Nematode Anticoagulant Protein c2 Reveals a Site on Factor Xa That Is Important for Macromolecular Substrate Binding to Human Prothrombinase*

Sai K. Buddai‡, Larisa Toulkohonova‡, Peter W. Bergum§, George P. Vlasuk§, and Sriram Krishnaswamy†††

From the ‡Joseph Stokes Research Institute, Children’s Hospital of Philadelphia and §Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and ¶CORVAS International Inc., San Diego, California 92121

The binding of recombinant nematode anticoagulant protein c2 (NAPc2) to either factor X or Xa is a requisite step in the pathway for the potent inhibition of VIIa tissue factor. We have used NAPc2 as a tight binding probe of human Xa to investigate protein substrate recognition by the human prothrombinase complex. NAPc2 binds with high affinity ($K_d \sim 1 \text{ nM}$) to both X and Xa in a way that does not require or occlude the active site of the enzyme. In contrast, NAPc2 is a tight binding, competitive inhibitor of protein substrate cleavage by human Xa incorporated into prothrombinase with saturating concentrations of membranes and Va. By fluorescence binding studies we show that NAPc2 does not interfere with the assembly of human prothrombinase. These are properties expected of an inhibitor that blocks protein substrate recognition by targeting extended macromolecular recognition sites (exosites) on the enzyme complex. A weaker interaction ($K_d = 260–500 \text{ nM}$) observed between NAPc2 and bovine X was restored to a high affinity one in a recombinant chimeric bovine X derivative containing 25 residues from the COOH terminus of the proteinase domain of human X. This region implicated in binding NAPc2 is spatially adjacent to a site previously identified as a potential exosite. Despite the weaker interaction with bovine Xa, NAPc2 was a tight binding competitive inhibitor of protein substrate cleavage by bovine prothrombinase as well. Extended enzymic surfaces elucidated with exosite-directed probes, such as NAPc2, may define a unique region of factor Xa that is modulated following its assembly into prothrombinase and in turn determines the binding specificity of the enzyme complex for its protein substrate.

The proteolytic activation of prothrombin is catalyzed by the prothrombinase complex of coagulation (2–5). Prothrombinase assembles through membrane-dependent interactions between the serine proteinase, factor Xa, and the protein cofactor, factor Va (2, 3). Although solution-phase Xa is a competent enzyme, its incorporation into prothrombinase yields a profound increase in the rate of thrombin formation (2, 3).

Prothrombin is the only known protein substrate cleaved efficiently by prothrombinase (2, 6). Such stringent selectivity is not evident in the action of factor Xa on oligopeptidyl substrates, nor is the rate of peptidyl substrate hydrolysis significantly enhanced upon assembly of factor Xa into prothrombinase (7, 8). Thus, the narrow and defined specificity of prothrombinase toward its protein substrate is unlikely to be solely explained by the specific recognition of residues surrounding the scissile bond by the active site of factor Xa within the enzyme complex.

Mechanistic studies of bovine prothrombinase function have borne out this suggestion (9–11). A series of studies indicate that recognition of the biological substrate is achieved through stepwise interactions of the protein substrate at an extended macromolecular recognition site (exosite) in prothrombinase removed from the catalytic site of Xa, followed by docking of elements surrounding the scissile bond with the active site of the enzyme and subsequent cleavage (9–11). The initial exosite-binding step dominates the affinity and binding specificity of the enzyme for the protein substrate. This interaction serves to tether the substrate to the enzyme, thereby directing ordered active-site docking and cleavage at two spatially distinct sites in the zymogen (11). Thus, surfaces on the enzyme complex, removed from the active site of factor Xa, contribute in a dominant way to the productive recognition of the protein substrate.

Although comparable mechanistic information is lacking in the human system, studies with human prothrombin derivatives have implied a principal role for an interaction between (pro)thrombin and factor Va, mediated by the (pro)fibrinogen-binding site in the substrate, in human prothrombinase function (12–14). However, findings with bovine proteins are not in complete agreement with these conclusions. Proteolytic derivatives of prothrombin lacking the fibrinogen-binding site apparently retain the ability to participate in exosite interactions with prothrombinase (10). A monoclonal antibody (αBFX-2b)1 directed against a region of the proteinase domain of factor Xa, spatially distinct from the active site, appears to block exosite

1 The abbreviations used are: αBFX-2b, monoclonal antibody directed against the proteinase domain of factor Xa; APMSF, p-amidinophenylmethylsulfonfonylfluoride; dansyl, 5-dimethylaminonaphthalene 1-sulfonyl; EGR, ethylene glycol succinimimidyl glycyglycine chloromethyl ketone; ITC, isothermal titration calorimetry; PC, phosphatidylcholine; PS, phosphatidylserine; NAPc2, recombinant nematode anticoagulant peptide c2; NAPc2-biotin, NAPc2 covalently modified with biotin; O$_2$-hXa, Xa inactivated with ethiothiocacetyl-L-glutamylglycyglycyl-L-arginine chloromethyl ketone and modified with Oregon Green 488 maleimide following thioester hydrolysis; PEG, polyethylene glycol with average molecular weight of 8000; X$_{Gly/Arg}$, chimeric recombinant derivative of bovine factor X containing proteinase domain residues 240–264 from human factor X; S2328, $H_2$-phenylalanylalanine-$p$-nitroanilide; SpXa, methoxy carbonyl-L-cyclohexylglycylglycyl-$p$-nitroanilide; TF, tissue factor; Xa, human Xa covalently inactivated with APMSF; Xa-L, Xa saturated with PCPS; Xa-Va-L, Xa incorporated into prothrombinase with saturating concentrations of Va and PCPS; MBS, 4-morpholinoethanesulfonic acid.

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† To whom correspondence should be addressed: Joseph Stokes Research Institute, Children’s Hospital of Philadelphia, 310 Abramson, 3516 Civic Center Blvd., Philadelphia, PA 19104. Tel.: 215-590-3346; Fax: 215-590-2320; E-mail: skrishna@mail.med.upenn.edu.
binding of the protein substrate to bovine prothrombinase without affecting the active site (15). These points along with the necessary caveats associated with the findings highlight uncertainties in the field and cast doubt on the validity of directly extrapolating findings in the bovine system to explain the mechanism of action of human prothrombinase on its protein substrate.

