Cooperative Interaction of Divalent Metal Ions, Substrate, and Tissue Factor with Factor VIIa†

Saulius Butenas, Jeffrey H. Lawson,‡ Michael Kalafatis, and Kenneth G. Mann*

Department of Biochemistry, Health Science Complex, University of Vermont, Burlington, Vermont 05405

Received October 21, 1993; Revised Manuscript Received January 18, 1994®

ABSTRACT: Factor VIIa–tissue factor (TF) complex formation in the presence of EDTA or divalent cations (Me^{2+}) was investigated. The influence of Me^{2+} on the amidolytic activity of factor VIIa and factor VIIa–TF complex was evaluated using low molecular weight synthetic substrates possessing substituted aminonaphthalenesulfonamides as detecting groups. Factor VIIa expressed low amidolytic activity in the presence of EDTA. In the presence of EDTA and saturating concentrations of TF, the amidolytic activity of factor VIIa was increased approximately 90-fold. Gel electrophoresis and sedimentation velocity studies demonstrated complex formation between factor VIIa and TF in the presence of EDTA. Substrate titration curves obtained at fixed factor VIIa and TF concentrations gave sigmoidal shapes, indicating that substrates influenced factor VIIa amidolytic activity in the presence of TF. In the absence of Me^{2+}, the $K_{D,app}$ of the factor VIIa–TF complex was influenced by substrate structure and varied from 3.9 to 34 nM. All Me^{2+} used increased the amidolytic activity of factor VIIa approximately 8-fold compared with experiments in the presence of EDTA. The $K_{D,app}$ values for factor VIIa–Ca^{2+} complex and factor VIIa–Mn^{2+} complex were independent of substrate and were 270 and 40 μM, respectively. The $K_{D,app}$ for factor VIIa–Mg^{2+} complex varied from 3 to 12 mM and was substrate structure dependent. The presence of TF had no influence upon the $K_{D,app}$ for the factor VIIa–Ca^{2+} complex. The amidolytic activity of factor VIIa was enhanced by TF significantly in the presence of Ca^{2+}, and similar results were obtained with Mg^{2+} and Mn^{2+}. The $K_{D,app}$ observed for the factor VIIa–TF complex was influenced by metal ions. In the presence of Ca^{2+}, this constant was found to be 1.1 nM, whereas in the presence of Mn^{2+} it was 5.1 nM. The $K_{D,app}$ was independent of substrate structure for both metal ions. TF and Me^{2+} had no significant influence on the K_M for substrate hydrolysis. These data indicate that (1) complex formation between factor VIIa and TF while Me^{2+} dependent does not have an essential requirement for metal ions, (2) the amidolytic activity of the factor VIIa–TF complex is increased in the presence of Me^{2+}, and (3) cooperative interactions exist between factor VIIa, Me^{2+}, substrate, and TF.

Factor VIIa is a two-chain serine protease which initiates the coagulation cascade by cleavage of the natural substrates factor X and factor IX (Jesty & Silverberg, 1979; Lawson & Mann, 1991). Tissue factor (TF), calcium ions, and a supporting surface (phospholipid or cell surface) are required for this process (Broze, 1982; Nemerson, 1988; Almus et al., 1989; Komiyama et al., 1990; Paborsky et al., 1991; Ruf et al., 1991a; Le et al., 1992; Krishnaswamy, 1992). All of these accessories significantly increase the proteolytic activity of factor VIIa with natural substrates (Komiyama et al., 1990; Bom & Bertina, 1990). The transmembrane domain of tissue factor is bound to the supporting surface, and this surface may affect the factor VIIa–tissue factor complex interaction with factor X and factor IX (Osterud & Rapaport, 1977; Ploplis et al., 1987; Ruf et al., 1991a; Lawson & Mann, 1991; Krishnaswamy, 1992). However, neither the supporting surface (Lawson et al., 1992; Krishnaswamy, 1992) nor the transmembrane domain of tissue factor (Ruf et al., 1991b; Shigematsu et al., 1992) is required for enhancement of the amidolytic activity of factor VIIa.

The EGF-1 domain (Toomey et al., 1991; Clarke et al., 1992; Schiodt et al., 1992) and residues 195–220 (Wildgoose et al., 1990, 1992; Ruf et al., 1991b) of factor VIIa have been reported to be involved in its Ca^{2+}-dependent binding to TF. Residues 157–167 of tissue factor were reported to be required for optimal recognition and cleavage of protein substrates by the factor VIIa–TF complex (Roy et al., 1991; Rehemtula et al., 1991, 1992; Ruf et al., 1992a,b). None of these or any other residues of TF were reported as participating directly in factor VIIa binding. Moreover, mutations in these regions did not influence the factor VIIa–TF complex amidolytic activity (Ruf et al., 1992a,b). Recent studies showed that the entire extracellular domain of TF may be essential for factor VIIa binding and functional activity (Konigsberg et al., 1993; Ruf et al., 1993).

The dissociation constant reported for factor VIIa–TF complex has varied over a wide range (Bach et al., 1986; Ruf et al., 1991, 1992a,b; Rehemtula et al., 1991b; Toomey et al., 1991; Krishnaswamy, 1992; Lawson et al., 1992) from 0.26 nM (Krishnaswamy, 1992) to 21 nM (Toomey et al., 1991). There is also little agreement concerning the Ca^{2+} concentration required to reach maximal factor VIIa amidolytic activity (Bom & Bertina, 1990; Komiyama et al., 1990; Pedersen et al., 1991).

In our previous experiments, we found the apparent dissociation constant for the factor VIIa–TF complex measured by TF saturation of synthetic substrate hydrolysis to range from 1 to 2 nM in the presence of 5 mM calcium ions (Lawson et al., 1992). In the present study, we evaluate the influence

† This work was supported by Grant PO1 HL46703 from the National Institutes of Health and by Grant 5-26387 from Haematologic Technologies Inc. A portion of this work was presented in a preliminary form at the XIVth Congrees of the International Society on Thrombosis and Haemostasis, New York, July 1993.

* To whom correspondence should be addressed.

‡ Present address: Department of Surgery, Duke University Medical Center, Durham, NC 27710.

® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

0006-2960/94/0433-3449$04.50/0

of the divalent metal ions on factor VIIa and factor VIIa–TF complex amidolytic activities. Four different peptidylamide substrates possessing different substituted 6-amino-1-naphthalenesulfonamide leaving groups and having relatively high hydrolytic efficiencies by factor VIIa and the factor VIIa–TF complex (Butenas et al., 1993) were used for this investigation. We show that the factor VIIa amidolytic activity in the presence of TF is influenced by divalent metal ions (Me^{2+}) and by substrate choice as well. From these data, we conclude that the dissociation constants of the factor VIIa–TF complex observed using functional substrate hydrolysis are dependent upon both the divalent metal ion and the substrate. Furthermore, we show that factor VIIa and TF display high-affinity binding in the absence of divalent metal ions.

