Production of thrombin at macroscopic surfaces

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Production of Thrombin at Macroscopic Surfaces

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Production of Thrombin at Macroscopic Surfaces

proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Limburg te Maastricht, op gezag van de
Rector Magnificus, Prof. Mr. M.J. Cohen, volgens het besluit
van het College van Dekanen, in het openbaar te
verdedigen op vrijdag 10 april 1992 om 14.00 uur

door

Peter Leonardus Arnoldus Giesen

geboren op 8 februari 1963 te Tilburg

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.....

In een doorzichtige tent
staat intussen, voor mij gebouwd,
een chroom en grijs instrument
waarvan ik haast menselijk houd.

In de vroegte kom ik zijn duistere
windstille labkamer binnen
en mag tegen hem fluisteren.
Dan knip ik drie schakelaars aan
en groene golven licht beginnen
door stalen buizen te gaan;
dan zak ik in een stoel
en denk God!, hoewel niet zeker
wie van ons ik bedoel.
Een kleine rechthoekige beker
water breng ik hem daarna;

....

Leo Vroman

fragment uit "de reactie van plasmathromboplastin
antecedent (PTA) met Hagemanfactor (HF)"

Voorwoord

Dit proefschrift zou nooit tot stand gekomen zijn zonder de steun van een hele reeks mensen. Met het risico een aantal mensen over te slaan poog ik toch de meest op de voorgrond tredende personen te noemen.

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En als laatste bedank ik Mieke, lieve Mieke bedankt voor al je geduld en steun die ik nodig had om dit proefschrift te kunnen maken.

A handwritten signature in black ink, appearing to read 'Peter', with a horizontal line underneath.

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Chapter 1

Introduction

BLOOD COAGULATION

The classical clotting cascade

In order to produce a stable haemostatic plug after vascular injury, a series of complex reactions occur in plasma and on the injured vessel wall. The basic reaction, repeated several times, is the activation of a zymogen. These zymogens are inactive precursor proteins that become active enzymes, serine proteases, upon activation. They are indicated by Roman numbers, followed by "a" for the activated form. The classical description of blood coagulation by MacFarlane [1] consists of a cascade following two distinct pathways, the intrinsic and the extrinsic one. Both pathways converge to the activation of factor X (see Fig.1). The intrinsic pathway is initiated when plasma comes into contact with artificial surfaces such as glass, kaolin or other surface-active materials.

FIG. 1 The classical blood coagulation cascade

A complicated series of surface-mediated reactions, involving the coagulation factors XII and XI and the plasma components high molecular weight kininogen (HMWK) and prekallikrein, then results in the activation of factor IX and factor X. The extrinsic pathway starts when plasma comes into contact with the so called tissue factor. Tissue factor is a membrane protein, present in cells outside the vascular wall. It forms a complex with factor VII, the tissue factor - factor VIIa (TF-VIIa) complex is capable

of factor X activation. The process is also surface-mediated but in this case the surface consists of naturally occurring phospholipid membranes, originating from the damaged tissue or from activated platelets. The activation of factor VII is unclear, recently it was thought that factor VII exhibits some catalytic activity thereby promoting the activation of its substrate X. Xa is able to activate VII to VIIa which is much more active. However, this initiation of coagulation is still disputed.[2]

Factor Xa, then converts prothrombin (factor II) to thrombin (factor IIa) and thrombin splits fibrinogen (factor I) into fibrin monomers (factor Ia) that rapidly form a network of fibrin polymer chains. A first layer of platelets adheres to the exposed interstitium (collagen). Thrombin also activates platelets and the activated platelets stick to each other and to the site of the lesion, thus forming a first barrier preventing further blood leakage from the damaged vessel. This first loose clot is stabilized by the fibrin network, and by cross-linking of the fibrin chains by thrombin-activated factor XIIIa. Although considerably modified, the cascade model still forms the backbone of our picture of blood clotting today.

Cofactor activation and inhibitory mechanisms

Several enzymes involved in blood clotting need a nonenzymatic protein cofactor. Inactive forms of these cofactors are present in plasma and platelets after activation, they form a complex with the enzyme. This complex is far more active than the enzyme itself. The enzyme product, or a product formed later in the cascade, often activates the cofactor which results in strong autocatalytic effects.

An example of the increase in activity due to a cofactor is the Xa-Va complex. One molecule of factor Xa converts prothrombin with a velocity of about 1 molecule every minute. To reach this velocity, a concentration of about 130 μM of prothrombin is required. Since the actual prothrombin concentration in plasma is more than 60 times less, the conversion velocity will be about 1 molecule per hour. The complete prothrombinase complex, that is, the complex of factor Xa and its cofactor factor Va adsorbed on a lipid bilayer, needs only about 5-10 nM of prothrombin to reach a velocity of about 1000 conversions per minute (see Chapters 2 and 3). Another example is the formation of the "tenase" complex of the enzyme factor IXa, the activated cofactor factor VIIIa, phospholipids and calcium, which activates factor X much more efficient than factor IXa [3]. Autocatalytic enhancement of thrombin production occurs, because thrombin activates both cofactors, factor V and factor VIII.

Formation of thrombin cannot go on indefinitely, or any small vessel injury would result in complete thrombosis. Therefore, various mechanisms for the inhibition of thrombin generation exist. Plasma contains extrinsic pathway inhibitor (EPI), a protein that forms a complex with factor Xa and this complex is able to inactivate the TF-VIIa complex [4]. Plasma also contains antithrombin III [5], a protein that binds and inactivates thrombin, especially efficient in the presence of heparin. Furthermore thrombin is inactivated after complex formation with circulating α_2 -macroglobulin

and α_1 -antitrypsin. Thrombin not only stimulates its own formation, by activation of the cofactors V and VIII, but also promotes the inhibition of its own generation. Together with the membrane protein thrombomodulin, thrombin is able to activate protein C [6]. Activated protein C, together with protein S, destroys factor Va [7]. The result is that after a trigger of tissue damage, the rate of thrombin formation in plasma initially increases, followed by increasing inhibition until the original situation is restored.

The clotting cascade updated

Over the years, the original clotting cascade has been considerably modified and a more up to date picture is shown in Fig.2. The intrinsic pathway is not longer considered as important in acute hemostasis after vessel injury but the process is assumed to be initiated by the TF-VIIa complex, directly activating factor X [8]. Factor Xa then converts factor IX into the nonenzymatic intermediate factor IX α , and factor IX α forms a good substrate for TF-VIIa to be converted to active factor IXa [9]. Factor Xa also converts some prothrombin into thrombin and thrombin activates the cofactors V and VIII. Factor VIIIa forms the tenase complex with factor IXa, that now converts factor X much more rapidly, as indicated with a bold arrow in Fig.2. Factor Va forms the prothrombinase complex with factor Xa, greatly enhancing the production of thrombin. Both complexes require binding to phospholipid membranes, indicated by broken lines in Fig.2. As can be concluded from Fig.2, this cooperative mechanism will cause an "explosion" of thrombin, only limited by the inhibitory mechanisms discussed in the preceding section.

The prothrombinase complex

In the sixties it was discovered [10, 11, 12] that efficient conversion of prothrombin requires the prothrombinase complex which consists of the activated coagulation factors Xa and Va, adsorbed to a phospholipid surface. Binding of factor Xa and prothrombin to the surface is dependent on calcium and presumably requires the formation of calcium bridges between γ -carboxyglutamic amino acid residues in the proteins and the polar headgroups of membrane phospholipids. The cofactor Va also binds to negatively charged phospholipid membranes but it does not contain γ -carboxyglutamic acid residues, and needs no calcium for binding. Binding is even inhibited by calcium concentrations exceeding about 10 mM. However, at calcium concentrations below about 10 μ M, factor Va is inactivated because of spontaneous dissociation into its heavy and light chain.

FIG. 2 The blood coagulation cascade updated

Factor Xa on its own it is not an effective enzyme. At 37 °C, the value of k_{cat} (the catalytic constant, or the maximal number of substrate molecules converted per enzyme molecule per second) in free solution is about 0.02 s^{-1} , and the value of K_m (the Michaelis constant, or the substrate concentration at which the conversion rate is half-maximal) is $131 \text{ }\mu\text{M}$ [13]. The presence of calcium hardly influences these reaction constants. Binding of factor Xa to phospholipids greatly decreases K_m and also increases k_{cat} to some extent. Addition of factor Va then allows the formation of the complete prothrombinase complex [14, 15], which increases k_{cat} with about three orders of magnitude to about 30 s^{-1} , and results in a final K_m value of about 10 nM (this thesis).

Two models for membrane-mediated thrombin production

A phospholipid membrane that binds the Xa-Va complex as well as the substrate prothrombin induces considerable decrease of the K_m . The phenomenon can actually be explained by two models. According to the so called bound-substrate model, a shell of concentrated substrate is created on the membrane surface. This greatly increases the collision rate between prothrombinase complexes and prothrombin molecules diffusing across the phospholipid surface [13, 16]. According to the so-called free substrate model, the formation of the prothrombinase complex induces a large increase in substrate affinity, making the concentration of free prothrombin molecules in the

buffer solution sufficiently high for effective substrate conversion. In this model, freely moving prothrombin molecules from the buffer are converted after collision with the prothrombinase complex on the phospholipid surface, in a bimolecular rate process [17]. Mixed versions of both models are also considered, in which the membrane promotes the rate of collisions between substrate and complex but also induces a conformational change in the complex, thereby making it more susceptible to the substrate [16].

Several observations must be taken into account to make a choice between the models. It is observed that K_m is dependent on the binding affinity of prothrombin to the phospholipid membrane. A phospholipid with a higher affinity for prothrombin also causes a larger decrease in K_m . When decarboxylated prothrombin is used, not containing γ -carboxyglutamic acid residues, thrombin formation is greatly reduced [19, 20]. These observations seemed to favour the bound-substrate model. On the other hand it was found that, in the presence of factor Va, phospholipids with a low binding affinity for prothrombin still may have a low K_m value [21, 22]. Further it was observed that K_m increases with the amount of phospholipid used [23], and high lipid concentrations will deplete the buffer of substrate. Therefore, the reaction rate seemed dependent on the free substrate concentration.

In the present study it will be demonstrated that the conversion of prothrombin, as well as the assembly of factor Xa and factor Va in the prothrombinase complex, proceeds according to the bound-substrate model. The observations suggesting a free-substrate model can be explained by the fact that most observations were performed in suspensions of small unilamellar phospholipid vesicles. Due to the very high catalytic efficiency (k_{cat}/K_m) of the prothrombinase complex, and the usual prothrombin concentrations of less than a few micromoles per litre, even a single Xa-Va complex will almost immediately convert any prothrombin molecule landing on a small vesicle. Thus the conversion rate is collisionally limited and becomes dependent on the buffer concentration of prothrombin.

FIG. 3 The ellipsometer

MEASUREMENT OF PROTEIN BINDING BY ELLIPSOMETRY

The instrument

Ellipsometry is an optical technique that measures binding of thin (0.1-100 nm) protein or lipid films to a reflecting surface. Due to reflection, the polarization state of light is altered and these changes are strongly influenced by the presence of minute amounts of various dielectric substances deposited on the reflecting surface. The ellipsometer (Fig. 3) consists of a laser that produces light with a wavelength λ of 632.8 nm which is transformed to circular polarized light and then plane-polarized again by the polarizing prism P (polarizer) in a plane at arbitrary angle to the plane of incidence. The light passes a quarter-wavelength plate (compensator) with a fast axis fixed at 45 degrees to the plane of incidence, and is reflected against a silicon slide in a quartz cuvette filled with buffer solution. After reflection the light passes a second polarizing prism A (analyzer) and the final light intensity is measured by a photodiode. When plane-polarized light passes a linear polarizing prism, this prism can be rotated such that the light intensity becomes minimal. The polarizer P is adjusted such that the phase change introduced by the compensator is exactly compensated during reflection. After reflection the light is thus again plane-polarized and the light intensity can be minimized by adjustment of the analyzer A. A computer that receives the signal of the diode also controls the position of P and A that are rotated by stepping motors. Normally, prisms P and A are sequentially rotated such that light intensity remains minimal. When one of the prisms P or A rotates, light intensity increases until a certain threshold is reached, then the rotation direction is reversed and intensity decreases and increases again until the threshold is reached again. Then the prism is moved toward the position with the lowest light intensity, right between the two threshold positions. In this manner light intensity is always kept minimal (null-ellipsometry). From the readings of P and A at minimal light intensity, mass on the reflecting surface can be calculated. The calculation of adsorbed protein mass is described in a separate section at the end of this chapter.

Estimation of the dissociation constant K_d

A set of protein concentrations was chosen and added to the cuvette. The corresponding equilibrium values Γ_{eq} of adsorbed protein, measured by ellipsometry, were plotted versus the buffer concentration of protein C_{bulk} . The data were fitted according to the relation:

$$\Gamma_{eq} = \Gamma_{max} \cdot C_{bulk} / (K_d + C_{bulk})$$

with $\Gamma_{\max}$ the maximal adsorbed mass and K_d the dissociation constant. The range of values of K_d that can thus be measured has certain limitations. Very high values will require high protein concentrations in the buffer and such large quantities are not always available. Very low values will introduce the problem that the period of time needed to reach Γ_{eq} can become very long. For instance at a concentration of 10 pM of factor Va ($= 0.00168 \mu\text{g/ml}$) and a fractional adsorption rate of $0.0015 \text{ cm}\cdot\text{s}^{-1}$ (see Ch.6) it would take about 20 hours before 50% of the value of $\Gamma_{\max} = 0.175 \mu\text{g}\cdot\text{cm}^{-2}$ is reached.

Off-null ellipsometry

A property of silicon as a reflecting surface is that increased mass on the surfaces will mainly change the position of the polarizer P and only minimally changes the position of the analyzer A. This feature is used in off-null ellipsometry. When the position of P is fixed at, for instance, 12 degrees from the position with minimal light intensity and A is kept in the minimal position, protein adsorption causes an increase in light intensity that is nearly linear with the adsorbed mass. This gives the opportunity to measure the adsorbed mass at 20 ms interval since the time-consuming rotation of the prisms is no longer required. Such short time intervals allow accurate measurement of rapid adsorption kinetics. This advantage is used in the experiments presented in Chapter 4 where the adsorption velocity is used as a measure for the free concentration of protein in the cuvette. The bound concentration on the vesicles can then be determined ($C_{\text{bound}} = C_{\text{total}} - C_{\text{free}}$), and a value of K_d for protein binding to vesicles can be determined. In this way a direct comparison of binding on vesicles and on planar bilayers was made.

