Kinetics of the Interaction between the Human Factor VIIIa Subunits: Effects of pH, Ionic Strength, Ca^{2+} Concentration, Heparin, and Activated Protein C-Catalyzed Proteolysis

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Received April 20, 1995; Revised Manuscript Received July 6, 1995⊗

ABSTRACT: Coagulation factor VIIIa consists of a heterotrimer in which the A2 subunit is bound to the A1/A3C1C2 dimer. The dissociation of this complex causes the spontaneous and reversible decay of factor VIIIa activity. In order to characterize the kinetics and affinity of the interaction between A2 and A1/A3C1C2, as well as the influence of different parameters on the interaction, the subunits were chromatographically separated and reassembled in a BIAcore instrument (Pharmacia Biosensor). In the binding experiments, A2 was free in solution, whereas A1/A3C1C2 was immobilized on the dextran surface by direct coupling or captured on an immobilized monoclonal anti-C2 antibody. At our chosen standard condition (pH = 6.0, I = 0.12, and $[Ca^{2+}]$ = 2 mM), the association rate constant, dissociation rate constant, and resulting equilibrium dissociation constant were ca. 1.4×10^4 M^{-1} s^{-1}, 2.1×10^{-4} s^{-1}, and 16 nM, respectively. Increasing the ionic strength or Ca^{2+} concentration resulted in both slower association and faster dissociation. At 0.3 M NaCl or 25 mM Ca^{2+}, the dissociation constant was >1 μM. This implies that electrostatic forces involved in the interaction contribute at least one-fourth of the total binding energy. Increasing pH caused a similar effect, yielding a dissociation constant of ca. 0.9 μM at pH 7.5. In those cases where the equilibrium dissociation constants had been determined from solution phase experiments [Fay, P. J., & Smudzin, T. M. (1992) *J. Biol. Chem. 267,* 13246−13250; Lollar, P., Parker, E. T., & Fay, P. J. (1992) *J. Biol. Chem. 267,* 23652−23657], these constants agreed well with our results. In addition, direct immobilization of A1/A3C1C2 or capture on an antibody gave very similar rate constants, indicating that neither the immobilization *per se* nor the mode of immobilization affected the subunit interaction. Limited proteolysis of A1/A3C1C2 by activated protein C abolished its ability to bind A2, supporting the involvement of the negatively charged region containing residues 337−372. Heparin prevented A2/A1/A3C1C2 heterotrimer formation, presumably by binding to and blocking basic regions of importance for the interaction.

Blood coagulation factor VIII circulates as a procofactor in complex with von Willebrand factor (Kane & Davie, 1988; Mann et al., 1988). Factor VIII is a mosaic protein comprising a heavy chain (A1A2B) and a light chain (A3C1C2) (Vehar et al., 1984; Toole et al., 1984). The three A and two C domains structurally relate factor VIII to factor V, ceruloplasmin, and a few other proteins including receptor protein tyrosine kinase (Vehar et al., 1984; Toole et al., 1984; Church et al., 1984; Karn et al., 1993), whereas the B domain is unique but not required for biological activity (Burke et al., 1986; Toole et al., 1986). After limited proteolysis by thrombin, factor VIII becomes a powerful cofactor for the factor IXa-catalyzed activation of factor X (van Dieijen et al., 1981). The activation process removes the B domain, hydrolyzes a peptide bond between the A1 and A2 domains, and liberates an activation peptide from the N-terminus of the light chain which in turn releases factor VIII from von Willebrand factor (Eaton et al., 1986; Lollar et al., 1988; Hill-Eubanks et al., 1989). In the resulting active form of factor VIII, factor VIIIa, A1 and A3C1C2 form a high-affinity complex linked by a divalent metal ion, whereas A2 binds the A1/A3C1C2 dimer with an equilibrium dissociation constant (K_d)[1] of ca. 0.26 μM under physiological conditions (Lollar & Parker 1989, 1990, 1991; Fay et al., 1991; Fay & Smudzin, 1992; Lollar et al., 1992). The assembled heterotrimer is required for expression of procoagulant activity. The relatively weak interaction between A1/A3C1C2 and A2 explains the spontaneous decay of factor VIIIa in the absence of inactivators such as activated protein C (APC). The A2 subunit presumably binds the A1 component of A1/A3C1C2, and the acidic region C-terminal of A1, comprising residues 337−372, appears to be of importance for the interaction (Fay et al., 1991a,b, 1993; Fay & Smudzin, 1992). Substitution of porcine A2 for the human subunit results in a stronger interaction indicating that properties of A2 govern the affinity (Lollar & Parker, 1991; Lollar et al., 1992). The effects of pH, ionic strength, and Ca^{2+} concentration on the activity of factor VIIIa have been studied by measurements of procoagulant activity of subunit mixtures in solution (Fay

