was eluted with a linear gradient of increasing NaCl in the same buffer (0.15 to 1.0 M) developed over 12 min. Tocacin eluted in a sharp peak at 0.6 M NaCl well resolved from other contaminants. Fractions containing trocinarin were pooled, concentrated by amnioum sulfate fractionation (80% saturation), dissolved in 50% glycerol, and stored at −20 °C. Recombinant t-α-phosphatidylcholine peptide (rTAP) was expressed in *Pichia pastoris* and purified as described (30). Monoclonal antibody eBFX-2b, prepared using bovine factor X as antigen, was either plasma or gift from Dr. William Church, University of Vermont, or purchased from Hematologic Technologies (Essex Junction, VT). All protein preparations were quality-controlled by SDS-PAGE analysis (31). Concentrations were determined using the following mo-lecular weights and extinction coefficients (ε_280, 1 mg/ml): bovine X, 56,000, 1.24; bovine Xa, 45,300, 1.24 (32, 33); bovine Va, 168,000, 1.74 (34, 35); bovine prethrombin 2, 37,400 (36); 1.95; human X, 58,900, 1.16; human Xa, 46,000, 1.16 (37); trocinarin, 45,000, 1.24; eBFX-2b, 150,000, 1.4.

**Steady State Fluorescence Measurements**—Steady state intensities were measured using a PFI QuantaMaster fluorescence spectrophotometer (PTI, Lawrenceville, NJ) using stirred 1 × 1 cm² quartz cuvettes maintained at 25 °C. Analog ratiometric fluorescence intensity was measured using λ_ex = 485 nm and λ_em = 515 nm with a KV-500 long pass filter (Schott, Duryea, PA) in the emission beam. Samples (2.0 ml) prepared either in assay buffer or in assay buffer containing 100 μM EDTA instead of 2 mM Ca²⁺ were titrated with incremental microliter additions of the indicated reagent. After each addition, samples were maintained in the dark for 5 min, and then the intensity was measured by averaging 15 readings obtained at 1-s intervals. The cycle of incubation and measurement was periodically repeated throughout the titration to ensure that the initial incubation period was sufficient to obtain a limiting signal. Three samples were used to derive data for each titration as follows: Sample A, 25 μM OG_350-Xa titrated with incremental additions of eBFX-2b; Sample B, 25 μM OG_350-Xa titrated with incremental additions of buffer; and Sample C, 25 μM Xa titrated with incremental additions of eBFX-2b. The primary fluorescence signal (Sample A) was corrected for dilution (Sample B) and minor scattering effects (Sample C) by the expression shown in Equation 1,

\[
\frac{F}{F_0} = \frac{F_0 - F_s}{F_0 - F_e} - 1
\]

where \(F_0\) is the corrected ratiometric signal in the absence of tritant.

**Kinetic Measurements with Peptidyl Substrates and Active Site-directed Ligands**—Steady state kinetic constants for the cleavage of SpXa were determined in assay buffer as described (10), using prothrombinase preassembled with 0.5 μM Xa, 50 μM PCPS, and 20 nM Va. For measurements in the presence of eBFX-2b, the enzyme solution was preincubated with 200 nM antibody for 1–3 h at ambient temperature prior to substrate addition.

The influence of eBFX-2b on the inhibition of prothrombinase by PAB was determined in an analogous approach using increasing concentrations of SpXa and different fixed concentrations of PAB corresponding to 0, 15, 30, 60, and 120 μM. The KM value for the inhibition of Xa by rTAP was measured following incubation of reaction mixtures containing 0.5 μM Xa and increasing concentrations of rTAP in assay buffer either in the presence or absence of 200 nM eBFX-2b. Following incubation for 1 h at ambient temperature, the reaction mixtures were initiated with 100 μM SpXa.

**Kinetic Measurements of Protein Substrate Cleavage**—Steady state, initial velocities of prethrombin 2 cleavage by prothrombinase were determined from discontinuous measurements of thrombin formation as detailed previously (10). Reaction mixtures (200 μl) contained 1 nM Xa, 50 μM PCPS, 25 nM Va, and increasing concentrations of eBFX-2b preincubated in assay buffer. Following incubation for 1–3 h at ambient temperature, prethrombin 2 cleavage was initiated by the addition of 50 μM of substrate solution to achieve a final concentration of 1.4 μM, and initial velocities were determined as described (10). The stoichiometry for inhibition was independently assessed using a 10-fold higher concentration of reagents in the initial incubation mixture. The enzyme/antibody mixture was then diluted 10-fold into a solution of 1.4 μM prethrombin 2 followed by initial rate measurements. Destabilizing effects of eBFX-2b on the assembly of prothrombinase were investigated using concentrations of factor Va fixed at 25, 50, and 100 nM. Possible competitive effects with substrate were assessed following initial velocity measurements using 1.4, 2.8, and 5.6 μM prethrombin 2 to initiate the reaction.