THE INFLUENCE OF FLOW CONDITIONS

Intrinsic rate-limited versus transport-limited adsorption

The velocity of adsorption of a protein can be limited in two different ways. Either a protein with a high affinity for the phospholipid binding sites is transported with the maximal velocity allowed by diffusion and convection, almost completely depleting the buffer from protein directly at the surface, or a protein with a low intrinsic adsorption rate is transported so slowly towards the surface that no appreciable concentration gradient at the surface results. The first case is called transport-limited adsorption and the second case intrinsic rate-limited adsorption. The range between these two extremes, in which the adsorption rate depends both on the transport conditions and on the intrinsic adsorption rate, is called the intermediate range of adsorption kinetics. The influence of the intrinsic adsorption rate constant

becomes clear from a typical adsorption curve of mass in time that goes through two stages. First, the protein adsorbs transport-limited on the empty surface. And secondly, as the surface concentration grows and more binding sites are occupied, the adsorption rate slows down. This is predicted by classical Langmuir theory, which describes a linear decrease of the adsorption rate at increasing surface coverage. In reality, however, the adsorption rate drops much faster. Due to surface-exclusion effects and molecular interactions, the adsorption rate is often lowered by several orders of magnitude when the surface concentration has reached only 20-30% of maximal surface coverage [26].

Coagulation occurs mainly at macroscopic surfaces such as a plug of activated platelets or a vessel wall. From this point of view, it is interesting to study prothrombinase activity at such surfaces and compare the results with measurements in vesicle suspensions. However, due to the high catalytic efficiency of the prothrombinase complex, the conversion process may easily become transport-limited. Hence it is important that the amount of enzyme bound to the surface has less converting capacity than the transport capacity of prothrombin toward it. Otherwise the intrinsic K_m , that is, the local substrate concentration in the solution directly adjacent to the surface at which the conversion rate becomes half-maximal, cannot be measured. The amount of enzyme on the surface can be manipulated by varying the adsorption time. Since the adsorption rate at a given factor Va concentration in the buffer is known, it can be calculated how much Va is transported towards the surface during the adsorption period. After this period the cuvette can be rapidly flushed in order to stop adsorption and the desorption of factor Va can be neglected on the time scale of the experiments. The known surface-bound amount of factor Va can subsequently be saturated with excess factor Xa and the result is a known amount of prothrombinase on the surface. Prothrombinase activity can then be measured by adding various concentrations of prothrombin to the free solution and measure thrombin generation from serial samples as described in Chapters 2 and 5.

Uniformly accessible surfaces

A useful model to describe protein adsorption on macroscopic surfaces is the unstirred layer model. Fig 4 shows a bulk solution separated from a protein adsorbing surface by a layer of stagnant fluid with thickness d . Proteins can only pass through this layer by diffusion. At time $t=0$ the perfectly mixed bulk solution suddenly contains a protein concentration C_{bulk} . Due to adsorption, the concentration of protein directly adjacent to the surface is lowered to C_0 (or $C(0)$, the concentration at zero distance from the surface) and a linear concentration gradient is established within a second [24].

FIG. 4 The unstirred layer model

This phenomenon can be understood from general principles. The transport capacity of diffusion and the capacity for adsorption of the surface are both large compared to the quantity of protein in the boundary layer. Under these circumstances a steady state concentration profile will be rapidly established in the unstirred layer and a linear gradient is the only possible steady state compatible with the diffusion equation.

Due to the uniform thickness d of the boundary layer, the adsorption at different sites will proceed at identical rates, the surface is "uniformly accessible". This is in contrast to the situation for most experimental configurations used in experiments on protein adsorption. For the usual laminar flow cells, for instance, the buffer becomes progressively depleted of protein and an equivalent unstirred layer model would require increasing thickness of the boundary layer in the downstream direction. The same is true for the configuration, also used in the present study, of a rotating stirrer opposite a flat adsorbing plate. As demonstrated in Ch.6, the surface is only uniformly accessible in a small region extending from the site directly opposite the stirrer to a small distance upstream. Non-uniform accessibility considerably complicates a quantitative description of protein conversion at macroscopic surfaces. The overall conversion rate in such experiments is measured from the appearance of thrombin in samples taken serially from the buffer. In order to relate this production to the surface process, however, the site-dependency necessitates a complex integration of the rate expression over the whole surface. Therefore, values of k_{cat} and K_m of prothrombinase were measured in Chapter 5 on a rotating disc, a classical example of a uniformly accessible surface [27].

Analysis of adsorption kinetics

As discussed in Chapter 5, the rate of protein adsorption on a uniformly accessible surface can be described with an equivalent unstirred layer model and can be described by [28]:

$$\frac{d\Gamma(t)}{dt} = \Delta [C_{\text{bulk}} - C_0(t)] \quad (1)$$

where $\Gamma(t)$ is the surface concentration of protein at time t , $C_0(t)$ is the buffer concentration at the surface at time t and C_{bulk} is the bulk concentration of protein in the buffer. In the unstirred layer formalism, the concentration gradient of protein at the surface equals $(C_{\text{bulk}} - C_0)/d$ and the diffusional flux towards the surface is equal to the product of the diffusion constant D and this gradient. Comparison with Equation 1 then shows that $\Delta = D/d$. For the rotating disc and the rotating stirrer opposite the slide, Δ also becomes a function of the fluid viscosity and the rotation rates (see Chapters 5 and 6).

For reversible binding to independent binding sites one has:

$$\frac{d\Gamma(t)}{dt} = k_{\text{on}} [\Gamma_{\text{max}} - \Gamma(t)] C_0(t) - k_{\text{off}} \Gamma(t) \quad (2)$$

where Γ_{max} is the maximal surface concentration and k_{on} and k_{off} are the intrinsic rate constants of adsorption and desorption respectively. Elimination of $C_0(t)$ from Equations 1 and 2 results in:

$$\frac{d\Gamma}{dt} = k_{\text{on}}^{\text{app}} (\Gamma_{\text{max}} - \Gamma) C_{\text{bulk}} - k_{\text{off}}^{\text{app}} \Gamma \quad (3a)$$

with

$$k_{\text{on}}^{\text{app}} = [\Delta / \{ \Delta + k_{\text{on}} (\Gamma_{\text{max}} - \Gamma) \}] k_{\text{on}} \quad (3b)$$

$$k_{\text{off}}^{\text{app}} = [\Delta / \{ \Delta + k_{\text{on}} (\Gamma_{\text{max}} - \Gamma) \}] k_{\text{off}} \quad (3c)$$

Some interesting conclusions follow from these equations. For small values of k_{on} , that is, $k_{\text{on}} \cdot \Gamma_{\text{max}} \ll \Delta$, there is no influence of diffusion on sorption kinetics and

the apparent values $k_{\text{on}}^{\text{app}}$ and $k_{\text{off}}^{\text{app}}$ equal the intrinsic values k_{on} and k_{off} . Also, for a high surface coverage Γ , close to Γ_{max} , one has $(\Gamma_{\text{max}} - \Gamma) \approx 0$, and the differences between apparent and intrinsic rates disappear. On the other hand, for high values of k_{on} together with low surface coverages, $k_{\text{on}}^{\text{app}}$ approaches Δ . In that case Equation 3a equals Equation 1 and the initial adsorption velocity is transport limited.

FIG. 5 The prothrombinase-complex and the items discussed in this thesis, numbers refer to the chapters.

MAIN OBJECTIVES OF THIS THESIS

The main objective of this thesis is to elucidate the assembly and mode of action of the prothrombinase complex at macroscopic surfaces. Figure 5 shows a schematic view of the investigated items. Interactions between phospholipid-bound prothrombinase and prothrombin, and the issue of free-substrate versus bound-substrate conversion, are dealt with in Chapter 2. The assembly rate of factors Xa and Va, and the role of the lipid surface in this process is described in Chapter 3. Chapter 4 presents a comparison between the dissociation constants and maximal surface coverages of factor Xa and prothrombin, on vesicles and on planar bilayers. The properties and the possibilities of the rotating disc are described in Chapter 5, whereas the rotating stirrer opposite an adsorbing slide is studied in Chapter 6.

APPENDIX

Calculation of adsorbed protein mass

The ratio of the reflection coefficients for light polarized parallel and perpendicular to the plane of incidence (R^p and R^s respectively) is given by [24, 25]:

$$R^p/R^s = \tan \Psi \cdot \exp(i \cdot \Delta) \quad (4)$$

where Ψ and Δ follow directly from the readings of P and A (see "The instrument"), and $i = \sqrt{-1}$.

FIG. 6 The three reflecting systems

Figure 6 shows the three reflecting systems that are normally measured in each experiment. First, the reflecting surface - usually a silicon slide - is placed inside the cuvette filled with buffer, and the positions of P and A corresponding to minimal light intensity at the photodiode are measured. For a single transition between two optically different media 1 and 2, the values of R^P and R^S equal the Fresnel coefficients $r_{1,2}^P$ and $r_{1,2}^S$ given by:

$$r_{1,2}^P = \tan(\phi_1 - \phi_2) / \tan(\phi_1 + \phi_2) \text{ and } r_{1,2}^S = -\sin(\phi_1 - \phi_2) / \sin(\phi_1 + \phi_2)$$

These relations are supplemented by Snell's law: $n_1 \cdot \sin \phi_1 = n_2 \cdot \sin \phi_2$.

In these relations, n_1 and n_2 are the refractive indices of medium 1 and 2, ϕ_1 is the angle of incidence and ϕ_2 is the angle of refraction between the transmitted light beam and the line perpendicular to the surface (see Fig 6). The refractive index n_2 of silicon has a real and a complex part, the latter determined by the adsorbance of light in silicon.

The refractive index of the buffer solution, measured by refractometry at 20 °C, was $n_1 = n_b = 1.3357$ and an angle of $\phi_1 = \phi_b = 68$ degrees was chosen. Substituting these values into the equations, the real and the complex part of the refractive index of silicon were calculated from the real and the complex part of Equation (4). Mean values thus found were: $\text{Re}(n_{si}) = 3.99$ and $\text{Co}(n_{si}) = -0.13$

The slide is then covered with a phospholipid bilayer of thickness d_{ph} (Fig 6, middle panel) and the ratio R^P/R^S is measured again. In this case we have the relation:

$$R^P = [r_{b,ph}^P + r_{ph,si}^P \exp(i\eta)] / [1 + r_{b,ph}^P \cdot r_{ph,si}^P \cdot \exp(i\eta)]$$

with an identical expression for R^S and $\eta = -4\pi \cdot n_{ph} \cdot \cos(\phi_{ph}) d_{ph} / \lambda$.

Substitution of these expressions into Equation (4) yields an equation that can be written as: $C_1[\exp(i\eta)]^2 + C_2[\exp(i\eta)] + C_3 = 0$, where C_1 , C_2 and C_3 are functions of n_b , n_{ph} , n_{si} , Ψ and Δ . Inserting these values, with an arbitrary (real) value for n_{ph} , this equation will generally yield a complex value for d_{ph} . The correct value of d_{ph} , however, must be real. So the equation was solved by an iterative procedure in which n_{ph} is adjusted such that the complex part of d_{ph} vanishes.

Protein adsorption on the phospholipid membrane is analyzed according to the system presented in the lower panel of Fig. 6. Values of R^P and R^S are calculated for

the slide covered with phospholipid and are used as $r_{pr,ph}^p$ and $r_{pr,ph}^s$. The remaining analysis is similar to the previous one. Accordingly, values of n_{pr} and d_{pr} are obtained.

The surface concentration Γ on the slide, can be calculated from the refractive index n and the thickness d of the adsorbed layer, according to the relation:

$$\Gamma = 3d(n^2 - n_b^2) / [(n^2 + 2)\{r(n_b^2 + 2) - v(n_b^2 - 1)\}] \quad (5)$$

with n_b the refractive index of the buffer, r the specific refractivity, and v the partial specific volume of the adsorbed substance. This equation is based on the Lorenz-Lorentz relation [25] and was experimentally validated for phospholipids and proteins. It is valid if the adsorbed substance is mixed with buffer in which case the value of n remains below the refractive index of the pure substance. If n approaches this latter value, the equation for a pure substance should be used: $\Gamma = d(n^2 - 1) / [r(n^2 + 2)]$.

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Chapter 2

Production of Thrombin by the Prothrombinase Complex is Regulated by Membrane-mediated Transport of Prothrombin

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SUMMARY

Production of thrombin by phospholipid-bound prothrombinase complexes has been described as being regulated by the prothrombin concentration in the buffer (free-substrate model) as well as by the concentration of prothrombin adsorbed to the phospholipid surface (bound-substrate model).

We studied simultaneous adsorption and conversion of prothrombin on planar bilayers consisting of 20% dioleoyl-phosphatidylserine (DOPS) and 80% dioleoyl-phosphatidylcholine (DOPC). A transport limitation in the conversion of prothrombin was prevented by using a very low ($0.3 \text{ fmol}\cdot\text{cm}^{-2}$) amount of prothrombinase on the bilayer. The Michaelis and catalytic constants thus found were $K_m = 5.8 \pm 0.7 \text{ nM}$ and $k_{cat} = 33 \pm 1 \text{ s}^{-1}$ (mean $\pm$ SD). The apparent bimolecular rate constant $k_{cat}/K_m = 5.7 \times 10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$ exceeds the theoretically maximal value for the free-substrate model. In contrast, k_{cat}/K_m is within the range expected for a diffusion-controlled bound-substrate model.

A similar mechanism for prothrombin conversion in suspensions of phospholipid vesicles would imply increasing k_{cat}/K_m values for increasing vesicle diameter. This prediction was tested and a threefold increase in k_{cat}/K_m values was indeed found for vesicles of 60-80 nm diameter compared to vesicles of 20-30 nm diameter. It is concluded that thrombin production is dependent on protein fluxes rather than on protein concentrations.

INTRODUCTION

Efficient conversion of prothrombin (factor II) to thrombin (factor IIa) requires the so called prothrombinase complex, consisting of the activated coagulation factors Xa and Va adsorbed on a phospholipid membrane. Binding of prothrombin to this membrane is also essential [1, 2, 3]. This process is usually studied in suspensions of small phospholipid vesicles and different mechanisms have been proposed for the conversion process in this situation. According to the so called free-substrate model, freely moving prothrombin molecules in the buffer solution are converted after collision with the prothrombinase complex in a bimolecular rate process [4]. Binding of prothrombin to the membrane is only a determinant of substrate affinity. According to the so called bound-substrate model such binding creates a shell of concentrated substrate on the membrane surface [5]. This greatly increases the collision rate between prothrombinase complexes and prothrombin molecules diffusing across the phospholipid surface.