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: K_d, equilibrium dissociation constant; k_a, association rate constant; k_d, dissociation rate constant; RU, resonance unit; F-25, a monoclonal antibody against the A2 domain; 56 IgG, a monoclonal antibody against the FVIII light chain; ESH 8, a monoclonal antibody against the C2 domain; APC, activated protein C.

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et al., 1991, 1993; Fay & Smudzin, 1992; Lamphear & Fay, 1992a). However, this approach does not distinguish between the inhibition of A2/A1/A3C1C2 complex formation and the inhibition of A2/A1/A3C1C2 heterotrimer activity. In this report, we present the results of a detailed, real-time biospecific interaction analysis of the protein−protein interaction between A1/A3C1C2 and A2 using surface plasmon resonance. With this approach, we obtained not only the K_d but also the rate constants for association (k_a) and dissociation (k_d), and the effects of various parameters on the individual events could be assessed.

MATERIALS AND METHODS

Proteins and Standard Procedures. B-domain-deleted recombinant factor VIII was expressed and purified by a standard procedure (Mikkelsen et al., 1991; Ezban et al., 1993). Factor VIII (0.3 μM), in 20 mM Hepes, pH 7.2, containing 0.1 M NaCl, 5 mM $CaCl_2$, 10% (v/v) glycerol, and 0.02% (v/v) Tween 80, was activated with 50 nM human thrombin (Boehringer Mannheim) for 30 min at 37 °C. A2 and A1/A3C1C2 were isolated by chromatography on a Mono S HR 5/5 column (Pharmacia Biotech) essentially as described by Lollar and Parker (1989). A2 and A1/A3C1C2 were localized by SDS−PAGE on 4−15% gradient gels (PhastSystem, Pharmacia Biotech). Residual A2 was removed from the A1/A3C1C2 pool by adsorption to F-25-Sepharose, and residual A1/A3C1C2 was removed from the A2 pool by adsorption to 56 IgG-Sepharose. F-25 (anti-A2) and 56 IgG (anti-light-chain) are in-house monoclonal antibodies. After completed purification, the subunits were analyzed by SDS−PAGE on 10% gels (Laemmli, 1970) followed by silver staining (Morrissey, 1981). The procoagulant activity of the subunit preparations was measured in a one-stage clotting assay using factor VIII-deficient plasma (Organon Teknika). The protein concentrations of the purified subunits were determined in a Bio-Rad protein assay using BSA as the standard. Reconstitution of factor VIIIa trimer from isolated A2 and A1/A3C1C2 was performed as previously described (Fay et al., 1993). Human protein C (Öhlin & Stenflo, 1987) was activated with 3% (w/w) thrombin for 3 h. A1/A3C1C2 was proteolytically modified by APC (10 μg/mL) as described (Fay et al., 1991b). The incubation time was extended to 51 h, and APC was added in three portions evenly distributed during the incubation. Protein transfer to nitrocellulose membrane (BA 85, Schleicher & Schuell) was carried out using a semidry electroblotter (Ancos). A1 was blotted with a polyclonal mouse anti-human factor VIII heavy chain antiserum and A3C1C2 with ESH 8 (30 μg/mL), using alkaline phosphatase-conjugated rabbit anti-mouse Ig (DAKOPATTS) as detection antibody. ESH 8 (anti-C2) was from Bioscot. Unfractionated heparin was from Novo Nordisk.