Recombinant nematode anticoagulant protein c2 (NAPc2) is an 85-residue polypeptide originally isolated from the hemaphagous hookworm, *An Neclostoma caninum* (16). NAPc2 is a potent inhibitor (*K*<sub>I</sub> = 10 pM) of factor X activation by the extrinsic Xase complex composed of VIIa and tissue factor (TF) (16). A critical component reaction in the inhibitory pathway is the initial interaction between NAPc2 and either factors X or Xa which facilitates delivery and greatly enhances the affinity of NAPc2 for the VIIa-TF complex (16, 17). As NAPc2 binds tightly to human Xa in a way that is apparently independent of the active site of the enzyme (17), we have further characterized these interactions and used NAPc2 as a small, tight-binding probe to investigate the function of human prothrombinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hen egg 1,α-phosphatidylincholine (PC) and bovine brain 1,α-phosphatidyl-2-sine (PS) were from Avanti Polar Lipids (Alabaster, AL). Heps, MES, and p-amidinophosphonothiolane fluoride (APMSF) were from Sigma. Methoxycarbonyl-n-cyclohexylglycyl-glycyl-ter, AL). Hepes, MES, and paired basic amino acid-converting enzyme. Hepes, MES, and paired basic amino acid-converting enzyme-furin were from Sigma. Methoxycarbonyl-n-cyclohexylglycyl-glycyl-ter, AL). Hepes, MES, and paired basic amino acid-converting enzyme (Essex Junction, VT). pCMV4-ss-pro-II-fX, pCDNA3 containing recombinant cDNAs for factors V, X, and Xa which were expressed in HEK293 cells using the pCMV4 vector previously described (20). Phospholipid concentrations are expressed in terms of the initial interaction between NAPc2 and either factors X or Xa which facilitates delivery and greatly enhances the affinity of NAPc2 for the VIIa-TF complex (16, 17). As NAPc2 binds tightly to human Xa in a way that is apparently independent of the active site of the enzyme (17), we have further characterized these interactions and used NAPc2 as a small, tight-binding probe to investigate the function of human prothrombinase.

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human factor X has been described (23). For the recombinant bovine derivatives, stable clones selected with G418 were identified by a functional assay in which conditioned medium was treated with the purified factor X activator from Russell’s viper venom followed by measurements of the initial rate of SpXa hydrolysis. Selected clones were expanded into 1.5–2.0 ml cultures in 150 cm2 flasks and harvested in 5–20 ml of buffer. Two stable cell lines producing each derivative of bovine factor X were separately expanded into cell factories (Nalge-Nunc, Naperville, IL) for long term protein expression in serum-free medium supplemented with vitamin K (Abbott) (23).

Conditioned medium (20 liters) containing either bovine X derivative was expanded into a 200-liter (6.4 × 6.0 × 10.0 m3) Bio-Rad fermentor using a MCS-ITC instrument (Microcal, Cambridge, MA) stabilized at 37 °C, pH 7.5. Samples were further purified using a HiPrep S-200 column (2.6 × 60 cm, Amersham Biosciences) equilibrated in the same buffer. Fractions containing factor X, identified by a functional assay, were pooled and dialyzed into 20 mM MES, 0.15 M NaCl, 1 mM benzamidine, pH 7.5. Bound protein was eluted (10 ml/min, 90 min) with a gradient of increasing NaCl (0.15 to 0.6 M) prepared in the same buffer. Fractions containing factor X identified by a functional assay were pooled and dialyzed into 20 mM MES, 0.15 M NaCl, 1 mM benzamidine, pH 6.0. A fraction (~20%) of the resulting material was applied to a 10 × 1-cm Poros HQ/M column (Applied Biosystems) equilibrated in the same buffer. Bound protein was eluted (2 ml/min) with a gradient of increasing Ca2+ developed with buffer B: 20 mM MES, 0.15 M NaCl, 1 mM benzamidine, pH 6.0 (0–100% buffer B, 40 min). Fractions containing factor X functional activity were pooled from repeated runs, precipitated by the addition of (NH4)2SO4 (80% saturation), collected by centrifugation (56,000 × g, 20 min), and dissolved in a small volume of 20 mM Hepes, 0.15 M NaCl, 1 mM benzamidine, pH 7.5. The sample was further purified using a HiPrep 5-200 column (2.6 × 60 cm, Amersham Biosciences) equilibrated in the same buffer and developed at 0.2 ml/min. Fractions containing factor X were pooled, precipitated with (NH4)2SO4 (80% saturation), collected by centrifugation (56,000 × g, 20 min), dissolved in 50% (v/v) glycerol, and stored at −20 °C. A representative yield of ~34 mg of purified product was obtained from 20 liters of conditioned medium.

All protein preparations were quality-controlled by SDS-PAGE (34). Concentrations were calculated using the following molecular weights and extinction coefficients (ε, nm−1 cm−1): human Xa, 45,300, 1.16 (35); human or bovine Va, 168,000, 1.74 (36, 37); human or bovine prethrombin 2, 37,400, 1.89 (29); bovine Xa, 45,300, 1.24 (38); NAPc2, 9,649 atomic mass units, 0.54. A systematic overestimate in the reported concentration for the second run was adjusted to yield offset values for the different fixed concentrations of PCPS and Va. The concentrations of prethrombin 2 and NAPc2 in assay buffer were initiated by the addition of 5 µM Va (10 µl, 100 nM). Aliquots (10 µl) withdrawn either before the addition of Xa or serially at t = 30, 60, 90, 120, and 180 s following initiation were quenched and further diluted, and the initial velocity of S2238 hydrolysis was determined by continuously monitoring the absorbance at 405 nm following the addition of 200 µM S2238 as described previously (9, 20). The concentration of thrombin formed as a function of time was determined by interpolation from the linear dependence of the rate of S2238 hydrolysis on known concentrations of human thrombin determined in each experiment. The initial steady state rate of thrombin formation in the initial reaction mixture was determined from the linear appearance of thrombin with time.

Fluorescence Anisotropy—Steady-state fluorescence anisotropy was measured using a customized fluorescence spectrophotometer (RSM-1000, OLIS, Bogart, GA) operated in T-format with Glan-Thompson polarizers. Samples (2.0 ml) were maintained at 25 °C in 1-cm2 stirred quartz cuvettes, and anisotropy was measured using λex = 490 and λem = 520 nm with long pass filters (KV500, Schott, Duryea, PA) in the emission beams. With the exception of the instrumentation, all measurements including control experiments and scattering corrections were performed exactly as described in detail for similar measurements with bovine prothrombinase (10).