In vivo, thrombin is usually produced at macroscopic surfaces such as the endothelial vessel wall or a plug of activated platelets. Situations with a relatively large surface area per prothrombinase complex, for instance several square micrometer, may thus arise. The differences between free and bound-substrate models will then become more important because the total prothrombin flux to the macroscopic surface may become overwhelmingly greater than the flux to the small areas occupied by prothrombinase complexes. However, this situation is often complicated by transport limitations. Due to incomplete mixing, the buffer directly adjacent to the surface may become depleted of protein and the rate of thrombin production may become transport-limited, that is, determined by diffusion and the flow conditions [6]. The rate constants for adsorption and conversion may then assume apparent values much lower than the intrinsic ones [7].

In the present study we measured thrombin production at a macroscopic surface, a silicon slide covered with a phospholipid bilayer, containing such low prothrombinase activity that prothrombin conversion was only slightly influenced by transport conditions. This allowed measurement of the intrinsic values of K_m and k_{cat} . The value of K_m thus found was more than 25 times lower than the value found for the usual suspension of 25 nm vesicles. From these data, and from a comparison of thrombin production in suspensions of differently sized phospholipid vesicles, it is concluded that thrombin production will generally be regulated by surface diffusion of prothrombin. In vesicle suspensions, however, this process will usually appear to be a collisional-limited bimolecular reaction.

MATERIALS AND METHODS

Proteins

Bovine factors II, V and X were purified respectively according to refs [8], [9] and [10]. Factor V was activated with thrombin [9] and factor X with Russels viper venom [11]. Concentrations of factor Xa were determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate hydrochloride (pNGB) purchased from ICN, Nutritional Biochemicals [12]. Concentrations of factor II were determined similarly after complete activation to thrombin with Echis Carinata venom (Sigma Chemical Co). Concentrations of factor Va were determined by titration with factor Xa in a kinetic assay [9].

Phospholipid vesicles and planar bilayers

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) was purchased from Sigma and converted to the corresponding phosphatidylserine (DOPS) by enzymatic conversion [13]. Phospholipid concentrations were determined by phosphate analysis [14]. Suspensions of small (20-30 nm) unilamellar vesicles were prepared by sonication of a 20% DOPS/80% DOPC mixture as described [15]. Suspensions of large unilamellar (60-80 nm) vesicles were prepared by lipid extrusion under moderate pressure, as described [16], using polycarbonate filters of 80 nm pore size.

Silicon wafers (Wacker Chemie, type n, phosphor doped) were cut into slides which were made hydrophilic by treatment with 30% chromic sulfuric acid for 24 hours. They were stored in 50% ethanol and thoroughly cleaned with detergent and water before use. The slides were covered with a mask leaving 0.5 cm^2 of the surface uncovered and placed in a vessel containing 0.05 M Tris-HCl buffer, pH = 7.5, 0.1 M NaCl and 3 mM CaCl_2 . Sonicated (20-30 nm) vesicles were added to a final lipid concentration of 50 μM and the buffer was rapidly stirred for 3 minutes. During this period a lipid bilayer is formed on the uncovered surface. The slide was then flushed with a large volume of buffer, the mask was removed and it was transported to the ellipsometer cuvette, preventing exposure of the bilayer to air. It was verified that the bilayers thus prepared have identical mass ($0.42 \pm 0.03\text{ }\mu\text{g}\cdot\text{cm}^{-2}$) and produced identical adsorption and conversion of prothrombin as the bilayers deposited with a film balance [7].

Measurement of prothrombin adsorption and production of thrombin

Adsorption of prothrombin was measured by ellipsometry as described in refs [7] and [17]. Surface concentrations Γ are expressed in $\text{pmol}\cdot\text{cm}^{-2}$. Experiments were performed at 25 °C in a cuvette with 4 ml of 0.05 M Tris-HCl buffer, pH = 7.5,

containing 0.1 M NaCl, 3 mM CaCl₂ and 0.5 g·litre⁻¹ ovalbumin (Sigma). Prothrombin was incubated for 10 min in this buffer before addition to the cuvette. The phospholipid-covered area of 0.5 cm² was positioned 2 mm from a rapidly rotating magnetic stir. The flow conditions of this system have been described [7].

Thrombin production was measured by serially taking 100 µl samples from the ellipsometer cuvette and adding these samples to 100 µl 0.05 M Tris-HCl buffer, pH = 7.9, containing 0.1 M NaCl, 6 mM EDTA, 0.5 g·litre⁻¹ ovalbumin and 0.1 g·litre⁻¹ soybean trypsin inhibitor (Sigma). Amidolytic activity of this mixture was measured in an automated spectrophotometer (Cobas Bio System, Hoffmann-La Roche) at 37 °C and a wavelength of 405 nm. Samples of 80 µl were assayed by adding 5 µl of the chromogenic substrate S-2238 (Kabi Diagnostica) to a final concentration of 240 µM.

Analysis of data

Maximal flux of prothrombin towards the slide- Using estimated values for the sorption rate constants of prothrombin, it has been calculated that within a second after addition of prothrombin to the cuvette a linear prothrombin gradient is established in the boundary layer of buffer at the slide [7] and one has:

$$\frac{d}{dt} \text{IIa} = \Delta (C_{\text{bulk}} - C_0) \quad (1)$$

with IIa the thrombin production expressed in moles per cm² of lipid, C_{bulk} the bulk concentration of prothrombin and C_0 the prothrombin concentration in the buffer directly adjacent to the surface, both expressed in mol·cm⁻³. The validity of this equation can also be understood from general principles. The capacity of protein transport towards the slide is large compared to the protein content of the boundary layer and a steady-state will thus be rapidly established. A linear concentration gradient is however the only steady-state solution of the diffusion equation. The constant Δ is determined by the diffusion constant of prothrombin, the buffer viscosity and the flow conditions. The catalytic effectivity of the prothrombinase complex is so high that thrombin production will generally be transport-limited with a value of C_0 that can be neglected compared to C_{bulk} [6]. This property was used to estimate Δ from measurements of $d\text{IIa}/dt$, as a function of prothrombin concentrations, after incubation of the phospholipid-covered slide for 10 minutes in 1 nM factor Va and 2 nM factor Xa. The rate of thrombin production increased linearly with the buffer prothrombin concentration up to 1 µM of prothrombin. Apparently, the capacity for thrombin production of the bilayer is so large that the value of C_0 remains below the K_m value, i.e. 5.8 nM (cf. below), even for $C_{\text{bulk}} = 1 \mu\text{M}$. Equation (1) can thus be approximated as $(d\text{IIa}/dt) = \Delta \cdot C_{\text{bulk}}$ and the value of Δ obtained was $\Delta = (5.2 \pm 0.2) \times 10^{-4} \text{cm} \cdot \text{s}^{-1}$.

Maximal rate of prothrombin conversion in the free-substrate model- The maximal, collisional-limited, steady state transport rate constant of prothrombin molecules diffusing towards a sphere of radius R in a buffer solution is equal to $4\pi DR$, with D the diffusion constant of prothrombin [18]. This expression assumes that the sphere is relatively large and immobile compared to the prothrombin molecule. Multiplication of this expression by Avogadro's number N gives the theoretically maximal conversion rate constant:

$$k_{\max} = 4 \pi N D R \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1} \quad (2)$$

For the prothrombinase complex on a phospholipid surface this value must be divided by 2 because prothrombin can only approach from one side. Inserting also the values $N = 6.0 \times 10^{23}$, $D_{20,w} = 4.8 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ [19] and $R \approx 10 \text{ nm}$, one obtains $k_{\max} = 1.8 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, i.e. maximally $k_{\max}$ mole of thrombin can be converted per s/mole of prothrombinase/mole of prothrombin.

This expression can be compared to the conversion rate of prothrombin according to the generally accepted Michaelis Menten activation mechanism: $v = k_{\text{cat}} \cdot E_0 \cdot C_{\text{bulk}} / (K_m + C_{\text{bulk}})$, with E_0 the enzyme concentration, which for low substrate concentrations, ($C_{\text{bulk}} \ll K_m$), can be approximated by $v = (k_{\text{cat}}/K_m) E_0 \cdot C_{\text{bulk}}$. It follows that the theoretically maximal value, i.e. assuming 100% collisional efficiency, of k_{cat}/K_m is about $1.8 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Maximal rate of prothrombin conversion in the bound-substrate model- The steady state solution of the diffusion equation on a surface for diffusion of prothrombin with a surface concentration $\Gamma(r)$ at a distance r from a prothrombinase complex with radius R located at $r=0$ is $\Gamma(r) = \Gamma_0 \cdot \ln(r/R) \text{ mol} \cdot \text{cm}^{-2}$. This implies that the maximal conversion rate of prothrombin by a single prothrombinase complex is $2\pi \cdot R \cdot D' [d\Gamma(r)/dr]_{r=R} = 2\pi \cdot D' \cdot \Gamma_0$, with D' the diffusion constant of prothrombin in the phospholipid layer. Multiplication with Avogadro's number N again yields the maximal conversion rate constant:

$$k_{\max} = 2\pi N D' \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1} \quad (3)$$

Inserting $N = 6.0 \times 10^{23}$, $D' \approx 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ [20, 21], one obtains $k_{\max} = 3.8 \times 10^{16} \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1}$

RESULTS

Experiments on planar bilayers

As mentioned in the Methods, thrombin production will be transport-limited unless the bilayer has very low prothrombinase activity. Transport-limitation precludes the measurement of true kinetic constants and one thus needs a reproducible method to deposit minute amounts of prothrombinase on the bilayer. To this end, 10 pM of factor Va was added to the buffer and after 60 seconds the cuvette was rapidly flushed with buffer. The initial adsorption of factor Va is transport-limited and desorption is negligible [22]. The amount adsorbed to the slide during the 60 seconds can thus be calculated as $60 \times \Delta \times [\text{Va}] = 60 \text{ s} \times (5.2 \pm 0.2) \times 10^{-4} \text{ cm} \cdot \text{s}^{-1} \times 10^{-14} \text{ mol} \cdot \text{cm}^{-3} = (0.31 \pm 0.01) \text{ fmol} \cdot \text{cm}^{-2}$. To be sure that all factor Va adsorbed to the slide was transformed to prothrombinase, excess (100 pM) factor Xa was added to the cuvette and incubated for 5 minutes before various concentrations of factor II were added and the resulting adsorption was followed by ellipsometry. After 1, 2, 4, 8 and 12 minutes a sample was taken from the cuvette for the determination of thrombin. It was verified that thrombin production by factor Xa alone, either adsorbed to the lipid or free in the buffer solution was negligible.

In spite of the very low prothrombinase activity on the bilayer, the rate of thrombin production still became an appreciable fraction of the maximal transport rate in the low range of prothrombin concentrations (*cf.* Fig. 1). This implies that the concentration C_0 close to the slide was different from the bulk concentration C_{bulk} . Equation (1) was used to calculate C_0 from $d\text{IIa}/dt$, Δ and C_{bulk} . For instance in Fig. 1, the rate of thrombin production is about half of the maximal transport-limited rate and the concentration C_0 will thus be about half of C_{bulk} .

FIG. 1 Simultaneous measurement of surface concentration of prothrombin (O) and thrombin (Δ) after addition of 7.5 nM of prothrombin to the ellipsometer cuvette at zero time. PreadSORption of factor Va (10 pM; 60 seconds) and factor Xa (100 pM; 300 seconds) did not result in measurable surface concentrations of these proteins. Maximal, transport-limited, adsorption is indicated by a straight line. The lower panel presents thrombin concentrations, with the best-fit straight line, during the whole period of observation.

Fig. 2 presents the thrombin production as a function of the prothrombin concentrations C_0 close to the surface. A nonlinear least squares fit of the rate equation $v = V_{\max} \cdot C_{\text{bulk}} / (K_m + C_{\text{bulk}})$ to these data yielded values of $K_m = 5.8 \pm 0.7$ nM and $V_{\max} = 5.10 \pm 0.05$ fmol $\cdot$ s $^{-1}$. This latter value, divided by the total amount of prothrombinase activity 0.5×0.31 fmol present at the bilayer, yields a value of $k_{\text{cat}} = 32.9 \pm 1.0$ s $^{-1}$. It follows from these values that the apparent bimolecular rate constant $k_{\text{cat}}/K_m = 5.7 \times 10^9$ M $^{-1}$ ·s $^{-1}$ exceeds the theoretically maximal value for the free-substrate model. As further discussed below, the production of thrombin at the slide cannot be explained by a free-substrate model.

FIG. 2 Rate (mean $\pm$ SD) of thrombin production, expressed as a percentage of $V_{\max}$, for planar bilayers (Δ), 60-80 nm vesicles ($\bullet$) and 20-30 nm vesicles ($\circ$).

If the observed value of k_{cat} is to be explained by collisions between molecules diffusing across the bilayer it follows from Equation 3 that $3.8 \times 10^{16} \cdot \Gamma_0 = 33$ s $^{-1}$ or $\Gamma_0 = 8.7 \times 10^{-16}$ mol $\cdot$ cm $^{-2}$. The amount of prothrombinase of 0.31 fmol $\cdot$ cm $^{-2}$ on the slide implies a mean distance of about $1 \mu\text{m}$ between the complexes. Using the expression $\Gamma(r) = \Gamma_0 \ln(r/R)$ (cf. "Materials and Methods") with $r = 1 \mu\text{m} = 10^{-4}$ cm and $R = 10$ nm = 10^{-6} cm, it follows that the prothrombin concentration at $1 \mu\text{m}$ distance from the prothrombinase complex must be at least 4.0 fmol $\cdot$ cm $^{-2}$. From the binding

parameters of prothrombin to 20% DOPS/ 80% DOPC bilayers, i.e. $\Gamma_{\max} = 3.1$ pmol·cm⁻² and $K_d = 1.6 \times 10^{-7}$ M [23], it follows that for a buffer concentration of prothrombin equal to $K_m = 5.8$ nM, a quantity of 108 fmol·cm⁻² will be bound in equilibrium. Thus it is concluded that the observed values of K_m and k_{cat} can indeed be adequately explained by surface diffusion in a bound-substrate model.