EXPERIMENTAL PROCEDURES

Materials. Fluorogenic substrates, 6-(D-Phe-Pro-Arg)-amino-1-naphthalenecyclohexylsulfonamide (FPR-ncs), 6-(D-Phe-Pro-Arg)-amino-1-naphthalenepropylsulfonamide (FPR-nps), 6-(*tert*-butyloxycarbonyl-D-Phe-Val-Arg)-amino-1-naphthaleneethylsulfonamide (b-FVR-nes), and 6-(D-Phe-Pro-Arg)-amino-1-naphthalenebutylsulfonamide (FPR-nbs), were synthesized by the methods described previously (Butenas et al., 1992, 1993).

Proteins. Recombinant human coagulation factor VIIa was purchased from NOVO Pharmaceuticals. Recombinant human tissue factor 1–242 (TF) was provided as a gift from Dr. Shu-Len Liu, Hyland Division, Baxter Healthcare Corp. The concentration of rVIIa was calculated using a molecular weight of 50 000 and an extinction coefficient ($E^{0.1\%}_{280nm}$) of 1.39 (Bajaj et al., 1981). The concentrations of TF preparations were determined by amino acid analyses.

Factor VIIa Assays. Factor VIIa assays were conducted in 20 mM HEPES/150 mM NaCl, pH 7.4 (HBS), at 37 °C. The final volume for all reactions was 1.2 mL. The substrates were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. These stock solutions were diluted in HBS to a working 40 μM concentration prior to all assays. The salts of metals were dissolved in HBS to stock concentrations of 5–100 mM. The final concentration of factor VIIa used in this study for kinetic experiments was 10 nM, and tissue factor concentration was 15 nM. In TF titration experiments, the final concentration of TF was 5 nM. In experiments designed to evaluate the reactivity of factor VIIa in the absence of metal ions, the assays were performed in the presence of 10 mM EDTA. Preincubation of factor VIIa with Me^{2+}, substrates, and TF was accomplished as described previously (Butenas et al., 1993). The Michaelis–Menten constant (K_M), catalytic constant (k_{cat}) assays, and TF titration by factor VIIa were performed in the presence of 2 mM Ca^{2+} or Mn^{2+}, or 10 mM Mg^{2+}. Substrate hydrolysis was measured over a period of 5 min. The rate of substrate hydrolysis was evaluated as nanomolar per second by the change in fluorescence over time which corresponded to the generation of the corresponding 6-amino-1-naphthalenesulfonamide from the provided substrate. This fluorescence was continuously monitored by excitation at 352 nm and emission at 470 nm using a Perkin-Elmer fluorescence spectrophotometer, Model MPF-44B, equipped with a standard chart recorder and a thermostated sample chamber at 37 °C.

Kinetic constants were determined using the nonlinear least-squares fitting program ENZFITTER (Elsevier-BIOSOFT, Cambridge, United Kingdom). This program was used to calculate the kinetic constants by iteratively fitting initial rates of substrate hydrolysis to the ligand binding (TF titration by factor VIIa) or Eadie plot (factor VIIa titration by TF) for the apparent dissociation constants ($K_{D,app}$), and to a Michaelis–Menten equation for the maximum velocity of hydrolysis, V, or transition midpoints $K_{D,app}$. The K_M's for substrate hydrolysis by factor VIIa in the presence of TF and EDTA were established using the Hill plot (Montgomery & Swenson, 1976).

Analytical Ultracentrifugation of Factor VIIa, TF, and Factor VIIa–TF Complex. Factor VIIa, TF, and the factor VIIa–TF complex in the presence and absence of Ca^{2+} were evaluated by sedimentation velocity analysis using a Beckman Model E photoelectric scanning analytical ultracentrifuge equipped with mirrored optics and a cylindrical lens. The photomultiplier carriage position and absorbance signal were accumulated and digitized using an ISAAC data acquisition system (Cyborg Corp., Newton, MA) interfaced to an Apple IIe microcomputer (Apple Computer Inc., Cupertino, CA). Measurements were made using a 4° double-sector cell that housed sapphire windows. All sedimentation velocity measurements were made using 400-μL samples at 60 000 rpm (260000g) at 21 °C. The final concentration of factor VIIa and/or TF in each experiment was 2 μM. All experiments were performed in HBS buffer, pH 7.4, which contained either 10 mM $CaCl_2$ or 20 mM EDTA. All experiments which contained TF also contained 0.023% *n*-dodecyl octaethylene glycol to ensure the solubility of this protein. Scanning intervals varied from 4 to 8 min depending on the nature of the experiment. Scans were initiated after depletion of the meniscus of protein. Sedimentation velocity of the protein mixture in each experiment was monitored by absorbance scanning each cell at 280 nm. Sedimentation coefficients were calculated from these data by linear least-squares regression analysis of the boundary position versus time. Boundary position was calculated by both second-moment and midpoint analysis and was used to calculate sedimentation velocity (Tanford, 1961; Cantor & Schimmel, 1980; Pryzdial & Mann, 1991).

Gel Electrophoresis. Native gels (gradient 2.5–27%) were performed as described (Stinson, 1984; Hawrylak & Stinson, 1987). Nonionic detergent (0.2% of Triton X-100 or NP-40) was included in the gel. The gels were subjected to preelectrophoresis for 18 h, 200 V, at 5 mA/gel. When the effect of EDTA on the factor VIIa–tissue factor interaction was studied, the proteins were preincubated in 10 mM EDTA (15 min, 25 °C) at a concentration of 1.6 μM, and EDTA was included in the gel solutions (2 mM), the running buffer (2 mM), and the sample buffer (2 mM). Following incubation of factor VIIa with tissue factor, the solutions were incubated in 0.09 M Tris/0.08 M boric acid, 2 mM EDTA, pH 8.5, and electrophoresis was performed at 4 °C for 24 h at 200 V (5 mA/gel). The final concentrations of proteins applied on the gel were 1.31 μM for factor VIIa and tissue factor alone and 0.66 μM for the VIIa–tissue factor complex. Similar experiments were performed in the presence of 2 mM $CaCl_2$. In the latter experiments, EDTA was omitted from all the solutions, and 2 mM $CaCl_2$ was included in all the solutions. When indicated, the gels were stained with silver nitrate as described (Merril et al., 1981). High molecular weight markers for native gels were from Pharmacia—LKB.

