The Association of Coagulation Factor Xa and Factor Va*

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The binding of factor Xa to factor Va in the presence of Ca²⁺ ions and phospholipid is fundamental for the activation of prothrombin to thrombin. Nevertheless, the biochemistry of the intrinsic association between factors Xa and Va is poorly understood. In the present study we have measured the formation of the protein-protein complex in the absence of phospholipid by using analytical ultracentrifugation. Factor Xa or factor Va were respectively modified with a chromophore-peptidyl-chloromethyl ketone or a thiol-specific chromophore, which permitted selective evaluation of the sedimentation of either component by virtue of its unique absorbance properties. Regardless of which protein was labeled, a factor Xa-Va complex ($
\theta_\infty = 9.8$) was formed. The interaction is specific and reversible. In 2 mM Ca²⁺ and at 20 °C, the dissociation constant for the binding of factor Xa to factor Va is 0.8 nM with a 1:1 stoichiometry. The association has multiphasic Ca²⁺ dependence. At concentrations of Ca²⁺ below 1 mM or above 2 mM, a weaker protein-protein equilibrium is maintained.

The association of the serine protease factor Xa and its cofactor Va is a central event of the procoagulant pathway, and is fundamental to the activation of prothrombin to thrombin. Although factor Xa can generate thrombin from prothrombin, the incorporation of factor Xa into the prothrombinase complex is necessary for rapid prothrombin activation. In the presence of a suitable cellular surface (or acidic phospholipid vesicles) and calcium ions, factor Va mediates a 10³-fold increase in the rate of thrombin production by factor Xa (for reviews see Jackson, 1984; Mann et al., 1988, 1990; Jenny and Mann, 1989). Deficiencies in the circulating precursor of either factor Xa or factor Va (factor X and factor V, respectively), lead to significant bleeding disorders (Owen, 1947; Mannen, 1983; Tracy and Mann, 1987).

Both factors Xa and Va are known to possess Ca²⁺ binding sites that are associated with the functions of these proteins. The binding of Ca²⁺ by factor X/Xa is complicated, and somewhat controversial. The protein has been reported to contain as many as 20 cooperative (Henrikson and Jackson, 1975; Lindout and Hemker, 1978) or 39 noncooperative sites (Yue and Gertler, 1978) for Ca²⁺ interaction, and dissociation constants ranging from 0.05 to 4 mM have been reported. Although ambiguity exists regarding the details of factor X/Xa-Ca²⁺ binding, there is general consensus that Ca²⁺ mediates a structural transition that is detectable by hydrodynamic (Radcliffe and Barton, 1972, 1973a, 1973b), spectral (Nelsestuen, 1976), proteolytic (Morita and Jackson, 1986; Morris et al., 1978), and antigenic (Church et al., 1989) changes. Calcium ions have no effect on the ability for factor Xa to hydrolyze chromogenic substrates (Morita and Jackson, 1986), but are known to enhance factor Xa reactivity toward prothrombin (Bajaj and Mann, 1973; Nesheim et al., 1978). The effect on prothrombin activation may involve requirements for Ca²⁺ interaction with factor Xa, prothrombin, or both. Factor V contains an extremely tight Ca²⁺ binding site, and two sites with $K_d = 6 \times 10^{-5} \text{M}$ (Hibbard and Mann 1980). Factor Va contains one Ca²⁺ binding site with $K_d = 2.4 \times 10^{-5} \text{M}$ and several sites of lower affinity (Giunto and Esmon, 1982). Calcium ions are required to stabilize the interaction between the heavy (factor VaH; 94 kDa) and light (factor VaL; 74 kDa) subunits of factor Va (Esmon, 1979). In the presence of excess Ca²⁺ the factor Va chains associate tightly ($K_d = 5.9 \times 10^{-4} \text{M}$) (Krishnaswamy et al., 1989).

The association of both factor Xa and factor Va with phospholipid vesicles requires accessible acidic polar head groups (e.g. phosphatidylserine). In the case of factor Xa, the interaction requires Ca²⁺ (Nelsestuen, 1976; Nesheim et al., 1981a) and involves the $\gamma$-carboxyglutamic acid (Gla)-rich domain (Skogen et al., 1984; Morita and Jackson, 1986). The dissociation constant for factor Xa-phospholipid binding has been measured by equilibrium and kinetic techniques to be in the 10⁻⁴ to 10⁻³ M range (Nesheim et al., 1981a; Krishnaswamy et al., 1988) The most reliable value is 1.1 × 10⁻⁴ M. The affinity of factor Va toward phospholipid is independent of added Ca²⁺. The dissociation constant for this interaction ($K_d = 2.7 \times 10^{-4} \text{M}$) (Krishnaswamy and Mann, 1988) is considerably lower than that of factor Xa, and is exclusively facilitated by the light chain (Higgins and Mann, 1983; Tracy and Mann, 1983; van de Waart et al., 1983; Krishnaswamy and Mann, 1988, Kalafatis et al., 1990).

Factor Va enhances the interaction of factor Xa with phospholipid by approximately 2 orders of magnitude ($K_d = 1.0 \times 10^{-4} \text{M}$) (Nesheim et al., 1979; Krishnaswamy et al., 1988; Krishnaswamy, 1990). However, factor Xa has no distinguishable effect on the energetics of factor Va-phospholipid complexes (Krishnaswamy, 1990). Fluorescent protein measurements have indicated that when membrane-bound factors Xa and Va associate, the active-site environment of factor Xa is altered (Tucker et al., 1983a; Husten et al., 1987; Krishnaswamy et al., 1988). It has been speculated that this alteration in factor Xa may account for the 3,000-fold increase in $k_{cat}$ that is observed (Rosing et al., 1980).

The factor Xa binding site is believed to involve both subunits of factor Va. This conclusion is consistent with the following observations published from several laboratories:
(a) specific monoclonal antibodies for either the 94- or the 74-kDa chain have been shown to inhibit factor Xa binding to factor Va (Tucker et al., 1983b; Annamalai et al., 1987) in immobilized factor Xa was observed to interact with intact factor Va but not with either purified subunit (Giunto and Esmon, 1984); (c) the platelet-bound 74-kDa subunit of factor Va was shown to interact with factor Xa (Tracy and Mann, 1983); and (d) differences in the proteolytic cleavage sites of APC and factor Xa on factor Va suggest a factor Xa heavy chain involvement in the association with factor Xa (Giunto and Esmon, 1984; Odegaard and Mann, 1987). The binding site for factor Va on factor Xa is known to be independent of the NH₂-terminal Glu-domain, which can be removed from factor Xa by limited chymotryptic digestion (Morita and Jackson, 1986).