Experiments with vesicles

Prothrombinase activity was obtained by addition of 1 pM of factor Xa and 100 pM of factor Va to 2 μ M of lipid. No excess of factor Xa was used because of the contribution to thrombin production of lipid-bound Xa, not complexed with Va. This complication did not occur in the experiments on slides because of the low phospholipid concentration of about 0.06 μ M used in these experiments. In contrast, those experiments could not be started with low Xa because of desorption of this protein during flushing of the cuvette. From the values of $K_d = 1.6 \times 10^{-7}$ and $\Gamma_{\max} = 3.1$ pmol·cm⁻² it can be verified that for the lipid concentration of 2 μ M, less than 10% of added prothrombin is bound to the lipid layer. After incubation of the prothrombinase mixture for 5 minutes, experiments were started by addition of prothrombin in concentrations of 1, 5, 10, 25, 50, 100, 200, 400 and 800 nM. The final reaction mixture volume was 200 μ l and thrombin was assayed in two 50 μ l samples taken after 2 and 4 minutes. Apparent values of K_m were 170 ± 37 nM and 42 ± 3 nM, respectively for the 20-30 nm and 60-80 nm vesicles. The corresponding apparent values of k_{cat} were 16.2 ± 1.3 s⁻¹ and 12.7 ± 0.2 s⁻¹.

The large shift in the K_m values, compared to the value of $K_m = 5.8$ nM found for the slides, and the lowering of the K_m values for increasing vesicle diameter can be explained by assuming that all prothrombin adsorbing to a vesicle is rapidly converted after a short time of diffusing across the vesicle surface towards the prothrombinase complex.

Using Equation 2 for vesicles of radius R we obtain the following data:

$$R = 12 \text{ nm: } k_{\max} = 4.3 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$k_{\text{cat}}/K_m = 0.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}.$$

$$R = 35 \text{ nm: } k_{\max} = 12 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$k_{\text{cat}}/K_m = 0.3 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}.$$

It is observed that the increase with a factor 3 in k_{cat}/K_m between vesicles with $R = 12$ nm and $R = 35$ nm is predicted by theory. Comparison of the apparent k_{cat} values found in the vesicle suspensions with the value found for the slides, shows that only about 40% of total factor Xa activity in the vesicle suspensions is transformed to prothrombinase. Taking account of this correction it follows from these figures that the values of k_{cat}/K_m are about 20 times lower than the theoretically maximal values of k_{max} .

Agreement between theory and experiment would thus imply a collisional efficiency of 5%, i.e. only 5% of the total number of collisions results in actual adsorption of the prothrombin molecule to the vesicle. Measurement of adsorption kinetics of prothrombin to 20-30 nm vesicles with stopped-flow light scattering has indeed produced values between 3% and 20% for the collisional efficiency at 10 °C [24] and it is concluded that prothrombin conversion in vesicle suspensions can usually be described as a transport-limited bimolecular reaction between prothrombin molecules and reactive vesicles.

DISCUSSION

After it had been established that adsorption of factor Xa, factor Va and prothrombin to phospholipid membranes is essential for effective thrombin production [1,2] most authors intuitively assumed that the membrane enhanced catalytic activity by concentration of substrate on its surface and by restriction of molecular movement and orientation, the so called "reduction of dimensionality" [25]. This bound-substrate model [5] was challenged when it was demonstrated that thrombin production in vesicle suspensions remains unchanged after drastic lowering of the surface concentration of prothrombin [4]. It was also shown that activation of factor X by the tissue factor-factor VIIa complex on lipid membranes, assumed to proceed analogous to prothrombin activation, was increased after displacement of lipid-bound factor X from the vesicles to the buffer solution by means of adsorption of prothrombin fragment 1 [26]. Both studies demonstrated that the rate of conversion was determined by the substrate concentration in the buffer, irrespective of the surface concentration on the vesicle. A similar conclusion was drawn from a study on the kinetics of prothrombin activation in vesicle suspensions [27]. These studies indicated a free-substrate model.

The present study explains these results. Even a single prothrombinase complex will rapidly deplete the surface concentration of prothrombin on a small vesicle. Thereafter, prothrombin can only be supplied from the buffer and the conversion will appear to be a diffusion-controlled bimolecular reaction between vesicles and prothrombin molecules in bulk solution. The very low surface concentrations of prothrombin required for half-maximal conversion rates, in measurements of K_m

values, are not detectable with the usual light scattering techniques [24, 28] or with ellipsometry. On planar bilayers this situation can be prevented by using extremely low surface coverage with prothrombinase. The surface area per prothrombinase complex then becomes large enough to ensure that prothrombin transport from the buffer to the bilayer exceeds the conversion capacity. The membrane acts as a kind of funnel, guiding the protein flux through its relatively large aperture towards the prothrombinase complex. In this way one prevents a transport limitation and obtains the intrinsic values of K_m and k_{cat} , after correction for the boundary layer gradients.

It was shown that the intrinsic value of k_{cat}/K_m exceeds the maximal theoretical value for the free-substrate model by a factor of 3 but in view of the approximations made in the calculations, this result is not compelling. However, the calculations assumed 100% collisional efficiency while, as discussed, the true value is in the range of 5%. So the discrepancy is a factor of 60. Other enzymatic conversions also remain orders of magnitude below the theoretically rate because of low collisional efficiency. Chymotrypsin, for instance, hydrolyses various amino acids with $k_{cat}/K_m = 5 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ [29]. The small peptide substrate S-2238, used to assay thrombin in the present study, is converted with a value of $k_{cat}/K_m = 5 \times 10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$ [30]. It can be concluded that the observed value of $5.7 \times 10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$ exceeds any realistic value in the free-substrate model by several orders of magnitude.

The lag phase in the production of thrombin, shown in Fig. 1, demonstrates that the membrane only produces thrombin after adsorption of prothrombin. It was calculated in the Results that a surface coverage of about $4 \text{ fmole}\cdot\text{cm}^{-2}$ would already explain the observed rate of thrombin production but, as shown in Fig. 1, more time is required before the thrombin production reaches its steady-state value. Apparently some time is needed for the build-up of the thrombin gradient at the surface, or some of the initially produced thrombin may remain attached to the surface. Although, as calculated in the Results, the surface transport of prothrombin across the bilayer is not rate-limiting, some factors could alter this situation. For instance, the binding affinity of prothrombin for the lipid can be substantially lower if the bilayer contains a lower percentage of phosphatidylserine [4]. Also, the diffusion constant of prothrombin in the bilayer may become much lower, in phospholipid mixtures of lower fluidity. In the limiting case of prothrombin molecules immobilized on the surface ($D' = 0$) one would obviously obtain a free-substrate model. This example shows that prothrombin conversion should not be analyzed in terms of concentrations of prothrombin but in terms of fluxes of prothrombin, i.e. the flux in the buffer towards the reactive surface and the flux across the surface towards the prothrombinase complex.

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Chapter 3

Membrane-mediated Assembly of the Prothrombinase Complex

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SUMMARY

Prothrombinase assembly was studied on macroscopic planar bilayers consisting of 20% dioleoyl-phosphatidylserine (DOPS) and 80% dioleoyl-phosphatidylcholine (DOPC). The dissociation constant for the binding of factor Xa to the bilayer, measured by ellipsometry, was $K_d = 47 \pm 8$ nM (mean $\pm$ SEE) and this value was lowered to $K_d = 2.2 \pm 0.3$ pM by pre-adsorption of factor Va. This latter value was determined from direct measurement of steady-state thrombin production. A comparable value of $K_d = 1.0 \pm 0.1$ pM was found by repeating these experiments in suspensions of phospholipid vesicles and it was verified that prothrombinase assembly was not influenced by the addition of prothrombin.

Using a minute amount (0.094 fmol $\cdot$ cm $^{-2}$) of preadsorbed factor Va, it was found that the rate of prothrombinase assembly exceeds the rate of collisions between Xa molecules from the buffer and the sparse Va molecules on the bilayer. Apparently, factor Xa adsorbs first to the membrane and then associates rapidly with factor Va by lateral diffusion. The data indicate almost instantaneous equilibrium of this complex formation on the surface with a lower limit for the bimolecular rate constant of $k_{on} = 2.8 \times 10^{13}$ (mol/cm 2) $^{-1}\cdot$ s $^{-1}$.

In suspensions of small phospholipid vesicles, prothrombinase assembly is collisionally limited and the value of k_{on} should be proportional to vesicle diameter. This was verified with a method for estimation of k_{on} values from thrombin generation curves. Values of 0.36×10^9 and 1.6×10^9 M $^{-1}\cdot$ s $^{-1}$ were found for vesicles of respectively 20-30 nm and 60-80 nm diameter.

INTRODUCTION

After it had been established [1, 2] that adsorption of the coagulation factors Xa, Va and prothrombin on phospholipid membranes is essential for efficient conversion of prothrombin by factor Xa, it was intuitively assumed that the membrane facilitates this process. Such facilitation could be the result of concentration of proteins on the membrane surface and restriction of molecular movement to the plane of the membrane. This concept has been called "reduction of dimensionality" [3]. Not only the directions of movement will be restricted, but also molecular orientation and this may enhance collisional efficiency.

Reduction of dimensionality was not only supposed to promote prothrombinase assembly but, by the same mechanism, also to enhance the rate of prothrombin conversion. A number of studies, however, challenged this concept [4, 5, 6]. It seemed as if the converted molecules arrived directly from the buffer solution, without previous adsorption to the membrane (free substrate model). As a result it was also doubted whether the principle played a role in prothrombinase assembly.

Recently, however, it was shown that binding of factors Xa and Va to phospholipid vesicles indeed precedes the assembly of the prothrombinase complex [7]. The rate of this assembly appeared to be limited by the frequency of collisions between Xa-containing vesicles and Va molecules in the bulk solution. Because of this collisional limitation it was not possible to estimate the assembly rate constant of membrane-bound factors Xa and Va.

In the present study, this assembly is studied on planar macroscopic bilayers. Using less than $0.1 \text{ fmol}\cdot\text{cm}^{-2}$ of adsorbed factor Va and picomolar buffer concentrations of factor Xa, it is directly demonstrated that the rate of prothrombinase production exceeds the frequency of collisions between Xa molecules from the buffer and Va molecules on the bilayer. Any significant contribution of free factor Xa molecules to prothrombinase assembly can thus be excluded. Moreover, under these circumstances the total flux of factor Xa molecules towards the surface exceeds the rate of prothrombinase formation, which allows estimation of a lower limit for the bimolecular rate constant of the assembly process on the membrane.

MATERIALS AND METHODS

Proteins and lipids

Bovine prothrombin (factor II) and coagulation factors X and V were obtained as described in respectively refs [8], [9] and [10]. Factor X was activated with Russel's viper venom (Sigma) [11] and factor V with thrombin [10]. Concentrations of factor Xa were determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate

hydrochloride (ICN Nutritional Biochemicals) [12]. Concentrations of prothrombin were determined similarly after complete activation to thrombin with Echi carinata venom (Sigma). Concentrations of factor Va were determined by titration with factor Xa in a kinetic assay [10]. Thrombin concentrations were measured at 37 °C as described [13], using the chromogenic substrate S2238 (Kabi Diagnostica) and a turnover number of thrombin for S2238 of $k_{cat} = 162 \text{ s}^{-1}$.

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Sigma and converted to the corresponding phosphatidylserine (DOPS) by enzymatic conversion [14]. Phospholipid concentrations were determined by phosphate analysis [15]. Planar phospholipid bilayers were deposited on silicon slides (Wacker Chemie, type n) as described [13]. After preconditioning of the surface, an area of 0.5 cm^2 was covered with a 20% DOPS / 80% DOPC bilayer by immersion of the slide in a vesicle suspension. Bilayer mass, measured by ellipsometry, was $0.42 \pm 0.03 \mu\text{g cm}^{-2}$ (mean $\pm$ SD). Suspensions of small (20-30 nm diameter) vesicles were prepared by sonication of a 20% DOPS / 80% DOPC mixture [16]. Suspensions of larger (60-80 nm diameter) vesicles were prepared by lipid extrusion, using poly-carbonate filters of 80 nm pore size [17]. Unless stated otherwise, all experiments were performed in 0.05 M Tris-HCl buffer, pH= 7.5, containing 0.1 M NaCl, 3 mM CaCl_2 and 0.5 g-liter⁻¹ ovalbumin (Sigma).

Experiments with planar bilayers

Adsorption of factor Xa on planar phospholipid bilayers was measured with an automated ellipsometer as described [18, 19]. Experiments were performed at 25 °C in a cuvette containing 4 ml of buffer with a rotating stirrer at 2 mm from the slide. Initial adsorption rates of factors II and Va are transport-limited, that is, proportional to the protein concentration in the buffer and a constant Δ , determined by the diffusion constant of the protein, the buffer viscosity and the flow conditions [19, 20]. For prothrombin, a value of $\Delta = (5.2 \pm 0.2) \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ was estimated from steady-state thrombin production of a bilayer containing excess prothrombinase [13]. The diffusion constant of prothrombin is $D_{II} = 4.8 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ [21]. For factor Va, a Stokes radius of $R = 5.05 \text{ nm}$ has been determined from gel filtration [22]. Using the Stokes-Einstein relation $D = kT/6\pi\eta R$, a diffusion constant of $D_{Va} = 4.3 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ is found at 25 °C. This small difference in diffusion constants for two molecules with molecular weights of respectively 72,000 and 168,000 is thought to reflect the highly asymmetrical form of prothrombin [23]. The value of $D = 5.2 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ was therefore also used for factor Va. The diffusion constant of factor Xa was estimated from ellipsometric measurements of initial adsorption rates of factors II and Xa on 100% DOPS bilayers, as described [19]. For identical buffer concentrations, factor Xa adsorbed 1.22 times faster than prothrombin, so a value of $\Delta = 6.3 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ was

used for factor Xa. The initial adsorption rate is proportional to $D^{2/3}$ [24], so $1.22 = (D_{Xa}/D_{II})^{2/3}$. Inserting D_{II} one obtains $D_{Xa} = 6.5 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and, from the Stokes-Einstein equation, $R_{Xa} = 3.5 \text{ nm}$.

Experiments with vesicle suspensions

Proteins were added to vesicle suspensions in 1.5 ml sealable plastic tubes (Eppendorf type 3810) and mechanically mixed for 5 s (Janke & Kunkel Vibrofix VF1). Final reaction volumes were 0.4 - 1 ml. During the experiments tubes were kept in a waterbath at 25 °C. Fresh vesicle suspensions were prepared each day and stored at -20 °C until use.

