Binding of blood coagulation Factor VIII and its light chain to phosphatidylserine/phosphatidylcholine bilayers as measured by ellipsometry

Joke SPAARGAREN,* Peter L. A. GIESEN,† Marie P. JANSSEN,† Jan VOORBERG,* George M. WILLEMS† and Jan A. VAN MOURIK*‡

*Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P.O. Box 9190, 1006 AD Amsterdam, The Netherlands and †Cardiovascular Research Institute Maastricht, University of Limburg, Maastricht, The Netherlands

Factor VIII is a plasma protein which plays an essential role in the coagulation system. When assembled with the enzyme Factor IXa on a phospholipid membrane, it functions as a cofactor in the enzyme complex that cleaves the zymogen Factor X to Factor Xa. We studied the binding of both Factor VIII and the Factor VIII light chain to planar phospholipid bilayers consisting of 25% dioleoylphosphatidylserine and 75% dioleoylphosphatidylcholine (PSPC) by ellipsometry. Equilibrium-binding studies revealed that both Factor VIII and its light chain bind with high affinity to PSPC bilayers. The binding affinity of Factor VIII, with a dissociation constant K_d of 0.24 nM, was comparable with that of the Factor VIII light chain (K_d 0.49 nM). Maximal binding was 2.3 mmol of protein per mol of PSPC for Factor VIII and 7.1 mmol of protein per mol of PSPC for the Factor VIII light chain. Adsorption kinetics of both Factor VIII and its light chain conformed to the classical Langmuir adsorption model yielding dissociation constants calculated from the rates of adsorption that were similar to those obtained by equilibrium-binding studies. In contrast, measurements of rates of desorption revealed a deviation from those expected for a single class of binding sites. The desorption rate of Factor VIII increased with increasing residence time on the lipid membrane. This indicates transition of Factor VIII to a configuration with a lower binding affinity. As this time-dependent change in affinity could affect the validity of the measurement of binding parameters, in particular equilibrium-binding determinations carried out on a long timescale, binding affinity was also estimated from adsorption kinetics at half-maximal surface coverage, a relatively rapid procedure for the determination of the affinity. A K_d of 0.087 nM was obtained under these conditions. Measurement of equilibrium binding to small PSPC vesicles, a system in which equilibrium is rapidly attained, resulted in similar binding parameters (K_d = 0.13 nM and a maximal binding of 2.8 mmol of protein per mol of PSPC). These data confirm the results of equilibrium binding to planar bilayers. Taken together, our results indicate that Factor VIII, by means of its 80 kDa light chain, binds to PSPC bilayers with a dissociation constant below the concentration of Factor VIII in plasma and therefore may readily bind to exposed phospholipid membranes under physiological conditions.

INTRODUCTION

Factor VIII functions as a cofactor in the Ca^{2+}- and phospholipid-dependent conversion of Factor X to Factor Xa by activated Factor IX [1,2]. The severe bleeding disorder haemophilia A is associated with the functional absence of Factor VIII, demonstrating its essential role in the intrinsic pathway of coagulation. The amino acid sequence of Factor VIII deduced from its cDNA has revealed that it consists of several discrete domains, denoted A1–A2–B–A3–C1–C2 [3,4]. After synthesis as a single-chain polypeptide, proteolytic processing at the B–A3 junction results in an 80 kDa light chain comprising the domains A3–C1–C2 and a 180 kDa heavy chain comprising the domains A1–A2–B [5,6]. Additional processing of the B domain results in size heterogeneity of the heavy-chain species. In plasma, von Willebrand factor binds non-covalently to Factor VIII, thereby protecting it from proteolytic inactivation [7,8]. A binding site for von Willebrand factor has been located on the acidic region at the N-terminus of the Factor VIII light chain [9–11], but the C-terminal C2 domain of the light chain also appears to be involved in the binding of Factor VIII to von Willebrand factor [12]. Interestingly, the Factor VIII light chain, in particular the C2 domain, has also been implicated in binding to phospholipids [12–15]. Employing synthetic peptides corresponding to various parts of the Factor VIII light chain, Foster and co-workers have shown that amino acid residues 2303 to 2332, at the C-terminus of the C2 domain of Factor VIII, are involved in binding of Factor VIII to phospholipids [16].

The aforementioned studies have defined the interaction of Factor VIII with phospholipids in a qualitative manner. Quantitative estimation of the interaction between Factor VIII and dansyl-labelled phospholipid vesicles revealed a dissociation constant of 2–4 nM [17]. Other studies, employing lipid bilayers on glass microspheres and fluorescein-labelled Factor VIII, yielded similar dissociation constants [18]. Interaction of Factor VIII with phospholipids is also strongly dependent on the presence of phosphatidylserine [13,19]. Detailed analysis of the kinetic parameters of Factor VIII adsorption on phospholipid bilayers indicated a complex binding process, comprising a fast initial binding of Factor VIII to phospholipids followed by a second slow binding event [20]. Inspection of the binding parameters of Factor VIII to activated platelets or microparticles revealed similar binding characteristics to those observed for artificial phospholipid bilayers, with dissociation constants of 2–10 nM and 2.9 nM for thrombin-activated platelets [21,22].