Inhibition of mi1AFL cleavage was investigated using an equivalent strategy except that product formation was monitored continuously using the fluorescent derivative, mi1AFL, as previously detailed (11). The initial incubation of prothrombinase constituents with increasing concentrations of eBFX-2b was conducted in a final volume of 150 μl in wells of a 96-well plate. Residual activity was measured following the addition of 50 μl of mi1AFL to achieve a final concentration of 0.8 μM. Product formation was monitored continuously using λ_ex = 450 nm and λ_em = 520 nm with a 500 nm long pass filter in the emission beam in a kinetic fluorescence plate reader (Gemini, Molecular Devices, Sunnyvale, CA), and initial rates were calculated as described previously (11).

Slow and tight binding by eBFX-2b during ongoing catalysis was assessed using progress curves for thrombin formation obtained by the addition of 150 μl of assay buffer containing eBFX-2b and prethrombin.
2 to an equal volume of preassembled prothrombinase (0.4 nM factor Xa, 40 nM factor Va, and 100 μM PCPS in assay buffer). Data sets were obtained at final concentrations of 1.4, 5.6, and 9.8 μM prothrombin 2 in the presence of 0, 2, 6, and 16 nM aBFX-2b. Aliquots (10 μL) were withdrawn serially from the reaction mixtures and quenched by mixing with 10-fold excess of aprotinin, 0.15 M NaCl, 0.3% SDS, 1 μg/ml RNase, pH 7.4. The concentration of thrombin formed in the quenched samples was determined from initial velocity measurements as described (10).

**Western Blot Analysis**—Samples were subjected to 10% SDS-PAGE either before or after disulfide bond reduction by either the methods of Laemmli (5) or Schagger and von Jagow (6). Electrophoretic transfer to Immobilon-P® membranes was achieved in 20 mM Tris, 20 mM glycine, 10% (v/v) methanol, pH 8.3, in a semidy blotting apparatus (Hoefer Scientific Instruments, San Francisco) at 0.04 mA cm⁻² for 15 h. Membranes were blocked with 0.1% (w/v) bovine serum albumin and reacted for 1 h with a solution of aBFX-2b (5 μg/ml). Following washes to remove unbound antibody, aBFX-2b was detected using the Vectastain ABC Elite kit following the instructions of the manufacturer and visualized using 2.6 mg/ml 4-chloronaphthol, 0.038% (v/v) H₂O₂ in 20 mM HEPES, 0.15 M NaCl, 37.5% (v/v) methanol, pH 7.5.

**Clavage of Xa with CNBr and Isolation of Fragments**—Factor X (2 mg/ml) was reduced and alkylated with [¹⁴C]iodoacetamide or bovine serum albumin, and digested with CNBr (100 μg/ml) in 100 mM HCOOH containing 10 mg/ml CNBr. Following digestion for 11 h at room temperature in the dark, the samples were dried by centrifugal evaporation, redissolved in 3.2 ml of 20 mM HEPES, 0.15 M NaCl, 0.3% (w/v) guanidine HCl, 12 mM dithiothreitol, pH 7.5, and fractionated by reversed phase high pressure liquid chromatography using a 100 × 4.6-mm Aquapore RP300 (Applied Biosystems, Foster City, CA) column equilibrated in 0.05% (v/v) trifluoroacetic acid. Bound protein fragments from a digest of human Xa were eluted with a gradient of increasing CH₃CN (3.3 ml/min) programmed as follows: 0–30%, 5 min; 30–35%, 25 min; and 35–100%, 5 min. For the separation of bovine Xa fragments, the gradient was as follows: 0–23%, 5 min; 23–45%, 25 min, and 45–100%, 5 min. Dot blot analysis of the eluted peaks with aBFX-2b using described procedures (26) identified immunoreactive material eluting at 31.6–32.1% for the human fragments and at 29.4–31.4% in the case of the bovine derivative. Fractions were pooled, concentrated by rotary evaporation, subjected to SDS-PAGE, and transferred to Immobilon-P® membranes in 10 mM CAPS, 10% (v/v) MeOH, pH 11. Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, and bands of interest identified by comparison to a Western blot performed in parallel were visualized by sequencing.