The maximal rate constant for the assembly of adsorbed factor Va and factor Xa from the buffer

The maximal, collisional-limited steady-state rate constant of assembly of factor Va and factor Xa molecules in buffer solution is $k_{\max} = 4\pi N(D_{Xa} + D_{Va})(R_{Xa} + R_{Va}) \text{ mol}^{-1} \cdot \text{cm}^3 \cdot \text{s}^{-1}$ [25], with N for Avogadro's number. For the relatively immobilized factor Va molecules on the lipid surface, D_{Va} can be neglected and a factor of 1/2 must be added because Xa molecules can only approach from one side. Inserting $N = 6.0 \times 10^{23}$ and the values of D and R mentioned before, one obtains $k_{\max} = 2.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The maximal rate constant for the assembly of adsorbed factors Va and Xa

It is assumed that for a factor Va molecule, situated at the origin $r = 0$, a free circular lipid area is available with radius R_0 . The steady-state equation for diffusion of factor Xa on the lipid surface towards the factor Va molecule can be described as $D' \cdot r^{-1} \cdot \delta / \delta r [r \cdot \delta \Gamma(r) / \delta r] = 0$, with $\Gamma(r)$ the surface concentration of factor Xa at a distance r from the factor Va molecule and D' the sum of the diffusion constants for lateral diffusion of factor Xa and factor Va on the lipid bilayer. The maximal flow towards the factor Va molecules is obtained by assuming that the factor Va molecule acts as a perfect sink for factor Xa or $\Gamma(R_c) = 0$, with $R_c = R_{Xa} + R_{Va}$. Using this condition the diffusion equation has the solution $\Gamma(r) = \Gamma_0 \cdot \ln(r/R_c)$. The arbitrary parameter Γ_0 can be expressed in the average surface concentration of factor Xa, defined as $\Gamma_{av} = (\int 2\pi r \Gamma(r) \delta r) / (\pi R_0^2)$. Performing this integration from $r = R_c$ to $r = R_0$ and using the condition $R_c \ll R_0$ one obtains $\Gamma_{av} \approx \Gamma_0 \cdot \ln(R_0/R_c)$. The solution of the diffusion equation thus becomes $\Gamma(r) = \{\Gamma_{av} / \ln(R_0/R_c)\} \ln(r/R_c)$.

The maximal transport rate of factor Xa towards the factor Va molecule then becomes $2\pi R_c \cdot D'(\delta\Gamma(r)/\delta r)_{r=R_c} = 2\pi D' \Gamma_{av}/\ln(R_0/R_c)$. Multiplication with Avogadro's number N and division by Γ_{av} gives the maximal assembly rate constant/mol of adsorbed factor Va $k_{\max} = 2\pi ND'/\ln(R_0/R_c) \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1}$. For a surface concentration of $0.094 \text{ fmol} \cdot \text{cm}^{-2}$, the mean distance between factor Va molecules is 1400 nm and $R_0 = 700 \text{ nm}$. Inserting also the values $R_{Xa} = 3.5 \text{ nm}$, $R_{Va} = 5.05 \text{ nm}$, $N = 6.0 \times 10^{23}$ and $D' \approx 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ [26, 27], one obtains $k_{\max} \approx 10^{16} \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1}$.

Analysis of data

Values of the dissociation constant K_d and maximal surface concentration $\Gamma_{\max}$ were obtained from the equilibrium surface concentrations Γ_{eq} , as a function of buffer concentrations C_{bulk} of protein, using the equation:

$$\Gamma_{eq} = \Gamma_{\max} \cdot C_{\text{bulk}} / (C_{\text{bulk}} + K_d) \quad (1)$$

If a low concentration of factor Va in excess lipid is equilibrated with various concentrations of factor Xa and excess prothrombin is added, thrombin production will be proportional to the amount of factor Xa bound to factor Va on the lipid surface. This is only true, of course, if production of thrombin by factor Xa not included in prothrombinase complexes, can be neglected. In that case one has:

$$d\Pi_a/dt = v_{\max} \cdot Xa / (Xa + K_d) \quad (2)$$

with $v_{\max}$ the rate of thrombin production for excess factor Xa. Equation 2 was used to estimate K_d from steady-state thrombin production.

The kinetic constants k_{on} and k_{off} of prothrombinase complex formation in vesicle suspensions were estimated as follows. If a low concentration of factor Xa is added to excess lipid, prothrombin and factor Va, the concentration of prothrombinase complexes changes according to:

$$dP/dt = k_{\text{on}} \cdot Va(Xa - P) - k_{\text{off}} \cdot P$$

Integration of this expression gives the amount of prothrombinase formed up to time t : $P(t) = P_{eq}\{1 - \exp(-kt)\}$ with $k = k_{\text{on}} \cdot Va + k_{\text{off}}$ and $P_{eq} = k_{\text{on}} \cdot Va \cdot Xa/k$. Multiplication with the turnover number of prothrombinase k_{cat} and integrating again, one obtains the amount of thrombin formed up to time t :

$$IIa(t) = v_{eq}\{t - 1/k + \exp(-kt)/k\} \text{ with } v_{eq} = k_{cat} \cdot P_{eq} \quad (3)$$

Parameters were obtained from non-linear least square fits of pooled data to the model equations 1, 2 and 3, using a standard computer program (BMDP Statistical Software Inc., procedure BMDP-3R, version 1988). Errors are expressed as standard error of estimate (SEE), that is, the standard deviation of the estimated parameter.

RESULTS

Influence of preadsorbed factor Va on the binding of factor Xa to planar bilayers

Equilibrium concentrations of adsorbed factor Xa as a function of the buffer concentrations are presented in Fig. 1. Fitting Equation 1 (see "Materials and Methods") to these data resulted in values of $K_d = 46.5 \pm 7.9$ nM and $\Gamma_{max} = 10.3 \pm 0.8$ pmol·cm⁻² or 0.45 ± 0.035 µg·cm⁻² (mean $\pm$ SEE). Preadsorption of factor Va largely increases the binding affinity for factor Xa and K_d drops below the nanomolar range. Such low values are difficult to measure directly by ellipsometry because of the extremely long adsorption times for such low concentrations. For instance, for a buffer concentration of 1 nM ($= 1 \times 10^{-12}$ mol·cm⁻³) factor Xa and a value of $\Delta = 6.3 \times 10^{-4}$ cm·s⁻¹ (see "Materials and Methods") it would take 10^4 seconds before 6.3 pmol·cm⁻² has adsorbed. Therefore K_d was measured from steady state rates of thrombin production as explained under "Materials and Methods". In order to prevent transport limitation, the bilayer should contain very low prothrombinase activity. To this end, the bilayer was exposed during 1 min to a buffer solution containing 3 pM ($= 3 \times 10^{-15}$ mol·cm⁻³) of factor Va and the cuvette was then rapidly flushed with fresh buffer. Initial adsorption of factor Va is transport limited [20] and the amount adsorbed in 1 min will thus be $60 \times \Delta \times C_{bulk} = 60 \times (5.2 \times 10^{-4}) \times (3 \times 10^{-15}) = 0.094$ fmol·cm⁻². Desorption of factor Va during and after the flushing of the cuvette is negligible [20]. Excess (200 nM) prothrombin and various concentrations (1-20 pM) of factor Xa were now added. The minute amount of factor Va is rapidly equilibrated with factor Xa, even for factor Xa concentrations in the picomolar range (see Fig. 3), and Fig. 2 shows the resulting steady state rates of thrombin production. Fitting equation 2 (see "Materials and Methods") to these data one finds $K_d = 2.15 \pm 0.30$ pM. For a final prothrombinase concentration on the surface equal to the total factor Va concentration of 0.094 fmol·cm⁻², it follows from these data that $k_{cat} = 32.3 \pm 1.3$ s⁻¹.

FIG.1 Binding isotherm of factor Xa on planar 20% DOPS/80% DOPC bilayers. Mean values of 6 experiments with best fit of the model equation are shown and SD is indicated.

This result is only valid if the contribution to thrombin production of free factor Xa, not complexed to factor Va, can be neglected. From the values of $K_d = 46.5$ nM and $\Gamma_{\max} = 10.3$ pmol·cm⁻² for the binding of factor Xa to the lipid bilayer it follows that for the highest concentration of 20 pM factor Xa used in Fig. 2 about 4 fmol·cm⁻² of factor Xa will be lipid-bound. This is about 40 times more factor Xa than present in the form of prothrombinase, but k_{cat} for the factor Xa-lipid complex is about 1000 times lower than for prothrombinase [28, 29]. The contribution of lipid-bound free factor Xa to thrombin production in Fig. 2 will thus be less than 4 %.

The rate of prothrombinase assembly by lateral diffusion of factor Va and factor Xa on planar bilayers.

Fig. 3 presents the initial phase of the experiments with 1 pM factor Xa shown in Fig. 2. The thrombin production rate increases during about 4 min until the steady state rate is obtained.

FIG.2 Steady-state rates of thrombin production after addition of factor Xa to planar 20% DOPS/80% DOPC bilayers containing 0.094 fmol·cm⁻² of factor Va and incubated in 200 nM of prothrombin. Mean results of 4 experiments with best fit of the model equation are shown and SD is indicated.

FIG.3 Kinetics of thrombin production by planar 20% DOPS/80% DOPC bilayers containing 0.094 fmol·cm⁻² of factor Va. Bilayers were incubated for 5 minutes in 200 nM of prothrombin and, at zero time, 1 pM of factor Xa was added to the buffer. Mean results of 3 experiments are shown and SD is indicated.

If v_c is the initial rate of formation of prothrombinase complexes, the amount of prothrombinase formed at time t will be $v_c \cdot t$ and the rate of thrombin production $k_{cat} \cdot v_c \cdot t$ (excess prothrombin). Integrating this expression, it is found that total thrombin production up to time t , as shown in Fig. 3, equals $0.5 \cdot k_{cat} \cdot v_c \cdot t^2$. Fitting the initial three minutes of Fig. 3 to this expression and using the value of $k_{cat} = 32.3 \text{ s}^{-1}$ it was found that $v_c = 0.20 \times 10^{-3} \text{ fmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$. For the factor Va concentration of $0.094 \text{ fmol} \cdot \text{cm}^{-2}$ and the factor Xa concentration of 1 pM , this result implies a value of $2.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the bimolecular rate constant of complex formation. However, this value is an underestimation by more than one order of magnitude because, due to the gradient in the boundary layer, the factor Xa concentration close to the surface is much lower than the buffer concentration. During the initial adsorption phase, the relation between the buffer concentration C_{bulk} and the concentration close to the surface C_0 is given by [19]: $C_0/C_{bulk} = \Delta/(\Delta + k_{on} \cdot \Gamma_{max})$. For factor Xa we have $\Delta = 6.3 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ (cf. "Materials and Methods"), $\Gamma_{max} = 10.3 \text{ pmol} \cdot \text{cm}^{-2}$ and $k_{on} = 0.4 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (cf. "Results"). The latter value was expressed per mole of factor Va or per mole of vesicles, because each vesicle will carry (less than) one factor Va molecule. From the vesicle radius of 12.5 nm and the value of Γ_{max} , a value of 118 binding sites for factor Xa per vesicle is calculated. So, expressed per mole of binding sites, $k_{on} = 3.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} = 3.4 \times 10^9 (\text{mol}/\text{cm}^3)^{-1} \cdot \text{s}^{-1}$. Inserting these values, one finds $C_0/C_{bulk} = 0.02$. The rate of complex formation thus exceeds the maximal theoretically possible value of $2.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (see "Materials and Methods") with a factor of about 50. It is concluded that direct binding of factor Xa molecules, from the buffer to factor Va molecules adsorbed on the lipid surface, contributes only marginally to the observed rate of prothrombinase formation (see "Discussion"). The only alternative mechanism of prothrombinase formation is complex formation between molecules meeting by lateral diffusion on the bilayer. An estimate of the bimolecular rate constant for complex formation between lipid-bound molecules can be obtained as follows. After two minutes, the total amount of factor Xa adsorbed on the surface is $120 \times (6.3 \times 10^{-4}) \times 10^{-15} = 0.076 \text{ fmol} \cdot \text{cm}^{-2}$. From the factor Va concentration of $0.094 \text{ fmol} \cdot \text{cm}^{-2}$ and the observed rate of prothrombinase formation of $v_c = 0.2 \times 10^{-3} \text{ fmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$, a bimolecular reaction rate constant of $2.8 \times 10^{13} \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1}$ is calculated. This is an underestimation because, due to formation of prothrombinase complexes, the actual concentrations of free factor Va and factor Xa on the surface will be lower than indicated (see "Discussion").

FIG.4 Steady-state rate of thrombin production in suspensions of 20% DOPS/80% DOPC vesicles of 20-30 nm diameter. Open and closed circles indicate respectively 0.5 pM of factor Va titrated with factor Xa, and 0.5 pM of factor Xa titrated with factor Va. Mixtures were incubated for 10 min in 2 μ M of lipid before 400 nM of prothrombin was added. Mean results of 8 experiments with best fits of the model equation are shown and SD is indicated.

It is interesting to calculate the surface concentration of factor Xa corresponding to a buffer concentration of 2 pM, that is for half-saturated bound factor Va. Applying equation 1 with $\Gamma_{max} = 10.3 \text{ pmol}\cdot\text{cm}^{-2}$, $K_d = 46.5 \text{ nM}$ and $C_{bulk} = 2 \text{ pM}$, one obtains $\Gamma_{eq} = 0.44 \text{ fmol}\cdot\text{cm}^{-2}$, which is the K_d value expressed in the surface concentration.

Estimation of the dissociation constant from steady state thrombin production in vesicle suspensions

The value of $K_d = 2.15 \text{ pM}$ for the dissociation constant of the prothrombinase complex, obtained in the preceding section, could be influenced by the physical state of the lipids deposited on a solid surface. Similar measurements were therefore performed in vesicle suspensions. A low concentration of factor Va was incubated for 10 min with excess lipid and various concentrations of factor Xa. The reaction was started by addition of excess prothrombin. The results, presented in Fig. 4, were similar if factors Xa and Va were interchanged. Fitting these results again to equation 2, a value of $K_d = 1.0 \pm 0.1 \text{ pM}$ was obtained, after correction for the fraction of factor Xa (50%) bound to the lipid.

FIG.5 Thrombin production in suspensions of 20% DOPS/80% DOPC vesicles with 20-30 nm diameter, containing 1 pM of factor Xa, 5 pM of factor Va, 400 nM of prothrombin and 2 μ M of lipid. Mixtures were incubated for 10 min before the reaction was started with factor Va (●), factor Xa (o) or prothrombin (Δ).