RESULTS

Enhancement of Amidolytic Activity of Factor VIIa by Me^{2+}. Ca^{2+}, Mn^{2+}, or Mg^{2+} enhances the activity of factor VIIa (Figure 1). At 10 nM factor VIIa in the presence of 10 mM EDTA, the amidolytic activity of factor VIIa was 8–13%

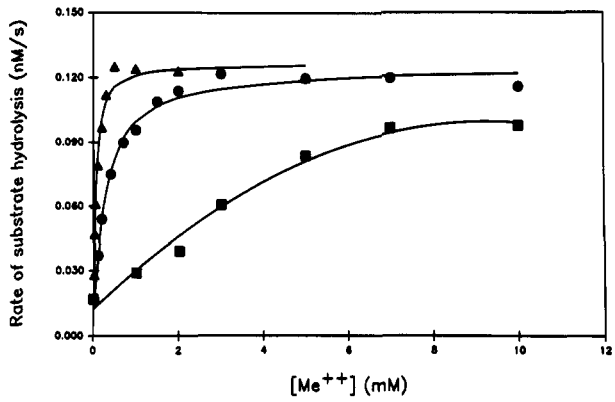

FIGURE 1: Factor VIIa Me^{2+} ion titration. The fluorogenic substrate FPR-ncs was preincubated in HBS at 37 °C for 5 min with increasing concentrations of $CaCl_2$ (circles), $MnCl_2$ (triangles), or $MgCl_2$ (squares). Factor VIIa was added, and the initial rates of substrate hydrolysis were evaluated as described under Experimental Procedures.

of the maximal rate observed in the presence of saturating concentrations of Me^{2+}. Substrate hydrolysis increased as a function of metal ion concentrations and reached transition midpoints ($K_{D,app}$) at 0.27 mM (Ca^{2+}), 40 μM (Mn^{2+}), or 3.3–12.2 mM (Mg^{2+}) (Table 1). The ultimate rates of substrate hydrolysis were similar for all Me^{2+} evaluated. In the case of Mg^{2+}, the concentration at which $K_{D,app}$ was observed is dependent upon the substrate structure. In the presence of the substrates FPR-ncs or FPR-nps, which possess similar structures, the $K_{D,app}$ were similar (3.3 and 4.4 mM, respectively). In the presence of the substrate b-FVR-nes, which is significantly different in structure, the Mg^{2+} concentration required to reach $K_{D,app}$ increased to 12.2 mM.

The Michaelis–Menten kinetic constants (K_M and k_{cat}) for substrate hydrolysis are presented in Table 2. There are not significant differences in the K_M observed in the presence of various Me^{2+} or EDTA. In contrast, the catalytic and second-order rate (k_{eff}) constants are significantly enhanced in the presence of divalent metal ions. The differences in the ultimate k_{eff} observed for various Me^{2+} are a consequence of the saturation levels of factor VIIa by these ions at the concentrations used in these experiments. Thus, the highest second-order rate constant observed was in the presence of Mn^{2+} at 2 mM concentration. This concentration was 50-fold higher than the $K_{D,app}$ for Mn^{2+}. For Ca^{2+}, the excess ratio was 8-fold $K_{D,app}$. For Mg^{2+}, 0.8–3-fold $K_{D,app}$ metal ions were used. From these experiments, we may conclude the following: (1) Metal ions are not essential elements of the factor VIIa active site. (2) Metal ions positively influence the amidolytic activity by increasing the substrate turnover with little effect on substrate binding to enzyme. In each case tested, the Me^{2+} produce similar rates of hydrolysis. (3) The interaction of protein, substrate, and Mg^{2+} appears to be a cooperative process.

Enhancement of Amidolytic Activity of Factor VIIa by Me^{2+} in the Presence of TF. TF increased the amidolytic activity of factor VIIa for all substrates. The data presented in Figure 2 illustrate that the amidolytic activity of factor VIIa toward the FPR-ncs substrate in the presence of TF increased with increasing concentrations of Me^{2+}. Each metal ion increased the rate of FPR-ncs hydrolysis, but in contrast to the results obtained for factor VIIa alone, the various Me^{2+} influenced the catalytic process at different ranges and to different extents. Ca^{2+} ions were most effective and resulted in the highest V, k_{cat}, and k_{eff} of substrate hydrolysis observed for the factor VIIa–TF complex (Tables 1 and 2). Mg^{2+} ions provided only 20% of the rate achieved with Ca^{2+} while Mn^{2+} gave approximately 60% of the rate achieved with Ca^{2+}. The Michaelis–Menten constants (Table 2) did not depend upon divalent metal ions, and were slightly higher in the presence of TF. The Me^{2+} $K_{D,app}$ values observed for all substrates are tabulated in Table 1. The average value of 0.28 mM is observed for Ca^{2+}. For Mn^{2+} and Mg^{2+}, the $K_{D,app}$ values are substrate dependent and range from 0.028 to 0.20 mM for

Table 1: Maximal Rate, V (nM/s), and Transition Midpoints, $K_{D,app}$ (mM), of Substrate Hydrolysis in the Presence of Various Divalent Metal Ions

	Ca^{2+}				Mn^{2+}				Mg^{2+}			
	VIIa		VIIa–TF		VIIa		VIIa–TF		VIIa		VIIa–TF	
substrate (40 μM)	V	$K_{D,app}$	V	$K_{D,app}$	V	$K_{D,app}$	V	$K_{D,app}$	V	$K_{D,app}$	V	$K_{D,app}$
FPR-ncs	0.13	0.26	10.45	0.34	0.13	0.052	6.94	0.11	0.13	3.28	2.54	1.05
FPR-nps	0.11	0.25	6.76	0.17	0.09	0.027	3.62	0.028	0.10	4.40	1.22	0.52
b-FVR-nes	0.10	0.31	5.12	0.34	0.09	0.041	2.47	0.20	0.13	12.19	0.65	3.23
FPR-nbs	0.21	0.25	6.16	0.28								
average		0.27		0.28		0.040						

Table 2: Kinetic Constants[a] for Substrate Hydrolysis

	EDTA		Ca^{2+}		Mn^{2+}		Mg^{2+}		average	
substrate	VIIa	VIIa–TF	VIIa	VIIa–TF	VIIa	VIIa–TF	VIIa	VIIa–TF	–TF	+TF
FPR-ncs										
K_M	95	208[b,c]	152	329	56	321	82	313	96	293
k_{cat}	0.0095	3.17	0.054	8.37	0.024	5.06	0.022	3.54		
k_{cat}/K_M	100	15200	355	25400	430	15800	269	11300		
FPR-nps										
K_M	82	108[c]	138	131	72	144	65	103	89	122
k_{cat}	0.0042	0.43	0.039	2.82	0.025	1.61	0.016	0.55		
k_{cat}/K_M	51	4000	283	21500	347	11200	246	5300		
b-FVR-nes										
K_M	ND[d]	ND	55	91	39	82	71	83	55	85
k_{cat}	ND	ND	0.017	1.34	0.014	0.51	0.015	0.22		
k_{cat}/K_M	ND	ND	309	14700	359	6200	211	2700		

[a] K_M, μM; k_{cat}, s^{-1}; k_{cat}/K_M, M^{-1} s^{-1}. [b] [TF], 120 nM. [c] Calculated from Hill's plot. [d] Not determined.

