Sakata, Y., & Aoki, N. (1982) *J. Clin. Invest. 69*, 536-542.
Siefring, G. E., Apostol, A. B., Velasco, P. T., & Lorand, L. (1978) *Biochemistry 17*, 2598-2604.
Slife, C. W., Dorsett, M. D., & Tillotoson, M. L. (1986) *J. Biol. Chem. 261*, 3451-3456.
Takahashi, N., Takahashi, Y., & Putnam, F. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8019-8023.
Tsunasawa, S., & Sakiyama, F. (1984) *Methods Enzymol. 106*, 165-170.
Williams-Ashman, H. G. (1984) *Mol. Cell. Biochem. 58*, 51-61.
Yoshida, A., & Lin, M. (1972) *J. Biol. Chem. 247*, 952-957.
Yoshikawa, M., Goto, M., Ikura, K., Sasaki, R., & Chiba, H. (1982) *Agric. Biol. Chem. 46*, 207-213.

Interaction of Clotting Factor V Heavy Chain with Prothrombin and Prethrombin 1 and Role of Activated Protein C in Regulating This Interaction: Analysis by Analytical Ultracentrifugation†

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ABSTRACT: Changes in the affinity of the heavy subunit of blood coagulation factor Va (Vh) for prothrombin are thought to be important in regulating the rate of thrombin production. Using analytical ultracentrifugation, we have measured the affinity of bovine Vh for prothrombin and for the prethrombin 1 fragment of prothrombin at 23.3 °C, pH 7.65, in 50 mM tris(hydroxymethyl)aminomethane, 0.1 M NaCl, 0.1 mM benzamidine, and either 2 mM Ca^{2+} or 2 mM ethylenediaminetetraacetate (EDTA). Under these conditions a 1:1 complex of Vh with prothrombin is formed that is governed by a dissociation constant (K_d) of 10 μM, regardless of whether the buffer contains Ca^{2+} or EDTA. An identical K_d is observed when prethrombin 1 is substituted for prothrombin. This indicates that the fragment 1 portion of prothrombin, containing the γ-carboxyglutamic acid residues, does not influence the association. Substitution of human prethrombin 1 for the bovine molecule also results in a 1:1 Vh–prethrombin 1 complex governed by a slightly weaker K_d (27 μM). Discrete proteolysis of bovine Vh by the anticoagulant activated protein C converts the Vh to a form with little or no affinity for prethrombin 1 ($K_d > 1$ mM), without detectable change in the mass of the Vh.

Previous studies have shown that factor Va is a critical regulatory protein in the factor Xa catalyzed activation of prothrombin (Mann, 1984). At least two separate roles have been postulated for factor Va function: (1) binding to factor Xa resulting in subsequent alteration of factor Xa conformation and improved catalytic efficiency (Mann, 1984; Nesheim et al., 1979; Tracy et al., 1981; Husten et al., 1987; Krishnaswamy et al., 1987; Rosing et al., 1980) and (2) binding to the prothrombin, thereby enhancing affinity for membrane surfaces and/or altering the conformation of the substrate (Hemker et al., 1967; Esmon et al., 1973; Van de Waart et al., 1984b; Guinto & Esmon, 1984). Support for the concept that prothrombin binding plays a role in the activation complex comes from the kinetics studies cited above, as well as the observation that factor V activation leads to the formation of a substrate binding site and that factor Va inactivation leads to the loss of this site (Esmon et al., 1973; Guinto & Esmon, 1984). Available information suggests that the major binding site for prothrombin is in the factor Va heavy chain (Vh)[1] and that calcium is not obligatory for the binding. However, no quantitative studies have been performed to evaluate the affinity of the interaction or to determine the role of the metal in this process (Guinto & Esmon, 1984). Further, while it is known that activated protein C proteolysis of Vh prevents binding of this chain to immobilized prothrombin, the extent the association is perturbed by proteolysis is not known.

We have used sedimentation equilibrium analysis to quantitate the structure–function relationships in the interaction between Vh and prothrombin. In these studies we have examined both prothrombin, which contains the γ-carboxyglutamic acid residues required for Ca^{2+} binding, and prethrombin 1, which lacks these residues. Further, we have examined the influence of activated protein C proteolysis of Vh

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; rms, root mean square; rpm, revolutions per minute; K_d, molar dissociation constant; Vh, heavy subunit of thrombin-activated blood coagulation factor V.

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on the affinity of this interaction.

Materials and Methods

Solution Reagents. All buffer components were of reagent grade. Calcium chloride hydrate (99.99+%) was purchased from Aldrich Chemical Co. Dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone and D-phenyl-L-prolyl-L-arginine chloromethyl ketone were purchased from Calbiochem.