As demonstrated by the constant rate of thrombin production in the upper curve of Fig. 5, adsorption of prothrombin to the lipid surface does not influence the assembly of prothrombinase. Although the lipid will almost instantaneously be saturated with prothrombin, formation of extra prothrombinase activity from the very low factor Xa and Va concentrations in the buffer would require several minutes, as shown in the lower curves of Fig. 5. These latter curves show little effect of starting the reaction with either factor Xa or factor Va. The number of vesicles is more than 100 times larger than the number of Va molecules and factor Va will bind rapidly. In both cases one essentially observes the much slower binding of factor Xa to the relatively few vesicles containing a factor Va molecule.

Collisionally limited kinetics of prothrombin assembly in vesicle suspensions

As explained under "Materials and Methods", the values of k_{on} and k_{off} for the formation of prothrombinase can be obtained from measurement of thrombin production after addition of a low factor Xa concentration to vesicles containing factor Va. It also follows from the preceding sections that, for vesicles with small surface areas ($< 1 \mu\text{m}^2$), k_{on} is determined by the frequency of collisions between vesicles and factor Xa molecules and by the collisional efficiency, that is the probability that the colliding factor Xa molecule will stick to the vesicle. If the collision indeed results in

adsorption of the factor Xa molecule to the lipid surface it will almost immediately be converted to prothrombinase, even if the vesicle contains only a single factor Va molecule. This implies that k_{on} will be strongly dependent on vesicle size because, as explained under "Materials and Methods", the frequency of collisions is proportional to vesicle radius. Moreover, by comparison of calculated collision rates and observed values of k_{on} , one may directly estimate the collisional efficiency. These predictions were checked in suspensions of vesicles of 20-30 nm and 60-80 nm diameter. A mixture of 2 μ M vesicles and 400 nM prothrombin was incubated for 10 min with 2.5, 5.0, 7.5 and 10 pM of factor Va and production of thrombin was started by addition of 1 pM of factor Xa. The upper part of Fig. 6 shows the results of these experiments. Fitting these curves to Equation 3 (see "Materials and Methods") one obtains values of $k = k_{on} \cdot Va + k_{off}$. The lower part of Fig. 6 shows a plot of k as a function of factor Va concentrations from which the slope k_{on} and the cut-off k_{off} were estimated by linear regression. Values obtained were $k_{on} = (0.36 \pm 0.03) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for 25 nm vesicles and $k_{on} = (1.6 \pm 0.19) \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for 70 nm vesicles, using again a correction of 50% lipid-bound factor Xa. Estimated values of k_{off} were respectively $(1.0 \pm 0.4) \times 10^{-3} \text{ s}^{-1}$ and $(1.6 \pm 2.3) \times 10^{-3} \text{ s}^{-1}$ and values of $K_d = k_{off}/k_{on}$ respectively 2.6 pM and 1.0 pM. It is concluded that larger vesicles indeed have higher values of k_{on} and that values of K_d obtained by this kinetic method are in agreement with the values found from steady state thrombin production. The theoretically maximal value of k_{on} can be estimated as (cf. "Materials and Methods") $4\pi N(D_{Xa} + D_{vesicle})(R_{Xa} + R_{vesicle}) \text{ mol}^{-1} \cdot \text{cm}^3 \cdot \text{s}^{-1}$. Using the Stokes-Einstein relation with $R_{vesicle} = 12.5 \text{ nm}$ one finds $D_{vesicle} = 1.7 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$, and inserting also the values $D_{Xa} = 6.2 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and $R_{Xa} = 3.5 \text{ nm}$ (cf. "Materials and Methods"), a value of $k_{max} = 9.5 \times 10^9 \text{ mol}^{-1} \cdot \text{cm}^3 \cdot \text{s}^{-1}$ is obtained. Comparison of this value with k_{on} demonstrates a collisional efficiency of about 4%, that is, about 6 times lower than observed in binding experiments with stopped-flow light scattering (7). This difference is probably caused by the coverage of vesicle surface with prothrombin, because a 2.5 times higher value of k_{on} was found when the prothrombin concentration was lowered to 50 nM, instead of 400 nM (result not shown).

FIG.6 Thrombin production in suspensions of 20% DOPS/80% DOPC vesicles of 20-30 nm (O) and 60-80 nm (□) diameter. The upper part shows mean results, with best fitting model curves and SD, of 5 experiments with 1 pM of factor Xa added to a mixture of 2.5 pM factor V_a and 400 nM prothrombin, incubated for 10 min in 2 μ M lipid. The lower part shows values of $k = k_{\text{on}} \cdot V_a + k_{\text{off}}$, as a function of factor V_a concentrations (see text).

DISCUSSION

The influence of preadsorbed factor V_a on the adsorption of factor Xa

The value of $K_d = 47$ nM for the dissociation constant of factor Xa on a 20% DOPS, 80% DOPC planar bilayer is in reasonable agreement with the values of 100-114 nM that were obtained under comparable conditions (2-3 mM CaCl_2 and 25%

phosphatidylserine 75% phosphatidylcholine) for factor X and factor Xa binding to small unilamellar vesicles as measured by light scattering [7, 30]. For planar bilayers as well as for vesicles, the K_d value of factor X and Xa is 3-5 times lower, than for prothrombin [30, 31]. The maximal surface concentration of $0.45 \mu\text{g}\cdot\text{cm}^{-2}$ for factor Xa is more than twice as high as for prothrombin [31]. This high surface mass implies that the factor Xa molecules are protruding relatively far from the surface, as also concluded in studies using light scattering [23] and fluorescence energy transfer [32].

In contrast to the situation for factor X or Xa and prothrombin, there is much confusion in the literature about the binding parameters of factor V and Va. It seems as if, even under comparable conditions such as 1-3 mM calcium and mixtures of phosphatidylcholine with 20 - 25% phosphatidylserine, different techniques give different answers. Values of $K_d = 10^{-7} - 10^{-8}$ M have been calculated from data on protein depletion [33] and steady-state light scattering [34]. Values of $K_d = 3 \times 10^{-9}$ M were obtained from fluorescence polarization [35] and stopped-flow light scattering in the presence of excess prothrombin fragment 1 [7]. Values of $K_d \approx 10^{-11}$ M were found from stopped-flow light scattering in buffer [33] and ellipsometry [20].

Part of these discrepancies may be explained by some inherent limitations of the various techniques used [37], but the main differences are probably caused by large variation in surface concentrations. It has been shown that, due to surface exclusion effects and molecular interactions, K_d values will generally increase rapidly for increasing surface concentrations and will be strongly influenced by the presence of other adsorbed proteins [38]. For instance, the saturation value of 42 mol of lipid per mol of Va, reported in some studies [7, 35], corresponds to about $2 \mu\text{g}$ of Va/ cm^2 of lipid surface, which is 6 times more than the value of $\Gamma_{\text{max}} = 0.35 \mu\text{g}\cdot\text{cm}^{-2}$ found for factor Va in the present study (data not shown) and more than 5 orders of magnitude higher than the $0.094 \text{ fmol cm}^{-2}$ of adsorbed Va used for the prothrombinase assembly experiments.

It follows from the present study that, for such low surface concentrations, preadsorption of factor Va lowers the value of K_d for the adsorption of factor Xa from 47 nM to 2.2 pM, that is, with a factor of 25,000. This "receptor" effect is much stronger than reported in two recent studies by Krishnaswamy et al. In those studies, prothrombinase assembly was measured from assembly-induced fluorescence changes in dansyl-glutamyl-glycylarginyl chloromethyl ketone-Xa [7], and from the kinetics of thrombin production [39]. Both studies indicate values of $K_d \approx 10^{-9}$ M for the dissociation of the prothrombinase complex, that is almost 3 orders of magnitude higher than presently reported. This is consistent with the difference in the dissociation constant of factor Va, just mentioned, and could thus also be caused by the large differences in surface concentrations. The fact that thrombin production yielded a similar value of K_d [39] as direct measurement of complex formation in the absence of

prothrombin [7], supports the conclusion, obtained from Fig. 5, that prothrombin does not contribute to the binding affinity of the prothrombinase complex. The conversion process does not involve a so-called conformational cage effect [40].

Analogy with membrane-mediated conversion of prothrombin.

The formation of the prothrombinase complex from the activated coagulation factors Xa and Va, phospholipids and calcium, increases the catalytic efficiency of factor Xa with five to six orders of magnitude [28, 29] and this tremendous effect strongly suggests that the principle of reduction of dimensionality is operative in the conversion of prothrombin [41]. Some doubts were raised, however, by several studies [4, 5] presenting evidence for direct conversion of prothrombin from the buffer. It was shown that the conversion could be described as a bimolecular rate process in bulk solution.

Recently, these seemingly conflicting results were reconciled. It was shown [13] that reduction of dimensionality indeed occurs. This makes the conversion process so efficient that even a single prothrombinase complex will convert almost instantaneously all prothrombin molecules landing on a small phospholipid vesicle. This implies that in suspensions of small unilamellar vesicles, as usually studied, the conversion rate will be limited by the arrival of new prothrombin molecules on the vesicle surface and thus will appear to be a bimolecular bulk reaction between prothrombin molecules and prothrombinase-containing vesicles. Only for larger surface areas per prothrombinase complex, for instance on macroscopic planar bilayers [13], one may prevent such transport limitation.

A similar situation exists with respect to prothrombinase assembly [7]. The assembly will usually appear to be a collisionally-limited bimolecular rate process between vesicles, containing one of the components, and the other component in bulk solution. Only by bringing the surface area per adsorbed factor Va molecule in the range of square micrometers, as in the present study, one can obtain a situation in which the total transport of factor Xa molecules towards the membrane surface may exceed the rate of complex formation.

Quantitative aspects of membrane-mediated prothrombinase assembly.

The values of $K_d = 47 \text{ nM}$ and $\Gamma_{\max} = 10.3 \text{ pmol}\cdot\text{cm}^{-2}$ for the binding of factor Xa to the lipid membrane, allow estimation of the membrane-induced increase of the rate of prothrombinase generation. For a given bulk concentration of $C_{\text{bulk}} = 1 \text{ pM}$ factor Xa, it follows from equation 1 that $\Gamma_{\text{eq}} = 0.2 \times 10^{-3} \text{ pmol}\cdot\text{cm}^{-2}$. From the values for the maximal rate constants, given under "Materials and Methods", it then follows that per mole of adsorbed factor Va, the maximal rates will then be $2.0 \times 10^{-3} \text{ s}^{-1}$ for assembly from bulk solution and 1.6 s^{-1} for assembly by lateral diffusion on the

membrane. So the maximal rate of prothrombinase generation will be increased by three orders of magnitude. This implies that the lateral diffusion constant of factor Xa could be lowered by about two orders of magnitude, for instance due to lower fluidity of the bilayer, without losing the membrane-induced enhancement.

It should be realized that the value calculated for the bimolecular rate constant on the surface $k_{on} = 2.8 \times 10^{13} \text{ mol}^{-1} \cdot \text{cm}^2 \cdot \text{s}^{-1}$, is probably an underestimation. One min after addition of 1 pM ($= 10^{-15} \text{ mol} \cdot \text{cm}^{-3}$) of factor Xa to the buffer a quantity of $10^{-15} \times 60 \times 6.3 \times 10^{-4} = 0.038 \text{ fmol} \cdot \text{cm}^{-2}$ of factor Xa has adsorbed. If this adsorbed factor Xa would be in equilibrium with the $0.094 \text{ fmol} \cdot \text{cm}^{-2}$ of adsorbed factor Va, it follows from the value of $0.44 \text{ fmol} \cdot \text{cm}^{-2}$ calculated for the surface concentration of factor Xa at half-saturation (see "Results") that the prothrombinase concentration would be approximately $0.007 \text{ fmol} \cdot \text{cm}^{-2}$. But in one min, and for the observed rate of prothrombinase assembly of $0.2 \times 10^{-3} \text{ fmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$, a quantity of $0.012 \text{ fmol} \cdot \text{cm}^{-2}$ is formed and this is even more than the estimated equilibrium concentration. So the data are roughly consistent with almost instantaneous equilibrium of adsorbed factors Xa and Va, which would imply a much higher bimolecular association constant.

These conclusions are only valid, of course, for the conditions used in the present study. Reduction of the residence time of proteins on the surface, for instance by lowering of the percentage of acidic phospholipids, or "freezing" of the proteins on the surface by use of lipids with a high melting temperature, could change the situation. This may explain effects of phospholipid composition on the generation of prothrombinase activity [42, 43]. Such effects could also be produced more indirectly. For instance, a change in phospholipid composition could alter protein binding parameters and thereby, depending on the lipid concentration, the bulk concentration of proteins. Use of different phospholipid preparations could also cause differences in vesicle size and thereby, as illustrated in the present study, influence the kinetics of prothrombinase assembly.

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Chapter 4

Measurement of Factor Xa and Prothrombin Binding to Phospholipid Vesicles by Monitoring of Unbound Protein with Off-null Ellipsometry

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SUMMARY

Experiments on the binding of proteins to small unilamellar phospholipid vesicles (SUV), normally do not allow direct determination of the concentration of unbound protein, because the vesicles cannot be separated routinely from the bulk solution. In the present study, this limitation was overcome with a supported planar phospholipid bilayer positioned in a cuvette containing a vesicle-suspension. Ellipsometric measurement of the adsorption velocities on this bilayer after addition of protein allowed determination of the concentrations of unbound protein. At high protein concentrations the adsorption is rapidly completed and the usual null-ellipsometry is too slow to obtain well-defined initial adsorption rates. Therefore, a new off-null technique was developed, allowing measurements of the adsorbed protein mass at time intervals of 20 ms. From the concentration of unbound protein thus determined, binding of factor Xa and prothrombin was measured in SUV suspensions prepared from a 20% dioleoyl-phosphatidylserine (DOPS) and 80% dioleoyl-phosphatidylcholine (DOPC) phospholipid mixture. For factor Xa, a dissociation constant $K_d = 49.6 \pm 6.3$ nM (mean $\pm$ SE) and maximal surface concentration $\Gamma_{\max} = (23.0 \pm 1.4) \times 10^{-3}$ mole of protein per mole of lipid, were obtained. For prothrombin, these values were $K_d = 140 \pm 27$ nM and $\Gamma_{\max} = (8.9 \pm 0.8) \times 10^{-3}$ M.M⁻¹. These binding parameters are similar to those obtained earlier for planar bilayers. Apparently, the binding of factor Xa and prothrombin is not dependent on surface curvature.