The aim of the present study was to assess binding of both Factor VIII and its 80 kDa light chain, the phospholipid-binding moiety of Factor VIII, to macroscopic, planar phospholipid membranes, a condition that could mimick the interaction of Factor VIII with phospholipid membranes under physiological

Abbreviations used: PS, 1,2-dioleolyl-*sn*-phosphatidylserine; PC, 1,2-dioleolyl-*sn*-glycero-3-phosphatidylcholine; SUVs, small unilamellar vesicles.

‡ To whom correspondence should be addressed.

conditions. As an experimental approach we used ellipsometry [23–27] to characterize the interaction of Factor VIII and its light chain with planar bilayers. This technique allows the characterization of Factor VIII binding under a variety of conditions, including equilibrium binding, adsorption and desorption kinetics and equilibrium binding to small unilamellar vesicles (SUVs). The results indicate that both Factor VIII and its light chain bind to macroscopic phosphatidylserine/phosphatidylcholine (PSPC) bilayers with a dissociation constant that is lower than the concentration of Factor VIII in plasma.

EXPERIMENTAL

Materials

All chemicals used were purchased from Merck (Darmstadt, Germany) unless specified otherwise. BSA (essentially fatty acid free) and benzamidine were obtained from Sigma (St. Louis, MO, U.S.A.). Column materials were purchased from Pharmacia–LKB (Uppsala, Sweden). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylserine (PS) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids.

Phospholipid vesicles and planar bilayers

Suspensions of SUVs were prepared by sonication of 25% PS/75% PC (PSPC) mixtures essentially as described previously [28]. Silicon slides (Wacker Chemie) were rendered hydrophilic by treatment with 25% H_2SO_4 containing 80 g/l $K_2Cr_2O_7$ and extensively rinsed with water before use. A phospholipid bilayer was deposited on the silicon slide by exposure to a stirred PSPC vesicle suspension (30 μM) as described previously [27]. The phospholipid-covered slides were transferred to the ellipsometer cuvette without exposure to air.

Purified proteins

Human Factor VIII was purified essentially as described previously [29]. After immunoaffinity chromatography, the Factor VIII-containing fractions were diluted in 20 mM Tris/HCl, pH 7.2, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 10% (v/v) glycerol, loaded on to Q-Sepharose FF and eluted with a linear gradient of 0.1–0.8 M NaCl. Fractions containing Factor VIII were combined and dialysed against 20 mM Tris/HCl, pH 7.2, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 55% (v/v) glycerol and stored at −20 °C. The specific activity of the preparations obtained ranged from 4500 to 6500 units/mg.

Factor VIII light chain was purified from EDTA-treated Factor VIII as described previously [30]. The product was dialysed against 20 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl and 55% (v/v) glycerol and stored at −20 °C. The purified protein migrated as a doublet of 80 kDa on SDS/PAGE under reducing conditions.

Quantification of proteins and lipids

Protein concentrations were determined by the method of Bradford [31]. Factor VIII light chain was measured using an ELISA based on a previously described method [32]. Factor VIII cofactor activity was determined spectrophotometrically, with a chromogenic Factor Xa substrate, purified bovine coagulation factors (Coatest Factor VIII; Chromogenix AB, Mölndal, Sweden) and pooled normal human plasma as standard. One ml of pooled plasma contains 1 unit of Factor VIII which corresponds to approx. 0.1 μg of protein. Phospholipid concentrations were determined by phosphate analysis [33].

Measurement of Factor VIII adsorption and desorption to planar phospholipid bilayers

Binding of Factor VIII or its light chain to planar bilayers was measured by ellipsometry as described previously [23–27]. This technique is based on the accurate measurement of changes in the polarization state of light after reflection, which is strongly influenced by the adsorption of even very thin layers of protein to a lipid bilayer deposited on a reflecting silicon slide. Measurements were performed at room temperature in a 4 ml trapezoidal quartz cuvette, filled with 50 mM Tris/HCl buffer, containing 0.1 M NaCl, 3 mM $CaCl_2$ and 5 g/l BSA (pH 7.5). BSA was added and cuvettes were made hydrophobic (Sigmacote; Sigma Chemical Co., SL-2) to avoid protein depletion. The solution was stirred with a magnetic stirring bar (8 mm × 2 mm) rotating at 1800 rev./min in front of the slide as described previously [25]. Adsorption was started by addition of protein to the cuvette and, in long-lasting (up to 6000 s) equilibrium-binding studies, the contents of the cuvette were continuously replaced by a flow of buffer (1 ml/min) containing Factor VIII at the final concentration. Desorption of protein was initiated, after adsorption of the desired amount of protein, by flushing the cuvette rapidly (30 s) with 30 ml of buffer. In order to avoid accumulation of protein in solution originating from the surface, the buffer in the cuvette was refreshed at a constant flow rate of 1 ml/min during the experiment.