Proteins. Four samples of bovine Vh were prepared as described previously (Esmon, 1979). Two preparations of activated protein C inactivated Vh were prepared as described by Guinto and Esmo (1984), except that the cleaved Vh was separated from the activated protein C by chromatography on a Pharmacia Mono-Q column developed with a linear gradient from 0.1 to 0.6 M NaCl in 0.02 M Tris-HCl (pH 7.5). Two samples of bovine prethrombin 1 and one sample of human prethrombin 1 were prepared as described (Owen et al., 1974). All proteins were used within 2 weeks of preparation. Protein integrity, both before and after centrifugation experiments, was assessed by SDS gel electrophoresis (Laemmli, 1970). Protein concentrations were determined according to synthetic boundary methods (Chervenka, 1970). All samples were dialyzed for a minimum of 72 h at 4 °C with stirring against three changes (1000:1 v/v) of buffer A [50 mM Tris-HCl (pH 7.65), 0.1 M NaCl, 0.1 mM benzamidine] containing either 2 mM EDTA or 2 mM Ca^{2+}. Prior to dialysis the samples were brought to 100 nM with dansyl-Glu-Gly-Arg chloromethyl ketone to inhibit protease activity (Kettner & Shaw, 1981). Later experiments, and those experiments involving prothrombin, also were made 100 nM with D-Phe-Pro-Arg chloromethyl ketone (Kettner & Shaw, 1981) prior to dialysis. Earlier studies showed that reproducible results were obtained only when the windows and centerpieces were treated to extensive cleaning, including treatment with these protease inhibitors (Luckow, 1988).

Sedimentation. Equilibrium sedimentation experiments were conducted with interference optics, an RTIC temperature controller, and an electronic speed control. Some experiments used a He–Ne laser light source (Williams, 1972), while others used the conventional AH-6 mercury source. Optics were refocused for the laser, and the refractive increment was adjusted according to the formula $dn/dc = (dn/dc)_0(0.940 + 2\,000\,000/\lambda^2)$, where dn/dc is the refractive increment in mL/g, $(dn/dc)_0$ is the refractive increment at a wavelength of 578 nm (0.1872 mL/g), and λ is the wavelength of interest in Å, as recommended by Perlman and Longsworth (1948). Interferograms were made on Kodak Plus-X film (AH-6 source) or Kodak Technical Pan (laser source).

High-speed experiments were carried out as described by Yphantis (1964). Three solution/solvent pairs were examined in each experiment using a six-channel, 12 mm thick, charcoal-filled epon centerpiece and sapphire windows. Experiments were conducted at a constant temperature of 23.3 °C and at speeds of 12 000, 16 000, 20 000, 24 000, and 28 000 rpm. Three interferograms were made at 30-min intervals following the estimated equilibrium time ($\epsilon = 0.001$; Yphantis, 1964). Blanks, with distilled water, were made before and after each run and used to correct the fringe displacement data (Yphantis, 1964). Before the cell was loaded, all proteins were spun in a microfuge at top speed for 5 min to remove particulates. Short-column equilibrium sedimentation was performed as described by Yphantis (1960). Four solution/solvent pairs were examined in each experiment using an eight-channel, 12 mm thick, charcoal-filled epon centerpiece and sapphire windows. Analyses were performed at 12 000, 16 000, 20 000, 24 000, 28 000, and 32 000 rpm and at a constant temperature of 23.3 °C.

Data Analysis. From 50 to 200 fringe displacements for each of 5 fringes were measured from each interferogram at a radial spacing (in cell coordinates) of 50 μm in the meniscus region, decreasing to 10 μm in the high-gradient regions. The data were edited with the REEDIT program (kindly provided by David Yphantis). Data were truncated to minimize Weiner skewing by excluding fringe gradients above 15 mm/cm^2, corresponding to 375 fringes/cm at a radius of 6.5 cm. The edited, blank corrected fringe displacements were analyzed with the computer program NONLIN to provide values and the 65% confidence intervals for the fitted parameters (Johnson et al., 1981). Data from one or more channels, at different loading concentrations, radial positions, and possible angular velocities may be fit simultaneously. The confidence intervals reflect the precision of the fit of a particular model to the experimental data and do not necessarily reflect the accuracy of the determination.

For the heteroassociations (Vh with prethrombin 1 or Vh with prothrombin), two models were found useful for fitting the data. The first model consists of a monomer–dimer (or higher oligomer) equilibrium with a hypothetical monomer having a molecular weight that is the approximate weight-average molecular weight of the mixture of reacting species:

$$Y(r) = \delta + \exp\{\ln \alpha + \sigma\xi - 2B(Y(r) - \delta)\} + \exp\{N[\ln \alpha + \sigma\xi - 2B(Y(r) - \delta)] + \ln K\} \quad (1)$$

where $Y(r)$ is the fringe displacement (in mm) at a given radius, δ is the base-line offset, α is the activity of the monomer at the (arbitrary) reference radius, σ is the reduced molecular weight (see below), $\xi = (r^2 - r^{\circ 2})/2$ (where r is the radius and r° is the reference radius), B is the nonideality coefficient, N is the stoichiometry of the association, and K is the formation constant for the complex (Johnson et al., 1981). The interpretation of the virial coefficient is treated as described by Johnson et al. (1981). Accurate results are expected from this model when there is no significant fractionation of the reacting components by the gravitational field.