Assessment of Competition between bFX-2b and NAPc2—Reaction mixtures (150 µl), prepared in assay buffer, contained 1.1 nM Xa, 67 µM PCPS, and increasing concentrations of NAPc2 in the presence of different fixed concentrations of bFX-2b. Following prolonged incubation for 30 min at ambient temperature, initial rates were determined for the interaction of Xa with bFX-2b. For both experiments, final reactant concentrations were reported even though a final equilibrium was unlikely to have been established during the brief initial velocity determination.

Affinity Chromatography on a Heparin Matrix—High performance heparin affinity chromatography was performed using a 0.46 × 10-cm Deep-Torr H5/20 column (Applied Biosystems) equilibrated in 20 mM Hepes, 0.15 M NaCl, 1 mM benzamidine (pH 7.5) prepared in the same buffer containing either 5 µM factor X or 5 µM factor X plus 7.5 µM NAPc2 were applied to 2 ml/min. Following washing (8 ml), bound protein was eluted with a gradient of increasing NaCl (0–1.0 M, 10 min) prepared in the same buffer.

Ligand Blotting—Protein samples (5 µg each) were subject to SDS-PAGE, transferred to Immobilon membranes (Millipore, Milford, MA) with a semi-dry apparatus (Hoefer, San Francisco, CA) using 20 mM Tris, 20 mM glycine, 10% (v/v) MeOH, pH 8.3, at 100 mA for 60 min. The membrane was blocked with blot buffer (20 mM Hepes, 0.15 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) containing 0.1% (v/v) BSA for 30 min, washed 3 times with blot buffer, and incubated with 82 nM NAPc2-biotin in blot buffer for 1 h. The membrane was washed 3 times with blot buffer and incubated (30 min) with avidin-biotinylated horseradish peroxidase complex (ABC Elite reagent, Vector Laboratories, Burlingame, CA) prepared according to the manufacturer’s directions. Excess reagent was removed by 3 washes using blot buffer followed by 2 washes with blot buffer lacking Tween 20. Reactive bands were visualized following the addition of ECL reagent (Amersham Biosciences), and chemiluminescence was detected by exposure to X-1 film (Eastman Kodak).

Data Analysis—In kinetic studies using factor Xa either in the presence of PCPS or in the presence of factor Va plus PCPS, reactant concentrations were chosen to ensure that the concentration of factor Xa was limiting, and the concentrations of PCPS and Va were saturating and well above the Kd values for the individual interactions (see
below) (40, 41). In each case, the concentration of catalyst (E) was considered equivalent to the limiting concentration of factor Xa incorporated satura-
torially into the Xa-PCPS binary complex or the Xa-Va-PCPS ternary complex (41).

All constants were obtained by fitting data to the indicated explicit expressions using nonlinear least squares regression analysis (42). The fitted constants are presented ±95% confidence limits.

Heat flow traces obtained by ITC measurements were integrated, corrected for background, and analyzed using Origin Scripts provided by Microcal (Northampton, MA) using the expressions for the binding of tritator to identical and non-interacting sites (45–45). When necessary, non-linear least squares routines accommodated the simultaneous analysis of two or more data sets obtained with a staggered injection sequence. Analysis by either approach, using 1/V weighting, yielded fitted values for ΔH, Kd, and stoichiometry. ΔG and ΔS were calculated using ΔG = RT lnKd and ΔG = ΔH – TΔS.

Steady state kinetic constants for the hydrolysis of SpXa either in the absence or in the presence of a saturating concentration of NAPc2 were determined by nonlinear least squares regression analysis according to the Henri-Michaelis-Menten equation.

Analysis according to Scheme I was done by combining nonlinear error minimization with numerical solution of rate and equilibrium expressions using the program Dynafit obtained as a gift from Petr Kasemir (Northampton, MA) (46). This approach used the rapid equilibrium assumption and assumed a stoichiometry (moles of NAPc2/moles of Xa) of 1. Changes in initial velocity with increasing concentrations of NAPc2 at a single concentration of SpXa were analyzed by the “local” method in which the independently measured kinetic constants in the absence and presence of NAPc2 were taken as known values for KES, NAPc2, KES, N, S, and KES, N, S. Local analysis yielded fitted values for KES, N and KES, N, S. Data sets obtained using increasing concentrations of SpXa at different fixed concentrations of NAPc2 were analyzed “globally” to yield fitted values for KES, N, S, kcat, kcat, NAPc2, kcat, NAPc2, kcat, NAPc2, kcat, NAPc2, and KES, N, S, was calculated from Equation 1,

KES, N, S = KES, N · KES, S, N
(Eq. 1)

The errors reported for the numerical analyses correspond to linear approximations of the 95% confidence limits. Uncertainty in KES, N, S, was established by error propagation (42).

Kinetic constants describing tight binding competitive inhibition of prethrombin 2 cleavage by prothrombinase were extracted by analysis of the rate data according to Equations 2 and 3,

Vmax = \frac{V_{max} \cdot S}{S + K_{m, S} \cdot I} + I
(Eq. 2)

I = I_{0} - \frac{(1 + n \cdot E + K_{i}) - \sqrt{(1 + n \cdot E + K_{i})^{2} - 4 \cdot n \cdot E \cdot I}}{2}
(Eq. 3)

where v, Vmax, and Km refer to the usual meaning. S, E, and I refer to total concentrations of substrate, enzyme, and inhibitor, and I0 refers to the free concentrations of I. Inhibition results from the interaction of I with E with an equilibrium dissociation constant of Ki and stoichiometry n. Analysis according to Equations 2 and 3 yielded fitted values for Vmax, Km,Kn, and Ki.