A construct expressing a fusion protein bearing residues 16–264 of human thrombin was spliced to the sequence encoding 103–116 of human factor Xa and inserted into a vector. PCR was conducted using Xa DNA (39) as a template. PCR was conducted using Phu polymerase, a forward primer (primer 1) containing an Ndel restriction site (5′-CCGGGTGTGCATA-GACCAGCCACTCGAGCTCTCCAAACTGATC-3′) and a reverse primer (primer 2) containing an XhoI restriction site (5′-GACCGACCTGCAGCTTAACTGGAGGA-3′). The resulting product was digested with the two enzymes, gel-purified, and ligated into the pTYB-2 vector digested previously with Ndel and XhoI. After transformation of XL-1 blue cells, the amplified product was isolated, and insert identity was verified by sequencing. A construct expressing a fusion protein bearing residues 16–116 of human factor Xa was prepared using primer 1 (above) and a reverse primer (5′-GACCGACCTGCAGCTTAACTGGAGGAAGTG-3′) designed to amplify the truncated sequence of interest. For the expression of the catalytic domain of thrombin (residues 16–245), PCR was performed using cDNA for human prothrombin as a template and appropriate forward (5′-CCGGGTGTGCATATGGTGGGAGGC-3′) and reverse (5′-GACCGACCTGCAGCTTAACTGGAGGAAGTG-3′) primers. The construct expressing a fusion protein of a mutant form of the catalytic domain of human Xa containing residues 92–102 from thrombin was prepared in three PCR steps. DNA fragments encoding residues 16–91 of human Xa and 92–102 of human thrombin were spliced to the sequence encoding residues 103–264 of human thrombin by using splicing by overlap extension (40). All resulting inserts in the pTYB-2 vector were verified by sequencing.

The constructs were transformed into E. coli 2566 cells, and the resulting single colonies were picked and amplified, and protein production was induced following the instructions provided with the Impact-CN kit. Thirty minutes following induction with 1 mM isopropylthiogalactopyranoside, the medium was supplemented with 20 μg/ml rifampicin, and cultures were maintained in shake flasks at 37 °C for 3.5 h at 37 °C. Inclusion bodies, isolated by sonication and differential centrifugation, were either directly analyzed by SDS-PAGE and Western blot analysis or were dissolved in 20 mM Hepes, 0.15 M NaCl, 1 mM EDTA, 0.1% (v/v) Tween 20, 5 μg/ml guanidine HCl, pH 7.5. The protein solution was diluted 10-fold in buffer lacking guanidine, and fusion proteins were captured using chitin-agarose and eluted with 20 mM Hepes, 0.5 M NaCl, 6 μg/ml guanidine HCl, pH 7.5. Eluted material was dialyzed extensively into assay buffer, concentrated by ultrafiltration using PM10 membranes (Amicon, Danvers, MA), and subjected to SDS-PAGE and Western blot analysis.

**Data Analysis**—The prothrombinase complex was assembled using limited concentrations of factor Xa and saturating concentrations of PCPS and factor Va. Because the concentrations of PCPS and Va were high relative to the dissociation constants for the discrete interactions with factor Xa which lead to prothrombinase assembly (41), the concentration of prothrombinase was considered to be equal to the limiting concentration of factor Xa.

Kinetic and equilibrium constants were obtained by fitting data sets to the indicated rate or equilibrium expressions by non-linear least squares analysis using the Marquardt algorithm (42). Fitted values are listed ±95% confidence limits.

Fluorescence titrations were analyzed according to Equation 2, assuming that the perturbation in fluorescence of OGA₄₄₀-Xa arises from the binding of aBFX-2b to equivalent and non-interacting sites,

\[
F - F' = 1 - \frac{n \cdot K_d}{1 + K_d} \cdot \frac{(n \cdot X_a + K_d)}{1 + (n \cdot X_a + K_d)}
\]

where \(X_a\) and \(K_d\) represent total concentrations of OGA₄₄₀-Xa and aBFX-2b, respectively, \(K_d\) is the equilibrium dissociation constant for the binding of n mol of aBFX-2b per mol of Xa; \(F/F'\) is the normalized fluorescence intensity in the presence of titrant, and \(F/F'_0\) is the normalized signal at infinite aBFX-2b. Analysis of the data according to Equation 2 yielded fitted values of \(n\), \(K_d\), and \(F/F'_0\).

For inhibition studies following prolonged incubation of aBFX-2b with enzyme components followed by a modest dilution by the addition of assay buffer, the short period of the initial velocity measurements was considered insufficient to perturb the pre-established equilibrium between antibody and enzyme. This assumption is justified by a series of experimental observations (below). Consequently, inhibition data were analyzed according to the expressions described previously (30), using enzyme and antibody concentrations present in the initial incubation mixture to derive fitted values for the equilibrium dissociation constant \(K_d\), stoichiometry \(n\), velocity in the absence of inhibitor \(v\), and velocity at infinite concentrations of inhibitor \(v_i\).