The second model explicitly describes the total fringe displacement as the sum of the displacements due to the individual components and their reversible complex (all assumed to be ideal):

$$Y(r) = \delta + \exp\{\ln \alpha_A + \sigma_A\xi\} + \exp\{\ln \alpha_B + \sigma_B\xi\} + \exp\{\ln \alpha_A + \ln \alpha_B + (\sigma_A + \sigma_B)\xi + \ln K\} \quad (2)$$

where $Y(r)$ is the fringe displacement (in mm) at a given radius δ is the base-line offset, α_A and α_B are the monomer activities of the A and B species, respectively, at the (arbitrary) reference radius, σ_A and σ_B are the reduced molecular weights of the monomeric species, ξ is as defined above, and K is the formation constant for the A·B complex. This model was modified to allow for association stoichiometries other than 1:1; however, it was found that the 1:1 stoichiometry was sufficient to describe all of the data (Teller et al., 1969).

The simplest model that adequately fits the experimental data was used to obtain the values presented here. More complex schemes were considered useful and necessary only if they significantly decreased the variance of the fit and decreased any systematic variations in the residuals (Johnson et al., 1981). In this case, the first model (eq 1) contains fewer molecular components (hence, fewer fitting parameters) and is simpler.

The difference between the reduced molecular weights (σ) of the reacting species determines which model is most useful for determining K_d. The reduced molecular weight of a component is defined as $\sigma = M(1 - \bar{v}\rho)\omega^2/RT$, where M is

Table I: Properties of the Isolated Components

protein	cell loading concn (μM)[a]	buffer addition[b]	speed ($\times 10^{-3}$ rpm)[c]	M_r[d]	K_d (μM)[e]	*BM* (mL/g)[f]	rms (μm)[g]
bovine prethrombin 1	11.5, 3.8, 1.3	EDTA (50 μM)	24, 28, 32	52 500 (52 100–53 200)	*h*	0[i]	14
	11.5, 3.8, 1.3	Ca^{2+} (2 mM)	28, 32	52 700 (52 100–53 300)	*h*	0[i]	13
bovine prothrombin	7.2, 2.4, 0.84	EDTA (50 μM)	20, 24	70 400 (69 600–71 200)	1500 (871–2900)	6 (0–8)	9
		Ca^{2+} (2 mM)	20, 24	72 900 (72 000–73 700)	640 (520–795)	3 (0–6)	7
		Ca^{2+} (10 mM)	20, 24	72 700 (71 200–73 900)	52 (44–62)	3 (2.8–5.5)	8
bovine Vh_i[j]	1.6, 0.8	none	20, 24, 28	91 000 (90 900–91 800)	*h*	0[i]	9

[a] Simultaneous fit to data acquired at the protein loading concentrations listed. [b] Dialysis performed against buffer A containing the addition listed. [c] Simultaneous fit to data acquired at the rotor speeds listed. [d] Molecular weight and 65% confidence interval (in parentheses). [e] Apparent dissociation constant for a monomer–dimer equilibrium. [f] Product of the second virial coefficient and the fitted molecular weight. [g] Square root of the variance of the fit. [h] No higher oligomers detectable. [i] Value held constant during fit. [j] Activated protein C inactivated Vh.

the molecular weight of the component, $\bar{v}$ is its partial specific volume, ρ is the solution density, and ω is the rotor angular velocity (Yphantis & Waugh, 1956). The difference between the reduced molecular weights for two components, A and B, is then $\Delta\sigma = \sigma_A - \sigma_B$. For these experiments it was found that if $\Delta\sigma$ was less than 0.65 cm^{-2}, only the simpler model could fit the data. When fits with the more complex model were attempted, NONLIN failed to converge. This was because the similarities in the component σ's lead to near-perfect correlation of the remaining component-specific fitting parameters. At rotor speeds that resulted in $\Delta\sigma$ greater than 0.65 cm^{-2}, but less than 2.5 cm^{-2}, either model provided nearly identical estimates for the association constant. Finally, at rotor speeds that resulted in $\Delta\sigma$ greater than 2.5 cm^{-2}, only the more complex model provided an adequate fit (Luckow, 1988).

Conversion of Units. Conversion from the fringe displacement scale to other concentration scales was made by using a refractive increment of 0.190 mL/g at 546 nm (Sober, 1970) and 0.185 mL/g at 632 nm (Perlman & Longsworth, 1948). The reduced molecular weights (Yphantis, 1964) returned by NONLIN were converted to the corresponding molecular weights by use of $\bar{v} = 0.72_5$ mL/g for Vh (Laue et al., 1984), 0.73 mL/g for prothrombin and prethrombin 1 (Bajaj et al., 1975), and 0.73 mL/g for the complex. The molar association constants were determined from the best-fit values of the association constant, ln K, returned by NONLIN. The units of this association constant were converted from reciprocal fringe displacement units (inverse millimeters) to reciprocal molarity units (1/M) by using the best-fit molecular weights for each case and the appropriate protein refractive increment (above). For monomer–dimer self-associating systems

$$K_M = K_{mm}(M_a/2)(Y_T/C_T) \quad (3)$$

where K_M is the association constant on the molar concentration scale, K_{mm} is the association constant on the millimeters of fringe displacement scale, M_a is the monomer molecular weight, and Y_T/C_T is the specific fringe displacement (1.169 mm·L/g at 546 nm and 1.090 mm·L/g at 632.8 nm for the experiments reported here). For the (A + B)-type association, the corresponding equation is

$$K_M = K_{mm}(M_aM_b/M_{ab})(Y_T/C_T) \quad (4)$$

where M_a, M_b, and M_{ab} are the molecular weights of A, B, and the A·B complex, respectively. The dissociation constants are calculated as the reciprocal of the determined association constants.