Fluorescence titrations obtained at different fixed concentrations of OG488-hXa, a fixed, saturating concentration of PCPS, and increasing concentrations of Va were analyzed according to the model and experimental considerations previously developed in detail for bovine prothrombinase (41). Analysis according to Equation 2 of Betz and Krishnaswamy (10) yielded fitted parameters for the equilibrium dissociation constant for the interaction between OG488-hXa and Va on the membrane surface (Kd, moles of Va bound per mol of OG488-hXa) and the increase in anisotropy at saturation (n, anisotropy in the absence of Va (r0), and the increase in anisotropy at saturation (Δr)). Competition measurements performed using a fixed concentration of OG488-hXa, PCPS, and two fixed concentrations of Va with increasing concentrations of Xa were analyzed by fitting to the implicit cubic Equation 17 of Olson et al. (47) solved iteratively by the Newton-Raphson method (42). Nonlinear least squares analysis yielded fitted constants for the indicator interaction (Kp, n, Δr) as well as the equilibrium dissociation constant for the membrane-mediated interaction between Xa and Va (KxVa,comp) and moles of Va bound per mol of Xa, at saturation (θVa,comp).
**Exosite-directed Inhibition of Human Prothrombinase**

**TABLE I**

Thermodynamic parameters for the binding of NAPc2 to human X and Xa. Thermodynamic constants were determined by isothermal titration calorimetry as illustrated in figure 1.

| Ligand *a* | $K_E \pm S.E. \, ^b$ | $k_{cat,ES} \pm S.E.$ | $K_{ES,N} \pm S.E.$ | $k_{cat,ENS} \pm S.E.$ | $K_{E,N} \pm S.E.$ | $K_{ENS,N} \pm S.E.$ |
|-----------|------------------|-----------------|------------------|------------------|-----------------|------------------|
| X (2 mM Ca$^{2+}$) | 0.87 ± 0.11 | 0.96 ± 0.03 | −13.82 ± 0.05 | −12.36 | −4.9 |
| X (no Ca$^{2+}$) | 1.58 ± 0.07 | 0.97 ± 0.05 | −13.65 ± 0.03 | −12.00 | −5.54 |
| Xa (2 mM Ca$^{2+}$) | 0.78 ± 0.04 | 1.03 ± 0.03 | −17.09 ± 0.03 | −12.42 | −15.68 |

*a* Measurements were performed at 298 K in 20 mM Hepes, 0.15 mM NaCl, pH 7.4, with or without 2 mM Ca$^{2+}$. The cell contained either human X or Xa inactivated with APMSF (Xa).

*b* Fitted constants are listed with 95% confidence limits.

**TABLE II**

Kinetic and inferred equilibrium constants for the modulation of peptidyl substrate cleavage by NAPc2.

| Enzyme species *a* | Analysis method *b* | $K_{ES,N} \pm S.E.$ | $k_{cat,ENS} \pm S.E.$ | $K_{E,N} \pm S.E.$ | $K_{ENS,N} \pm S.E.$ |
|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Xa Local | 98.1 ± 6 | 172 ± 4 | 53.9 ± 7 | 169 ± 5 | 0.56 ± 0.08 | 0.31 ± 0.01 |
| Xa-L Global | 98.3 ± 3 | 181 ± 5 | 43.3 ± 3 | 42 ± 8 | 0.59 ± 0.09 | 0.26 ± 0.04 |
| Xa Va-L Global | 205 ± 10 | 179 ± 5 | 63.8 ± 2 | 86 ± 1 | ND | ND |
| Xa Va-L Global | 200 ± 6 | 169 ± 2 | 64.1 ± 12.2 | 92 ± 2 | 4.9 ± 0.7 | 1.57 ± 0.22 |

*a* The enzymic species represent Xa alone, Xa saturated with PCPS (Xa-L), and Xa saturated with both Va and PCPS (Xa Va-L).

*b* In the local method of analysis, steady state kinetic constants for the cleavage of SpXa were independently determined in the absence of NAPc2 ($K_{ES,N}$ and $k_{cat,ENS}$) or in the presence of 40 nM NAPc2 ($\sim 10–80 \times K_E$) to yield estimates of $K_{ENS,N}$ and $K_{E,N}$ and $K_{ENS,N}$ were determined separately by numerical analysis of velocity changes as a function of increasing concentrations of NAPc2 (e.g. Fig. 2A) according to Scheme I, using the determined steady state kinetic constants. In the global method, data sets obtained with increasing concentrations of SpXa in the presence of different fixed concentrations of NAPc2 (e.g. Fig. 2B) were globally analyzed according to Scheme I to yield all listed constants.

SpXa hydrolysis catalyzed by Xa, Xa saturated with PCPS (Xa-L), or Xa saturated with both PCPS and Va (Xa Va-L) (Table II, local analysis). In each case, saturating concentrations of NAPc2 modestly decreased the $K_{ES}$ value for SpXa. An associated decrease in $k_{cat}$, was observed for Xa-L and Xa-Va-L. The findings suggest that NAPc2 binds to factor Xa regardless of the presence of other prothrombinase constituents and modestly perturbs active site function. The slightly enhanced affinity for SpXa in the presence of NAPc2 implies that this probe does not occlude the active site of the enzyme.

The findings are consistent with a kinetic model (Scheme I) in which NAPc2 (N) acts as a modulator of SpXa (S) hydrolysis catalyzed by Xa, Xa-L, or Xa Va-L (E). N may bind to E or the ES complex and modestly alter steady state kinetic constants for the cleavage of S.

**FIGURE 2**

Binding of NAPc2 to Factor Xa and Prothrombinase—Equilibrium dissociation constants for the binding of NAPc2 to Xa, Xa-L, or Xa Va-L were inferred from steady state kinetic studies and analysis according to Scheme I. When feasible, $K_{E,N}$ and $K_{ENS,N}$ were “locally” determined from the change in initial velocity observed with increasing concentrations of NAPc2 (e.g. Fig. 2A), using the independently determined steady state kinetic constants in the absence and at saturating NAPc2 as known parameters. Alternatively, initial velocities measured at increasing concentrations of SpXa at different fixed concentrations of NAPc2 were used to globally extract all kinetic and equilibrium constants illustrated in Scheme I (e.g. Fig. 2B). Both approaches yielded a series of internally consistent constants (Table II). The inferred equilibrium dissociation constants ($K_{E,N}$) for the binding of NAPc2 to either solution-phase Xa or Xa saturated with PCPS were equivalent to each other and in tolerable agreement with constants derived from ITC measurements (Table I). However, $K_{ENS,N}$ was increased by a factor of −10 when Xa was incorporated into prothrombinase with saturating concentrations of PCPS and Va (Table II). In each case, equilibrium dissociation constants inferred for the binding of NAPc2 to enzyme saturated with substrate ($K_{ENS,N}$) were modestly decreased. Taken together with the thermodynamic measurements, these data provide quantitative support for the conclusion that NAPc2 binds with comparable affinity to factors X and Xa, in a manner that neither requires a functional active site nor one that leads to the occlusion of the active site of the proteinase. Assembly of factor Xa into prothrombinase leads to a modulation in affinity for NAPc2, evident as a 8–10-fold in the equilibrium dissociation constant.