Analysis of equilibrium binding

Equilibrium-binding experiments were analysed using the Langmuir model for independent binding sites [34]:

$$\Gamma_{eq.} = \Gamma_{max.}\frac{C_{bulk}}{K_d + C_{bulk}} \quad (1)$$

which relates the amount of bound protein ($\Gamma_{eq.}$) to the unbound protein concentration (C_{bulk}), the maximal protein adsorption ($\Gamma_{max.}$) and the dissociation constant (K_d). The K_d represents the solution concentration of protein resulting in half-maximal occupation of the binding sites, i.e. $\Gamma_{eq.} = 0.5\Gamma_{max.}$. The values of K_d and $\Gamma_{max.}$ were estimated from experimental data of $\Gamma_{eq.}$ as a function of C_{bulk} using a least-squares fit. Data are presented as estimate ± standard error of the estimate (S.E.E.).

Analysis of adsorption and desorption kinetics

The following equation was used in the analysis of adsorption and desorption experiments [35]:

$$\frac{d\Gamma}{dt} = \Delta\left[C_{bulk} - K_d\left(\frac{\Gamma}{\Gamma_{max.} - \Gamma}\right)\right] \quad (2)$$

where Δ is the mass-transfer coefficient which depends on the diffusion coefficient of the protein and the flow conditions near the surface [25]. The value of $\Gamma_{max.}$ obtained in the equilibrium-binding experiments was used and the values of Δ and K_d were estimated by a least-squares fit of the (numerical) solution of eqn. (2) to the measured data. Data are presented as mean ± S.E.M. When $C_{bulk} = 0$, rearrangement of eqn. (2) results in the relation:

$$K_d = -\frac{1}{\Delta}\left(\frac{\Gamma_{max.} - \Gamma}{\Gamma}\right)\frac{d\Gamma}{dt} \quad (3)$$

which allows estimation of K_d from the desorption rate once $\Gamma_{max.}$ and Δ have been determined.

Eqn. (2) also provides the basis for a more rapid procedure for the determination of the dissociation constant once $\Gamma_{max.}$ is

known. For $\Gamma = 0.5\Gamma_{max.}$, the dissociation constant K_d is equal to the protein concentration C_{bulk} that corresponds to equilibrium $d\Gamma/dt = 0$. An experimental set-up was chosen in which Factor VIII was allowed to adsorb to $0.5\Gamma_{max.}$. Subsequently, the cuvette was rapidly flushed and the desorption rate was measured for 15 min. Thereafter a minute amount of Factor VIII was added (final concentration 0.1–0.2 nM) and the adsorption rate was measured again. The velocities were plotted against the concentration and the K_d was determined by linear interpolation as the concentration corresponding to $d\Gamma/dt = 0$.

Measurement of Factor VIII binding to SUVs

Eqn. (2) shows that the initial adsorption rate equals the mass-transfer coefficient multiplied by the free protein concentration: $d\Gamma/dt = \Delta C_{bulk}$. From initial adsorption rates at known protein concentrations, Δ can be estimated. Subsequently this relation can be used to determine C_{bulk} from the initial adsorption rates at unknown protein concentrations. Previously, we have taken advantage of this relationship for measuring concentration of unbound prothrombin, Factor Xa and annexin V in a mixture of these proteins with SUVs [26,36]. It was shown for these proteins that protein bound to SUVs does not contribute to the adsorption to a PSPC bilayer and therefore the initial adsorption rate observed after addition of a mixture of protein and PSPC SUVs reflects the concentration of unbound protein. Factor VIII binding to PSPC SUVs was measured as follows: Factor VIII was first incubated with various concentrations of PSPC SUVs. After preincubation, this mixture was infused into the ellipsometer cuvette in order to measure the concentration of unbound Factor VIII. The amount of Factor VIII bound to the vesicles then equals Factor $VIII_{total}$ − Factor $VIII_{unbound}$. For further details, see the Results section.