INTRODUCTION

Lipid-protein interactions play a significant role in blood coagulation and most experiments in this area of research have been performed with suspensions of small unilamellar phospholipid vesicles (SUV). Such vesicles are prepared by sonication of phospholipid mixtures and have 15-30 nm diameter with a high surface curvature [1, 2]. After the introduction of (stopped-flow) light scattering in the study of binding of coagulation factors X and prothrombin to SUV by Nelsestuen and coworkers [3, 4, 5, 6], most studies on lipid-protein interactions in blood coagulation have been performed with this technique. However, a comparison with results from techniques based on planar membranes, such as ellipsometry or adsorption of radiolabeled proteins to Langmuir-Blodgett layers, revealed conflicting data concerning the measured values of the binding constants [7]. Also, several studies demonstrated effects of vesicle size on the binding parameters of proteins [8, 9, 10].

Recently, several reports have been published concerning activation of coagulation factors on macroscopic phospholipid-coated surfaces [11, 12, 13, 14, 15]. These studies have produced binding and activation parameters considerably differing from those obtained with SUV. Although part of these differences could be satisfactory explained by transport limitations, the question whether the binding of coagulation factors to SUV may proceed differently from the binding to planar macroscopic bilayers was raised again.

Protein binding as measured by light scattering in SUV suspensions is based on a considerable enhancement of the scattering when vesicle mass is increased by protein adsorption. This technique does not allow direct measurement of the concentration of unbound protein, because SUV cannot be separated routinely from the buffer solution. In the present study this limitation is overcome by use of ellipsometry. On macroscopic phospholipid-coated surfaces, factor Xa and prothrombin both show transport-limited initial adsorption, with an adsorption rate proportional to the free protein concentration and a mass transfer coefficient Δ , depending on the flow conditions and the protein diffusion constant [15, 16]. Measurement of the adsorption rate of protein on a planar phospholipid bilayer, deposited on a silicon slide, thus allows direct measurement of the buffer concentration of protein. In the presence of SUV the amount of protein bound to vesicles equals the added protein concentration minus the measured unbound buffer concentration.

Normally, ellipsometry is a null-method with two polarizer prisms, P and A, rotated to positions resulting in maximal extinction of a laser beam [16, 17]. These null-positions change during protein adsorption and are continuously adjusted in order to keep the light intensity minimal. The adjustment procedure requires 5-10 s for a single determination of adsorbed protein mass and, at high protein concentrations, this is too slow to measure initial adsorption rates. The silicon slide, however, has the special property that protein adsorption changes the null-position of P but hardly

affects the null-position of A. This property was used by fixing A in its null-position and keeping P also in a fixed position, 12 degrees off-null. In this setting, the resulting light intensity becomes a sensitive function of the adsorbed protein mass and, using a null-calibration before and after each experiment, the change in adsorbed protein mass can be determined from the change in light intensity, sampled at 20 ms intervals. In this way, initial adsorption rates could be accurately determined from measurements during only a few seconds.

Application of this method to SUV prepared from a 20% DOPS / 80% DOPC mixture demonstrated similar binding parameters as published earlier for the binding of factor Xa and prothrombin to planar mono- and doublelayers. It is concluded that the binding mechanisms of these coagulation factors, which are calcium-dependent and probably do not require penetration of protein into the lipid bilayer, are not dependent on surface curvature.

MATERIALS AND METHODS

Proteins

Bovine factor X and prothrombin were purified according to [18] and [20] respectively. Factor X was activated with Russell's viper venom (Sigma Chemical Company) [19]. Concentrations of factor Xa ($M_r = 44,000$) were determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate hydrochloride (ICN Nutritional Biochemicals) [21]. Concentrations of prothrombin ($M_r = 72,000$) were determined similarly after complete activation to thrombin with factor Xa, factor Va and phospholipid. All experiments were performed at room temperature (20-22 °C) in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 3 mM CaCl_2 and 0.5 g·litre⁻¹ bovine serum albumin (Sigma).

Phospholipid vesicles and planar bilayers

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and the corresponding phosphatidylserine (DOPS) were purchased from Sigma and Avanti Polar Lipids, respectively. Phospholipid concentrations were determined by phosphate analysis [22]. Suspensions of small unilamellar vesicles (SUV) were prepared by sonication of 20% DOPS / 80% DOPC and 40% DOPS / 60% DOPC mixtures as described [1]. Silicon slides were obtained and treated as described [14]. The slides were covered with a 40% DOPS / 60% DOPC bilayer by exposure to a vesicle suspension, as described [14]. This higher percentage of DOPS in the planar bilayer facilitates the determination of adsorption velocities, as explained below.

Off-null ellipsometry

Ellipsometry is based on the measurement of changes in light polarization due to reflection [23]. These changes are strongly influenced by the presence of thin (0.1-10 nm) phospholipid and protein films on the reflecting surface. As described earlier [14], the reflecting surface is a phospholipid-coated silicon slide placed in a quartz cuvette with buffer. Normally, the changes in light polarization are measured by null-ellipsometry: A laser beam passes a polarizing prism P, is reflected by the silicon slide, and then passes through a second polarizing prism A. The prisms P and A are rotated to positions such that the final light intensity, measured by a photodiode, is kept minimal. A computer, receiving the signal of the photodiode, controls the rotation of P and A with two stepping motors.

From the changes in null-positions of P and A, during adsorption of protein to the slide, the adsorbed protein mass can be determined by means of the Lorentz-Lorenz relation [16, 17]. However, one cycle of adjustments of P and A takes 5-10 seconds. For the binding experiments in the present study, protein concentrations up to 300 nM were used. For a value of the mass transfer coefficient $\Delta = 2 \times 10^{-3} \text{ cm}\cdot\text{s}^{-1}$ (see below) and a protein concentration of 300 nM, the adsorption of $1 \text{ pmol}\cdot\text{cm}^{-2}$ of protein (25% of Γ_{max} for prothrombin) will take 1.7 seconds. Apparently, much faster sampling is required.

For a phospholipid-coated silicon slide, protein adsorption affects mainly the null-position of P, while the null-position of A is hardly changed. This property was used in the following measuring procedure (see Fig.1):

- (1) The null-positions P_s and A_s at the start of the experiment were determined for the silicon slide covered with the lipid bilayer, before any protein was adsorbed.
- (2) The position of A was kept fixed at $A_0=A_s$ and the position of P was fixed at a position P_0 , usually at 12 degrees below P_s . The light intensity I_s was then measured.
- (3) Protein adsorption was started by addition of protein to the cuvette and the light intensity $I(t)$, as a function of time t , was recorded during ongoing adsorption.
- (4) When a stable final intensity level I_e was attained, the final null-positions P_e and A_e were again measured by null-ellipsometry.

FIG.1 Principles of off-null ellipsometry (see text).

The light intensity $I(t)$, as a function of polarizer and analyzer positions P_0 and A_0 and the time-dependent null-positions $P_n(t)$ and $A_n(t)$, is given by [23]:

$$I(t) = K \{ \sin^2[A_0 - A_n(t)] + \sin[2A_0] \cdot \sin[2A_n(t)] \cdot \sin^2[P_0 - P_n(t)] \}$$

where K is an instrument-dependent constant.

Because $A_n(t)$ hardly changes from $A_0 = A_s$, the first term can be neglected and, using the equality $2 \cdot \sin^2 x = 1 - \cos[2x]$, the expression can be approximated by:

$$I(t) = \frac{1}{2} K \cdot \sin^2[2A_s] \cdot \{ 1 - \cos[2(P_0 - P_n(t))] \}$$

The value of $\cos[2(P_0 - P_n(t))]$ is thus a linear function of $I(t)$ and can be obtained by linear interpolation:

$$\cos[2(P_0 - P_n(t))] = \frac{I(t) - I_s}{I_e - I_s} \cos[2(P_0 - P_e)] + \frac{I_e - I(t)}{I_e - I_s} \cos[2(P_0 - P_s)] \quad (1)$$

Equation 1 allows the calculation of the null-position $P_n(t)$ from the light intensity $I(t)$, and the adsorbed protein mass Γ was then calculated from the change in polarizer position, as $\Gamma(t) = 0.085 \times [P_n(t) - P_s] \mu\text{g} \cdot \text{cm}^{-2}$. This latter relation is an approximation

of the full Lorentz-Lorenz relation (16, 17), valid for the optical constants of the silicon surface and for small changes in $A_n(t)$.

Analysis of adsorption kinetics

The protein content of the boundary layer at the surface is very small compared to the adsorption capacity of the surface and the transport capacity of diffusion. As a result, within a fraction of a second after addition of protein to the cuvette, a quasi-steady state will be formed at the surface with $d\Gamma/dt = (C_{bulk}-C_0)$, with C_{bulk} the buffer concentration of protein, C_0 the concentration of protein at the surface and Δ an experimental constant depending on the flow conditions and the diffusion constant of the protein (16). For initial adsorption rates, that is, for low surface coverage, local equilibrium can be described by the classical Langmuir expression [24] with a dissociation constant $K_d = C_0(\Gamma_{max}-\Gamma)/\Gamma$. Eliminating C_0 from these expressions one obtains:

$$\frac{d\Gamma(t)}{dt} = \Delta [C_{bulk} - \Gamma(t) \cdot K_d / (\Gamma_{max} - \Gamma(t))] \tag{2}$$

First, Equation 2 was used to determine the values of Δ and K_d from a separate series of experiments, without SUV and with known values of C_{bulk} , using a standard non-linear least squares fit (BMDP Statistical Software, Inc. Los Angeles, Routine 3R, version 1988). The value of Γ_{max} was not obtained from this fit but from separate measurements with high protein concentrations. Using these values of Δ , K_d and Γ_{max} , Equation 2 was again applied in the binding experiments with SUV, in order to estimate the concentration C_{bulk} of unbound protein in the buffer. It should be realized that, in these experiments, C_{bulk} is the only important parameter, because the parameters K_d and Γ_{max} in Equation (2) describe the binding to the 40% DOPS / 60% DOPC bilayer on the silicon slide and are not relevant for the binding to SUV.

From the values of C_{bulk} obtained in this way, and the total protein concentrations added, the equilibrium concentrations Γ_{eq} of bound protein were calculated. The parameters K_d and Γ_{max} for the binding of proteins to SUV were then obtained from a non-linear fit, using the expression:

$$\Gamma_{eq} = \Gamma_{max} \cdot C_{bulk} / (C_{bulk} + K_d) \tag{3}$$

RESULTS

Experiments without vesicles

Some experimental complications are associated with off-null ellipsometry. Addition of protein or a vesicle suspension to the cuvette causes irregularities due to mixing, increased light scattering, temperature effects etc., and these mixing effects are recorded, due to the high sampling frequency. In order to minimize these irregularities, the dilutions were chosen such that 1 ml of protein solution was added to a cuvette filled with 4 ml of buffer, up to final concentrations of 25-150 nM of factor Xa and 25-600 nM of prothrombin. This relatively high sample volume promotes rapid mixing.

FIG.2 Effect of SUV on protein adsorption, measured with off-null ellipsometry. Upper curve: adsorption of 25 nM prothrombin in the absence of SUV. Lower curve: adsorption in the presence of 50 μ M SUV.

Even so, the first 0.3 s were discarded in the fits with Equation 2. An example of the adsorbed mass as a function of time is shown in the upper curve of Fig.2. Values of $\Gamma_{\max}$ for the 40% DOPS / 60% DOPC bilayer were determined from saturating protein concentrations. Values of respectively $\Gamma_{\max} = 10.5 \text{ pmol}\cdot\text{cm}^{-2}$ and $\Gamma_{\max} = 3.7 \text{ pmol}\cdot\text{cm}^{-2}$ were obtained for factor Xa and prothrombin respectively. Inserting these values into Equation 2 the initial adsorption rates $(d\Gamma/dt)_{t=0} = \Delta\cdot C_{\text{bulk}}$ could be determined, as a function of C_{bulk} , from fits restricted to low surface coverages ($< 25\%$ of $\Gamma_{\max}$) for which the adsorption can be described with the Langmuir formalism and Equation 2 remains valid.

FIG.3 Determination of initial adsorption rates as a function of buffer concentrations of protein. Upper curve: factor Xa. Lower curve: prothrombin.

Fig.3 shows the results from which values of Δ were determined. For the experimental conditions used, a stirrer of 1 cm diameter rotating at 1.2 mm distance from the adsorbing slide with 2300 rpm, values of $\Delta = (22.2 \pm 1.7) \times 10^{-4} \text{ cm}\cdot\text{s}^{-1}$ and $\Delta = (19.6 \pm 1.9) \times 10^{-4} \text{ cm}\cdot\text{s}^{-1}$ (mean $\pm$ SE) were obtained for Factor Xa and prothrombin respectively. The dissociation constants for binding to the 40% DOPS / 60% DOPC bilayer, obtained as a byproduct from Equation 2, were $K_d = 13.3 \pm 1.8 \text{ nM}$ for factor Xa and $K_d = 31.6 \pm 3.9 \text{ nM}$ for prothrombin. These values are lower than for 20% DOPS / 80% DOPC (see below) and this implies that Equation 2 departs less rapidly from linearity and allows better determination of initial adsorption rates. For this reason, a DOPS content of 40% was chosen for the bilayer on the silicon slide.

Experiments with SUV and factor Xa

Fig.2 also shows the large effects of addition of SUV on the adsorption rates. These experiments were performed with total protein concentrations in the range of 25-300 nM, combined with 3, 6 or 9 μM of lipid, such that the concentrations of bound and unbound protein were of the same order of magnitude, as required for accurate estimation of the bound protein concentration from the total concentration and C_{bulk} . In order to minimize mixing effects, the protein/vesicle mixture was incubated for 10 min before it was added to the cuvette, choosing again 1 ml of sample on 4 ml of buffer. For protein concentrations exceeding 300 nM, aggregation of vesicles became apparent from turbidity in the cuvette. From the total buffer

concentrations of lipid and protein, and the concentrations C_{bulk} of unbound protein, the concentrations of bound protein, expressed as moles of protein per mole of lipid ($\text{M} \cdot \text{M}^{-1}$) were calculated and the results are shown in Fig.4 where each square represents one experiment. Fitting these results to Equation 3, values of $K_d = 49.6 \pm 6.3 \text{ nM}$ and $\Gamma_{\text{max}} = (23 \pm 1.4) \times 10^{-3} \text{ M} \cdot \text{M}^{-1}$ were obtained.

FIG.4 Binding isotherm of factor Xa on SUV, prepared from 20% DOPS / 80% DOPC.