**Inhibition of Protein Substrate Cleavage by Prothrombinase**—In contrast to the small effects of NAPc2 on catalytic function assessed with SpXa, initial experiments revealed that this probe was a complete inhibitor of prothrombin activation by prothrombinase, in agreement with previous observations (16). Because of the known difficulties in the meaningful interpretation of the kinetics of prothrombin activation (20, 30), mechanistic information was further sought using prothrombin 2, established as an appropriate substrate analog for the first half-reaction of prothrombin activation (20, 30).

Initial velocity studies established NAPc2 as a tight binding inhibitor of prothrombin 2 cleavage by prothrombinase (Fig. 3). Inhibition was independent of prior incubation of NAPc2 with the enzyme (not shown) but depended on the concentration of...
substrate (Fig. 3). The data could be described by the rate expression for tight binding complete, competitive inhibition (Fig. 3) yielding a stoichiometry of $-1$ and a $K_i$ in agreement with the equilibrium dissociation constant determined from studies of SpXa cleavage by prothrombinase (Table II). The fitted steady state kinetic constants for prethrombin 2 cleavage were also in agreement with independently determined values of $K_m = 7.8 \pm 0.6 \mu M$ and $V_{max}/E_T = 2.62 \pm 0.03 \text{ s}^{-1}$. Alternative inhibition mechanisms could be excluded on the basis of the series of criteria described previously (15, 48). NAPc2 and prethrombin 2 therefore bind in a mutually exclusive manner to prothrombinase. As NAPc2 binding to Xa within prothrombinase does not occlude the active site of the proteinase, it follows that competitive inhibition of protein substrate cleavage is achieved by interfering with interactions at sites removed from the active site. These are properties expected of an exosite-directed inhibitor of prothrombinase (9).

**Influence of NAPc2 on the Assembly of Prothrombinase**—Deleterious effects of NAPc2 on the equilibrium dissociation constant for the interaction between Xa and Va could provide an explanation for the findings. Kinetic studies in the presence of different fixed and saturating concentrations of Va failed to provide obvious evidence for the destabilization of prothrombinase by NAPc2 (not shown). Nevertheless, this possibility was further evaluated by binding studies.

Bovine Xa modified with dansyl-EGR-CH$_2$Cl has proved a powerful fluorescent probe for thermodynamic and kinetic studies of the assembly of bovine prothrombinase (49, 50). Because an equivalent approach with human proteins fails to yield a reliable change in probe intensity, anisotropy, or excited powerfully fluorescent probe for thermodynamic and kinetic studies of the assembly of bovine prothrombinase (49, 50).

**FIG. 2.** Kinetic analysis of the binding of NAPc2 to active Xa. A, initial velocities were determined using 0.5 nM Xa, increasing concentrations of NAPc2, and 45 $\mu M$ SpXa in assay buffer. Means and S.D. from duplicate measurements are presented. The line was drawn following local analysis according to Scheme I to yield fitted values of $K_{E,N}$ and $K_{E,Va}$ (Table II). $B$, initial velocities were determined using reaction mixtures 1 nM Xa plus 50 $\mu M$ PCPS and increasing concentrations of SpXa and NAPc2 fixed at 0 ($\bullet$), 0.5 ($\bigcirc$), 1 ($\bigtriangleup$), 2 ($\bigtriangleup$), 5 ($\bigtriangledown$), 10 ($\bigcirc$), and 20 nM (■). The lines are drawn following global analysis according to Scheme I with the fitted parameters listed in Table II.

**FIG. 3.** Inhibition of prethrombin 2 cleavage by prothrombinase. Initial velocities for thrombin formation were determined using reaction mixtures containing 5 nM Xa, 25 nM Va, 50 $\mu M$ PCPS (5 nM prothrombinase), increasing concentrations of NAPc2, and 1.4 ($\bullet$), 2.8 ($\bigcirc$), 4.2 ($\bigtriangleup$) and 8.4 $\mu M$ ($\bigtriangledown$) prethrombin 2 in assay buffer. The lines are drawn according to tight binding, complete competitive inhibition (Equation 3) using the fitted constants $K_{E,N} = 6.9 \pm 0.4 \mu M$, $V_{max}/E_T = 2.06 \pm 0.07 \text{ s}^{-1}$, $K_i = 5.11 \pm 0.3 \text{ nM}$, and $n = 1.19 \pm 0.2 \text{ mol of NAPc}$ 2/mol of $E$ at saturation.

(Fig. 4A). No change in anisotropy was observed in buffer containing EDTA in place of Ca$^{2+}$ (Fig. 4A). Titration of reaction mixtures containing OG$_{488}$-hXa, Va, and saturating concentrations of PCPS with increasing concentrations of a non-fluorescent, inactivated Xa derivative (Xai) reduced the anisotropy (Fig. 4A, inset). Based on the model previously developed for the analysis of binding interactions in the assembly of bovine prothrombinase (41), the titration data yielded $K_d = 4.34 \pm 0.56 \text{ nM}$ for the interaction between OG$_{488}$-hXa and human Va on the membrane surface and a stoichiometry of $1.17 \pm 0.04 \text{ mol}$ of Va bound per mol of OG$_{488}$-hXa at saturation. Analysis of the competition data (Fig. 4A, inset) yielded comparable equilibrium parameters for the binding of either OG$_{488}$-hXa or Xa, to human Va, thereby documenting the reversibility and authenticity of the binding interactions monitored using OG$_{488}$-hXa. In keeping with extensive work performed with bovine prothrombinase (10, 41), these points outline the basic and quantitative features of the assembly of human prothrombinase and establish OG$_{488}$-hXa as an appro
NAPc2 was found to decrease the maximum anisotropy change observed following the assembly of OG488-hXa into prothrombinase. Despite the decreased amplitude of the signal, approximately equivalent equilibrium dissociation constants and stoichiometries for the assembly of prothrombinase were obtained from titration curves in the absence and presence of a vast excess of NAPc2 (Fig. 4B). Therefore, the decreased anisotropy change in the presence of NAPc2 does not result from a disruption of prothrombinase but rather an altered signal associated with the assembly of OG488-hXa into prothrombinase in the presence of NAPc2. The results support the interpretation that NAPc2 does not interfere, in an obvious way, with the membrane-dependent interaction between Xa and Va. Thus, selective inhibition of protein substrate cleavage by NAPc2 does not arise from a destabilization of the interaction between Xa and Va. The 10-fold reduction in the affinity of NAPc2 following the incorporation of Xa into prothrombinase cannot be explained by a matched decrease in the interaction between Xa and Va from the simple consideration of two linked binding interactions. The decreased affinity of NAPc2 for prothrombinase could arise from a Va-dependent modulation of surfaces in factor Xa that follows the interaction between OG488-hXa and Va detected by the anisotropy change.