BIAcore Measurements. All experiments were performed in duplicate or triplicate on a BIAcore instrument (Pharmacia Biosensor) at 25 °C and a flow rate of 5 μL/min. Using this instrument, it is possible to monitor the binding event and the subsequent dissociation in real time (Jönsson et al., 1991). Detection is based on the surface plasmon resonance phenomenon (Liedberg et al., 1983; Cullen et al., 1987), which measures the change in refractive index due to association of an analyte to the immobilized ligand or due to the dissociation of a formed complex. The resulting signal is proportional to the amount of protein bound, and 1000 resonance units (RUs) correspond to 1 ng/mm^2 (Jönsson et al., 1991). The CM5 sensor chips and reagents for activation (EDC, NHS) and deactivation (ethanolamine) of the dextran surface were from Pharmacia Biosensor. When studying the effects of pH, APC-catalyzed modification of A1, and heparin, the antibody ESH 8 (20 μg/mL) in 10 mM sodium acetate, pH 4.5, containing 0.02% Tween 80 was coupled to the sensor chip dextran layer via primary amino groups using 7-min activation, 7-min adsorption, and 7-min deactivation (Johnsson et al., 1991). A1/A3C1C2 (10 μg/mL, $\approx$85 nM) in 50 mM Hepes, pH 7.5, containing 0.1 M NaCl, 2 mM $CaCl_2$, and 0.02% Tween 80, was captured on ESH 8 during a 7-min injection. Alternatively, A1/A3C1C2 (12 μg/mL, $\approx$100 nM) was coupled directly to the dextran according to the same procedure after addition of 2 mM $CaCl_2$ to the buffer. In the studies of the effects of ionic strength and Ca^{2+} concentration, A1/A3C1C2 was coupled directly to the dextran. In all experiments where A2 was allowed to bind A1/A3C1C2, the association and dissociation phases lasted for 7 and 10 min, respectively. The flow cell was perfused with an A2 solution during the association phase and with buffer alone during the dissociation phase. In the pH study, A2 (310 nM) in 10 mM MES (pH 6.0 or 6.5) or 10 mM Hepes (pH 7.0 or 7.5), all containing 0.1 M NaCl, 2 mM $CaCl_2$, and 0.02% Tween 80, was pumped over the immobilized A1/A3C1C2. The experiments with APC-modified A1/A3C1C2 ($A1^{336}$/A3C1C2) and the study of the heparin effect were performed at the above conditions and pH 6.0 using 340 nM A2. In the ionic strength study, A2 (310 nM) was in 10 mM MES, pH 6.0, containing 2 mM $CaCl_2$, 0.02% Tween 80, and 0.1, 0.2, or 0.3 M NaCl. In the Ca^{2+} study, A2 (420 nM) was in 10 mM MES, pH 6.0, containing 0.1 M NaCl, 0.02% Tween 80, and 2, 10, or 25 mM $CaCl_2$. A set of experiments was performed in the same flow cell, and between experiments the surface was regenerated with 50 mM Tris, pH 8.0, containing 0.1 M NaCl, 2 mM $CaCl_2$, and 0.02% Tween 80, which is known to rapidly dissociate A2/A1/A3C1C2 complexes. At the end of a series of experiments, the initial experiment was repeated to check the stability of the binding capacity of the immobilized A1/A3C1C2. Results of $\geq$85% were obtained. For all experiments, parallel injections of A2 over a blank surface were performed, and no binding of A2 in the absence or presence of immobilized ESH8 could be detected at any of the experimental conditions.

Analysis of Binding Data. From sensorgram data output at 0.5-s intervals by the BIAcore instrument (Figures 2A, 3A, 4A, 5A, and 6B), representative data points (open symbols in Figures 2B, 3B, 4B, 5B, and 6C), taken as an average over 5 s of output data, were selected for analysis using software provided with the instrument. The small percentage of A2 stock solution buffer in the injected samples results in a change in the bulk refractive index of the solution, giving a net upward shift in signal during the association phase. These refractive index changes were estimated from injection of the sample over a blank surface or by measuring the jumps directly in the sensorgram itself, and the shift was subtracted from the sensorgram association phase data. The resulting curve represents the true magnitude of the interaction signal, so that we were able to use a method of analysis taking this into account.