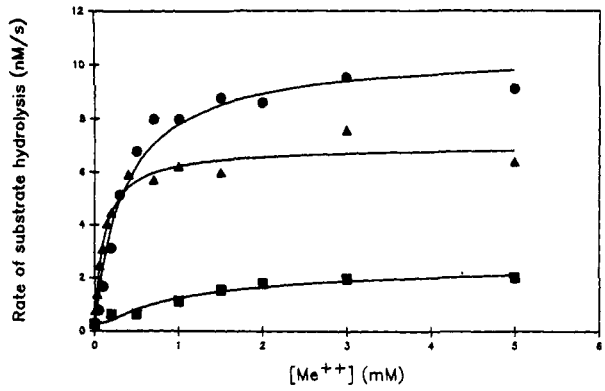

FIGURE 2: Factor VIIa Me^{2+} ion titration in the presence of TF. Factor VIIa, TF, and increasing concentrations of $CaCl_2$ (circles), $MnCl_2$ (triangles), or $MgCl_2$ (squares) were preincubated in HBS at 37 °C for 10 min. The fluorogenic substrate FPR-ncs was added, and the initial rates of substrate hydrolysis were evaluated.

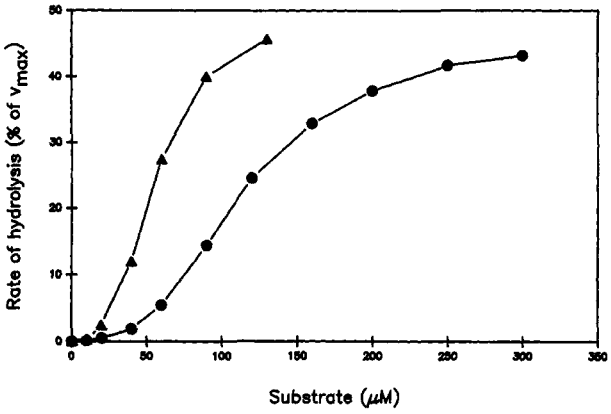

FIGURE 3: Factor VIIa substrate titration in the presence of TF and EDTA. Factor VIIa, TF, and EDTA were preincubated in HBS at 37 °C for 10 min. Increasing concentrations of the substrate FPR-ncs (circles) or the substrate FPR-nps (triangles) were added, and the initial rates of substrate hydrolysis were evaluated.

Mn^{2+} and from 0.52 to 3.23 mM for Mg^{2+}. On the basis of these data, we see that the saturating Ca^{2+} concentration does not depend upon the presence or absence of TF (see Table 1). In the case of Mn^{2+}, higher concentrations are required to achieve saturation in the presence of TF, whereas for Mg^{2+} lower saturating concentrations were observed. When Mn^{2+} or Mg^{2+} was used to estimate factor VIIa–TF complex formation, the saturating Me^{2+} concentrations used were dependent upon substrate structure. The highest Me^{2+} concentrations were for the substrate b-FVR-nes. Tissue factor enhanced the rate of substrate hydrolysis even in the presence of EDTA, indicating that metal ions are not essential for tissue factor–VIIa complex formation in the presence of the sulfonamide substrates.

Enhancement of Factor VIIa Amidolytic Activity by Synthetic Substrates in the Presence of TF and EDTA. Factor VIIa displays amidolytic activity in the absence of cofactors. The rate of substrate hydrolysis increases with increasing substrate concentration according to Michaelis–Menten kinetics. In the presence of TF and EDTA, however, the substrates influence factor VIIa activity in a cooperative fashion. In the absence of Me^{2+}, the substrate titration curves observed were sigmoidal (Figure 3). At low substrate concentrations (<10–20 μM), the rate of hydrolysis was negligible. At substrate concentrations of 20–80 μM, the amidolytic activity of factor VIIa–TF was substrate augmented. At higher substrate concentrations, saturation kinetics were observed. At saturating TF concentrations in the presence of EDTA, the maximum velocities observed were one-third of those in the presence of saturating Ca^{2+} concentration. The calculations accomplished on the basis of a Hill's plot (Montgomery & Swenson, 1976) suggest that two molecules of substrate are bound to the factor VIIa–TF complex.

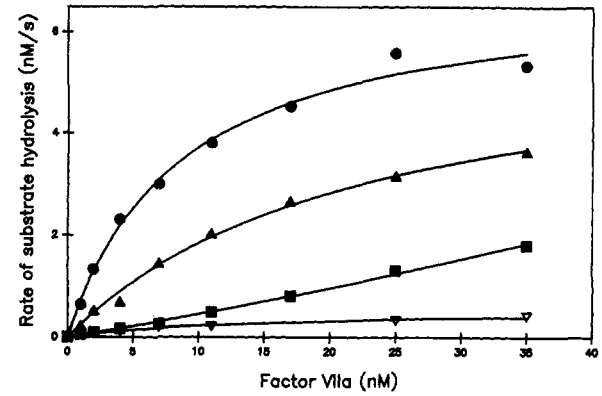

FIGURE 4: TF factor VIIa titration. TF, 2 mM $CaCl_2$ (filled circles), 2 mM $MnCl_2$ (filled triangles), 10 mM $MgCl_2$ (filled squares), or 10 mM EDTA (open triangles) and increasing concentrations of factor VIIa were precincubated in HBS at 37 °C for 10 min. The fluorogenic substrate FPR-nps was added, and the initial rates of substrate hydrolysis were evaluated.

Table 3: Apparent Dissociation Constants ($K_{D,app}$) of the Factor VIIa–TF Complex (nM)

	EDTA				
substrate	A[a]	B[b]	Ca^{2+}	Mn^{2+}	Mg^{2+}
FPR-ncs[c]	19.0 ± 4.9	36.4 ± 10.1	1.3 ± 0.3	4.5 ± 0.9	52.3 ± 12.0
FPR-nps[d]	3.9 ± 1.2	8.3 ± 1.6	1.1 ± 0.5	7.0 ± 1.2	ND[e]
b-FVR-nes[d]	34.1 ± 5.0	45.8 ± 14.1	0.9 ± 0.3	3.9 ± 0.7	ND
FPR-nbs			1.2 ± 0.6		
average			1.1 ± 0.2	5.1 ± 1.6	

[a] TF titration by factor VIIa. [b] Factor VIIa titration by TF. [c] [TF], 2.8 nM; [Ca^{2+}], 5 mM; [Mn^{2+}] 5 mM; [Mg^{2+}] 100 mM. [d] [TF], 5.0 nM; [Ca^{2+}], 2 mM; [Mn^{2+}], 2 mM; [Mg^{2+}], 10 mM. [e] Not determined.