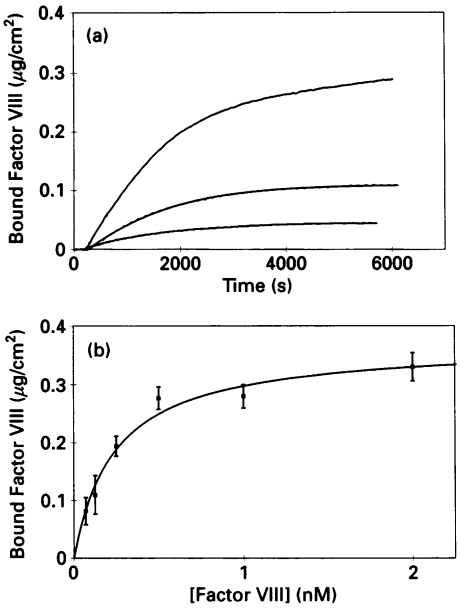

Figure 1 Ellipsometric measurement of Factor VIII binding to PSPC bilayers

(**a**) Adsorption of Factor VIII as a function of concentration. The concentrations of Factor VIII were 0.5 nM (top curve), 0.25 nM (middle curve) and 0.125 nM (bottom curve). The fitted model curves according to eqn. (2) are represented by dashed lines which coincide with the solid lines. (**b**) Equilibrium-binding isotherm of Factor VIII. Indicated are the mean (±S.E.M.) equilibrium adsorptions of at least three experiments. The solid line represents the best-fitting model isotherm according to eqn. (1).

RESULTS

Binding of Factor VIII to phospholipid membranes

Figure 1(a) shows the adsorption of Factor VIII to PSPC bilayers as a function of time at several Factor VIII concentrations. The initial adsorption rate $d\Gamma/dt$ is proportional to the concentration of protein in the cuvette in agreement with eqn. (2). After the initial transport-limited phase, the adsorption rate declines with increasing coverage of the surface with protein until equilibrium is reached. The long duration of the adsorption at low concentrations of Factor VIII can be seen.

Figure 1(b) shows the equilibrium adsorptions, $\Gamma_{eq.}$, plotted as a function of the Factor VIII concentration. Least-squares fitting of eqn. (1) to these data resulted in values (estimate ± S.E.E.) of $K_d = 0.24 \pm 0.06$ nM and $\Gamma_{max.} = 0.37 \pm 0.03$ μg/cm². Assuming a molecular mass of 300 kDa, the maximal adsorption is 1.23 pmol/cm² and the area of phospholipid surface occupied per Factor VIII molecule is approximately 130 nm². The mass of the PSPC bilayer, as measured by ellipsometry, was 0.42 ± 0.03 μg/cm² [25] which, as the molecular mass of PS and PC is 786 Da, corresponds to 0.53 nmol/cm². Therefore the maximal binding corresponds to 2.3 mmol of Factor VIII per mol of PSPC, i.e. 430 phospholipid molecules per Factor VIII binding site.

The kinetics of Factor VIII adsorption were analysed using eqn. (2) and the value $\Gamma_{max.} = 0.37$ μg/cm² as determined in the equilibrium-binding experiments. The fit of this model to the adsorption curves as presented in Figure 1(a) resulted in values (mean ± S.E.M.) of $\Delta = 9.4(\pm 0.8) \times 10^{-4}$ cm/s and $K_d = 0.40 \pm 0.06$ nM. A good agreement between model and data is apparent from the coincidence of the measurements and model curves in Figure 1(a).

Desorption kinetics of Factor VIII bound to PSPC membranes

To relate the kinetics to equilibrium binding we next investigated the kinetics of desorption. Figure 2 shows the adsorption of relatively high Factor VIII concentrations (1–5 nM). After about

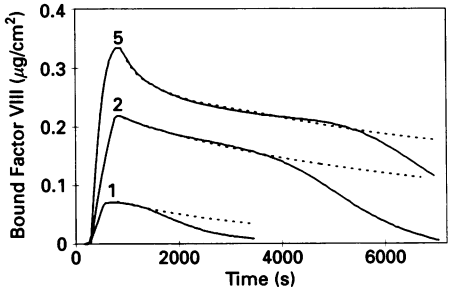

Figure 2 Desorption kinetics of Factor VIII

Adsorption of Factor VIII (1, 2 and 5 nM; lower, middle and upper curves, respectively) and desorption after a short residence time (1000 s). Desorption was initiated after 6000–8000 s by flushing the cuvette with buffer. The predicted desorptions, as obtained by a fit of eqn. (2) to the initial phase of these desorptions, are represented by dashed lines.