Synthetic Data. Data were generated to simulate the concentration dependence of the point-average z-average molecular weight on the basis of the fitting parameters returned by NONLIN and by assuming a mensicus positioned radially at 6.15 cm, the cell base at 6.229 cm, and a cell loading concentration of 3.4 μM. These conditions approximate those in the most centripetal compartment of the multichannel cell.

Estimates of the Second Virial Coefficient. The second virial coefficient used for Vh was (5–10) $\times 10^{-5}$ mol·mL/g^2 (Laue et al., 1984). Second virial coefficients for prothrombin and for prethrombin 1 were estimated from the equations given by Tanford (1961) and under the assumption that the magnitude of the net charge on the molecules in buffer A is in the range from 1 to 6 for prethrombin 1 and from 3 to 10 for prothrombin. By use of these values, the second virial coefficient in buffer A of prothrombin is (4–8) $\times 10^{-5}$ mol·mL/g^2 and that for either bovine or human prethrombin 1 is (5–8) $\times 10^{-5}$ mol·mL/g^2, where the range in values encompasses the uncertainty in the net charge. The complexes are assumed to exhibit a second virial coefficient that is the sum of those of the components. Although not strictly correct (Tanford, 1961), this assumption does not cause significant errors in the determined parameters.

Results

Component Sedimentation. Before determining the association of Vh with prothrombin or prethrombin 1, it was necessary to establish the extent of self-association of these molecules in the presence and absence of Ca^{2+}. The Vh used in this study had an M_r = 92 400 (Luckow, 1988), as assessed by short-column sedimentation equilibrium (Yphantis, 1960). Vh exhibited trace quantities of a very high molecular weight contaminant ($M_r > 10^6$) and showed no tendency to self-associate over the range of concentrations employed in these studies. These results agree with our previous analyses of Vh (Laue et al., 1984). Examination of the sedimentation behavior over the range of concentrations employed in the interaction studies failed to demonstrate significant self-association of bovine prethrombin 1, even in the presence of ultrapure Ca^{2+} (Table I) or when nonideality is included in the analysis. This observation greatly simplifies the analysis of interactions of prethrombin 1 with Vh.

Bovine prothrombin has been reported to undergo a reversible self-association in the presence of Ca^{2+} (Prendergast & Mann, 1977; Jackson et al., 1979, 1987), while others have not observed significant association (Ingwall & Scheraga, 1969; Nelsestuen et al., 1981). Such a self-association would complicate the analysis of the formation of the Vh–prothrombin complex by the methods used here. In our analyses of 20 samples, from four different bovine prothrombin preparations, only one preparation exhibited a weak, Ca^{2+}-dependent, reversible self-association. The K_d determined in 10 mM Ca^{2+} (Table I) is in agreement with that determined by Jackson et al. (1987). However, we find that the self-association is significantly weaker in either 50 μM EDTA or 2 mM Ca^{2+}, in contrast to the results of Jackson et al. (1987). Over the protein concentration range employed for studying the interaction with Vh and in buffers containing EDTA or 2 mM Ca^{2+}, the prothrombin self-association is sufficiently weak to be neglected.

Table II: Vh–Prethrombin 1 Dissociation Constant at 23.3 °C

condition	K_d (μM)	rms (μm)	notes
rotor speed (rpm)			[Vh] = 2.0 μM; [Pre-1] = 2.0 μM; buffer A + 2 mM EDTA
16 000[a]	11 (8.1–15)[c]	6	
20 000[a]	11 (8.6–16)	11	
24 000[a]	18 (15–21)	7	
28 000[b]	8.9 (7.3–10)	6	
cell loading concn (g/100 mL)			equimolar Vh and Pre-1; 28 000 rpm; buffer A + 2 mM EDTA
0.025[b]	7.6 (5.1–11)	7	
0.04[b]	10 (7.2–14)	11	
0.05[b]	10 (9.1–12)	8	
mole ratio of components, [Vh]:[Pre-1]			
1:1[b]	10 (8.8–12)	8	[Vh] = 2.0 μM; [Pre-1] = 2.0 μM; 28 000 rpm; 2 mM Ca^{2+}
1:2[b]	7.4 (4.9–10)	7	[Vh] = 2.5 μM; [Pre-1] = 5.0 μM; 28 000 rpm; 2 mM EDTA
1:3[b]	10 (7.1–14)	11	[Vh] = 3.4 μM; [Pre-1] = 10.0 μM; 28 000 rpm; 2 mM EDTA
buffer addition			cofit of data from cell loading concentrations of 0.025, 0.04, and 0.05%,
2 mM EDTA[b]	9.7 (8.1–11)	7	examined at 24 000 and 28 000 rpm
2 mM Ca^{2+}[b]	9.4 (7.2–12)	9	

[a] Modeled as a monomer–dimer self-association; see Materials and Methods for details. [b] Modeled as an A + B heteroassociation; see Materials and Methods for details. [c] 65% confidence interval.