Extensive pretreatment of factor VIIa with EDTA did not influence amidolytic activity. The rate of substrate FPR-ncs hydrolysis by factor VIIa was constant (0.024 nM/s) throughout the EDTA incubation. The amidolytic activity of the factor VIIa–TF complex (10–100 nM, respectively) decreased from 1.80 to 1.52 nM/s after 1 h of factor VIIa EDTA pretreatment. The rate of substrate hydrolysis after 48 h of experiment was 1.33 nM/s or 74% of the initial rate. In the presence of Ca^{2+} and TF, the initial rate of hydrolysis was 7.54 nM/s, 6.56 nM/s after 1 h, and 5.97 nM/s after 48 h.

Apparent Dissociation Constants for the Factor VIIa–TF Complex. As illustrated in Figure 4, increasing the factor VIIa concentration at fixed TF concentration in the presence of Me^{2+} lead to saturable increases in the rate of substrate hydrolysis. The apparent dissociation constants suggest that factor VIIa is most tightly bound to TF in the presence of Ca^{2+} (Table 3); the apparent K_D in this situation was 1.1 nM. Mn^{2+} gave a $K_{D,app}$ of 5.1 nM. We were able to evaluate the influence of Mg^{2+} only for the substrate FPR-ncs. The estimated $K_{D,app}$ for the TF–factor VIIa interaction in the presence of Mg^{2+} ions was 52 nM.

The apparent dissociation constants in the presence of EDTA were evaluated either at fixed factor VIIa concentration or at fixed TF concentration. These constants were dependent upon substrate structure and varied from 34 nM for the substrate b-FVR-nes to 3.9 nM for the substrate FPR-nps. In

Table 4: Sedimentation Velocity of TF and/or Factor VIIa as a Function of Complex Assembly

component(s)	s_{obs}[a]
TF/EDTA	1.83
TF/Ca^{2+}	2.30
VIIa/EDTA	2.39
VIIa/Ca^{2+}	3.85
TF/VIIa/EDTA	3.54
TF/VIIa/Ca^{2+}	9.52

[a] Observed sedimentation velocity of each protein species as determined by second-moment analysis.

the latter case, the $K_{D,app}$ was in the same range as for Mn^{2+} and only 4-fold lower than for Ca^{2+} (7.0 nM for Mn^{2+}, 1.1 nM for Ca^{2+}).

The unexpected apparent association of factor VIIa and TF in the absence of Me^{2+} lead us to conduct a preliminary direct assessment of the binding phenomenon. To further analyze the binding interaction of factor VIIa and TF directly, native gel electrophoresis and sedimentation velocity experiments containing factor VIIa, TF, and the factor VIIa–TF complex in the absence of Me^{2+} and substrate were evaluated. Data presented in Table 4 compare the observed sedimentation velocity (s_{obs}) of each protein or a mixture of proteins (TF and factor VIIa). These data demonstrate that Ca^{2+} directly and substantially alters the sedimentation properties of both factor VIIa and TF. TF demonstrated a 26% increase in the s_{obs} in the presence of 10 mM Ca^{2+} while the s_{obs} for factor VIIa increased 61% in the presence of calcium ions. When equimolar mixtures of TF and factor VIIa were investigated, the two proteins sedimented as a species with a single sedimentation boundary in both the presence and absence of calcium ions. However, the s_{obs} for the Me^{2+} mixture was substantially higher than that observed for the EDTA-containing mixture.

These data support the hypothesis that factor VIIa and TF have calcium-dependent and calcium-independent binding interactions. The s_{obs} of the factor VIIa–TF complex in the presence of 20 mM EDTA was 3.54. This value should be compared to factor VIIa in 20 mM EDTA (2.39) and TF in 20 mM EDTA (1.83). The average s_{obs} of the factor VIIa–TF complex in the presence of 10 mM Ca^{2+} was 9.52. This value should be compared to factor VIIa (3.85) and TF (2.30) under identical conditions of solvent and temperature. These data provide direct physical evidence that factor VIIa and TF form a complex(es) in the absence of Ca^{2+}. The increase in the s_{obs} identified for the complex in the presence of Ca^{2+} suggests that a higher level of protein aggregation is promoted in the presence of metal ions.

The analytical ultracentrifugation experiments were performed with a 2 μM aliquot of each protein. Virtually 100% factor VIIa and TF complex (a single boundary) were observed in both the presence and absence of Ca^{2+}. The apparent homogeneity of the sedimenting species is also suggested by the coincidence of sedimentation values estimated by both midpoint and second-moment analyses. These data are in accord with the conclusion that the factor VIIa–TF dissociation constant is well below 2 μM.

The data thus far accumulated are semiquantitative and allow us to conclude only that factor VIIa and TF form complexes in the presence and absence of Me^{2+}. Extensive studies are anticipated in the future to define the qualitative and hydrodynamic nature of these complexes.

To ascertain whether factor VIIa binds to TF in the presence of EDTA, we have developed a system which employs native