The predominance of information regarding the association of factors Xa and Va has been accumulated in the presence of phospholipid. In view of the stabilizing effect of the phospholipid, these experiments were designed to measure interactions that occur at approximately 10⁻⁵ M. Consequently, weaker phospholipid-independent interactions were indiscernible. Several laboratories have attempted to examine the solution-phase association of factors Xa and Va by following the kinetics of prothrombin activation. While informative, these studies are complicated by the fact that ternary complex formation (factor Xa-factor Va-prothrombin) is an essential part of such studies and may effect the nature of the factor Xa-Va interaction. The present study was therefore undertaken to evaluate the intrinsic association of factor Xa with factor Va using quantitative solution techniques. Analytical sedimentation was employed to enable the direct demonstration of the phospholipid-independent, fluid-phase interaction between these proteins.

MATERIALS AND METHODS

Chemicals and Reagents—Hepes (Sigma), EDTA (EM Science), EG Rck, and FPRck (Calbiochem), 5- (and 6) carboxy-X-rhodamine, succinimidyl ester (Molecular Probes) and the chromogenic substrate spectrozyme Xa (American Diagnostica Inc.) were obtained commercially. Bovine coagulation factors Va (Krishnaswamy and Mann, 1988; Krishnaswamy et al., 1986b), factor X, prothrombin (Krishnaswamy et al., 1986b), VaH and VaL (Haley et al., 1989) were purified as previously described. The isolation of the heavy and light chain, respectively. Bovine coagulation factors Va (Krishnaswamy and Mann, 1988) and 12.4 and 45,000 for factor Xa (Jackson et al., 1968; Fujikawa et al., 1974); 14.4 and 72,000 for prothrombin (Mann and Eliot, 1980); 13.7 and 54,000 for APC (Kisiel et al., 1976); 12.4 and 94,000 for factor Va (Higgins and Esmon, 1983; Krishnaswamy and Mann, 1988); and 72,000 for factor Va, (Higgins and Mann, 1983; Krishnaswamy and Mann, 1988). The optical density at 280 nm was corrected for Rayleigh light scatter by subtracting 1.7 times the absorbance at 320 nm, as described previously (Bloom and Mann, 1978).

Incorporation of Rhodamine-X-EGRck into Factor Xa—The preparation of rhodamine-X-modified EGRck (rx-EGRck) was accomplished according to the procedure of Williams et al. (1989), who reported the analogous synthesis of carboxyfluorescein-EGRck from the N-succinimidyl ester. A further chromatographic step was employed to isolate the rx-EGRck from the unreacted EGRck and rhodamine-X succinimidyl ester. This involved passage of the partially purified material in 10 mM HCl over a reverse-phase preparative C4 high pressure liquid chromatography column (Waters). The column was subsequently eluted with a linear gradient from 0 to 40% acetonitrile containing 0.05% trifluoroacetic acid. The elution was monitored at 214 and 580 nm. Rhodamine-X-EGRck was identified on several criteria, including the ability to inhibit the hydrolysis of chromogenic substrates by factor Xa; (b) the selective incorporation into factor Xa heavy chain as judged by reducing SDS-PAGE; and (c) the thin layer chromatographic mobility of the starting materials (high performance silica plates (Sigma) were used with an 80:20 methanol:water solvent system). The final rx-EGRck product was stored dry at −20 °C.

The incorporation of rx-EGRck (and other chloromethyl ketone) conjugates was less extensively in this study into the active site of factor Xa was accomplished by incubating factor Xa in 50 mM Tris, 150 mM NaCl, pH 7.4, with a 5-fold molar excess of rx-EGRck. The dry rx-EGRck was resuspended in 10 mM HCl immediately prior to use at a concentration that was sufficiently high to prevent the addition of more than 4% of the total reaction volume. Concentration determinations of rx-EGRck were based upon the assumption that the extinction coefficient of free rhodamine-X was identical to that of rx-EGRck at 582 nm (75,000 M⁻¹ cm⁻¹, R. P. Huggett, personal communication). (Molecular Probes Handbook). To follow the progress of factor Xa modification, the reactivity of the reaction mixture toward the hydrolysis of the chromogenic substrate spectrozyme Xa (200 μM) was compared to an identical concentration of native factor Xa. The incubation was allowed to proceed at room temperature until less than 2% of the original factor Xa activity was measurable (generally 45 min were adequate). At this stage a 5-fold molar excess of EGRck was used to block the active factor Xa that remained.

The reaction mixture was concentrated to approximately 20% of its original volume by ultrafiltration in a Centricron-30 concentrator (Amicon) and was loaded onto a G-25 (Sigma) column that had been previously equilibrated in HBS to remove nonincorporated rx-EGRck and EGRck. Following a 4-h dialysis against HBS the gel filtered rx-EGR-factor Xa was aliquoted and frozen at −20 °C. The lack of rhodamine fluorescence at the dye front of SDS-PAGE indicated that the rx-EGRck that was noncovalently associated with factor Xa had been removed by this procedure. Incorporation of rx-EGRck was exclusive to the heavy chain of factor Xa as indicated by SDS-PAGE under reducing conditions. This was expected since this subcomponent of factor Xa contains the active site histidine. The concentration of each rx-EGR-factor Xa preparation was assessed by a colorimetric assay (BCA assay; Pierce Chemical Co.) and by quantitative amino acid composition analysis (Bohlin, 1983), both of which were based on a known concentration of unmodified factor Xa as a standard.