Inspection of the dissociation phases showed that there was a rapid initial dissociation followed by a slower, near-

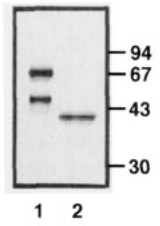

FIGURE 1: SDS−PAGE of the purified A1/A3C1C2 (lane 1) and A2 (lane 2) subunits. The proteins were analyzed in the nonreduced form on a 10% gel and visualized by silver staining. The positions of molecular mass markers (kDa) are shown to the right.

exponential dissociation phase. Therefore, the dissociation phase was analyzed in terms of a two-site binding model by fitting a double-exponential function to the data, thereby determining a fast and a slow dissociation rate parameter. These dissociation rates were held fixed thereafter, and the entire sensorgram was fitted by a two-site kinetic model with parameters k_{a_1}, k_{d_1}, r_{0_1}, k_{a_2}, k_{d_2}, and r_{0_2}, where k_a and k_d refer to the kinetic association and dissociation constants and r_0 refers to the total receptor number. Subscripts 1 and 2 refer to the low- and high-affinity binding sites, respectively. For the pH, NaCl, and Ca^{2+} experiments, r_{0_2} was estimated from a fit of the control sensorgram (pH 6.0, 0.1 M NaCl, 2 mM Ca^{2+}) and was held fixed in fitting the other sensorgrams of the set. For the heparin and APC experiments, r_{0_2} was a fitted parameter. All numerical analyses were done on a Sun workstation using standard nonlinear numerical methods (Press et al., 1986). We have found that parameters derived in this way are comparable to those obtained using the evaluation software provided with the BIAcore instrument (BIAevaluation 2.0, *Software Handbook*, Pharmacia Biosensor, 1994), except for sensorgrams with relatively low signals, where our method, which takes into account the magnitude of the association phase signal, gives more stable results.

This method also gives estimates of the parameters of the low-affinity component of binding. However, these estimates tend to have large variations and are therefore unreliable. The high-affinity parameter estimates, on the other hand, are more stable. Furthermore, the low-affinity component is a minor fraction (mean ± SD = 0.14 ± 0.09) of the total binding, so that the high-affinity parameters provide a reasonably accurate representation of the overall binding patterns.

RESULTS

Isolation of Factor VIIIa Subunits. The isolated A2 and A1/A3C1C2 subunits appeared to be homogeneous as judged by SDS−PAGE (Figure 1). Reconstitution of heterotrimer from isolated subunits (10 nM of each) increased the factor VIIIa coagulant activity more than 100-fold compared to that of the isolated subunits. This demonstrated that the purified subunits were virtually free of factor VIIIa heterotrimer.

Real-Time Analysis of the Interaction between A1/A3C1C2 and A2. The interaction between A2 and A1/A3C1C2 was studied in the BIAcore instrument. A1/A3C1C2 was chosen to be the stationary component because this was believed to reduce the risk of steric hindrance. Furthermore, a suitable antibody which recognizes an epitope in a domain (C2) not believed to be involved in the subunit interaction was available. The association of A2 to A1/A3C1C2 and the subsequent dissociation were recorded in a sensorgram, and

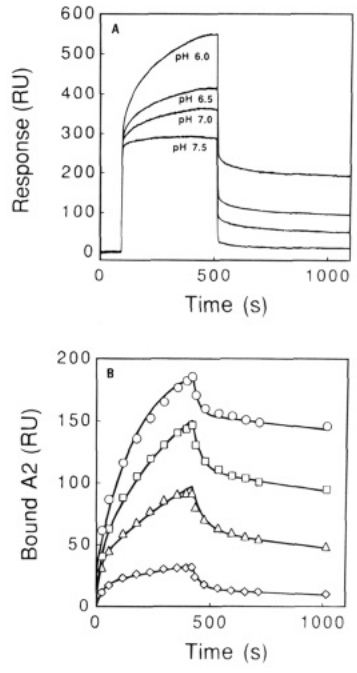

FIGURE 2: Kinetics at different pH values for the binding of A2 to A1/A3C1C2. A1/A3C1C2 was coupled to the BIAcore biosensor at a level of 8400 RUs (8.4 ng/mm^2). Sensorgram data output (A) from the BIAcore instrument, where the rapid rise at the start of the association phase and the corresponding drop at the end are due to refractive index changes caused by the small amount of stock buffer present when the samples are diluted in running buffer, are shown. These refractive index changes were subtracted to yield corrected sensorgrams used for kinetic analyses (B). The open symbols represent data points selected from the sensorgram data at pH 6.0 (○), 6.5 (□), 7.0 (△), and 7.5 (◇) subsequently fitted to a two-site binding model (solid curves).