Slow and tight binding inhibition data were analyzed by combining the numerical solution of the ordinary differential equations and equilibrium expressions relevant to Scheme I with error minimization techniques using the program DynaFit (43), provided as a generous gift by Petr Kuzmic (BioKin Ltd., Pullman, WA). In this approach, the rapid equilibrium assumption was applied to the substrate binding steps, and inhibitor binding and dissociation were considered to be slow. Analysis by this approach yielded fitted values for \(k_i\), \(k_i\), \(k_{cat}\), and \(k_{cat}\). The appropriate relationship between the observed steady state kinetic constants and the stepwise kinetic constants for the cleavage of prethrombin 2 is illustrated in Scheme I.

**RESULTS**

**Binding of aBFX-2b to Factor Xa**—Saturating concentrations of aBFX-2b were found to modestly quench the steady state fluorescence intensity of Oregon Green₄₄₀ tethered by a diacycloligohexyl-ethyl ketone to the N-terminal site of bovine factor Xa. Quenching could be reversed by the addition of excess factor Xa, and quenching was not observed with an isotype- matched control IgG (not shown). Equilibrium parameters for the binding of aBFX-2b to OGA₄₄₀-Xa inferred from a fluorescence titration with aBFX-2b in the presence of Ca²⁺ yielded \(K_d = 17.1 ± 5.6 \text{ nM, } n = 1.22 ± 0.24 \text{ mol of aBFX-2b/mo}l\) of
Inhibition of Prothrombinase by αBFX-2b—The binding of αBFX-2b to bovine prothrombinase was inferred from a series of functional measurements assessed following prolonged incubation of αBFX-2b with the preassembled enzyme complex. Concentrations of αBFX-2b as high as 0.5 μM did not detectably alter the initial rate of peptidyl substrate cleavage assayed at −K_m concentrations of SpXa (Fig. 2). Inhibition of protein substrate cleavage by prothrombinase was measured by initial velocity studies of the cleavage of either prethrombin 2 or meizothrombin des-fragment 1 (Fig. 2), previously established as valid substrates for the assessment of the kinetics of recognition and cleavage at the two individual sites in prothrombin by prothrombinase (25). Prolonged incubation of αBFX-2b with prothrombinase yielded potent inhibition of protein substrate cleavage (Fig. 2). Assuming that the equilibrium between prothrombinase and αBFX-2b established in the initial incubation was minimally perturbed during initial velocity measurements, analysis of the inhibition data yielded an overall equilibrium dissociation constant (K_i) of 0.55 ± 0.05 nM.

Inhibition studies using a fixed concentration of prothrombinase in excess of the measured K_i (Fig. 2, inset) yielded an empirically determined stoichiometry of 1.14 mol of αBFX-2b bound per mol of prothrombinase at saturation. Taken together with the fluorescence measurements, the data suggest that the binding of αBFX-2b to Xa is significantly enhanced, by a factor of ~30, when the proteinase is incorporated into the prothrombinase complex. αBFX-2b binds to Xa within prothrombinase and selectively inhibits protein substrate cleavage without interfering, in an obvious way, with active site function of the enzyme. These data establish the basic properties of this monoclonal antibody, in line with previous studies (20) that suggest its potential utility as a unique probe of macromolecular substrate interactions with prothrombinase.

The results of more detailed studies with ligands that target the active site of factor Xa are summarized in Table 1. Saturating concentrations of αBFX-2b had no obvious effect on the steady state kinetic constants for peptidyl substrate cleavage or the binding of PAB. The overall equilibrium dissociation constant for the inhibition of factor Xa by rTAP was not significantly affected by the presence of saturating concentrations of αBFX-2b. These findings provide quantitative support for the conclusion that the binding of αBFX-2b to factor Xa does not directly or indirectly occlude interactions at the active site of the enzyme. Because interactions between TAP and the active site as well as extended surfaces in factor Xa contribute to the measured K_i (30), the data imply that this antibody does not perturb, in an obvious way, interactions at the active site as

**Fig. 1.** Binding of αBFX-2b to factor Xa. Reaction mixtures containing 25 nM OG_488-Xa in assay buffer (■) or assay buffer containing 100 μM EDTA instead of 2 mM Ca^{2+} (○) were titrated with increasing concentrations of αBFX-2b in the same buffer. The fractional change in fluorescence intensity was calculated from Equation 1 using λ_ex = 485 nm and λ_em = 515 nm. The solid line was drawn following analysis according to Equation 2, using the fitted constants: K_f = 17.1 ± 5.6 nM, α = 1.22 ± 0.24 mol of αBFX-2b/mol of OG_488-Xa and F/F_0 = 0.78 ± 0.01. Residuals to the fitted line are illustrated in the upper panel. The dashed line was arbitrarily drawn.