Table III: Dissociation Constant for Interaction between Bovine Vh and Human Prethrombin 1 at 23.3 °C

conditions[a]	K_d (μM)	rms (μm)
buffer A + 2 mM EDTA; [Vh] = 1.3 μM; [Pre-1] = 1.3 μM; 20 000, 24 000, and 28 000 rpm	27 (17–35)[b]	7
buffer A + 2 mM Ca; [Vh] = 4.7 μM and [Pre-1] = 2.3 μM; [Vh] = 2.3 μM and [Pre-1] = 1.2 μM; 20 000, 24 000, and 28,000 rpm	25 (19–33)	8

[a] Dissociation constant obtained from the simultaneous fit of data from experiments conducted with the protein loading concentrations and at the rotor speeds listed. [b] 65% confidence interval.

Bovine Vh that has been inactivated by limited proteolysis by activated protein C exhibits a molecular weight (Table I) that is within 2% of that of intact Vh (Laue et al., 1984; Luckow, 1988). No evidence for smaller or larger molecular weight species was observed in these samples.

Association of Bovine Vh with Bovine Prethrombin 1. To examine the Vh interaction with prethrombin 1, we first established that we were observing a reversible association rather than irreversible aggregates or populations of incompetent molecules (Squire & Li, 1961; Yphantis et al., 1978). This was accomplished three ways: (1) by quantitation of the K_d of the heterodimeric complex over a wide range of rotor speeds, (2) by examination of the interaction over a range of protein loading concentrations, and (3) by alteration of the molar ratios of the interacting components. The results indicate that the interaction was reversible and governed by a K_d of about 10 μM (Table II). When the same analysis was performed in the presence of 2 mM Ca^{2+}, the dissociation constant was not altered (Table II).

Association of Bovine Vh with Human Prethrombin 1. Bovine Va is known to function in human plasma (Jobin & Esnouf, 1967), which indicates that bovine Va can interact with human prothrombin. When this interaction was studied under conditions similar to those described in Table II, complex formation was readily detectable and found to be reversible with a K_d of about 27 μM (Table III). As is the case with the bovine Vh–prethrombin 1 interaction, the K_d was independent of the presence of Ca^{2+} (Table III). The somewhat larger confidence interval for this interaction results from the relative weakness of this association and the lower protein loading concentrations employed for high-speed equilibrium analyses. Accordingly, these results were confirmed at higher protein loading concentrations by the determination of *z*-average molecular weights with short-column analyses (Figure 1). The experimental data points obtained at five different speeds agree with the predicted values of the *z*-average molecular weight (Arakawa & Yphantis, 1987).

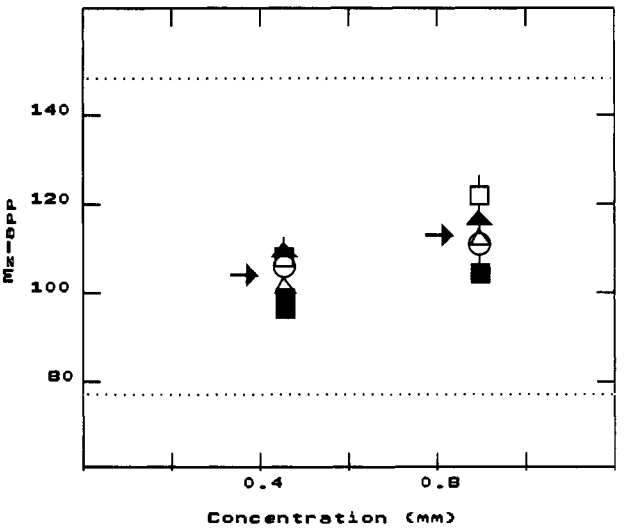

FIGURE 1: Apparent *z*-average molecular weight of solutions containing equimolar mixtures of bovine Vh and human prethrombin 1, in buffer A containing 2 mM EDTA. Samples were examined at 23.3 °C and at 12 000 (○), 16 000 (□), 20 000 (△), 24 000 (■), and 28 000 (▲) rpm. The horizontal lines indicate the expected *z*-average molecular weight in the absence of any association (M_r = 78 000) and in the case where the molecules were fully associated (M_r = 146 000). The arrows indicate the expected *z*-average molecular weight based on data synthesized as described under Materials and Methods and assuming a K_d = 27 μM. The abscissa is defined in units of mm of fringe displacement, with 1 mm corresponding to about 1 mg/mL.