Analytical Ultracentrifugation—Hydrodynamic studies were performed with a Beckman model E Photopolarimeter analytical ultracentrifuge that was equipped with mirror optics and a cylindrical lens. The photomultiplier carriage position and absorbance output signals were accumulated and digitized through an ISAAC data acquisition system (Cyborg Corp.) interfaced to a microcomputer. Digitization and analysis programs were developed by Drs. M. N. Blackburn and K. G. Mann. All measurements were made in 4° double-sector cells that housed sapphire windows. To selectively follow rx-EGR-factor Xa or total protein, the samples were scanned at 582 or 290 nm, respectively. The samples were extensively dialyzed against HBS, pH 7.4, prior to experimentation. The extinction coefficient of rx-EGRck that is resuspended in HBS was found to be approximately 15 ± 1 μM (duplicated sample) by use...
of a Nova ionized Ca++ analyzer. To bring the Ca++ to a measurable level, the HBS was concentrated 20-fold by lyophilization prior to analysis. No Ca++ was detectable in similarly concentrated milli-Q-treated H2O.

Sedimentation velocity measurements were made on 380-μl samples at 60,000 rpm (260,000 x g) and 20.0 °C. In all cases, a 4-min scan time was selected. Scans were initiated at the desired rotor speed after the meniscus had been depleted of protein generally 4–5 min after reaching 60,000 rpm in the case of factor Xa. Sedimentation coefficients were extracted from these data by linear least squares regression analysis of the natural logarithm of the boundary position versus time. Boundary positions were calculated either from the zero time moment or by midpoint analysis for background see Tanford, 1961; Cantor and Schimmel, 1980). Second moments (F) were calculated according to the following equation (Goldberg, 1953):

\[ F = \frac{(1/A_p) \int_0^\infty (dA/d\theta)^2 d\theta}{\int_0^\infty (dA/d\theta)^2 d\theta} \]

where A is absorbance, Ap is the absorbance of the plateau region, r is radial position, r0 is the radial position at an arbitrary point within the plateau region, and r2 is the radial position at a point that had been depleted of solute. Average boundary positions were obtained from the square root of the second moment. These yielded weight average sedimentation coefficients since the absorbance of only a single species was monitored and the extinction coefficient of the species was independent of the experimental conditions. Therefore, the weight average sedimentation coefficient (s) of rx-EGR-factor Xa in equilibrium with factor Va followed at 582 nm, is given by:

\[ S = (A_p s_p + A_p s_p'A_p) \]

where Ap is the absorbance due to free rx-EGR-factor Xa, Ap is the absorbance of factor Va-bound rx-EGR-factor Xa, Ap is the total absorbance of rx-EGR-factor Xa, s is the sedimentation coefficient of the rx-EGR-factor Xa-Va complex. When it was desirable to extract the sedimentation coefficient of a single species from a resolvable mixture (e.g. the sedimentation coefficient of rx-EGR-factor Xa-Va complex in the presence of uncomplexed rx-EGR-factor Xa), boundary positions were arrived at by determining the radial position of the particular boundary’s absorbance midpoint. (Sedimentation coefficients that were obtained in this manner are indicated in the text). Sedimentation coefficients were standardized to s, only when they were compared to values previously reported in the literature or for the calculation of hydrodynamic shape parameters (see Tables I and III, Fig. 5). In all other cases, the observed sedimentation coefficients are referred to. These values were obtained under internally consistent experimental conditions of solvent, and temperature. To minimize boundary spreading, the rotor was accelerated as quickly as possible to the target speed.

Molecular weights were determined using the high speed meniscus depletion method of Yphantis (1964), according to the equation:

\[ M_r = \frac{[2RT/\omega^2] \ln(A/2\omega^2)}{d \ln(A/\omega^2)} \]

where R is the gas constant, T is the temperature, ω is the angular velocity, d is the partial specific volume of the protein, ρ is the solvent density, A is the absorbance at 280 nm, and r is the radial position from the center of rotation. Rotor speeds were selected such that the effective reduced molecular weight (2 ln A/ω²d²) was approximately 4. Reduced specific volumes were determined by plotting the logarithm of absorbance against radial position squared and subsequently analyzed by least squares regression. The partial specific volume used for rx-EGR-factor Xa was 0.175 ml/g as determined for factor Xa (Radcliffe and Barton, 1972) and 0.719 ml/g for factor Va (Liu et al., 1984).

**Measurement of the Solution Interaction between Factors Xa and Va**—The phospholipid-independent association between factors Xa and Va was quantified by utilizing changes in the weight average sedimentation coefficient of rx-EGR-factor Xa as a function of factor Va added. It was indicated by the linear nature of the sedimentation progress curves (correlation coefficients greater than 0.994; see Fig. 1, inset) that the rx-EGR-factor Xa-Va equilibrium is not significantly perturbed during sedimentation and thus the dissociation rate is slow relative to the experiment. Therefore, changes in weight-average sedimentation coefficients are directly proportional to the fractional saturation of rx-EGR-factor Xa at equilibrium. Therefore these are mathematically related by rearranging the equation for the weight-average sedimentation coefficient of rx-EGR-factor Xa in the presence of factor Va and substituting Aa = Aa - Aa, to yield:

\[ (Aa/Aa') = (S - S_a)/(S_a - S_p) \]

A plot of the observed sedimentation coefficients, as calculated by second moment analysis versus concentration of factor Va, was fit by using a nonlinear least squares iterative computer program (Marquardt, 1963). A dissociation constant (K,) was extracted from these data by fixing the stoichiometry of the factor Xa-Va interaction at 1:1 (as was experimentally determined), according to the following equation (from Segal (1975) and Henderson (1973), as modified by Krishnaswamy et al. (1986b)):

\[ (Aa/Aa') = [Va]/([Va] + ([Va] + S)/K_s) \]

where [Va] is the total concentration of factor Va and [Xa], is the total concentration of rx-EGR-factor Xa. Although qualitative evidence (lack of time dependence) of s suggests that the equilibrium distribution is not affected over the duration of the experiment the derived dissociation constant must be considered apparent since physical separation of bound and free species is occurring through centripetal fractionation.

From the dissociation constants, the Gibbs free energy change (∆G) of the interaction was calculated by the equation:

\[ ∆G = -RT \ln K_s \]

where R is the gas constant, T is temperature in degrees Kelvin, and Ks is the inverse of the dissociation constant. The intrinsic binding energy of the incorporation of factor Xa into the prothrombinase in the absence of strain and losses due to translational or rotational entropy (△Gs) was estimated by the following equation (Jencks, 1981):

\[ ∆Gs = ∆Gx,va + ∆Gx,pcps + ∆Gx,phips \]

where ∆Gx, va is the free energy change associated with the factor Xa-Va interaction, ∆Gx,pcps is the free energy change associated with the factor Xa-phospholipid interaction, and ∆Gx,phips is the free energy change associated with the factor Xa-phospholipid interaction.