Modulation of NAPc2 Binding by Ligands Targeting the Proteinase Domain—The functional effects of NAPc2 on Xa and prothrombinase function parallel those previously described with the monoclonal antibody aBFX-2b (15). Prolonged incubation of Xa with aBFX-2b in the presence of NAPc2 progressively reduced the ability of NAPc2 to modulate the initial velocity of SpXa (Fig. 5A). The slow, tight binding interaction between aBFX-2b and Xa (15) precluded a quantitative analysis of the potentially competitive equilibria established in this reaction system. However, simulations yielded the preliminary conclusion that the interaction between aBFX-2b and Xa or NAPc2 and Xa reduces the affinity for the second ligand by at least a factor of 40 (Fig. 5A). The data therefore suggest that aBFX-2b somehow interferes with the binding of NAPc2 to Xa.

Similar observations with high concentrations of low molecular weight heparin suggested that the low affinity interaction between heparin and Xa (51) could also affect NAPc2 binding. This was qualitatively documented by affinity chromatography using a heparin matrix. Factor X was near-quantitatively retained by the resin and could be recovered following elution with increasing concentrations of NaCl (Fig. 5B). In contrast, premixing of factor X with 1.5 molar eq of NAPc2 blocked binding of factor X to the resin (Fig. 5B). Although the quantitative basis for this effect is presently unclear, NAPc2 appears to significantly affect the binding of both heparin and aBFX-2b to factor X. Because both heparin and aBFX-2b are known to bind to sites in the heavy chain of factor Xa (15, 51), the observations implicate a role for structures present in the proteinase domain in binding NAPc2.

Structural Studies of the Interaction of NAPc2 with Xa—A biotinylated derivative of NAPc2 (NAPc2-biotin) was found to reveal bands corresponding to human X in ligand blotting studies following SDS-PAGE without disulfide bond reduction. SDS-PAGE analysis of a series of plasma-derived and recombinant factor X derivatives along with human prethrombin 2 and human factor Va (Fig. 6, A and B) is compared with the corresponding ligand blot obtained with NAPc2-biotin without disulfide bond reduction (Fig. 6C). NAPc2-biotin could detect human factor X, both a and b forms of human Xa, as well as recombinant human factor X but not Va, prethrombin 2, and either plasma derived or recombinant bovine factor X (Fig. 6C). The data document the apparent specificity of NAPc2 for human factors X and Xa and confirm the previous suggestion (52) that NAPc2 binds weakly, if at all, to bovine factor X or even bovine Xa (not shown in Fig. 6). The sequences of human and bovine factor Xa are strikingly dissimilar in the 19–25 residues at the COOH terminus of the proteinase domain following residue 240 (53). A recombinant chimeric derivative of bovine factor X, in which heavy chain residues 240 onward were replaced with the sequence present in human factor X, bound...
NAPc2-biotin in the ligand blotting approach (Fig. 6C). Thus, the COOH terminus of the heavy chain of human factor X somehow contributes to the high affinity interaction with NAPc2, and differences in this region are at least partly responsible for the surprising species selectivity of NAPc2.

The quantitative bases for these findings were established by ITC (Table III). Recombinant human factor X yielded thermodynamic constants that were in agreement with those obtained in a parallel experiment with factor X isolated from human plasma. Both recombinant and naturally occurring forms of bovine factor X bound NAPc2 with 300–500-fold lower affinity. In contrast, the recombinant chimeric form of bovine factor X bearing the human X sequence at the COOH terminus had 100–1000-fold higher affinity.

Significance of the Selectivity of NAPc2 for Human Xa—The data establish exosite-mediated recognition of the protein substrate as an important feature of the function of human prothrombinase. As NAPc2 acts as a competitive inhibitor of protein substrate cleavage by human prothrombinase, it is possible that structural elements of human Xa that participate in binding NAPc2 also play a role in exosite-dependent macro-molecular substrate recognition. Because both human and bovine prothrombinase likely employ similar mechanisms for substrate recognition, the apparent selectivity of NAPc2 for human Xa raises concerns regarding the significance of such conclusions.

In agreement with the modest affinity observed for the interaction between NAPc2 and bovine X, no evidence was obtained for an interaction between bovine Xa in the absence or presence of saturating concentrations of PCPS and nanomolar concentrations of NAPc2 (not shown). However, NAPc2 was a potent inhibitor of prethrombin 2 cleavage catalyzed by bovine Xa assembled into prothrombinase with saturating concentrations of PCPS and bovine Va (Fig. 7). The data illustrate that NAPc2 acts as a competitive inhibitor of protein substrate cleavage by bovine prothrombinase with an affinity ($K_i = 1.4 \pm 0.09$ nM) slightly superior to that observed for the human enzyme. Therefore, the modest affinity of NAPc2 for bovine Xa is greatly enhanced following the assembly of bovine Xa into prothrombinase. Initial velocity measurements of SpXa hydrolysis indicate that NAPc2 has no obvious effect on active site function of bovine Xa within prothrombinase (Fig. 7). The data indicate that regardless of differences at the COOH terminus of the proteinase, NAPc2 binds with comparable affinity to both human and bovine Xa within prothrombinase at a site distinct from the active site. This binding interaction selectively blocks exosite-dependent protein substrate recognition by either enzyme and leads to equivalent functional consequences.

The large increase in affinity for NAPc2 observed upon assembly of bovine Xa into prothrombinase provides further evidence to support the initial suggestion that the interaction between Xa and Va modulates the proteinase at sites distinct from the active site, leading to changes in the affinity for NAPc2. This modulation could either occur at the COOH
Km is drawn following analysis according to Equations 1 and 2 assuming a 100-fold excess of the proteinase domain itself or at additional sites (17). The results derived from isothermal titration calorimetry at 298 K in 20 mM Hepes, 0.15 M NaCl, 2 mM Ca$^{2+}$, pH 7.4.