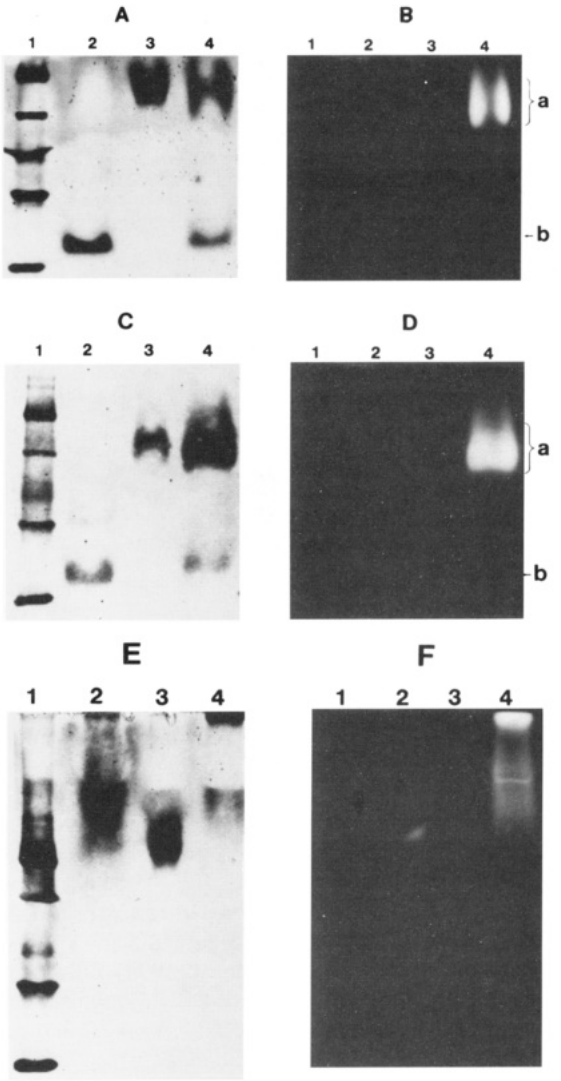

FIGURE 5: Gradient gel electrophoresis of factor VIIa, tissue factor, and the factor VIIa–tissue factor complex. The proteins were treated with 10 mM EDTA (panels A–D) or 2 mM $CaCl_2$ (panels E and F) and analyzed by gel electrophoresis using a native gel (gradient 2.5–25%) as described under Experimental Procedures. The native gels were incubated with a solution of the substrate FPR-nbs (10 μM in HBS) for 15 min, photographed through a 500-nm long-pass filter (panels B, D, and F), and silver stained (panels A, C, and E). Lane 1, molecular weight markers (from the top of the gel to the bottom: 669 000, 440 000, 232 000, 140 000, 67 000); lane 2, factor VIIa; lane 3, tissue factor; lane 4, factor VIIa–tissue factor. "a" indicates the migration of tissue factor or of the factor VIIa–tissue factor complex, and "b" denotes the position of free factor VIIa.

gel electrophoresis that allows for direct identification of amidolytic activity. This method was previously used to study the relationship between alkaline phosphatase's tetrameric and dimeric forms (Hawrylak & Stinson, 1987). Native gradient gels (2.5–27%) containing 0.2% nonionic detergent (NP-40 or Triton X-100) were performed according to previously described methodologies (Stinson, 1984; Hawrylak & Stinson, 1987). Following 24 h of electrophoresis, the native gels were incubated with a 10 μM solution of FPR-nbs which is a substrate previously described to be cleaved by factor VIIa or the factor VIIa–TF complex (Butenas et al., 1993). The substrate alone does not emit any fluorescence. The enzyme or the enzymatic complexes present in the gel will cleave the substrate locally, resulting in the emission of fluorescence. The positions of the fluorescence bands within the gels are detected by excitation with a long-wave UV light box, and the gels are photographed through a 500-nm long-

$$VIIa + TF \underset{}{\overset{K_1}{\rightleftharpoons}} VIIa{\bullet}TF$$

$$VIIa + Ca^{++} \overset{K_2}{\rightleftharpoons} VIIa{\bullet}Ca^{++}$$

$$TF + Ca^{++} \overset{K_3}{\rightleftharpoons} TF{\bullet}Ca^{++}$$

$$VIIa{\bullet}Ca^{++} + TF{\bullet}Ca^{++} \overset{K_4}{\rightleftharpoons} VIIa{\bullet}TF{\bullet}Ca^{++}$$

$$VIIa + S \overset{K_5}{\rightleftharpoons} VIIa{\bullet}S \xrightarrow{k_{cat_5}} VIIa + P$$

$$VIIa{\bullet}Me^{++} + S \overset{K_6}{\rightleftharpoons} VIIa{\bullet}Me^{++}{\bullet}S \xrightarrow{k_{cat_6}} VIIa{\bullet}Me^{++} + P$$

$$VIIa{\bullet}TF + S \overset{K_7}{\rightleftharpoons} VIIa{\bullet}TF{\bullet}S \xrightarrow{k_{cat_7}} VIIa{\bullet}TF + P$$

$$VIIa{\bullet}TF{\bullet}Me^{++} + S \overset{K_8}{\rightleftharpoons} VIIa{\bullet}TF{\bullet}Me^{++}{\bullet}S \xrightarrow{k_{cat_8}} VIIa{\bullet}TF{\bullet}Me^{++} + P$$

$K_5 \simeq K_6 \simeq K_7 \simeq K_8 = K_M$

$k_{cat_5} < k_{cat_6} < k_{cat_7} \leq k_{cat_8}$

FIGURE 6: Schematic representation of the protein complexes. Shown are the various factor VIIa complexes with Me^{2+}, TF, and substrates presented in this study. K_1–K_8 represent the dissociation constants for these complexes; k_{cat_5}–k_{cat_8} depict the amidolytic activity of each complex.

pass filter. The migration of the various proteins and complexes is identified by staining the gel with silver nitrate.

Visual confirmation of the Me^{2+}-free functional factor VIIa–TF complex is shown in Figure 5. When EDTA is present in the reaction mixture and in the gel solutions, there is complex formation between factor VIIa and TF since amidolytic activity is associated with TF when incubated with factor VIIa as compared with TF alone (Figure 5, panels A and B, lanes 3 and 4). In the presence of EDTA, factor VIIa alone also demonstrated amidolytic activity (Figure 5B, lane 2); however, as recently demonstrated (Butenas et al., 1993), the amidolytic activity of factor VIIa alone is 2 orders of magnitude lower than factor VIIa when complexed with TF. Similar results were obtained when EDTA-treated factor VIIa and TF were analyzed on a native gel in the absence of EDTA (Figure 5, panels C and D). When the same experiments were performed in the presence of Ca^{2+} (2 mM $CaCl_2$ was included in the reaction mixture, in the gel solutions, and in the running buffer), it was observed that factor VIIa and the factor VIIa–TF complex had altered migration, at a position consistent with that for a very high molecular weight complex (Figure 5, panels E and F). Together these data demonstrate that factor VIIa–TF complex formation is not essentially Ca^{2+} dependent and that in the presence of Ca^{2+}, factor VIIa in the presence and absence of TF forms a high molecular weight complex.

DISCUSSION

Data associated with a substantial number of factor VIIa complexes are presented in this study (Figure 6). The stability and functional properties of these complexes show a strong dependence upon various ligands involved in their formation. The complex of most significant functional importance is that formed by factor VIIa and TF. The evaluation of factor VIIa binding to TF in the presence of Ca^{2+} is reported in many publications; however, the formation of a factor VIIa–TF complex in the absence of Me^{2+} has not been previously reported. Data obtained in the presence of EDTA using three independent approaches provide direct evidence that factor VIIa and TF form a stable and functional enzymatic complex in the absence of Me^{2+}. This noncovalent complex is stable at micromolar protein concentrations and is tightly associated under conditions of ultracentrifugation and gel electrophoresis.

When factor VIIa, TF, and factor VIIa–TF mixture were evaluated by sedimentation velocity analyses, it was observed that factor VIIa and TF form a stable complex in the absence of divalent metal ions (20 mM EDTA) at pH 7.4. These data provide direct physical evidence that TF and factor VIIa form a stable enzymatic complex even in the absence of Me^{2+}. These data also support the conclusion that factor VIIa and TF have a Ca^{2+}-independent binding interaction. The protein concentration used in these studies was 2 μM for TF and factor VIIa. Because only one sedimentation boundary was observed for the TF–factor VIIa mixture under these conditions, it supports the conclusion that the K_D for both the calcium dependent- and calcium-independent TF–factor VIIa binding interaction is well below 2 μM. From the data reported above using fluorogenic substrate hydrolysis, the dissociation constant of the calcium-dependent binding site(s) falls in the range of 0.9–1.3 nM. The gel electrophoresis data provided unequivocally demonstrate the formation of a functional enzymatic complex in the absence of Me^{2+} (in the presence of EDTA) as well.