**RESULTS**

**Demonstration of the Solution Interaction between Factors Xa and Va**—Analytical ultracentrifugation was used in the present study to follow hydrodynamic changes that are associated with factor Xa-Va complex formation. The interpretation of the multicomponent data was simplified by chemically modifying factor Xa or factor Va with a chromophore reagent so that the sedimentation of either species could be monitored independent of the other. To incorporate a chromophore into factor Va, the active site-directed compound rhodamine-X Gru-Gly-Arg chloromethyl ketone (rx-EGRck) was used. The dansyl analogue of this reagent has been extensively employed in the past and shown to have no adverse effects on the interaction of factor Xa with factor Va in the presence of phospholipid (Neshim et al., 1981; Krishnaswamy et al., 1988). The rhodamine-X derivative was chosen for the present work because it has an extinction coefficient that is approximately 20-fold larger than that of the dansylated compound. In addition to the introduction of a spectral probe into factor Xa, rx-EGRck also functioned to prevent cleavage of factor Va by factor Xa (Tracy, et al., 1983; Odegaard and Mann, 1987). Factor Va was modified through the highly reactive sulfhydryl of the heavy chain of the molecule. The reagent employed was fluorescein-5-(6)-maleimide, which has been demonstrated previously to incorporate into factor Va without consequence to its cofactor activity or subunit association (Krishnaswamy et al., 1989).

Table I compares the previously reported s and molecular weights for factors Xa and Va obtained by centrifugation, to those of the native and modified proteins used in the current study. Since the present work utilized the photoelectric scanning system of the analytical ultracentrifuge, sufficiently low
levels of protein could be used to avoid concentration-dependent sedimentation. While the sedimentation velocity and molecular weight of factor Xa derived in our laboratory were virtually identical to those previously reported (Radcliffe and Barton, 1972), the values for factor Va are moderately lower than previous reports (Laue et al., 1984). The incorporation of the chromophore reagent into factor Xa or factor Va does not affect the hydrodynamic properties of either protein.

An illustration of a sedimentation velocity experiment that demonstrates the interaction between rx-EGR-factor Xa and factor Va is presented in Fig. 1. To selectively follow the transport of rx-EGR-factor Xa, the sample was scanned at the absorbance maximum of rhodamine-X (582 nm). The profile of free rx-EGR-factor Xa (panel A) indicates homogeneity of the sample because the sedimentation coefficient derived by second moment analysis is identical to that derived by midpoint analysis (s = 3.9). When an equimolar concentration of factor Va was included (Fig. 1, panel b), the rhodamine-X factor Xa signal clearly divides into a slow boundary corresponding to the free factor Xa (S = 3.9, midpoint), and a fast sedimenting species (s = 9.6, midpoint). The latter species represents the complex between rx-EGR-factor Xa and factor Va. In the presence of a 3-fold molar excess of factor Va (Fig. 1, panel C), virtually all of the rx-EGR-factor Xa can be accounted for in the fast sedimenting boundary (s = 9.6, midpoint). The total absorbance of rx-EGR-factor Xa at 582 nm is independent of the presence of factor Va, which demonstrates that the absorbance signal for rhodamine-X is not altered by the formation of the complex. The observed sedimentation coefficients were independent of the length of time (20–960 min) that the factor Xa/factor Va mixture was allowed to preincubate. These experiments confirmed that a 20-min preincubation was sufficient for equilibration of the sample.

Evidence for complex formation was also obtained with factor Xa labeled with fluorescein-EGRck, rhodamine-X-FPRck, or lissamine-rhodamine-FPRck. An interaction between factors Xa and Va was also observed when the sedimentation rate of fluorescein maleimide-modified factor Va (fl-5-factor Va) was monitored. The sedimentation coefficient of fl-5-factor Va increased in the presence of a nonsaturating concentration of EGR-factor Xa from s = 7.3 to s = 8.2. Unlike the chromophore labeled-factor Xa data, however, two distinct boundaries were not observed because of the relatively small change in sedimentation rate for the complex relative to free factor Va.

To ascertain whether the interaction between rx-EGR-factor Xa and factor Va was reversible and independent of the rhodamine-X chromophore, concentrations of rx-EGR-factor Xa (1.8 μM) and factor Va (2.0 μM) were chosen to reach approximately 60% saturation of the rx-EGR-factor Xa. When the rx-EGR-factor Xa was combined with an excess of EGR-factor Xa (10 μM) and then added to the factor Va, we observed a decrease in the proportion of chromophore-labeled factor Xa in the fast boundary (complex), with a corresponding decrease in the weight average sedimentation coefficient from s = 7.0 to s = 5.3 (Table II). The sedimentation coefficient of 1.8 μM rx-EGR-factor Xa was increased from s = 3.9 to s = 4.4 when combined with 10 μM EGR-factor Xa, presumably due to concentration-dependent self-association of factor Xa as reported by Radcliffe and Barton (1972). By taking into account that the factor Va is essentially 100% bound (as supported by the near saturation that is reached at considerably lower concentrations in Fig. 1, panel

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**TABLE I**

| Component        | s(mid) | M₀ × 10⁴ | Reference                      |
|------------------|--------|----------|--------------------------------|
| Factor Xa        | 3.90   | 48       | Radcliffe and Barton (1972)     |
| Factor Xa        | 4.10   | 45       | Present                        |
| rx-EGR-factor Xa | 4.15   | 45       | Present                        |
| Factor Va        | 8.18   | 174      | Laue et al. (1984)             |
| Factor Va        | 7.40   | ND       | Krishnaswamy et al. (1989)     |
| Factor Va        | 7.58   | 170      | Present                        |
| fl-5-Va          | 7.40   | ND       | Present                        |

* Determined at 2 mM Ca²⁺.
* Not determined.
C), the observed sedimentation rate of rx-EGR-factor Xa in the presence of factor Va and excess EGR-factor Xa was identical to that predicted ($\delta = 5.2$). The data support the premise of reversibility and illustrate that the incorporation of rhodamine-X peptide chloromethyl ketone into factor Xa is without consequence to the interaction. These inhibition data are consistent with a 1:1 molar stoichiometry for the factor Xa-factor Va interaction. The observation that the sedimentation coefficient of the fast species was limited to $s = 9.6$ (midpoint) at a large excess of factor Xa, eliminates the possibility of multiple factor Xa binding sites on factor Va. A univalent stoichiometry was therefore used for calculating a dissociation constant. The observation that increasing the amount of slow sedimenting species (free rx-EGR-factor Xa) does not affect the sedimentation of the faster species (rx-EGR-factor Xa-Va complex), also indicates that there are no contributions by Johnston-Ogsten effects at the concentration of protein used (Johnston and Ogsten, 1946).