Table 1: Calculated Values of k_a, k_d, and K_d for the Interaction between A2 and A1/A3C1C2 at Different Experimental Conditions

condition[a]	k_a (10^4 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_d (μM)
pH: 6.0	1.8	1.3	0.0073
6.5	0.82	2.8	0.034
7.0	0.37	4.5	0.12
7.5	0.07	6.3	0.94
NaCl: 0.1 M	1.8	2.1	0.012
0.2 M	0.30	1.7	0.058
0.3 M	0.12	40	3.3
Ca^{2+}: 2 mM	1.0	2.0	0.020
10 mM	0.20	8.1	0.40
25 mM	0.10	14	1.5

[a] See Materials and Methods for buffer details.

the acquired data were fitted by a two-site binding model. This type of model was chosen on the basis of the biphasic shape of the dissociation phase of the sensorgrams and gave a better fit than did a single-site model. However, only the high-affinity binding will be described further since the high-affinity component accounted for most of the binding (ca. 85%) and since the numbers derived for the rapidly dissociating component were less reliable.

The interaction between A2 and A1/A3C1C2 was studied at four different pH values ranging from 6.0 to 7.5 (Figure 2). Increasing pH resulted in lower affinity due to decreased association rate and increased dissociation rate (Table 1). The dissociation constant was 7.3 nM at pH 6.0 and 0.94 μM at pH 7.5, and there was a gradual change in this pH range. When investigating the effects of ionic strength and Ca^{2+} concentration on the interaction between A2 and A1/

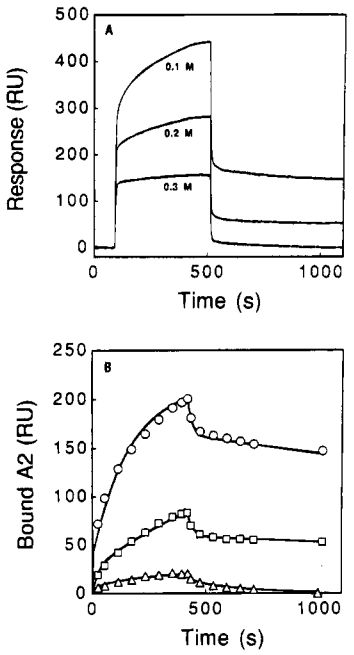

FIGURE 3: Kinetics at different NaCl concentrations for the binding of A2 to A1/A3C1C2 coupled to the BIAcore biosensor. A1/A3C1C2 was immobilized at a level of 9100 RUs (9.1 ng/mm^2). Raw (A) and corrected (B) sensorgrams, obtained as described in the legend to Figure 2, are shown. The curves show the best fits to a two-site binding model for the data acquired at 0.1 (○), 0.2 (□), and 0.3 (△) M NaCl, respectively.

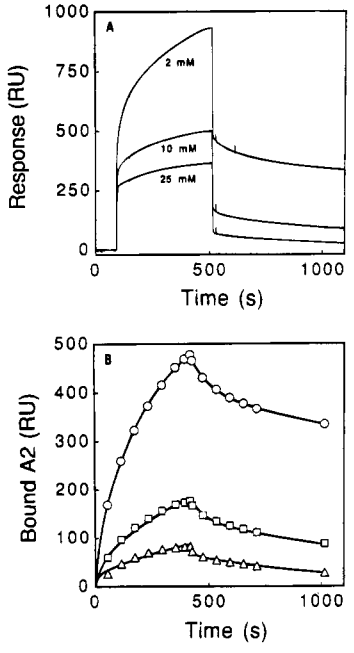

FIGURE 4: Kinetics at different Ca^{2+} concentrations for the binding of A2 to A1/A3C1C2 coupled to the BIAcore biosensor. A1/A3C1C2 was coupled at a level of 6500 RUs (6.5 ng/mm^2). Data output from the instrument (A) and corrected sensorgrams (B), obtained as described in the legend to Figure 2, are shown. The curves show the best fits to a two-site binding model for the data obtained at 2 (○), 10 (□), and 25 (△) mM $CaCl_2$, respectively.