**TABLE III**

| Derivative$^a$ | $K_a$ ± S.E. | $n$ ± S.E. | $\Delta H$ ± S.E. | $\Delta G$ ± S.E. | $\Delta S$ ± S.E. |
|----------------|--------------|------------|------------------|------------------|------------------|
| p-X$_{Hum}$   | 0.98 ± 0.07  | 1.06 ± 0.06| $-13.46$ ± 0.03  | $-12.28$ ± 0.03  | $-4.0$ ± 0.03   |
| r-X$_{Hum}$   | 0.78 ± 0.04  | 1.29 ± 0.03| $-13.06$ ± 0.03  | $-12.42$ ± 0.03  | $-2.1$ ± 0.03   |
| p-X$_{Bov}$   | 266 ± 4.2    | 0.99 ± 0.03| $-11.70$ ± 0.04  | $-8.96$ ± 0.04   | $-9.2$ ± 0.03   |
| r-X$_{Bov}$   | 500 ± 9.1    | 1.05 ± 0.03| $-11.72$ ± 0.05  | $-8.59$ ± 0.05   | $-10.5$ ± 0.05  |
| r-X$_{Bov/Hum}$| 2.06 ± 0.07  | 1.05 ± 0.01| $-15.82$ ± 0.03  | $-11.84$ ± 0.03  | $-11.5$ ± 0.03  |

$^a$ The derivatives of human X$_{Hum}$ or bovine factor X (X$_{Bov}$) are prefixed with p or r to denote the plasma derived or recombinant species. r-X$_{Bov/Hum}$ denotes the chimeric recombinant derivative of bovine X containing heavy chain residues 240–264 from human factor X.

terminus of the proteinase domain itself or at additional sites in the proteinase that play a role in binding NAPc2.

**DISCUSSION**

Extensive work has suggested a dominant role for extended macromolecular interactions in determining protein substrate binding specificity for bovine prothrombinase (9–11). One hallmark of this strategy is that ligands that block the exosite-mediated bimolecular interaction between enzyme and substrate are expected to act as competitive inhibitors of protein substrate cleavage without occluding the active site of the proteinase within the enzyme complex or interfering with the assembly of prothrombinase (10). By using NAPc2 as a tight binding probe, we now establish these formal criteria to apply to the function of human prothrombinase. Therefore, extended macromolecular interactions play a key role in substrate recognition by the human enzyme complex as well. The data further support the conclusion that occlusion of surfaces on factor Xa within prothrombinase, at sites distinct from the active site, is sufficient to interfere with exosite-dependent binding of the protein substrate.

NAPc2 was initially identified as a potent factor X- or Xa-dependent inhibitor of VIIa-TF (16). In agreement with previous work (17), our results support the idea that NAPc2 binds to human factor X and Xa equivalently and in a way that does not require or occlude the active site of the proteinase. Whereas these interactions appear essential for the delivery of NAPc2 to VIIa-TF (17), they also lead to tight binding inhibition of the action of prothrombinase on its protein substrate. Thus, NAPc2 targets two enzyme complexes of blood coagulation with unique strategies. However, inhibition of VIIa-TF by NAPc2 bound to either factors X or Xa occurs with far greater affinity than the inhibition of prothrombinase (17). The plasma concentrations of NAPc2 achieved in clinical studies suggest that the efficacy of NAPc2 as a therapeutic antithrombotic in humans (54, 55) probably derives from inhibition of VIIa-TF.

The monoclonal antibody aBFX-2b, directed against the proteinase domain of Xa, has been established as a prototypic exosite-directed inhibitor in previous work (15) with bovine prothrombinase. Equivalent observations, now described in a completely independent approach with a substantially smaller 85-residue polypeptide probe, greatly increase the confidence in interpretations from such work. It now appears less likely that the distinctive features of such exosite-inhibitors can be explained by generalized steric phenomena associated with the binding of a very large probe to factor Xa within the enzyme complex. The fact that aBFX-2b is able to interfere with the binding of NAPc2 to human Xa further indicates that the comparable properties of the two probes arise from related binding interactions with the proteinase.

The high affinity interaction between NAPc2 and the chimeric X$_{Bov/Hum}$ derivative implicates the COOH terminus of the proteinase domain of human factor X in NAPc2 binding. Autoproteolytic cleavages following Lys$^{251}$ and Lys$^{249}$ at the COOH terminus of the proteinase domain lead to the conversion of a human Xa to the β form (56), with the probable release of the COOH-terminal peptide(s) (22). The ability of NAPc2 to bind both these forms of Xa evident in ligand blotting studies and the absence of heterogeneity in ITC experiments with approximate equimolar mixtures of α and β Xa, implies that NAPc2 binds both species equivalently. Together, the data suggest that side chains present in the 240–251-residue region somehow contribute to NAPc2 binding.

X-ray structures of Xa are variably truncated or poorly resolved at the COOH terminus of the proteinase domain. Residues 240–245 in a human Xa derivative (57) are located on a face of Xa clearly removed from the active site of the enzyme but immediately adjacent to the discontinuous epitope delineated for the binding of aBFX-2b (15). Residue 240 has been implicated in binding heparin (51). These points establish structural correlates for the ability of heparin or aBFX-2b to interfere with the interaction between NAPc2 and human Xa and provide a plausible physical explanation for the equivalent way in which both aBFX-2b and NAPc2 affect enzymic function.

Altered binding of NAPc2 to Xa following its assembly into prothrombinase replicates observations made with aBFX-2b (15). Modulation in binding was evident as a modest decrease in affinity for NAPc2 following the assembly of human prothrombinase and as a large increase in affinity following the assembly of the bovine enzyme complex. Without ascribing significance to magnitude or direction of the change, the data support the previous proposal (15) that the interaction between Xa and Va on the membrane surface detectably perturbs struc-
REFERENCES

1. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475
2. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 913–956
3. Mann, K. G., Nesheim, M. E., Church, W. R., Raskob, G. E., and Shah, K. G. (1990) Blood 76, 1–16
4. Kalafatla, M., Swords, N. A., Rand, M. D., and Mann, K. G. (1994) Biochim. Biophys. Acta 1227, 113–129
5. Davies, E. W., Fujikawa, K., and Kiesel, W. (1991) Biochemistry 30, 10363–10370
6. Jackson, C. M., and Nemerson, Y. (1980) Annu. Rev. Biochem. 49, 765–811
7. Lottenberg, R., Hall, J. A., Paulier, F., Zapan, A., Christensen, U., and Jackson, C. M. (1986) Biochim. Biophys. Acta 874, 326–336
8. Walker, R. K., and Krishnaswamy, S. (1993) J. Biol. Chem. 268, 13920–13929
9. Friederich, P. W., Levi, M., Bauer, K. A., Vlasuk, G. P., Rote, W. E., and Page, L. M. (1997) Biochemistry 36, 12080–12088
10. Betz, A., and Krishnaswamy, S. (1998) J. Biol. Chem. 273, 10709–10718
11. Boskovic, D. S., and Krishnaswamy, S. (2000) J. Biol. Chem. 275, 38561–38570
12. Anderson, P. J., Nesset, A. M., Dharmawardana, K. R., and Bock, P. E. (2000) J. Biol. Chem. 275, 16435–16442
13. Dharmawardana, K. R., and Bock, P. E. (1998) Biochemistry 37, 13143–13152
14. Dharmawardana, K. R., Olsen, S. T., and Bock, P. E. (1999) J. Biol. Chem. 274, 18635–18643
15. Wilkens, M., and Krishnaswamy, S. (2002) J. Biol. Chem. 277, 9366–9374
16. Stassens, P., Bergum, P. W., Gansmansen, Y., Jespers, J., Laroche, Y., Huang, S. M., Maki, S., Messens, J., Lavens, P., Cappello, M., Hotz, P. J., Lasters, I., and Vlasuk, G. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2194–2195
17. Bergum, P. W., Cruikshank, A., Maki, S., Kelly, C. R., Ruf, W., and Vlasuk, G. P. (2001) Biotechnology 36, 10632–10671
18. Lottenberg, R., and Jackson, C. M. (1983) Biochim. Biophys. Acta 742, 558–564
19. Bock, P. E. (1993) Methods Enzymol. 222, 478–503
20. Krissanic, S., and Vlasuk, G. P. (1997) Biochemistry 36, 3319–3330
21. Gomori, G. (1942) J. Lab. Clin. Med. 27, 955–960
22. Baugh, R. J., and Krishnaswamy, S. (1996) J. Biol. Chem. 271, 16126–16134
23. Camire, R., Larson, P. J., Stafford, D. W., and High, K. A. (2000) Biochemistry 39, 14322–14329
24. Kim, D. J., and James, H. L. (1994) Biotechnol. Lett. 16, 549–554
25. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) J. Biol. Chem. 262, 32929–32936
26. Chase, T. J., and Shaw, E. (1987) Methods Enzymol. 19, 20–27
27. Baugh, R. J., Dickinson, C. D., Ruf, W., and Krishnaswamy, S. (2000) J. Biol. Chem. 275, 28826–28833
28. Nesheim, M. E., Krissanic, S., Traey, P. B., and Mann, K. G. (1981) Methods Enzymol. 60, 249–274
29. Mann, K. G., Ellion, J., Butowski, R. J., Downing, M., and Nesheim, M. E. (1981) Methods Enzymol. 80, 286–302
30. Wisker, R., and Krishnaswamy, S. (1994) J. Biol. Chem. 269, 27441–27450
31. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z., and Pease, L. R. (1993) Methods Enzymol. 217, 270–279
32. Degen, S. J., MacGillivray, R. T., and Dавide, R. W. (1988) Biochemistry 22, 2087–2097
33. Messier, T. L., Pittman, D. D., Long, G. L., Kaufman, R. J., and Church, W. R. (1991) Gene (Amst.) 99, 299–305
34. Laemmli, U. K. (1970) Nature 227, 680–685
35. De Scipio, R. G., Hermosade, M. A., and Davies, E. W. (1977) Biochemistry 16, 5253–5260
36. Krishnaswamy, S., and Mann, K. G. (1988) J. Biol. Chem. 263, 5714–5723
37. Luckow, E. A., Lyons, D. A., Ridgway, T. M., Esmon, C. T., and Laue, T. M. (1989) Biochemistry 28, 2348–2354
38. Jackson, C. M., Johnson, T. F., and Hanahan, D. J. (1968) Biochemistry 7, 4492–4505
39. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
40. Krishnaswamy, S., Field, K. A., Edgington, T. S., and Mann, K. G. (1992) J. Biol. Chem. 267, 26110–26120
41. Leavitt, S., and Freire, E. (2001) Curr. Opin. Struct. Biol. 11, 560–566
42. Indyk, L., and Fisher, H. F. (1998) Methods Enzymol. 295, 350–364
43. Davie, M. L. (1997) Curr. Opin. Biotechnol. 8, 31–35
44. Kuczm, P. (1996) Anal. Biochem. 236, 267–273
45. Olsen, S. T., Bock, P. E., and Sheffer, R. (1991) Arch. Biochem. Biophys. 286, 533–545
46. Strubner, M., and Johnson, M. L. (1992) Methods Enzymol. 210, 87–105
47. Nesheim, M. E., Kettnner, C., Shaw, E., and Mann, K. G. (1981) J. Biol. Chem. 256, 6537–6540
48. Krishnaswamy, S., Nesheim, M. E., Prydzell, E. L., and Mann, K. G. (1993) Methods Enzymol. 222, 260–280
49. Rezaie, A. R. (2000) J. Biol. Chem. 275, 3320–3327
50. Vlasuk, G. P., Maki, S., Cruikshank, A., Rote, W. E., and Bergum, P. (1998) Blood 92, 361
51. Jackson, C. M. (1984) Proc. Hemostasis Thromb. 7, 55–109
52. Lee, A., Agnelli, G., Buller, H., Ginsberg, J., Heit, J., Rote, W., Vlasuk, G., Costantini, L., Julian, J., Pagliari, P., Raskob, G., and Gent, M. (2001) Circulation 104, 74–78
53. Friederich, P. W., Levi, M., Bauer, K. A., Vlasuk, G. P., Rote, W. E., Brederveld, D., Keller, T., Spatzenegger, S., and Bock, B. E. (2001) Circulation 103, 2555–2559
54. Eby, C. S., Mullane, M. P., Porsche-Sorbet, R. M., and Miletich, J. P. (1992) Blood 80, 306
55. Brandstetter, H., Kuhne, A., Bode, W., Huber, R., von der Saal, W., Wirthschnitz, K., and Engh, R. A. (1996) J. Biol. Chem. 271, 29988–29992
56. Schulz, G. E., and Schirmer, R. H. (1979) Principles of Protein Structure, 1st Ed., pp. 27–45, Springer-Verlag, New York.