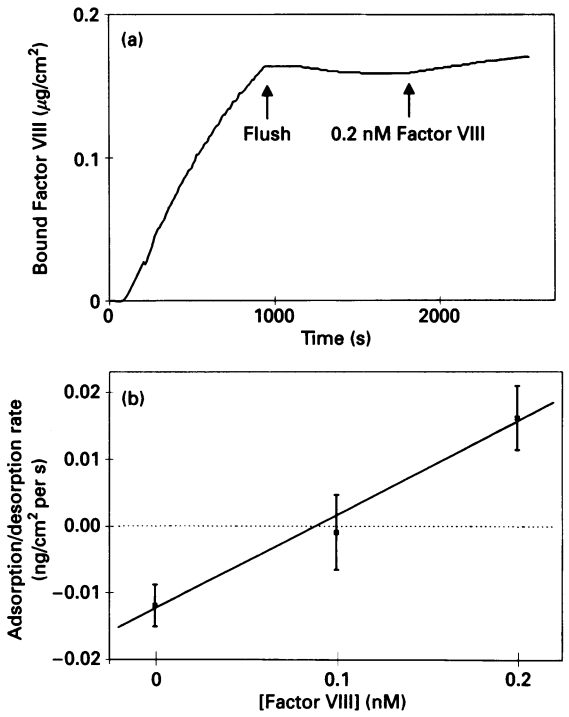

Figure 3 Adsorption and desorption of Factor VIII to PSPC bilayers covered with Factor VIII

(**a**) Adsorption and desorption of Factor VIII at $\Gamma = 0.5\Gamma_{max.}$. Factor VIII (1.0 nM) was allowed to adsorb up to half-maximal adsorption. Subsequently, at $t = 1000$ s the solution in the cuvette was flushed with buffer and the desorption rate was followed, again for about 1000 s. Thereafter 0.1 or 0.2 nM Factor VIII was added to the cuvette and the adsorption/desorption measured. (**b**) Adsorption/desorption rate as a function of the Factor VIII concentration in solution. Indicated are the means ± S.E.M. of at least three experiments. The solid line represents the best fit to these data.

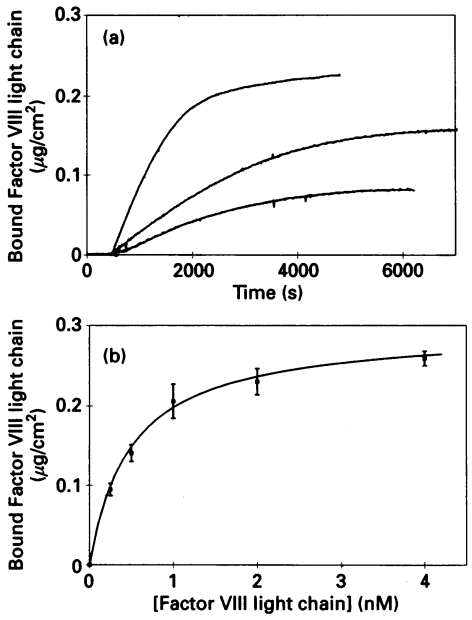

Figure 4 Binding of Factor VIII light chain to PSPC bilayers

(**a**) Adsorption of Factor VIII light chain as a function of concentration. The concentrations were: 2 nM (upper curve), 0.5 nM (middle curve) and 0.25 nM (lower curve). The dotted lines, which coincide with the solid lines, represent model curves obtained by a fit of eqn. (2) to the data. (**b**) Binding isotherm of Factor VIII light chain. Indicated are the equilibrium adsorptions (mean ± S.E.M.) of at least three experiments and the solid line represents the best fit of eqn. (1) to these data.

600 s, long before establishment of equilibrium, adsorption is interrupted and desorption is initiated by flushing the cuvette with buffer. The upper curve with Factor VIII binding close to $\Gamma_{max.}$ shows, as predicted by eqn. (2), a rapid initial phase of desorption. At Γ below 0.3 $\mu g/cm^2$ the desorption rate slows down. Apparent in all curves is the acceleration of the desorption rate after a delay of about 2000 s (lower curve), up to 5000 s (upper curve). This indicates that Factor VIII bound to the PSPC membrane slowly loses its high-affinity-binding capacity. Calculation of the K_d from the desorption rate after 10 min results in a K_d value (mean ± S.E.M.) of 0.092 ± 0.016 nM, whereas the final rapid phase results in a K_d of 1.0 ± 0.1 nM. The deviation from the model represented by eqn. (2) is apparent from the indicated fits to the initial phase of desorption.