**Fig. 2.** Inhibition of prothrombinase function by αBFX-2b. Reaction mixtures containing 1 nM Xa, 25 nM Va, and 50 μM PCPS were incubated with increasing concentrations of αBFX-2b (lower axis), and remaining activity was determined from initial velocity measurements of product formation with 1.4 μM prethrombin 2 (■) or 0.8 μM mIIaAFK (○). For studies of peptidyl substrate cleavage, the initial reaction mixture contained 0.5 nM Xa, 25 nM Va, and 50 μM PCPS in assay buffer incubated with increasing concentrations of αBFX-2b (upper axis). The data were analyzed according to the fitted constants K_i = 0.55 ± 0.05 nM, v_0 = 98.2 ± 1.8%, and v = 4.8 ± 2.3%. Inset, inhibition of prethrombin 2 cleavage following incubation of prothrombinase and αBFX-2b at 10-fold higher concentrations than those described above followed by dilution into substrate solution. Linear regression analysis of the limits of the data yielded a stoichiometry of 1.14 ± 0.03 mol of αBFX-2b/mol of prothrombinase.
well as at other extended surfaces in the proteinase domain implicated in TAP binding (44).

**Mechanism of Inhibition of Prothrombinase**—The findings raise the possibility that the binding of aBFX-2b to factor Xa within prothrombinase blocks protein substrate binding by interfering with exosite interactions between the enzyme complex and protein substrate. However, satisfactory documentation of this possibility requires that aBFX-2b and the protein substrate bind in a mutually exclusive fashion to prothrombinase and that selective inhibition of protein substrate cleavage is not achieved by interfering with the Xa-Va interaction on the membrane surface.

Qualitative evidence has been presented previously (20) to document a membrane-dependent interaction between factors Xa and Va even in the presence of aBFX-2b. Furthermore, the lower inferred equilibrium dissociation constant for the binding of aBFX-2b to factor Xa within prothrombinase relative to Xa in solution also implies that aBFX-2b is likely to enhance rather than weaken the interaction between Xa and other constituents of prothrombinase (30). Nevertheless, selective inhibition of protein substrate cleavage arising from modest changes in the affinity for the Xa-Va interaction was empirically assessed by examining the ability of aBFX-2b to inhibit prethrombin 2 cleavage at different fixed concentrations of factor Va (Fig. 3A). Initial velocity studies following prolonged incubation of increasing concentrations of aBFX-2b with prothrombinase preassembled with different fixed concentrations Va prior to initiation of catalysis by the addition of prethrombin 2 yielded comparable results (Fig. 3A). Because the fixed concentrations of Va were in excess of the concentration of factor Xa and well above the measured $K_d$ for prothrombinase assembly (41), the data indicate that even minor perturbations in the equilibrium dissociation constant for the Xa-Va interaction elicited by antibody binding cannot account for the observed inhibition of protein substrate cleavage.

Competition between aBFX-2b and protein substrate binding to prothrombinase was initially evaluated in a comparable approach in which inhibition by aBFX-2b was measured at different fixed concentrations of prethrombin 2 following prolonged incubation of increasing concentrations of aBFX-2b with prothrombinase (Fig. 3B). Equivalent results were obtained at fixed substrate concentrations ranging between $0.5$ and $2\times K_m$ (Fig. 3B). These data indicate that either aBFX-2b does not compete for prethrombin 2 binding to the enzyme complex or that the brief measurement period in the presence of the substrate is insufficient for a new equilibrium between prothrombinase, aBFX-2b, and prethrombin 2 to be established.

Further insights into the relationship between antibody and protein substrate binding to prothrombinase were obtained by treating the antibody as a slow and tight binding inhibitor (45). Inhibition by aBFX-2b during ongoing catalysis was determined by progress curve analysis of thrombin formation, following the initiation of reaction mixtures containing different fixed concentrations of prethrombin 2 and aBFX-2b by the addition of prothrombinase (Fig. 4). Global analysis of the progress curves by combining non-linear least squares error minimization with numerical solution of ordinary differential equations according to Scheme I yielded adequate fits (Fig. 4). Alternative inhibition mechanisms either yielded obviously poorer fits or yielded poorly determined parameters without a significant improvement in fit quality. The fitted steady state kinetic constants for prethrombin 2 cleavage are in agreement with values derived from more extensive studies (8, 10). The fitted rate constants for antibody binding and dissociation provide a rational explanation for the findings in the initial velocity studies following prolonged incubation of prothrombinase with aBFX-2b (Figs. 2 and 3). Furthermore, the calculated $K_i$ ($K_i = k_{b} / k_{a}$) value is in agreement with independently measured values. On these bases, we conclude that the inhibitory properties of aBFX-2b can adequately be described by a model.
bond reduction revealed that for proteins of either human or bovine origin, the proteinase domain of thezymogen and both α and β forms of factor Xa reacted with αBFX-2b (Fig. 5A). No evidence for antibody reactivity with the light chains of any of these species was obtained. In agreement with previous suggestions (20), the blotting results indicate that side chains present between residues 16 and 245 contribute significantly to the interaction with αBFX-2b.