The experiments of substrate hydrolysis in the presence of EDTA gave the initial suggestion that functionally active factor VIIa–TF complex formation occurs in the absence of Me^{2+}. Additionally, these data show that substrates themselves play a significant role in the elevation of factor VIIa amidolytic activity in the presence of TF. The estimation of saturating substrate concentrations for TF binding site(s) of factor VIIa is rather complicated. At least two processes occur simultaneously with increasing substrate concentration: (1) the augmentation of substrate hydrolysis caused by the elevation of factor VIIa amidolytic activity due to factor VIIa structure stabilization, and (2) the increase of substrate hydrolysis associated with Michaelis–Menten kinetics. Quantitative interpretation suggests that the saturating substrate concentrations depend upon their structure and are in the 20–80 μM range. At higher substrate concentrations, the hydrolysis followed Michaelis–Menten kinetics. The K_D in the range of 4–34 nM for the factor VIIa–TF complex formed in the presence of synthetic substrates is significantly higher than that obtained in the presence of Ca^{2+} (approximately 1 nM), but lower than that obtained in the presence of magnesium ions (52 nM). The apparent dissociation constant demonstrates strong dependence on the substrate structure in the absence of Me^{2+}.

The data presented above do not allow distinction of the possibilities either that factor VIIa contains distinct Ca^{2+}-dependent and Ca^{2+}-independent TF binding sites or that a single site exists on each protein, and that the Ca^{2+} alters or stabilizes the complex, resulting in an overall decrease in K_D.

A number of studies have previously evaluated the influence of Ca^{2+} on factor VIIa and factor VIIa–tissue factor amidolytic activity using natural (Bom & Bertina, 1990; Komiyama et al., 1990) and synthetic substrates (Pedersen et al., 1991; Higashi et al., 1992; Wildgoose et al., 1993). Heterogeneity among these data suggests that the Ca^{2+} concentrations required for saturation are influenced by the nature of the substrate utilized. In the presence of the natural substrate factor X, saturating Ca^{2+} concentrations between 2.5 and 6 mM were observed. For factor IX as substrate, saturation was reached at approximately 1 mM Ca^{2+}. In the case of the synthetic substrate S-2288 (D-IPR-pna), the transition midpoint of factor VIIa amidolytic activity was reached at approximately 0.1–0.4 mM Ca^{2+} concentration. The factor VIIa–Ca^{2+} dissociation constant calculated from the Tb^{3+} displacement experiment has been estimated to be 1.8 mM (Schiodt et al., 1992).

In the present experiments, we used four synthetic substrates possessing various 6-amino-1-naphthalenesulfonamide leaving groups to evaluate the influence of Me^{2+} on factor VIIa amidolytic activity. For all substrates studied, the Ca^{2+} affinity to factor VIIa was similar, displaying the transition midpoint ($K_{D,app}$) at 0.27 mM Ca^{2+}. Mn^{2+} displayed 7-fold higher affinity for factor VIIa than Ca^{2+}. Binding of Mg^{2+} to factor VIIa was substrate dependent, and these ions had significantly lower affinity for factor VIIa than Mn^{2+} or Ca^{2+}. The amidolytic activity of factor VIIa and factor VIIa–substrate complex formation were not influenced significantly by the nature of Me^{2+} used as it follows from similar constants of Michaelis–Menten kinetics. Moreover, Me^{2+} ions are not required for substrate binding to factor VIIa, because this process is not influenced by Me^{2+}. This observation is valid for factor VIIa in the presence of TF as well. TF by itself only slightly increases the K_M of substrate hydrolysis. Thus, the enzyme–substrate binding interaction is metal ion and TF independent. Similar results supporting the substrate binding independence of TF were presented in publications where *p*-nitroanilide (Higashi et al., 1992), 4-methylcoumarin-7-ylamide (Shigematsu et al., 1992), or factor X (Bom & Bertina, 1990) and factor IX (Komiyama et al., 1990) were used as factor VIIa substrate.

Factor VIIa demonstrated low amidolytic activity in the absence of Me^{2+} (EDTA). It was increased by all Me^{2+} to the same extent (6–9-fold), an effect similar to that reported in the publications mentioned above (3–11-fold). The effect of Me^{2+} on factor VIIa amidolytic activity was significantly lower than that obtained in the presence of TF. TF increased the amidolytic activity of factor VIIa approximately by 2 orders of magnitude. The factor VIIa–TF complex observed in the presence of Ca^{2+} had the highest amidolytic activity, while that observed in the presence of EDTA or Mg^{2+} had the lowest. Wildgoose and coauthors (Wildgoose et al., 1993) reported that saturating Ca^{2+} concentrations were a fewfold lower for the factor VIIa–TF complex than for factor VIIa alone. In constrast, our data suggest that tissue factor does not influence the Ca^{2+} concentration required to achieve maximal amidolytic activity of factor VIIa.

The K_D in the range of 0.9–1.3 nM for the factor VIIa–TF complex observed in the presence of Ca^{2+} was significantly lower than that reported in a majority of publications: 4.5 nM (Bach et al., 1986), 4.2 nM (Ruf et al., 1991b), 21 nM (Toomey et al., 1991), 7.8–13.8 nM (Ruf et al., 1992a). In all these studies, binding experiments were performed using ^{125}I- or ^{3}H-labeled factor VIIa. On the other hand, binding analyses performed to evaluate the factor VIIa–TF complex amidolytic activity toward *p*-nitroanilide substrates gave a K_D at 0.26 nM (Krishnaswamy, 1992). Thus, the value of the dissociation constant depends on the method used for determination.

In conclusion, the data provided in this study suggest that factor VIIa–TF complex formation and functional activity are Me^{2+} dependent but not Me^{2+} essential. The enhancement of factor VIIa activity is a cooperative effect generated by Me^{2+}, TF, and synthetic substrates *via* stabilization of factor VIIa structure. Tissue factor stabilizes factor VIIa to the highest extent, whereas divalent metal ions have the weakest stabilizing effect. All the ligands mentioned above may form binary, ternary, or quaternary complex with factor VIIa (Figure 6). Factor VIIa–Ca^{2+} complex and factor VIIa–substrate complex formation does not depend on the other ligands (TF or substrate and TF or Me^{2+}, respectively), whereas the affinity of TF for factor VIIa may be significantly enhanced by Me^{2+} or substrate. These ligands, most likely, increase the stability of the factor VIIa structure required for TF binding.