A3C1C2, direct immobilization of A1/A3C1C2 to the dextran had to be employed since the complex between A1/A3C1C2 and the capture antibody dissociated detectably at >0.2 M NaCl or ≥10 mM $CaCl_2$. Capturing on the monoclonal antibody ESH 8 yields uniformly oriented A1/A3C1C2 as compared with random coupling directly onto the dextran. This was reflected in a higher capacity of the antibody-captured A1/A3C1C2 to bind A2. Increasing NaCl or Ca^{2+} concentration affected the on and off rates in the same direction as did an increased pH value (Table 1 and Figures 3 and 4). Changing the NaCl concentration from 0.1 to 0.3 M or changing the Ca^{2+} concentration from 2 to 25 mM increased the K_d 50-fold or more. Similar constants were calculated from the first experiment of each set where A2 was injected on different sensor chips under identical conditions, which demonstrated that the binding kinetics were not significantly different for the two immobilization methods. Moreover, injecting A2 at 90, 200, and 420 nM gave almost identical constants (not shown), demonstrating that the derived values were not concentration dependent.

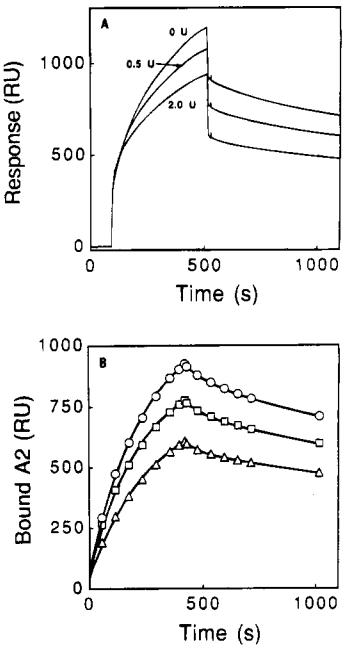

FIGURE 5: Kinetics at different heparin concentrations for the binding of A2 to A1/A3C1C2 captured on the monoclonal antibody ESH 8. The antibody was coupled to the BIAcore biosensor at a level of 4900 RUs, and subsequently 6000 RUs (6.0 ng/mm^2) of A1/A3C1C2 was bound to ESH 8. Data output from the instrument (A) and corrected sensorgrams (B), obtained as described in the legend to Figure 2, are shown. The curves show the best fits to a two-site binding model for the data acquired at 0 (○), 0.5 (□), and 2.0 (△) units/mL heparin.

Table 2: Calculated Values of k_a, k_d, K_d, and the Maximal Binding (RU_{max}) for the Interaction between A2 and A1/A3C1C2 Before and After Heparin Addition or APC-Catalyzed Proteolysis

condition[a]	k_a (10^4 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_d (nM)	RU_{max} (RUs)
heparin: none	1.1	3.1	28	1038
0.5 U/mL	1.2	3.2	26	854
2.0 U/mL	1.0	3.0	30	719
before APC cleavage	1.1	1.9	16	227
after APC cleavage	1.8	4.1	23	51

[a] See Materials and Methods for experimental details.

The inclusion of heparin in the reassembly experiments resulted in a decreased net binding of A2 to A1/A3C1C2 (Figure 5). Heparin did not affect the kinetics but acted by reducing the maximal binding, i.e., the available number of binding sites (Table 2). Thus the binding of heparin to A1/A3C1C2 and/or A2 appeared to prevent the formation of factor VIIIa heterotrimer.