Association of Bovine Vh with Bovine Prothrombin. Interactions of Vh with prethrombin 1 are simplified because prethrombin 1 lacks the fragment 1 region that contains the γ-carboxyglutamic acid residues of prothrombin (Stenflo et al., 1974) and most, if not all, of the moderately high-affinity Ca^{2+} binding sites that are largely responsible for metal-dependent conformational changes in the intact molecule (Furie et al., 1978; Nelsestuen, 1976; Bloom & Mann, 1978; Henriksen & Jackson, 1975). Since metal ions play a major role in prothrombin structure, we reexamined the Vh–prothrombin interaction in the presence and absence of Ca^{2+} (Table IV). Although Guinto and Esmon (1984) demonstrated previously that the interaction was not obligatorily dependent on Ca^{2+}, the methods employed would have been insensitive to even moderate alterations in affinity. Our equilibrium measurements reveal no effect of Ca^{2+} on the Vh–prothrombin in-

Table IV: Bovine-Vh/Bovine-Prothrombin Dissociation Constant at 23.3 °C

conditions[a]	K_d (μM)	rms (μm)
buffer A + 2 mM EDTA; [Vh] = 8.6 μM and [PT] = 5.8 μM; [Vh] = 2.9 μM and [PT] = 1.9 μM; [Vh] = 1.0 μM and [PT] = 0.6 μM; 20000 and 24000 rpm	13 (10–15)[b]	13
buffer A + 2 mM Ca^{2+}; [Vh] = 2.9 μM; [PT] = 2.4 μM; 20000 and 24000 rpm	8 (6–11)	7

[a] Dissociation constant obtained from the simultaneous fit of data from experiments conducted with the protein loading concentrations and the rotor speeds listed, modeled as an ideal monomer–dimer equilibrium; see Materials and Methods for details. [b] 65% confidence interval.

teraction (Table IV). There is no change in the values in Table IV even when the data are adjusted for the Ca^{2+}-dependent self-association of prothrombin (Table I) according to the methods described previously (Laue et al., 1984).

Effect of Activated Protein C Proteolysis on the Vh–Prethrombin 1 Interaction. Equimolar mixtures of activated protein C treated bovine Vh and bovine prethrombin 1 (at concentrations of 5.6, 1.9, and 0.6 μM for each chain) were analyzed in buffer A containing 2 mM EDTA at 24000 and 28000 rpm. Data from these experiments could not be fit to any model that involved association of the monomeric species. The lower limit on K_d is estimated to be at least 100-fold higher than that for the intact chains.

Discussion

Modulation of the strength of the interaction between Vh and prothrombin is thought to be important in the regulation of prothrombin activation (Hemker et al., 1967; Esmon et al., 1973; Guinto & Esmon, 1984; Van der Waart et al., 1984). The present studies quantitate the interaction of Vh with prothrombin with the direct physical technique of equilibrium ultracentrifugation. It is clear that these two proteins associate to form a 1:1 complex with a moderate affinity ($K_d \simeq 10$ μM). Furthermore, the binding interaction is similar for bovine and human prethrombin 1. The latter observation is consistent with the fact that bovine Va is functional in human V deficient plasma (Jobin & Esnouf, 1967). Although the activation of prothrombin and the stability of Va both require Ca^{2+}, the interaction of Vh with prothrombin is completely Ca^{2+} independent. This suggests that the γ-carboxyglutamic acid residues play no role in the Va–prothrombin interaction. This idea is further supported by the observation that the interactions of prethrombin 1 and prothrombin with Vh are indistinguishable and both are Ca^{2+} independent. The Ca^{2+} independence of this reaction is consistent with the previous observation (Guinto & Esmon, 1982) that Vh has no high-affinity Ca^{2+} binding sites.

The interaction of Va with prothrombin is likely important in prothrombin activation since the presence of Va allows optimal prothrombin activation, even with a low density of negatively charged phospholipids (Van Rijn et al., 1984). Direct Va–prothrombin interaction is probably involved since Va has been shown to enhance prothrombin binding to membrane surfaces (Van der Waart et al., 1984b). This binding interaction could contribute to the affinity of the activation complex for the substrate. Likewise, Vh binding of prothrombin could affect its orientation or alter its conformation as a substrate for Xa, in which case this interaction would not be reflected in the substrate binding but might be observed in the catalytic efficiency.

In a previous study we demonstrated (Guinto & Esmon, 1984) that activated protein C cleaves Vh at one major site and multiple minor sites, abolishing the ability of Vh to bind to immobilized prothrombin. Our studies demonstrate that the proteolytic fragments of activated protein C treated Vh remain tightly associated (Table I). Despite retention of most or all of the mass of Vh, the molecule is converted to a form with little or no affinity for prethrombin 1 ($K_d > 1$ mM). Loss of prothrombin binding affinity could be due either to a conformational change in the subunit or to cleavage of the peptide domain responsible for the prothrombin–Vh interaction. In the latter model, one would anticipate that prothrombin would protect Vh from inactivation by activated protein C. Previous experiments have failed to detect such protection (Walker et al., 1979; Nesheim et al., 1981). Given this observation, it is likely that proteolysis does lead to a conformational change that dramatically decreases the affinity of Vh for prothrombin. Despite inactivating the molecule and destroying the Vh–prothrombin interaction site, proteolysis does not result in loss of the capacity to form the heterodimeric structure of Vh·Vl (Guinto & Esmon, 1984; Van der Waart et al., 1984a).