A series of proteolytic and chemical cleavage approaches were initiated to further resolve regions within these proteins that may contribute to the interaction with αBFX-2b. The most informative results were obtained following cleavage of human and bovine Xa with CNBr (Fig. 5A). In the case of bovine Xa, cleavage at methionine yielded an immunoreactive fragment with $M_r = 24,000$ that could be reduced to a species of $M_r = 9,000$ upon disulfide bond reduction. An immunoreactive fragment with $M_r = 12,000$ was detected for human Xa regardless of disulfide bond reduction. In all cases, bands observed at apparently higher molecular weights reflect incomplete cleavage products of bovine and human Xa.

Protein fragments were purified from preparative CNBr digests of bovine and human Xa by reversed phase high pressure liquid chromatography following disulfide bond reduction. Im-

![Fig. 5. Identification of Xa/Xa fragments that bind αBFX-2b. A, the binding of αBFX-2b to bovine and human X/Xa and fragments was analyzed by Western blots following SDS-PAGE either before (left blot) or after (right blot) disulfide bond reduction. The lanes correspond to samples of human X (lanes 1 and 7), human Xa (lanes 2 and 8), CNBr-digested human Xa (lanes 3 and 9), bovine X (lanes 4 and 10), bovine Xa (lanes 5 and 11), and CNBr-digested bovine Xa (lanes 6 and 12). Mobility of molecular weight markers ($\times 10^3$) are illustrated in the left margin, and migration positions of X, Xa, and corresponding heavy chains are denoted in each blot. B, CNBr cleavage sites in bovine and human Xa are illustrated schematically. The light chain is denoted by a hatched bar. The shaded portion in each heavy chain illustrates the position of the immunoreactive fragment identified by N-terminal sequence analysis following high pressure liquid chromatography purification. C, alignment of residues 82–116 in factor Xa from different species. The consensus sequence was calculated using ClustalW (58) using Xa sequences from species known to bind αBFX-2b. The sequences of Trocarin and human thrombin which do not bind αBFX-2b are shown for comparison. Dark and light shading denotes identical or homologous side chains. Thrombin contains an additional residue (numbered 97A) in this region, illustrated in an offset way to maintain alignment.](http://www.jbc.org/content/383/9/9371.full.html)
munoreactive fragments were identified by blotting with αBFX-2b and placed in the primary structure by N-terminal sequence analysis. The fragment derived from human Xa yielded the N-terminal sequence of the proteinase domain (Fig. 5B). An internal sequence was obtained for the bovine derivative beginning at residue 82 (Fig. 5B). A second minor sequence was detected in this case starting at residue 88 and likely results from inefficient cleavage of Met87–Thr88 by CNBr as noted previously (46). Based on sequence results (Fig. 5B), mobility of the immunoreactive fragments on SDS-PAGE with and without disulfide bond reduction (Fig. 5A) and by results of mass spectrometry, heavy chain residues 82–156 in bovine Xa and 16–116 in the human counterpart, were concluded to contribute in a significant way to antibody binding. We therefore conclude that the intersection set of these two sequences, comprising heavy chain residues 82–116, contributes in a significant way to the ability of αBFX-2b to bind to Xa in the Western blotting approach.

In addition to factors X/Xa derived from human and bovine plasma, previous work (20) has established that αBFX-2b can also bind these proteins of rabbit, canine, and porcine origin. Trocarin, a factor Xa-like enzyme from the venom of T. carinatus, exhibits ~60% identity and ~70% homology with the proteinase domain sequences of factor Xa from a series of mammalian species (47). However, striking sequence divergence is evident in the region encompassing residues 74–100 (47). Western blotting with αBFX-2b failed to detect trocarin even in heavily loaded gels (not shown). Human thrombin also differs significantly from factor X in this region (Fig. 5C) and fails to react with αBFX-2b in the Western blotting approach (below). Alignment of the known sequences of mammalian factor X species, documented to bind this antibody, illustrates the high degree of equivalence in the 82–116 region (Fig. 5C). The lack of detectable binding of αBFX-2b to trocarin and the fact that the protein sequence of trocarin diverges markedly in this region (Fig. 5C) further supports a role of these residues in antibody binding.