From the molecular weight and sedimentation coefficients that were determined, information regarding the hydrodynamic shape and size of the factor Xa-Va complex can be obtained (Onceley, 1941). Presented in Table III are frictional coefficients ($f$) and frictional coefficient ratios ($f/f_{min}$) (Tanford, 1961; Cantor and Schimmel, 1980) that correspond to the complex and the individual constituent proteins at 2 mM Ca$^{2+}$. The anhydrous axial ratio of the prolate ellipsoid of revolution ($a/b$) that corresponded to a given ($f/f_{min}$) were read directly from Figure 19–3 in Tanford (1961). The data in Table III are consistent with factors Xa (3:1) and Va (9:1) being asymmetric, which confirms previous observations regarding factor Va (Laue et al., 1984). The rx-EGR-factor Xa-Va complex (6:1) has shape asymmetry intermediate to factors Xa and Va.

**Quantification of the Solution Interaction between Factor Xa and Factor Va**—Figure 2 represents the binding curve for rx-EGR-factor Xa as a function of factor Va, at 2 mM Ca$^{2+}$ and 20 °C. A dissociation constant of $0.8 \pm 0.2 \times 10^{-8}$ M was calculated by fitting the binding data to a quadratic equation at a fixed stoichiometry of 1:1. An identical dissociation constant was obtained when the binding of rx-FPR-factor Xa was similarly evaluated. The dissociation constant was also estimated by determining the concentration of bound and free rx-EGR-factor Xa from the absorbance at the plateau region of the resolvable boundaries (n = 3) from sedimentation profiles similar to that presented in Fig. 1, panel B. A dissociation constant of $0.4 \pm 0.1 \times 10^{-8}$ M was calculated. Although error due to boundary spreading and overlap was not taken into account when estimating the plateau heights, this value is in good agreement with the dissociation constant derived from the binding isotherm.

In addition to the calculation of standard error, the competency of the computer-generated fit was judged from the sedimentation coefficients of free rx-EGR-factor Xa ($\delta = 3.7$) and of the rx-EGR-factor Xa-Va complex ($\delta = 9.8$) that were extracted by the curve-fitting routine. Both of these values

**TABLE III**

Hydrodynamic parameters of the rx-EGR-factor Xa complex and constituent proteins

| Macronucleotide | $f_{max}$ | $M_r \times 10^{-8}$ | $a/b$ | $f/f_{max}$ | a/b |
|-----------------|------------|------------------|------|-------------|------|
| Factor Va       | 7.58       | 170              | 1.47 | 9.1         |      |
| rx-EGR-factor Xa| 4.15       | 45               | 1.13 | 3.1         |      |
| rx-EGR-factor Xa+Va | 9.8    | 215              | 1.93 | 6.1         |      |

* Determined at 2 mM Ca$^{2+}$.  
* Anhydrous prolata ellipsoid axial ratio.  
* Derived by midpoint analysis.

**Fig. 2.** Measurement of the phospholipid-independent interaction between rx-EGR-factor Xa and factor Va. The sedimentation of 18 $\mu M$ rx-EGR-factor Xa was monitored at 582 nm in the presence of various concentrations of factor Va. The resulting binding isotherm was fit iteratively using a univalent stoichiometry to derive a dissociation constant. All samples were in HBS, pH 7.4, 2 mM Ca$^{2+}$ at 20.0 °C.

**TABLE IV**

Effect of various derivatives of factor Va or prothrombin on rx-EGR-factor Xa sedimentation

| Component(s)     | $K_{on}$ |
|------------------|----------|
| 1.8 $\mu M$ rx-EGR-factor Xa | 3.95     |
| 1.8 $\mu M$ rx-EGR-factor Xa + 1.8 $\mu M$ factor Va | 7.30     |
| 1.8 $\mu M$ rx-EGR-factor Xa + 10.0 $\mu M$ factor Va | 4.81     |
| 1.8 $\mu M$ rx-EGR-factor Xa + 2.0 $\mu M$ factor Va | 3.85     |
| 1.8 $\mu M$ rx-EGR-factor Xa + 10.0 $\mu M$ prothrombin | 4.12     |
| 1.8 $\mu M$ rx-EGR-factor Xa + 2.0 $\mu M$ factor VIII | 4.09     |

* Determined at 2 mM Ca$^{2+}$.  

were within 5% of those that were measured directly.

The association of factor Xa with factor Va was further explored by testing the effect of AFC-treated factor Va (Va) (2 $\mu M$) on rx-EGR-factor Xa sedimentation (Table IV). We were unable to detect an interaction with factor Va, at this concentration; an observation consistent with previous observations (Guinto and Esmon, 1984). This negative result supports the specificity of the rx-EGR-factor Xa-Va association in our experimental system.

To test whether the constituent factor VaH chain and/or factor VaL chain contribute individually to the factor Xa binding site, each polypeptide (at 10 $\mu M$) was assessed for an interaction with rx-EGR-factor Xa by centrifugation. The data presented in Table IV suggest that each factor Va subunit individually participates in the interaction with factor Xa, since each was found to independently increase the sedimentation velocity of rx-EGR-factor Xa. Factor Va one stage clotting activity was residual to both subunit preparations, but was present at levels that would give rise to less than 2% of the observed increase in rx-EGR-factor Xa sedimentation. The extent of the sedimentation velocity increases are small (especially in the case of factor VaL) suggesting that the binding to each individual chain is present but considerably weaker than that to intact factor Va.