Most, if not all, of the prothrombin binding site on Va resides on Vh (Guinto & Esmon, 1984). Still, it would be useful to know whether or not Va binding of prothrombin is governed by the same K_d as that determined here. Unfortunately, the Va interaction with prothrombin is complicated by the fact that Va itself undergoes both a weak dissociation to Vl and Vh and a weak dimerization (Laue et al., 1984). The deconvolution of the Va interaction with prothrombin from these other interactions would be a formidable task with the methods employed here. This is especially true since the Va–prethrombin 1 complex differs in molecular weight by only 30% from that of Va alone. In the present work, there is a 60% difference in molecular weight between the Vh–prethrombin complex and Vh. The greater signal afforded by using Vh rather than Va helps in the quantitation of the K_d.

Previous reports indicate that bovine prothrombin undergoes a self-association to dimer (Tarvers et al., 1987), with the association governed by a $K_d \simeq 85$ μM, when measured in a buffer containing 10 mM Ca^{2+} (Jackson et al., 1987). Our results for one preparation of prothrombin (Table I) agree quantitatively with their observations. However, significant self-association was observed only when Ca^{2+} levels were 10 mM, and the apparent strength of the association decreased with lower Ca^{2+} concentrations (Table I). This suggests that there is a strong linkage between Ca^{2+} binding and self-association of prothrombin. These results differ with those of Jackson et al. (1987), who found that the dimer formation constant was only slightly smaller in a low-Ca^{2+} buffer. Finally, we find preparation-dependent self-association behavior for prothrombin. One preparation showed no tendency to self-associate, while two others exhibited low levels of aggregates that were not in rapid equilibrium with the $M_r = 73000$ species. Significant self-association was observed for only one of the four preparations examined. The inconsistency in self-association did not extend to other properties of the prothrombin preparations. All four preparations yielded identical (within 3%) molecular weights, showed the same extent of nonideality, and behaved identically on denaturing gels. Moreover, all four preparations showed identical capacities to bind phospholipid vesicles [1:4 (w/w) phosphotidylserine–phosphotidylcholine], as assessed by right-angle light scattering (Nelsestuen & Lim, 1977), and all exhibited calcium-dependent inhibition of thrombin cleavage to prethrombin 1 and fragment 1 (not shown). The cause for the inconsistent association behavior is not understood at this time, but it may

help explain why some groups observe dimer formation (Tarvers et al., 1982, 1986; Cox & Hannahan, 1970; Agrawal et al., 1977; Prendergast & Mann, 1977; Jackson et al., 1979, 1987) while others do not (Nelsestuen et al., 1981; Silversmith et al., 1983). Moreover, it suggests that the positive-cooperative metal ion binding exhibited by prothrombin may not result from ligand-induced oligomerization, as has been suggested (Jackson et al., 1987).

Many important biological processes involve protein–protein interactions of weak to moderate strength. Often these interactions are not amenable to kinetic analysis. Likewise, the proteins often are not available in sufficient quantities or are not suitable for analysis by other physical techniques. These studies illustrate the utility of analytical ultracentrifugation in the analysis of protein–protein interactions in the absence of requirements for protein modification.

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Registry No. Factor Va, 65522-14-7; factor XIVa, 42617-41-4; prothrombin, 9001-26-7; prethrombin, 69866-47-3.

References

Agarwal, G. P., Gallagher, J. B., Aune, K. C., & Armediades, C. D. (1977) *Biochemistry 16*, 1865–1870.

Arakawa, T., & Yphantis, D. A. (1987) *J. Biol. Chem. 262*, 7484–7485.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 2150–2156.

Bloom, J. W., & Mann, K. G. (1978) *Biochemistry 17*, 4430–4438.

Chervenka, C. H. (1970) in *A Manual of Methods for the Analytical Utracentrifuge*, pp 69–72, Spinco Division of Beckman Instruments, Palo Alto, CA.

Cox, A. D., & Hannahan, D. J. (1970) *Biochim. Biophys. Acta 207*, 49–64.

Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964–973.

Esmon, C. T., Owen, W. G., Duiguid, D. L., & Jackson, C. M. (1973) *Biochim. Biophys. Acta 310*, 289–294.

Furie, B., Provost, K. L., Blanchard, R. A., & Furie, B. C. (1978) *J. Biol. Chem. 253*, 8980–8987.

Guinto, E. R., & Esmon, C. T. (1982) *J. Biol. Chem. 257*, 10038–10043.

Guinto, E. R., & Esmon, C. T. (1984) *J. Biol. Chem. 259*, 13986–13992.

Hemker, H. C., Esnouf, M. P., Hemker, P. W., Swart, A. C. M., & MacFarlane, R. G. (1967) *Nature (London) 215*, 248–251.

Henriksen, R. A., & Jackson, C. M. (1975) *Arch. Biochem. Biophys. 170*, 149–159.

Husten, E. J., Esmon, C. T., & Johnson, A. E. (1987) *J. Biol. Chem. 262*, 12953–12961.

Ingwall, J. S., & Scheraga, H. A. (1969) *Biochemistry 8*, 1860–1869.

Jackson, C. M., Peng, C.-W., Brenkle, G. M., Jonas, A., & Stenflo, J. (1979) *J. Biol. Chem. 254*, 5020–5026.