Binding of αBFX-2b to Recombinant Derivatives of Human Factor X—Recombinant techniques were used to express fragments of the heavy chain of human Xa as C-terminal fusion products with thioredoxin in E. coli. A series of 12 fragments produced in this expression system yielded erratic results (not shown). Western blotting with these fragments yielded results consistent with the initial conclusion implicating residues 82–116 in binding αBFX-2b. However, detection of antibody binding to fragments bearing human Xa residues 82–116 was substantially enhanced following the initial cleavage and removal of the thioredoxin fusion partner and was eliminated by the inclusion of additional factor Xa residues extending beyond residue 116. These results yield the somewhat surprising suggestion that despite the ability of αBFX-2b to bind to cleaved fragments of factor Xa following disulfide bond reduction and SDS-PAGE, the antibody epitope is unlikely to be determined by a single linear peptidyl sequence.

Further studies were therefore conducted by bacterial expression of larger protein fragments as N-terminal fusions with an intein sequence linked to the chitin binding domain (48). Because of the difficulties in interpreting the quantitative contributions of specific side chains to the thermodynamics of protein-protein interaction (49), particularly in a system where proteins are initially produced as insoluble and aggregated products, we have deliberately chosen to focus on positive results obtained from Western blotting approaches assessed qualitatively and presented in Scheme II.

A fusion product containing the catalytic domain of human factor Xa reacted with αBFX-2b, whereas a comparable derivative bearing the catalytic domain of human thrombin did not (Scheme II). In agreement with the results of CNBr digestion, a fusion product containing human Xa-(16–116) reacted with αBFX-2b, and antibody reactivity was retained in a derivative bearing the catalytic domain of human Xa in which residues 92–1023 were replaced with equivalent residues found in human thrombin. Collectively, the data indicate that residues 82–91 and 102–116 within the catalytic domain of human factor Xa contribute in a significant way to the interaction with αBFX-2b.

Structural Significance of Residues Implicated in Binding αBFX-2b—The published x-ray structures of human and bovine Xa indicate that the 82–91 and 102–116 polypeptide sequences are largely surface-exposed (50). As illustrated using the x-ray structure of the proteinase domain of human factor Xa (Fig. 6), residues 82–116 form a loop extending from the Ca2+-binding site. Residues 82–91 and 102–116, implicated in binding αBFX-2b, form adjacent antiparallel structures on the surface on a face of the molecule clearly removed from the catalytic site and residues implicated in substrate-inhibitor interactions at the active site (50).

3 Residue 102 is Asp in all the serine proteinases of coagulation.
A series of studies have established a primary role for exosite interactions in determining the affinity and binding specificity of prothrombinase for its protein substrate (10–12). Ligands targeting such exosites within prothrombinase are expected to compete with protein substrate binding to the enzyme complex without interfering with complex assembly or active site function (10–12). Evidence developed in the present work establishes αBFX-2b as a prototypic exosite-directed inhibitor of prothrombinase. Our findings also suggest that the occlusion of sites on the surface of factor Xa within prothrombinase is sufficient to interfere with exosite-mediated recognition of the protein substrate.

Recent high resolution x-ray structures of analogous or related enzyme complexes in coagulation and fibrinolysis have provided indications that the cofactor or accessory protein likely provides an extended surface for binding the protein substrate (51–53). In most of these cases, such inferences remain to be correlated with functional studies assessing the precise energetic contributions of extended surfaces in the cofactor to the productive interaction between the protein substrate and the enzyme complex. In the case of prothrombinase, early studies (14) suggested a requisite interaction between the fragment 2 domain of the substrate and factor Va for efficient cleavage of prothrombin by the enzyme complex. Subsequent work with prothrombin derivatives lacking the fragment 2 domain has indicated that a direct binding interaction, of the type initially proposed, is unlikely to play a major contributing role to the function of factor Va within the prothrombinase complex (8). More recently, a series of studies (18) have provided evidence for an interaction between the substrate/product and factor Va, mediated by the fibrinogen-binding site present in the proteinase domain. However, proteolytic derivatives of prothrombin, lacking the fibrinogen-binding site, retain the ability to bind to prothrombinase with comparable affinity as the substrate and block exosite-mediated substrate binding (12). Consequently, the precise contribution of Va-substrate interactions to the productive pathway of substrate recognition by prothrombinase is uncertain. Our results now indicate that extended surfaces on the proteinase domain of factor Xa within the enzyme complex, and removed from the active site, play a significant role in the exosite-dependent binding of the protein substrate to prothrombinase. Although these findings do not rule out a contribution from direct interactions between the protein substrate and factor Va, it would appear that extended surfaces on factor Xa, within the enzyme complex, participate in determining the binding specificity for the protein substrate.