Since the prothrombinase complex recognizes prothrombin as a substrate, it was logical to test the ability of prothrombin to effect the sedimentation of rx-EGR-factor Xa (Table IV). It is known that factor Xa alone has catalytic activity toward prothrombin (Nesheim et al., 1979), which must involve an interaction between the two proteins. We were unable to observe complex formation between rx-EGR-factor Xa and prothrombin (10 $\mu M$). This observation is consistent with the $K_{on}$ of $84 \times 10^{-8}$ M previously reported for the solution-phase...
interaction (Rosing et al., 1980).

Factor VIII is structurally and functionally analogous to factor V (Church et al., 1984; Jenny and Mann, 1989; Mann et al., 1990) and participates with factor IXa in the procoagulant enzyme complex preceding the prothrombinase (tenase).

Factor VIII (obtained as a gift from Hyland Therapeutics) was observed to have two resolvable boundaries at 290 nm with s = 7.7 and s = 1.2 (midpoint) at a 4:1 ratio. Following treatment with 2 NIH units/ml of human thrombin for 20 min at 37 °C, the factor VIII sedimentation profile consisted of resolvable boundaries at s = 6.5 and s = 2.3 (midpoint) with a 3:2 ratio. Factor VIII (at 2 μM) could not be demonstrated to participate in an interaction with rx-EGR-factor Xa by sedimentation (Table IV). This points toward fidelity of factor Xa for factor Va within the procoagulant pathway. This result also supports the specificity of the rx-EGR-factor Xa-Va association in our experimental system.

Effect of Ca²⁺ Ions on the Solution Interaction between Factors Xa and Va—The sedimentation coefficient of rx-EGR-factor Xa demonstrated only a moderate Ca²⁺-dependence (Fig. 3, panel A). The sedimentation of factor Va (Fig. 3, panel B), was observed to substantially increase up to approximately 2 mM Ca²⁺ and then plateau at s = 7.5. In the presence of 2 mM EDTA, the dissociated factor Va subunits sediment at s = 5.2 with a M₁ of 79,000. In the presence of 2 mM CaCl₂, which facilitates fully associated factor Va according to the Ca²⁺ titration data (Fig. 3, panel B), the s and M₁ were 7.5 and 170,000, respectively. When no Ca²⁺ or chelator was added to the factor Va, s = 6.0 and M₁ was estimated to be 157,000, which indicated considerable subunit association. Partly assembled factor Va was not unexpected, since we found the residual concentration of Ca²⁺ (15 μM) to be within the range known to influence the factor V₁₁-V₁₁ association (Guinto and Esmon, 1982).

To study the effect of Ca²⁺ on complex formation between factors Xa and Va, changes in the sedimentation of rx-EGR-factor Xa were selectively followed in the mixture. The Ca²⁺ titration was performed (Figs. 3 and 4) at concentrations of protein that were determined in a preceding section (Fig. 2) to facilitate approximately 60% saturation of the rx-EGR-factor Xa in 2 mM Ca²⁺. A nonsaturated system was chosen to take into account that a stronger interaction between rx-EGR-factor Xa and factor Va may exist at higher concentrations of Ca²⁺. A triphasic Ca²⁺-dependence was observed (Fig. 4). This consisted of: (a) an increase in the sedimentation rate of the rx-EGR-factor Xa/factor Va mixture from residual (15 μM) to approximately 2 mM Ca²⁺; (b) a gradual decline in the sedimentation rate of rx-EGR-factor Xa/factor Va mixture which was complete by 10 mM Ca²⁺; and (c) a Ca²⁺-independent region (s = 5.1), which extends to (minimally) 18 mM Ca²⁺.

The first phase is coincident with the Ca²⁺ dependence of factor Va sedimentation. The binding of rx-EGR-factor Xa to factor Va was not observed unless Ca²⁺ was added. These data suggest that the Ca²⁺-mediated factor Va and/or factor Xa transitions are essential for factor Xa-Va complex formation.

The remainder of the Ca²⁺-dependent factor Xa-Va binding profile exhibited a decrease and then a leveling off of the observed sedimentation coefficient (Fig. 4). This process was associated with a decrease in the affinity between rx-EGR-factor Xa and factor Va, rather than a hydrodynamic change in the proteins, since (a) the sedimentation coefficient of the rx-EGR-factor Xa/Va complex (s = 9.6 midpoint) was independent of Ca²⁺ concentration; (b) the sedimentation of free rx-EGR-factor Xa was not appreciably affected by Ca²⁺ concentration; and (c) the proportion of rx-EGR-factor Xa that was complexed to factor Va was visibly reduced at high Ca²⁺ concentrations.

Complex Energetics—In the simplest model, the energetic driving force behind the incorporation of factor Xa into the prothrombinase would correspond to the sum of the free energy changes of the binary interactions (Jencks, 1981); these include the binding of factor Xa to factor Va, the binding of factor Xa to phospholipid and the binding of factor Va to phospholipid. Listed in Table V is the Gibbs free energy (ΔG) of the relevant bimolecular equilibria. A comparison of the sum of these to the experimentally determined value (from Nesheim et al., 1981a) indicates that the interaction between factor Xa and factor Va-phospholipid would be even tighter if it were a simple sum of the contributing binary interactions (Jencks, 1981).

**FIG. 3.** Effect of Ca²⁺ on the sedimentation of rx-EGR-factor Xa or factor Va. Panel A, the mass transport of 1.8 μM rx-EGR-factor Xa was monitored at 582 nm as a function of Ca²⁺. Panel B, the mass transport of 1.8 μM factor Va was monitored at 290 nm as a function of Ca²⁺. All samples were in HBS, pH 7.4, at 20.0 °C. “Zero Ca²⁺ added” contains levels of Ca²⁺ that are residual to buffers (approximately 15 μM) made with standardly deionized H₂O (Milli-Q, Millipore).

**FIG. 4.** Effect of Ca²⁺ on the phospholipid-independent interaction between rx-EGR-factor Xa and factor Va. The mass transport of 1.8 μM rx-EGR-factor Xa was monitored at 582 nm in the presence of 1.8 μM factor Va as a function of Ca²⁺. All samples were in HBS, pH 7.4, at 20.0 °C. “Zero Ca²⁺ added” contains levels of Ca²⁺ that are residual to buffers (approximately 15 μM) made with standardly deionized H₂O (Milli-Q, Millipore).