Adsorption and desorption kinetics at half-maximal surface coverage

The long duration of the equilibrium-binding experiments (see Figure 1) is a consequence of the low concentrations that must be considered in view of the high binding affinity and the limited mass-transfer rate for macroscopic surfaces. The observed slow decline in binding affinity of bound Factor VIII as observed in Figure 2 could complicate the interpretation of such equilibrium experiments. In order to assess to what extent our estimates of K_d are influenced by the duration of the experiments, we devised a method to assess the dissociation constant within a relatively short time span. To this end we took advantage of the knowledge that K_d corresponds to the solution concentration of protein in equilibrium with a half-maximally covered phospholipid surface, i.e. K_d is the Factor VIII concentration that is exactly sufficient to prevent net desorption for $\Gamma = 0.5\Gamma_{max.}$ [see eqns. (1) and (2)]. First 1 nM Factor VIII was allowed to adsorb rapidly (300–600 s) up to a surface coverage of $\Gamma = 0.5\Gamma_{max.}$. Next, the cuvette was rapidly flushed with buffer, and Factor VIII was allowed to desorb below $0.5\Gamma_{max.}$ for 15 min (Figure 3a). Finally, 0.2 nM Factor VIII was added which resulted in slow adsorption of Factor VIII. The same experiment was repeated, but this time 0.1 nM Factor VIII was added to the cuvette, which resulted in extremely slow desorption. Figure 3(b) shows that $d\Gamma/dt$ increases linearly with the concentration of added Factor VIII. Subsequently, the K_d can be calculated as the intersection of this line with the horizontal axis ($d\Gamma/dt = 0$), which represents the Factor VIII concentration required to maintain a constant Factor VIII binding of $0.5\Gamma_{max.}$. This resulted in a value (estimate ± S.E.E.) of 0.087 ± 0.015 nM for the dissociation constant of the Factor VIII binding to PSPC, which is only threefold lower than found in the equilibrium-binding experiments.

Binding of Factor VIII light chain to phospholipids

The phospholipid-binding site of the Factor VIII molecule has been localized on the light chain [13–16]. To examine whether the heavy chain modulates the phospholipid binding of Factor VIII we also studied the binding of Factor VIII light chain to PSPC. Adsorption at various concentrations of the Factor VIII light chain is shown in Figure 4(a). These experiments showed the same adsorption pattern as observed for the intact Factor VIII molecule. Fitting eqn. (1) to the equilibrium-binding data shown

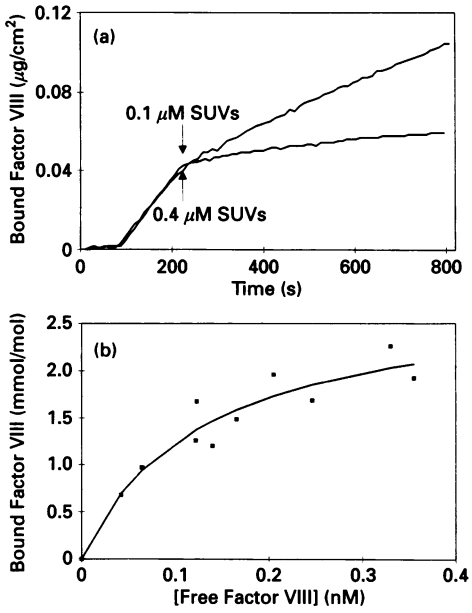

Figure 5 Ellipsometric measurement of binding of Factor VIII to unilamellar PSPC vesicles

(**a**) Effect of PSPC SUVs on the adsorption of Factor VIII to PSPC bilayer. Factor VIII (1.0 nM) was added to the cuvette and binding to a PSPC bilayer was monitored over 140 s in order to define the initial adsorption rate. At $t = 240$ s, PSPC SUVs were added (0.1 μM, upper curve; 0.4 μM, lower curve) and the effect on adsorption rate was measured. (**b**) Binding isotherm of Factor VIII to PSPC SUVs as determined from the initial adsorption rates for mixtures of Factor VIII (0.5 nM) and PSPC SUVs (0–0.675 μM). The solid line represents the best fit of eqn. (1) to these data.

in Figure 4(b) yields values (estimate $\pm$ S.E.E.) of $K_d = 0.49 \pm 0.09$ nM and $\Gamma_{max.}$ of 0.30 ± 0.02 μg/cm^2. Thus the binding affinities of Factor VIII and its light chain to PSPC are similar. The maximal surface coverage for Factor VIII light chain is, however, 7.1 mmol of protein per mol of phospholipid, which is about threefold higher than for the much larger intact Factor VIII molecule. This corresponds to binding of 1 molecule of Factor VIII light chain to 140 molecules of phospholipid. The adsorption kinetics of the light chain also conformed to the model of eqn. (2), and fitting this equation to the data gives values (mean $\pm$ S.E.M.) of $\Delta = 13.1(\pm 0.9) \times 10^{-4}$ cm/s and $K_d = 0.45 \pm 0.08$ nM.