Jackson, C. M., Brenkle, G. M., Hogg, P. J., & Winzor, D. J. (1987) *J. Biol. Chem. 262*, 13472–13475.

Jobin, F., & Esnouf, M. P. (1967) *Biochem. J. 102*, 666–674.

Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. R. (1981) *Biophys. J. 36*, 575–588.

Kettner, C., & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.

Krishnaswamy, S., Church, W. R., Nesheim, M. E., & Mann, K. G. (1987) *J. Biol. Chem. 262*, 3291–3299.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laue, T. M., Johnson, A. E., Esmon, C. T., & Yphantis, D. A. (1984) *Biochemistry 23*, 1339–1348.

Luckow, E. A. (1988) Masters Thesis, University of New Hampshire, Durham, NH.

Mann, K. G. (1984) *Prog. Hemostasis Thromb. 7*, 1–23.

Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648–5656.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.

Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Pletcher, C. H., & Bloomfield, V. A. (1981) *Biochemistry 20*, 351–358.

Nesheim, M. E., Taswell, J. B., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 10952–10962.

Nesheim, M. E., Canfield, W., Kisiel, W., & Mann, K. G. (1981) *J. Biol. Chem. 257*, 1443–1447.

Owen, W. G., Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594–605.

Perlman, G. E., & Longsworth, L. G. (1948) *J. Am. Chem. Soc. 70*, 2719–2724.

Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.

Rosing, F., Tans, G., Govers-Riemslag, J. W. P., van de Waart, P., Zwaal, R. F. A., & Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274–283.

Silversmith, R. E., Wei, J. G., & Nelsestuen, G. L. (1983) *Biochem. Biophys. Res. Commun. 111*, 213–218.

Sober, H. A., Ed. (1970) *Handbook of Biochemistry*, 2nd ed., pp 67–69, Chemical Rubber Publishing Co., Cleveland, OH.

Squire, P. G., & Li, C. H. (1961) *J. Am. Chem. Soc. 83*, 3521–3528.

Stenflo, J., Fernlund, P., Egan, W., & Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2730–2733.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 194–236, Wiley, New York.

Tarvers, R. C., Noyes, C. M., Roberts, H. R., & Lundblad, R. L. (1982) *J. Biol. Chem. 257*, 10708–10714.

Tarvers, R. C., Roberts, H. R., & Lundblad, R. L. (1984) *J. Biol. Chem. 259*, 1944–1950.

Tarvers, R. C., Noyes, C. M., Tarvers, C. K., & Lundblad, R. L. (1986) *J. Biol. Chem. 261*, 4855–4859.

Tarvers, R. C., Roberts, H. R., Straight, D. L., Featherstone, G. L., & Lundblad, R. L. (1987) *Arch. Biochem. Biophys. 257*, 439–443.

Teller, D. C., Horbett, T. A., Richards, E. G., & Schachman, H. K. (1969) *Ann. N.Y. Acad. Sci. 164*, 66–101.

Tracy, P. B., Nesheim, M. E., & Mann, K. G. (1981) *J. Biol. Chem. 256*, 743–751.

Van der Waart, P., Bruls, H., Hemker, H. C., & Lindhout, T. (1984a) *Biochim. Biophys. Acta 799*, 38–44.

Van der Waart, P., Hemker, H. C., & Lindhout, T. (1984b) *Biochemistry 23*, 2838–2842.

Van Rijn, J. L. M. L., Govers-Riemslag, J. W. P., Zwaal, R. F. A., & Rosing, J. (1984) *Biochemistry 23*, 4557–4564.

Walker, F. J., Sexton, P. W., & Esmon, C. T. (1979) *Biochim. Biophys. Acta 571*, 333–342.

Williams, R. C., Jr. (1972) *Anal. Biochem. 48*, 164–172.

Yphantis, D. A. (1960) *Ann. N.Y. Acad. Sci. 88*, 586–601.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.
Yphantis, D. A., & Waugh, D. F. (1956) *J. Phys. Chem. 60*, 623–629.
Yphantis, D. A., Correia, J. J., Johnson, M. L., & Wu, G.-M. (1978) in *Physical Aspects of Protein Interactions* (Castimpoolas, N., Ed.) pp 275–303, Elsevier/North-Holland, New York.

CORRECTIONS

Purification and Characterization of a CMP–Sialic:$LcOse_4Cer$ Sialyltransferase from Human Colorectal Carcinoma Cell Membranes, by Vis Liepkans,* Alain Jolif, and Goran Larson, Volume 27, Number 23, November 15, 1988, pages 8683–8688.

Page 8685. In column 1, lines 17 and 18, 10 microunits of *V. cholerae* sialidase should read 10 milliunits.

Tight-Binding Inhibition of Angiogenin and Ribonuclease A by Placental Ribonuclease Inhibitor, by Frank S. Lee, Robert Shapiro, and Bert L. Vallee*, Volume 28, Number 1, January 10, 1989, pages 225–230.

Page 228. In column 1, the sentence beginning on line 5 of the second paragraph should read as follows: Scheme I predicts that the value of β will increase linearly with increasing 2′-CMP concentration and that the absolute value of the x intercept in this plot will equal $K_{i,C}$.