**DISCUSSION**

Although it is clear that the biological functions of prothrombinase are expressed by phospholipid-bound factor Xa-Va complexes, the current study has focused on the phospholipid-independent association in order to further our understanding of the mechanisms by which prothrombinase is
TABLE V

Gibbs free energy of incorporation of factor Xa into prothrombinase

| Interactions | $K_a$ (M) | $\Delta G_i$ (kcal/mol) | Ref. |
|--------------|-----------|-------------------------|------|
| Factor Xa + factor Va | $8.0 \times 10^{-3}$ | $-8.2$ | Present |
| Factor Xa + phospholipid | $0.1 \times 10^{-6}$ | $-9.5$ | Krishnaswamy et al. (1988) |
| Factor Va + phospholipid | $2.7 \times 10^{-6}$ | $-11.7$ | Krishnaswamy and Mann (1988) |
| Factor Xa + factor Va + phospholipid | $0.7 \times 10^{-9}$ | $-12.4$ | Nesheim et al. (1981a) |
| Factor Xa + factor Va + phospholipid (predicted from sum of binary interactions) | $-29.4$ | |

We present a direct, quantitative measurement of the intrinsic solution-phase binding of factor Xa to factor Va. The sedimentation of factor Xa as a function of factor Va concentration was selectively followed in the mixture by using a site-directed chromophore reagent (rx-EGRck) (Williams et al., 1989). We derive a dissociation constant of $0.8 \times 10^{-6}$ M at 20 °C in 2 mM Ca$^{2+}$; a value 3 orders of magnitude larger than the value observed in the presence of phospholipid (10$^{-9}$ M) (Nesheim et al., 1981b; Krishnaswamy, 1980). In the simplest scenario, the enhanced affinity due to phospholipid is mediated exclusively through the formation of a ternary complex between factor Xa, factor Va, and phospholipid. The relatively weak interaction between the two proteins is insufficient to promote association when both proteins are in a solution phase at physiological concentrations. However, after both proteins have bound individually to the phospholipid surface the protein-protein interaction between the phospholipid-bound entities promotes complex formation. Thus the energetic driving force behind the incorporation of factor Xa into the prothrombinase should be predictable by summing the free energy change associated with factor Xa-Va, factor Xa-phospholipid and factor Va-phospholipid complex formation (Jencks, 1981). The Gibbs connection energy that is estimated by subtracting the predicted from the observed free energy change (from Nesheim et al., 1981b) for factor Xa incorporation into the prothrombinase is $+17.0$ kcal/mole. This indicates that the interaction of factor Xa with factor Va-phospholipid involves energetically demanding processes. Several have been proposed in the literature. These include: a loss in entropy due to macromolecular interactions and constraint at the phospholipid surface (Krishnaswamy, 1990); a conformational change, for which evidence exists that implicates the active-site environment of factor Xa (Tucker et al., 1983a; Husten et al., 1987; Krishnaswamy et al., 1988) and may conceivably include factor Va; and an enhancement in the affinity of factor Xa for phospholipid following the interaction with factor Va has been deduced from kinetic modeling (Krishnaswamy, 1990). It should be stressed that in this case, the calculation of the Gibbs connection energy is approximate, since the rigorous derivation of a dissociation constant describing the simultaneous equilibria between factor Xa, factor Va, and phospholipid requires the measurement of free factor Xa and free phospholipid-binding sites for factor Xa. At this time, these variables are inaccessible.

The observation of association between factors Xa and Va in the absence of phospholipid has been made previously. On a qualitative level, the interaction has been demonstrated by use of either immobilized factor Xa (Guinto and Esmon, 1984) or factor Va (Annamalai et al., 1987) in solid-phase assays. Several indirect demonstrations of the intrinsic solution-phase binding of factor Xa to factor Va have also been made by quantifying the rate of factor Va-enhanced conversion of prothrombin to thrombin by factor Xa (Nesheim et al., 1979; Rosing et al., 1980; Lindhout et al., 1982; Skogen et al., 1984; Morita and Jackson, 1986; Boskovitz et al., 1990). Of those that reported dissociation constants, Boskovitz et al. (1990) are in agreement with the direct solution-phase value that we derived in the present study (10$^{-6}$ M). The remaining two reports, however, showed dissociation constants in the 10$^{-4}$ to 10$^{-5}$ M range (Lindhout et al., 1982; Skogen et al., 1984). A potential explanation for this discrepancy is that tighter binding may have been observed in the earlier studies due to contaminating phospholipid.

In addition to measuring the binding of factor Xa and Va determined from kinetics, Boskovitz et al. (1990) demonstrated the interaction by following the external fluorescence of dansyl-Glu-Gly-Arg chloromethyl ketone-modified factor Xa. They found a dissociation constant (2.7 × 10$^{-6}$ M) comparable to the value currently reported. Also in concordance with our observations, these authors were unable to detect an interaction between factor Xa and prothrombin, and provide evidence to suggest that factor Va is required to mediate the association at a reasonable (plasma) prothrombin concentration.

From molecular weights and sedimentation coefficients, information regarding the hydrodynamic shape of macromolecules can be estimated (Onchley, 1941). Factors Xa and Va were determined to have anhydrolyzed prolactine ellipsoid axial ratios of 3:1 and 9:1, respectively. This suggests that factor Va is considerably more elongated than factor Xa. The nature of the factor Va shape confirms the previous report (Laue et al., 1984). Since the factor Xa-Va complex (a/b = 6:1) is less elongated than factor Va, the interaction is unlikely to be mediated by a simple end-to-end contact. When factor Xa (Husten et al., 1987) or factor Va (Isaacs et al., 1996) interact with a phospholipid vesicle, it is believed that they protrude longitudinally from the surface. In conjunction with the shape approximations presented here, these data indicate that the contact region between factors Xa and Va may be within the long axis of each protein.