Binding of Factor VIII to PSPC SUVs

In these experiments we measured the initial adsorption rate to PSPC bilayers to estimate the solution concentration of unbound Factor VIII in a suspension of PSPC SUVs. Figure 5(a) shows the effect of addition of PSPC SUVs (0.1 and 0.4 μM) on the adsorption of 1 nM Factor VIII on a PSPC bilayer. Before addition of SUVs the adsorption increases at a practically constant rate. Addition of SUVs results in a rapid decrease in the adsorption rate. Within 1–2 min a new lower quasi-steady-state adsorption rate is established. This second adsorption rate in the presence of 0.1 μM SUVs is 51% of the initial adsorption rate. With 0.4 μM SUVs the adsorption rate is reduced to less than 6%. Apparently nearly all added Factor VIII is bound to vesicles and this bound Factor VIII does not affect the rate of adsorption to the PSPC bilayer. The adsorption rate of a mixture of Factor VIII and SUVs therefore can be used to estimate the free Factor VIII concentration. In the experiments shown in Figure 5(b) the free Factor VIII concentration in a vesicle suspension containing 0, 0.075, 0.150, 0.225, 0.30, 0.45 and 0.675 μM PSPC was determined. The Factor VIII/vesicle mixture was preincubated for 15 min. Then a 4 ml cuvette was rapidly flushed with 12 ml of this mixture and the adsorption rate of Factor VIII to a PSPC bilayer was monitored for 600 s. Control experiments without PSPC vesicles showed that this procedure resulted in less than 6% dilution of the infusion mixture. The binding isotherm shown in Figure 5(b) gives values of $K_d = 0.130 \pm 0.038$ nM and $\Gamma_{max.} = 2.8 \pm 0.35$ mmol of Factor VIII per mol of PSPC.

DISCUSSION

The binding characteristics of the Factor VIII–phospholipid membrane interaction as measured under various experimental conditions by ellipsometry are summarized in Table 1. Measurement of the adsorption of Factor VIII to planar phospholipid bilayers under equilibrium conditions revealed that Factor VIII binds with high affinity ($K_d = 0.24$ nM). Similarly, analysis of binding of Factor VIII to PSPC bilayers under non-equilibrium conditions, including measurements of adsorption and desorption rates, revealed high-affinity binding with binding parameters of the same order of magnitude as found in the equilibrium-binding studies. Also the affinity of Factor VIII of PSPC SUVs as assessed by ellipsomety was of the same order of magnitude ($K_d = 0.13$ nM). Apparently, Factor VIII binds to both PSPC bilayers and PSPC SUVs with a K_d value that is below the Factor VIII concentration of plasma (0.3–0.7 nM, assuming a molecular mass of 300 kDa and plasma concentration of 0.1–0.2 μg/ml [37]).

The dissociation constant measured in the present study differs from the K_d values reported previously. In one study the dissociation constant measured was one order of magnitude lower [13], whereas in other studies 10-fold higher values were reported [17–19]. We suspect that these differences are due to the composition of the phospholipid membranes prepared and differences in the techniques used to assess the binding parameters. Ionic-strength differences could also account for the observed discrepancies, as it has been shown that the binding of Factor Va, another cofactor of the coagulation system that is both structurally and functionally very similar to Factor VIII, to phospholipid membranes is strongly affected by ionic strength (G. M. Willems, unpublished work; [38]).

Table 1 Binding of Factor VIII and Factor VIII light chain to PSPC

No. is the number of phospholipid molecules per Factor VIII or Factor VIII light chain binding site. N.D., Not determined.

	Factor VIII		Factor VIII light chain	
Experiments	K_d (nM)	No.	K_d (nM)	No.
Equilibrium binding*	0.24 ± 0.06§	430	0.49 ± 0.09§	140
Adsorption kinetics*	0.40 ± 0.06‖	–	0.45 ± 0.08‖	–
$1/2\Gamma_{max}$†	0.09 ± 0.02§	–	N.D.	–
PSPC vesicles‡	0.13 ± 0.04§	360	N.D.	N.D.

* Binding to planar PSPC bilayers.
† Kinetics of Factor VIII adsorption and desorption to planar PSPC bilayers.
‡ Equilibrium binding to SUVs.
§ Least-squares estimates $\pm$ S.E.E.
‖ Mean values $\pm$ S.E.M.

A point of interest is our observation that the affinity of Factor VIII for phospholipid bilayers appears to be dependent on the residence time of Factor VIII on the phospholipid surface. As illustrated in Figure 2, desorption of Factor VIII from planar phospholipid bilayers displays a time-dependent dissociation rate. A high-affinity-binding configuration (K_d = 0.1 nM) is slowly converted on the phospholipid bilayer into a form with a relatively low affinity (K_d = 1 nM). This observation extends previous data indicating that dissociation of Factor VIII from the phospholipid membrane is a complex biphasic or multistep process [20]. These complex dissociation kinetics could complicate the interpretation of both the equilibrium- and non-equilibrium data obtained in the present study. However, comparison of K_d values obtained by the different experimental approaches, which predominantly differ in terms of duration of the adsorption/desorption events, does not reveal substantial differences. For instance, the K_d derived from binding and desorption experiments performed at half-maximal surface occupation, experiments that are completed within a relatively short time span, is only 2–3-fold lower than that derived from long-lasting (up to 6000 s) equilibrium-binding studies. Similarly, assessment of the K_d of binding of Factor VIII to SUVs, a process that is completed within 120 s, is of the same order of magnitude. It appears therefore that the observed anomalous desorption behaviour of Factor VIII (Figure 2) has a limited effect on the equilibrium-binding experiment.