The findings are consistent with the interpretation that the binding of αBFX-2b to factor Xa within prothrombinase is sufficient to exclude protein substrate binding. As competitive inhibition of protein substrate cleavage can only be achieved by interfering with the bimolecular interaction between the substrate and the enzyme exosite (10, 12), one possible interpretation is that residues included in the binding site identified for αBFX-2b directly contribute to interactions with the protein substrate. However, alternative interpretations include the possibility that the binding of αBFX-2b perturbs residues distant from the antibody-binding site that are directly involved in interactions with the substrate or that selective inhibition of protein substrate cleavage arises from steric effects associated with the binding of this large probe to the enzyme. The finding that the Fab fragment of the antibody acts in an equivalent manner to the intact IgG (20) and the fact that αBFX-2b does not alter the binding of large ligands such as rTAP that involve extended interactions with the proteinase domain (44) can be offered as arguments against generalized steric phenomena. However, these arguments are not compelling, and we are presently unable to convincingly rule out such alternative interpretations. The present findings, however, provide a reasonable starting point for the further systematic investigation of structures present in the proteinase domain of factor Xa that may play a role in binding the protein substrate.

Despite the fact that the heavy chain of factors X and Xa and their proteolytic digestion products are all readily detected by αBFX-2b by Western blotting following disulfide bond reduction, this antibody surprisingly appears to bind to a discontinuous epitope on the protein. This point has adversely affected our ability to establish the structural basis for the interaction between αBFX-2b and factor Xa in a quantitative way. Thus, it is possible that additional structures neighboring residues 82–91 and 102–116 also contribute significantly to the interaction between αBFX-2b and factor Xa or that the antibody epitope is considerably smaller than proposed by our studies. The identified sequences are immediately adjacent to the Ca$^{2+}$-binding site in the proteinase domain (50, 54). Such proximity provides a plausible explanation for the Ca$^{2+}$-dependence of αBFX-2b binding observed in this and prior work (20). In the event that this region includes residues that directly contribute to the interaction with the protein substrate, it is possible that Ca$^{2+}$ binding to the proteinase domain of factor Xa may also modulate exosite-dependent tethering of the protein substrate to the enzyme complex.

Evidence has also been developed for a major contribution of exosite interactions in determining the productive recognition of factor X by the VIIa-tissue factor complex (19). An extensive body of information indicates that extended surfaces in both VIIa and tissue factor contribute to factor X binding. Structural studies of VIIa in complex with an inhibitory polypeptide have implicated extended surfaces that include the autolysis loop of VIIa in factor X activation (55). However, this inhibitor appears to modulate catalytic function rather than compete with the binding of the protein substrate (55). Additional evidence for a role of extended surfaces in VIIa in binding factor X has resulted from mutagenesis studies used to map the binding determinants of antibody probes that function as exosite-directed inhibitors of factor X activation (56). The residues identified are generally localized to the same face of the proteinase domain and include or are in the vicinity of the residues implicated in the present work for the binding of αBFX-2b to factor Xa. Thus, it is possible that both VIIa and Xa within their respective enzyme complexes employ equivalent structures in the proteinase domain to mediate exosite interactions with their protein substrates.

The incorporation of factor Xa into prothrombinase was found to enhance detectably the binding of αBFX-2b. This observation is in line with the suggestions of previous studies (30, 57) that surfaces in factor Xa, distinct from the active site, are modulated following the interaction between proteinase and cofactor. Such perturbations have been shown previously to lead to large changes in the affinity and kinetic mechanism of the interaction of macromolecular inhibitor probes directed toward Xa (30, 57). Although the significance of such changes to protein substrate recognition remains to be established, the data are consistent with the hypothesis that one consequence of the interaction of factor Xa with factor Va on the membrane surface is the modulation of extended macromolecular recognition sites on the proteinase that at least partly accounts for the exosite-mediated binding of the protein substrate to prothrombinase. These ideas and the present findings indicate a significant role for exosites present in the proteinase domain of factor Xa in determining binding affinity and specificity for the recognition of the protein substrate by prothrombinase.
Acknowledgments—We are grateful to Dr. William Church, University of Vermont, for the generous gift of monoclonal antibody αFX-2b. We are also grateful to Dr. George Vlasuk for critical reading of the manuscript and to Dr. Jan Pohl, Emory University Microchemical Facility, for N-terminal sequence analysis.

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The Contribution of Factor Xa to Exosite-dependent Substrate Recognition by Prothrombinase
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J. Biol. Chem. 2002, 277:9366-9374.
doi: 10.1074/jbc.M110848200 originally published online January 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110848200

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