Calcium ions are known to play an essential part in prothrombinase assembly. They do so through the direct interaction with both factors Xa and Va. In the case of factor Xa, Ca$^{2+}$-binding to the Glb-domain facilitates the association with phospholipid (Morita and Jackson, 1986). Factor Va requires Ca$^{2+}$ to maintain the interaction between its subunits (Nesheim and Mann, 1979; Esmon, 1979), which the present and other studies (Guinto and Esmon, 1982) have demonstrated is a prerequisite for optimal factor Xa binding. Furthermore, the deposition of the prothrombinase substrate, prothrombin, on the phospholipid surface is a Ca$^{2+}$-dependent event. In view of the multifaceted involvement of Ca$^{2+}$ in the generation of prothrombinase, complicated activity profiles as a function of Ca$^{2+}$ have been observed in the past (Lindhout et al., 1982; Rosing et al., 1980; Hibbard and Mann, 1980). These are typified by an increase in enzymatic activity up to an optimal Ca$^{2+}$ concentration of 2–3 mM. At higher levels of metal ion, two of these studies reported a decrease in activity (Lindhout et al., 1982; Hibbard and Mann, 1980), while the third reported a plateau region. It is difficult to interpret these results because many Ca$^{2+}$-dependent variables exist.

In the present study, we eliminated the two Ca$^{2+}$-mediated variables by following the effect of Ca$^{2+}$ on the solution-phase interaction between factors Xa and Va. Thus, the effect of Ca$^{2+}$ on phospholipid-binding of factor Xa and the effect of Ca$^{2+}$ on the proteolytic conversion of prothrombin to throm-
bin was avoided. The fact that we also observed a profile that increased and decreased with Ca\(^{2+}\) suggests that the binding of factor Xa to factor Va is the limiting Ca\(^{2+}\)-dependent aspect of total prothrombinase assembly. Our data are in sharp contrast to the indirect kinetic data published by Lindhout et al. (1982), who concluded that Ca\(^{2+}\) has no effect on the strength of the phospholipid-independent factor Xa-Va interaction (above 0.5 mM Ca\(^{2+}\)). These authors also reported a much lower dissociation constant for the binding of factor Xa to factor Va than we observe. Either inconsistency could be due to the presence of contaminating phospholipid in the earlier study, which could have resulted in 100% of the factor Xa to be bound to factor Va over the Ca\(^{2+}\) range that was assayed. Alternatively, the kinetically derived binding data (Lindhout et al., 1982) may be influenced by ternary complex formation between factor Xa, factor Va and prothrombin, and continuous turnover of substrate.

There are several mechanisms by which Ca\(^{2+}\) ions may influence the intrinsic association of factors Xa and Va. Summarized in Fig. 5 are the relevant protein-protein and protein-Ca\(^{2+}\) interactions including the available dissociation constants and \(s_{20,w}\) values. Both factor Xa and factor Va undergo hydrodynamic change in response to Ca\(^{2+}\). In the case of factor Va, the change is due to the association of the heavy and light subunits (Esmon, 1979; Krishnaswamy et al., 1989), although secondary conformational transitions cannot be ruled out. The induction of the Ca\(^{2+}\)-dependent factor Xa-Xa hydrodynamic change is conformational and was previously shown to involve Ca\(^{2+}\) binding exclusive to the Gla-domain (Sugo et al., 1984). Since the interaction between factors Xa and Va in solution was reported to be independent of the Gla-domain (Skogen et al., 1984), it is reasonable to hypothesize that the metal ion-driven conformational change in factor Xa is not necessary for factor Va binding. This does not preclude its importance to the membrane binding or catalytic efficiency of factor Xa.

At levels of Ca\(^{2+}\) exceeding 2 mM we observed a reduction in the affinity of factor Xa for factor Va. These data are consistent with either or both of two working models. The first advocates that the points of contact between factors Xa and Va are composed of both protein-protein interactions and a Ca\(^{2+}\) bridge(s). As more metal ions are made available to saturate binding sites that previously shared cations, the Ca\(^{2+}\) bridge(s) is broken. The outcome is that a weaker factor Xa-Va equilibrium is maintained through the contribution of the remaining protein-protein interactions. The second model suggests that a hydrodynamically indistinguishable conformational alteration is induced by high concentrations of Ca\(^{2+}\). This new conformation results in the observed reduction in affinity between factors Xa and Va.

To our knowledge, the interaction between factors Xa and Va represents the first study in which the optical selectivity of the analytical ultracentrifuge has been exploited to quantify heteromacromolecular complex formation. On a qualitative level, Krishnaswamy et al. (1989) utilized this technology to verify the kinetics of factor Va subunit reassociation. In view of the array of specific reagents that facilitate the introduction of covalent chromophore probes into proteins (and other macromolecules), it is probable that photometric analytical ultracentrifugation will be applied to a wide variety of biochemical systems. As an example, we are currently investigating the physical properties of ternary complexes within coagulation.

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\[ V_{a_2} (5.3 \text{ S}_{20, w}) + V_{a_2} (5.0 \text{ S}_{20, w}) \]
\[ V_{a_2} (7.6 \text{ S}_{20, w}) \]
\[ \frac{K_d = 24 \mu M}{Ca^{2+}} \]
\[ \frac{K_d = 0.7 \text{ nM}}{Ca^{2+}} \]
\[ V_{a_2} (9.8 \text{ S}_{20, w}) \]
\[ \frac{K_d = 0.8 \mu M}{Ca^{2+}} \]
\[ \frac{K_d = 0.8 \mu M}{Ca^{2+}} \]
\[ \frac{K_d = 0.05-4 \text{ mM}}{Ca^{2+}} \]
\[ V_{a_2} (13.6 \text{ S}_{20, w}) \]

Fig. 5. Schematic of the involvement of Ca\(^{2+}\) on the formation of factor Xa-Va complexes. Shown are the \(s_{20,w}\) values for each component determined at 2 mM Ca\(^{2+}\) (unless otherwise specified), and the available dissociation constants for Ca\(^{2+}\)-protein (shown in brackets) and protein-protein interactions. Both factors Xa and Va undergo a hydrodynamic transition in response to Ca\(^{2+}\). The hydrodynamic change in factor Xa is conformational, while that of factor Va is due to subunit association. No complex formation between factors Xa and Va is observed in the absence of Ca\(^{2+}\). At levels of Ca\(^{2+}\) in excess of 2 mM, the affinity between factors Xa and Va is weaker (as depicted by the notation: (XaVa)\(^2\)).

Laue et al. (1984), Gymnitz and Esmon (1982), Krishnaswamy et al. (1989), Henrikson and Jackson (1975); Lindhout and Henkem (1978); Yue and Gertler (1978), \(s_{20,w}\) determined at residual concentrations of Ca\(^{2+}\) (approximately 15 \(\mu M\)).
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