As outlined above the maximal surface occupation is about 1 molecule of Factor VIII per 430 phospholipid molecules, i.e. 1 molecule of Factor VIII per 215 lipid molecules in the outer leaflet of the lipid bilayer, a value that is in agreement with previously reported binding parameters of this interaction [17]. Electron-microscopic analysis of both porcine and human Factor VIII has revealed that the central B domain projects as a long thin rod up to 50 nm in length from a globular core which is about 14 nm in diameter [39,40]. This globular structure contains the A domains and the lipid-binding C domains. On the basis of this estimate, one Factor VIII molecule would occupy about 200 nm^2, a value that is in good agreement with the maximal surface coverage derived from the present study (one molecule per 130 nm^2). This suggests that maximal binding solely reflects the steric constraint of tight packing of a monolayer of protein on the membrane.

Also the light chain of Factor VIII, the 80 kDa C-terminal polypeptide that harbours the phospholipid-binding site [13–16], displays high-affinity phospholipid binding with K_d values comparable with that of the intact parent molecule, either measured under equilibrium conditions or assessed from initial-adsorption-rate measurements (Table 1). The calculated surface occupation at saturation was about one light chain molecule per 140 phospholipid molecules. It thus appears that heavy chain–light chain assembly does affect the maximal surface occupation. Also this observation suggests a close spatial relationship between these subunits at the phospholipid surface, a configuration that has also been ascribed to Factor VIII in solution [41]. Apparently heavy chain–light chain assembly does not affect the affinity of the light chain for phospholipids. This observation suggests that the light chain acts as an autonomous structural domain within the Factor VIII molecule. This view is supported by the observations that the Factor VIII light chain and the intact molecule are also equally effective in binding to both von Willebrand factor [10] and Factor IXa [30]. As the apparent affinities of the respective ligands for Factor VIII differ by not more than one order of magnitude, it would be of interest to know to what extent binding of Factor VIII, or its light chain, to von Willebrand factor, Factor IXa and phospholipid membranes are mutually exclusive. Accumulated data collected so far seem to indicate that von Willebrand factor at least is capable of preventing binding of either Factor IXa or phospholipid membranes to Factor VIII [8,30]. This is most clearly illustrated by the observation that thrombin-mediated cleavage of the N-terminal portion of the Factor VIII light chain, the polypeptide region that is involved in von Willebrand factor binding, apparently leads to exposure of both Factor IXa- and phospholipid-binding sites [18,30]. As such, von Willebrand factor plays a major role in controlling Factor VIII as a cofactor in Factor IXa-mediated Factor X activation. Whether the binding of Factor VIII to phospholipids and to Factor IXa are also mutually exclusive remains to be established.

A point of concern is the heterogeneity of the Factor VIII and Factor VIII light chain preparations used for this study, an intrinsic property of these molecular species that could result in erroneous estimation of the binding parameters. The heterogeneity of Factor VIII stems from limited proteolysis of the B domain and, as yet less well understood, microheterogeneity of the light chain [42]. Likewise the purified light chain consists of a number of polypeptides of similar size. As the K_d values of Factor VIII and its light chain do not differ (Table 1), the differences in charge and size apparently do not affect the binding kinetics of the different molecular species to a considerable extent. Pertinent to this point is the observation that the structural basis of the microheterogeneity of the Factor VIII light chain is not located within the phospholipid-binding C2 domain [43]. Also the light chain of Factor Va is heterogeneous in nature. However, in contrast with Factor VIII, this heterogeneity, which is most likely due to differences in glycosylation of the C2 domain, clearly affects the PSPC-binding properties of Factor Va [44].

Taken together, the present study indicates that Factor VIII readily binds to both PSPC bilayers and PSPC SUVs by means of its 80 kDa light chain, whereas the desorption kinetics of Factor VIII indicate a complex dissociation process. The physiological significance of this interaction remains to be established. The plasma concentration of Factor VIII is low (0.3–0.7 nM) and, consequently, adsorption proceeds at a low rate (Figure 1). In addition, under physiological conditions, Factor VIII is bound to von Willebrand factor, the carrier protein that prevents Factor VIII from binding to phospholipids. We suspect therefore that high concentrations of appropriate phospholipids are required together with thrombin-mediated dissociation of the Factor VIII–von Willebrand factor complex to ensure effective Factor VIII-dependent haemostasis at sites of vascular injury.

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