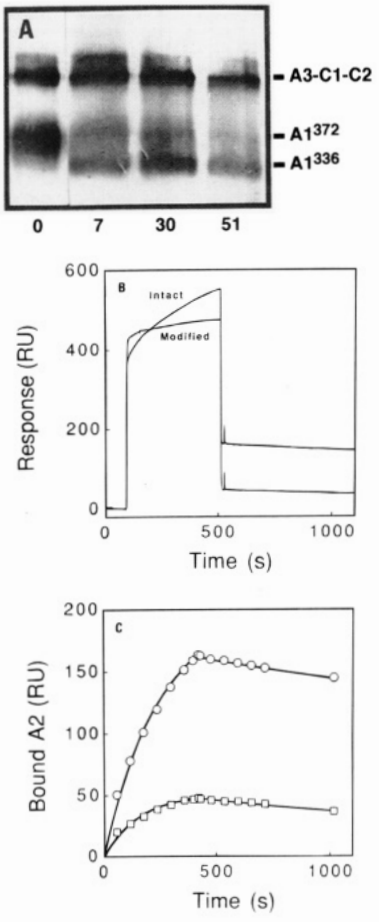

FIGURE 6: Proteolysis of A1/A3C1C2 by APC and the kinetics for the binding of A2 to $A1^{372}$/A3C1C2 or $A1^{336}$/A3C1C2 captured on the monoclonal anti-C2 antibody ESH 8. (A) Time course of the proteolysis of A1/A3C1C2 by APC monitored by Western blotting. A1 and A3C1C2 were visualized by using a polyclonal anti-heavy-chain antiserum and ESH 8 in combination. The numbers below each lane show the incubation time with APC in hours. The interaction of A2 with 2800 RUs (2.8 ng/mm^2) of $A1^{372}$/A3C1C2 (intact) captured on 4200 RUs of ESH 8 or 2400 RUs (2.4 ng/mm^2) of $A1^{336}$/A3C1C2 (modified) captured on 3700 RUs of ESH 8 was followed in the BIAcore instrument. The sensorgram data output from the instrument (B) and corrected sensorgrams (C), obtained as described in the legend to Figure 2, are shown. The curves in panel C show the best fits to a two-site binding model for the data acquired with intact (○) and APC-modified (□) A1/A3C1C2.

The physiological inactivator of factor VIIIa, APC, was used to proteolytically modify A1/A3C1C2. Western blotting after incubation with APC showed that A1 had a reduced mass and that A3C1C2 was intact (Figure 6A). The reaction was difficult to drive to completion, and some remaining intact A1 was observed. Binding of A2 to A1/A3C1C2 was severely impaired after APC treatment (Figure 6B, C). Thus the acidic region removed by APC, comprising residues 337−372, appears to be important for subunit recognition. Residual binding was most likely due to the presence of intact A1/A3C1C2 since the binding capacity was reduced with unaltered kinetic constants (Table 2).

DISCUSSION

The effects of pH, ionic strength, and Ca^{2+} on the interaction between A2 and A1/A3C1C2 seen in the BIAcore instrument are in general agreement with those observed in subunit reassembly experiments followed by procoagulant activity measurements (Fay et al., 1991a, 1993; Fay & Smudzin, 1992; Lamphear & Fay, 1992a). The K_d values calculated from solution phase experiments are also in good agreement with our data (Fay & Smudzin, 1992; Lollar et al., 1992). In addition to a more complete set of equilibrium constants, we obtained the on and off rates by measuring heterotrimer formation and dissociation directly in real time. Increasing pH or NaCl or Ca^{2+} concentration slowed down the association rate and speeded up the dissociation rate, resulting in an increase in K_d from ca. 10−20 nM at 0.1 M NaCl/2 mM Ca^{2+} to above 1 μM at the highest ionic strength or Ca^{2+} concentration and pH 6.0. Thus electrostatic forces appear to contribute significantly to the interaction since approximately one-fourth of the total binding energy is lost at 0.3 M NaCl or 25 mM Ca^{2+}. The importance of electrostatics may be even greater since hydrophobic interactions may be promoted at higher ionic strength, diminishing the observed net effect on the A2/A1/A3C1C2 complex formation. The effect of Ca^{2+} is greater than would be expected merely from its contribution to the ionic strength, suggesting that Ca^{2+} binds, although with low affinity, to specific sites in the A2 and/or A1/A3C1C2 subunit. Ca^{2+} has been shown to shield charges important for the interaction of a peptide comprising residues 337−372 with A2 (Fay et al., 1993), conceivably by bridging adjacent carboxylate moieties in the acidic peptide. It should be noted that the ionic strength and Ca^{2+} dependencies of factor VIIIa heterotrimer stability are completely different from those of factor VIII heavy and light chain assembly (Fay, 1988). The observation that pH changes affect the interaction between A2 and A1/A3C1C2 suggests that the protonation state of certain residues, conceivably histidines and terminal α-amino groups, influences the affinity (Fay & Smudzin, 1992; Lamphear & Fay, 1992a). However, since the pK_a depends on the environment of the individual residue, other groups may be affected.