ACKNOWLEDGMENT

We are grateful to Dr. Shu-Len Liu of Baxter Healthcare Corp. for providing recombinant human tissue factor. We thank John Robinson for helpful advice during initial experiments with native gels. We are grateful to Dr. Casey M. Lawler for advice in sedimentation data analysis.

REFERENCES

Almus, F. E., Rao, L. V. M., & Rapaport, S. I. (1989) *Thromb. Haemostasis 62*, 1067–1073.
Bach, R., Gentry, R., & Nemerson, Y. (1986) *Biochemistry 25*, 4007–4020.
Bajaj, S. P., Rapaport, S. I., & Brown, S. F. (1981) *J. Biol. Chem. 256*, 253–259.
Bom, V. J. J., & Bertina, R. M. (1990) *Biochem. J. 265*, 327–336.
Broze, G. J. (1982) *J. Clin. Invest. 70*, 526–535.
Butenas, S., Orfeo, T., Lawson, J. H., & Mann, K. G. (1992) *Biochemistry 31*, 5399–5411.
Butenas, S., Ribarik, N., & Mann, K. G. (1993) *Biochemistry 32*, 6531–6538.
Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry. Part II. Techniques for the Study of Biological Structure and Function*, pp 531–642, W. H. Freeman & Co., San Francisco.
Clarke, B. J., Ofosu, F. A., Sridhara, S., Bona, R. D., Rickles, F. R., & Blajchman, M. A. (1992) *FEBS Lett. 298*, 206–210.
Fair, D. S., & MacDonald, M. J. (1987) *J. Biol. Chem. 262*, 11692–11698.
Hawrylak, K., & Stinson, R. A. (1987) *FEBS Lett. 212*, 289–291.
Higashi, S., Kawabata, S., Nishimura, H., Funasaki, H., Ohyama, S., Miyamoto, S., Funatsu, A., & Iwanaga, S. (1990) *J. Biochem. 108*, 654–662.
Higashi, S., Nishimura, H., Fujii, S., Takada, K., & Iwanaga, S. (1992) *J. Biol. Chem. 267*, 17990–17996.
Jesty, J., & Silverberg, S. A. (1979) *J. Biol. Chem. 254*, 12337–12345.
Komiyama, Y., Pedersen, A. H., & Kisiel, W. (1990) *Biochemistry 29*, 9418–9425.
Konigsberg, W., Nemerson, Y., Fang, C., & Lin, T.-C. (1993) *Thromb. Haemostasis 69*, 1171 (Abstr.).
Krishnaswamy, S. (1992) *J. Biol. Chem. 267*, 23696–23706.
Lawson, J. H., & Mann, K. G. (1991) *J. Biol. Chem. 266*, 11317–11327.
Lawson, J. H., Butenas, S., & Mann, K. G. (1992) *J. Biol. Chem. 267*, 4834–4843.
Le, D. T., Rapaport, S. I., & Rao, L. V. M. (1992) *J. Biol. Chem. 267*, 15447–15454.
Merril, C. R., Danau, M. L., & Goldman, D. (1981) *Anal. Biochem. 110*, 201–207.
Montgomery, R., & Swenson, C. A. (1976) in *Quantitative Problems in the Biochemical Sciences*, pp 255–259, W. H. Freeman and Co., San Francisco.
Nemerson, Y. (1988) *Blood 71*, 1–8.
Osterud, B., & Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5260–5264.
Paborsky, L. R., Caras, I. W., Fisher, K. L., & Gorman, C. M. (1991) *J. Biol. Chem. 266*, 21911–21916.
Pedersen, A. H., Lund-Hansen, T., Komiyama, Y., Petersen, L. C., Oestergard, P. B., & Kisiel, W. (1991) *Thromb. Haemostatsis 65*, 528–534.
Ploplis, V. A., Edgington, T. S., & Fair, D. S. (1987) *J. Biol. Chem. 262*, 9503–9508.
Pryzdial, E. L. G., & Mann, K. G. (1991) *J. Biol. Chem. 266*, 8969–8977.

Rehemtulla, A., Ruf, W., & Edgington, T. S. (1991) *J. Biol. Chem. 266*, 10294–10299.
Rehemtulla, A., Ruf, W., Miles, D. J., & Edgington, T. S. (1992) *Biochem. J. 282*, 737–740.
Roy, S., Hass, P. E., Bourell, J. H., Henzel, W. J., & Vehar, G. A. (1991) *J. Biol. Chem. 266*, 22063–22066.
Ruf, W., Rehemtulla, A., & Edgington, T. S. (1991a) *J. Biol. Chem. 266*, 2158–2166.
Ruf, W., Kalnik, M. W., Lund-Hansen, T., & Edgington, T. S. (1991b) *J. Biol. Chem. 266*, 15719–15725.
Ruf, W., Miles, D. J., Rehemtulla, A., & Edgington, T. S. (1992a) *J. Biol. Chem. 267*, 6375–6381.
Ruf, W., Miles, D. J., Rehemtulla, A., & Edgington, T. S. (1992b) *J. Biol. Chem. 267*, 22206–22210.
Ruf, W., Schullek, J. R., Stone, M. J., & Edgington, T. S. (1993) *Thromb. Haemostasis 69*, 741 (Abstr.).
Shigematsu, Y., Miyata, T., Higashi, S., Miki, T., Sadler, J. E., & Iwanaga, S. (1992) *J. Biol. Chem. 267*, 21329–21337.
Shiodt, J., Harrit, N., Christensen, U., & Petersen, L. C. (1992) *FEBS Lett. 306*, 265–268.
Stinson, R. A. (1984) *Biochim. Biophys. Acta 790*, 268–274.
Tanford, C. (1961) in *Physical Chemistry of Macromolecules*, pp 194–233, J. P. Wiley & Sons, New York.
Toomey, J. R., Smith, K. J., & Stafford, D. W. (1991) *J. Biol. Chem. 266*, 19198–19202.
Van der Helm, D., & Willoughby, T. V. (1969) *Acta Crystallogr., Sect. B 25*, 2317.
Waxman, E., Laws, W. R., Laue, T. M., Nemerson, Y., & Ross, J. B. A. (1993) *Biochemistry 32*, 3005–3012.
Wildgoose, P., Kazim, A. L., & Kisiel, W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7290–7294.
Wildgoose, P., Jorgensen, T., Komiyama, Y., Nakagaki, T., Pedersen, A., & Kisiel, W. (1992) *Thromb. Haemostasis 67*, 679–685.
Wildgoose, P., Foster, D., Schiodt, J., Wiberg, F. C., Birktoft, J. J., & Petersen, L. C. (1993) *Biochemistry 32*, 114–119.