The equilibrium constants derived from the BIAcore instrument data are similar to those reported in earlier studies both at pH 6, 10−20 nM versus 28 nM (Fay & Smudzin, 1992), and at physiological pH, 0.9 μM versus 0.25−0.30 μM (Fay & Smudzin, 1992; Lollar et al., 1992). However, a discrepancy exists between the on and off rates at pH 7.4−7.5 determined using the biosensor and those measured using an activity assay in solution (Lollar et al., 1992), although they result in similar equilibrium constants. This may partly be explained by the different NaCl and Ca^{2+} concentrations employed, and the presence of factor IXa and phospholipids in the activity-based analyses presumably influences the results. In addition, there are methodological differences between the real-time BIAcore instrument approach and the methods used in the previous studies which may also explain the slightly different results. Firstly, one of the subunits is covalently immobilized or captured on an antibody in the BIAcore instrument in contrast to previous studies employing subunits in solution. However, our data using different modes of immobilization indicate that immobilization *per se* does not affect the subunit interaction. Secondly, analysis in the BIAcore machine is nonintervening, whereas the removal of a sample for activity measurement results in a dilution of the sample and a change of buffer composition. This disturbs the equilibrium and may affect the outcome of the activity measurements and in turn the calculated values. Thirdly, the constants determined from the solution

phase experiments may include an effect of pH on the specific A2/A1/A3C1C2 activity, whereas our data are true binding data. Taking these points into consideration, the results in the two types of studies are supportive. However, the great advantage with the real-time approach is the possibility to obtain continuous information about both association and dissociation in the absence of additional components, whereas the results of the activity measurements reflect an end point concentration of factor VIIIa heterotrimer.

This and previous studies have demonstrated the electrostatic contribution to the interaction between A2 and A1/A3C1C2. The obvious role for the acidic region (residues 337−372), inferred from the effects of peptides and antibodies (Fay et al., 1993) as well as $A1^{336}$/A3C1C2 (Fay et al., 1991b) on factor VIIIa reconstitution, has been corroborated by the direct demonstration of the inability of $A1^{336}$/A3C1C2 to bind A2 in the BIAcore instrument. The acidic character of residues 337−372, and in particular of the N-terminal part that was found to be responsible for the interaction with A2 (Fay et al., 1993), suggests that a positively charged patch on A2 is involved. Examination of the human A2 sequence reveals a basic region between residues 405 and 427 (net charge +8) which is extended toward the C-terminus with additional flanking positive charges in the porcine sequence (Lubin et al., 1994). The substitution of porcine A2 for the human subunit indeed results in increased stability of the factor VIIIa heterotrimer (Lollar & Parker, 1991; Lollar et al., 1992). Residues 466−499 also have a strong positive net charge (+7), although this is slightly reduced in porcine A2 (+6). However, the lack of three-dimensional structural information on factor VIII or any other protein containing a homologous A domain permits speculation on primary structure level only. It is possible that clusters of basic residues distant in the linear sequence are brought together in the tertiary structure. The observed heparin effect on factor VIIIa subunit assembly is presumably due to an interaction with such a basic region, the occupation of which precludes heterotrimer formation. Barrow et al. (1994) recently reported the inhibition of thrombin-catalyzed factor VIII activation by heparin. Although thrombin itself is known to bind heparin, the two effects of heparin may be consequences of the same interaction with factor VIII(a).

APC hydrolyzes two peptide bonds in factor VIIIa, cleavage at Arg-562 bisects A2 and at Arg-336 removes an acidic 37-residue peptide C-terminal of A1 (Fay et al., 1991b). APC-catalyzed inactivation of factor VIIIa appeared to correlate with proteolysis at Arg-562, presumably since this position was most susceptible to cleavage. However, in the presence of factor IXa the cleavage at Arg-562 was inhibited whereas the peptide bond at Arg-336 was hydrolyzed at the same rate (Regan et al., 1994). This suggests that the latter site is important for inactivation of factor VIIIa incorporated in the intrinsic factor Xase complex, especially in the absence of protein S. Indeed, cleavage at Arg-336 alone inactivates factor VIIIa since factor IXa-catalyzed cleavage at this position correlates with loss of factor VIIIa activity (Lamphear & Fay, 1992a,b; O'Brien et al., 1992). Thus the dissociation of A2 from A1/A3C1C2 following APC-catalyzed proteolysis at Arg-336, monitored in the BIAcore instrument as an inability of $A1^{336}$/A3C1C2 to bind A2, may provide a physiologically relevant means of inactivating factor VIIIa, especially considering the stabilization of the inherently labile factor VIIIa heterotrimer by factor IXa and phospholipid (Lollar et al., 1984; Lollar & Fass, 1984; Lamphear & Fay, 1992a).

ACKNOWLEDGMENT

We thank Anette Østergaard for skillful technical assistance and Professor Johan Stenflo for providing us with activated protein C.

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BI950884C