FIG.5 Binding isotherm of prothrombin on SUV, prepared from 20% DOPS / 80% DOPC.

Experiments with SUV and factor II

The total range of prothrombin concentrations was 25-500 nM combined with a phospholipid concentration of 50 μ M. Results are presented in Fig.5 and fitting these data to Equation 3 resulted in values of $K_d = 140 \pm 27$ nM and $\Gamma_{\max} = (8.9 \pm 0.8) \times 10^{-3}$ M·M⁻¹.

DISCUSSION

Comparison with binding parameters from the literature

The binding parameters found for SUV in the present study agree well with earlier published values for 20% DOPS / 80% DOPC planar membranes, measured by ellipsometry. For factor Xa, values of $K_d = 47$ nM and $\Gamma_{\max} = 20 \times 10^{-3}$ M·M⁻¹ were obtained from the equilibrium binding isotherm [15], and similar measurements resulted in values of $K_d = 160$ nM and $\Gamma_{\max} = 6 \times 10^{-3}$ M·M⁻¹ for prothrombin [38]. However, these values differ to some extent from the results of light scattering experiments. Using again only data obtained with SUV prepared from PS/PC mixtures with 20-25% PS, reported values were $K_d = 114$ -200 nM, $\Gamma_{\max} = (16$ -22) $\times 10^{-3}$ M·M⁻¹ for factor X or factor Xa [3, 25, 26], and $K_d = 300$ -500 nM, $\Gamma_{\max} = (11$ -15) $\times 10^{-3}$ M·M⁻¹ for prothrombin [3, 5, 25]. For factor Xa, this implies a similar maximal surface concentration, but a 2-4 times higher dissociation constant than found in the present study. For prothrombin, both the maximal surface concentration and the dissociation constant are a factor of about 2 higher than in the present study. Comparison of these values indicates systematic differences between results obtained with light scattering and with ellipsometry. However, as demonstrated in the present study, these discrepancies cannot be explained by differences between the binding properties of SUV and planar bilayers.

Lipid content and packing density of the bilayer leaflets in SUV

It follows from simple geometrical considerations that for small vesicles like SUV, the outer bilayer leaflet must contain considerable more lipid than the inner leaflet. Several studies have estimated this difference at a factor of 1.8-2.3 for SUV prepared from pure DOPC or egg PC [1, 27] and 2.3-3.2 for egg PC / brain PS [28, 27] and egg PC / phosphatic acid (PA) mixtures [29]. The high surface curvature of SUV may also introduce differences in the mechanical properties, for instance due to bending stiffness [30], or packing density of the bilayer. Indeed, lower phase transition temperatures were found in SUV, compared to large unilamellar vesicles (LUV)

prepared from the same lipids [39], and differences in packing density between SUV and planar bilayers have been suggested [2, 31]. Such effects were mainly attributed to restriction of the available surface area per headgroup in the inner leaflet [32, 33]. Higher binding affinities for protein binding to SUV, compared to LUV, has been reported for cytochrome b5 [8], the complement complex C5b-7 [9] and the light chain of factor Va [10]. Binding of these proteins may be associated with insertion of part of the protein into the hydrocarbon core of the membrane and a more loose packing of the lipids in the outer monolayer of SUV could facilitate such hydrophobic interactions. The present study indicates that for peripheral, calcium-dependent, binding of proteins like factor Xa and prothrombin, the differences between SUV and planar bilayers do not result in significantly altered binding affinities.

Asymmetry of PS/PC ratio in the bilayer leaflets of SUV

Conflicting data have been reported on the asymmetry of phospholipid distribution in the inner and outer bilayer leaflet of SUV. This phenomenon has been characterized with the asymmetry coefficient $k_{as} = (PS/PC)_{in} / (PS/PC)_{out}$. A value of $k_{as} = 2.1$ [28] for a 20% PS / 80% PC mixture may have been caused by preparation of SUV at pH = 5, because an earlier study [27] showed that k_{as} changed from 1.1 to 1.9 when the pH was shifted from 7 to 5. These results were obtained with NMR spectroscopy and differ from those obtained from dye binding or TNBS (2,4,6-trinitrobenzenesulfonic acid) adsorption [34], which showed preferential accumulation of PS in the outer monolayer. Such contradictory results were also reported for the distribution of PA and PC. Homogeneous distribution [29], and accumulation of PA in the outer layer [34] were both reported. The same is true for mixtures of phosphoglycerol (PG) and PC [36, 37]. The asymmetry coefficient also tends to give a false impression of the size of the effect. For instance, a value of $k_{as} = 2$ for SUV prepared from 20% DOPS / 80% DOPC, would imply a 15% DOPS / 85% DOPC in the outer monolayer, containing twice as much lipid as the inner monolayer. The value of $K_d = 140 \pm 27$ nM for binding of prothrombin on 20% DOPS / 80% DOPC SUV, found in the present study, is not significantly different from the value of $K_d = 160$ nM reported earlier for planar monolayers of the same composition [38]. In this earlier study, the DOPS / DOPC ratio was checked with radiolabeled DOPS and it was found that the value of K_d rapidly decreased for increasing percentage of DOPS. The value of $K_d = 31.6$ nM, found in the present study for the 40% DOPS / 60% DOPC bilayer, indicates the same phenomenon. This indicates that the DOPS / DOPC ratio in the outer monolayer of the SUV used in the present study, does not depart too much from the overall 20%/80% ratio, although this is a rather crude indication and the data could still be compatible with a considerable deviation of unity of k_{as} . For instance, even if all DOPS would be accumulated in the outer bilayer, the outside ratio would become

about 30% DOPS / 70% DOPC and this would still be compatible with the obtained value of K_d for prothrombin.

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Chapter 5

Adsorption and Conversion of Prothrombin on a Rotating Disc

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SUMMARY

In most flow systems, the rate of protein transfer from bulk solution to a macroscopic surface is site-dependent. In studies on surface-mediated protein conversion, this hampers the comparison of a proposed expression for the conversion process, for instance the Michaelis-Menten equation, with actually measured overall conversion rates. However, the rotating disc is a classical example of a uniformly accessible surface and was therefore used for a quantitative analysis of prothrombin conversion by the phospholipid-bound factor Xa / factor Va complex (prothrombinase complex).

A simple design of a rotating disc, adapted for ellipsometric measurement of protein adsorption, is presented. Agreement between experiment and theory was obtained for the influence of rotation velocity on the initial, transport-limited, adsorption rates of lysozyme, prothrombin and fibrinogen. Such measurements allow determination of diffusion constants, and values of $D_{20,w} = (6.8 \pm 0.2) \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ (mean $\pm$ SE) and $D_{20,w} = (5.3 \pm 0.1) \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ were obtained for factor Xa and factor Va respectively.

After coverage of the disc with a 20% phosphatidylserine / 80% phosphatidylcholine bilayer and preadsorption of factor Va, addition of excess factor Xa and prothrombin resulted in effective conversion of prothrombin. For low ($0.1\text{--}0.5 \text{ fmol}\cdot\text{cm}^{-2}$) surface concentrations of factor Va, the conversion rate dropped below the transport limit and the intrinsic kinetic parameters could be estimated at $K_m = 8.6 \pm 2.0 \text{ nM}$ and $k_{\text{cat}} = 25 \pm 1.8 \text{ s}^{-1}$ (20°C). The results also indicate that neither the assembly, nor the catalytic efficiency, of the prothrombinase complex is influenced by fluid shear rate.

INTRODUCTION

The influence of flow conditions on the adsorption kinetics of proteins from solution has been studied on various macroscopic surfaces. For a low binding rate of the protein to the surface, the transport capacity of convection and diffusion will generally be sufficient to maintain the concentration of protein in the boundary layer close to the bulk concentration. In that case one measures true "intrinsic" binding rates, not dependent on stirring rates, shear etc. Usually, however, the adsorption rates are higher and the boundary layer will show some degree of protein depletion. This is the intermediate range, with adsorption rates depending on both the intrinsic binding constant and transport conditions [1, 2, 3]. In the limit of very high intrinsic adsorption rates, the solution directly adjacent to the surface becomes almost completely protein-depleted, and one has transport-limited adsorption, with adsorption rates only determined by convection and diffusion [4, 5, 6].

The adsorbing surfaces in the quoted studies were not uniformly accessible, that is, diffusion and convection produced site-dependent transport rates of protein towards the surface. In a laminar flow cell [1, 5, 3], for instance, the width of the protein-depleted boundary layer increases progressively in the downstream direction and the transport rate of protein from bulk to surface will drop accordingly. For an adsorbing surface in front of a stirrer [4, 2, 6], the adsorption rate will be maximal at the site directly opposite the stirrer and will also drop in the downstream direction.

These aspects of protein adsorption kinetics are also relevant for protein conversion at macroscopic surfaces. The conversion of prothrombin by the lipid-bound prothrombinase complex, has been studied on lipid-coated planar slides [7, 8] and in similarly coated perfused capillaries [9]. The latter system was introduced to study the activation of factor X by the lipid-bound tissue factor / factor VIIa complex [10, 11]. Due to the non-uniform accessibility of the surfaces used in these studies, the overall conversion rates, estimated from accumulation of conversion products in the fluid phase, could only be related to the intrinsic values of the Michaelis-Menten constants K_m and k_{cat} for low surface activities [11, 8].

A classical example of a uniformly accessible surface is the rotating disc, one of the few three-dimensional flow problems for which the steady-state Navier-Stokes equations can be solved analytically [12, 13] and the flow field has the property that the flow component towards the surface only depends on the distance to the surface and not on the coordinates in the plane of the surface. Using this solution Levich [14] obtained an explicit solution for the convection-diffusion equation describing the mass transport towards the surface. It appears that this transport depends only on the diffusion coefficient and the flow component towards the surface. As a result, the mass transfer towards the surface is site-independent, despite of radially increasing shear rates. The rotating disc electrode has been extensively used in electrochemistry [30]

and was also used to study platelet adhesion [15], reaction kinetics of immobilized enzymes [16, 17] and protein adsorption [18].

In the present study, a simple rotating disc was constructed which could serve as the reflecting surface in ellipsometric measurements of protein adsorption. It is shown that protein adsorption rates are site-independent and that measurements of initial adsorption rates as function of the rotation velocity permit determination of protein diffusion constants. After coverage of the slide with a phospholipid bilayer and preadsorption of coagulation factors Xa and Va, a prothrombin-converting surface was obtained. This surface produced thrombin at a rate that could simply be calculated from the initial adsorption velocity of prothrombin, measured at an arbitrary site on the slide, and total surface area. Neither the assembly of the prothrombinase complex, nor the conversion of prothrombin, appeared to be influenced by fluid shear rate at the surface. Using a low surface coverage with prothrombinase, the conversion rate was no longer transport-limited and the intrinsic rate constants of conversion could be obtained. The conversion conformed to the classical Michaelis-Menten scheme and the difference between the apparent and intrinsic values of the kinetic constants could be explained quantitatively.

MATERIALS AND METHODS

Proteins

Human lyophilized fibrinogen and lysozyme were purchased respectively from Kabi Diagnostica (grade L; clottability > 90%) and Merck (chicken egg). Fresh solutions were prepared before each experiment and protein concentrations were determined by spectrophotometry (Cobas Bio System, Hoffmann-La Roche) at 280 nm, using $E_{280}^{1\%, 1\text{cm}}$ values of 13.6 for fibrinogen and 25.7 for lysozyme [19]. Bovine factors V, X and prothrombin were purified according to references [20], [21] and [22] respectively. Factor V was activated with thrombin [20] and factor X with Russel's viper venom [23]. Concentrations of factor Xa were determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate hydrochloride [24], purchased from ICN Nutritional Biochemicals. Concentrations of prothrombin were determined similarly after complete activation to thrombin with Echis carinata venom (Sigma Chemical Cy). Concentrations of factor Va were determined by spectrophotometry at 280 nm with $E_{280}^{1\%, 1\text{cm}} = 9.6$ [25]. Thrombin was measured by its amidolytic activity, using the chromogenic substrate S-2238, as described [8].

Ellipsometric measurement of protein adsorption

Ellipsometry is an optical technique for the measurement of changes in the polarization state of light. These changes are caused by reflection and are strongly influenced by the presence of thin (0.1-10 nm) films deposited on the reflecting surface. The instrument and analysis of data have been described [26, 2]. Calculation of the adsorbed mass of protein, using the Lorentz-Lorenz relation, requires the measurement of the optical constants of the reflecting surface and the values of the specific refractivity r and the partial specific volume v of the adsorbing protein [26, 2]. For the optical constants of the (lipid-covered) silicon slide, and the values of $r=0.24-0.25$ and $v=0.71-0.72 \text{ ml}\cdot\text{g}^{-1}$ for the proteins used in the present study, this calculation could be simplified to $\Gamma(t) = 0.085 \cdot \{P(t) - P_s\}$, where $\Gamma(t)$ is the adsorbed protein mass at time t , expressed in $\mu\text{g}\cdot\text{cm}^{-2}$, $P(t)$ is the position of the ellipsometer polarizer at time t , expressed in degrees, and P_s is the starting position of P . The reflecting surface is placed in a quartz cuvette (see Fig.1), filled with 5 ml of buffer solution, with a trapezoidal form in order to allow perpendicular transfer of incident and reflected light through the side walls, at an angle of incidence of 68 degrees. The incident light beam has a diameter of 2 mm, resulting in a reflecting spot of about 3 mm width and 2 mm height on the silicon disc. Adsorption experiments were performed at room temperature (20-22 °C) and were started by addition of protein to the buffer. Ellipsometer readings, allowing the calculation of adsorbed protein mass, were obtained at approximately 10 s intervals. For fibrinogen and lysozyme, the adsorptions were performed directly on the silicon surface. For prothrombin, factor Xa and factor Va, the surface was precoated with a phospholipid bilayer by exposure to a suspension of sonicated vesicles, prepared from a phospholipid mixture of 40% dioleoyl-phosphatidylserine (DOPS) and 60% dioleoyl- phosphatidylcholine (DOPC), as described [8]. Experiments were performed in 5 mM Tris-HCl buffer, pH = 7.5, containing 100 mM NaCl, 3 mM CaCl_2 . A 40% DOPS / 60% DOPC, instead of a 20% DOPS / 80% DOPC phospholipid mixture was chosen to enhance the total quantities adsorbed. In order to maintain comparability with the experiments on prothrombin conversion, the adsorptions of prothrombin, factor Xa and factor Va were performed in buffer containing $0.5 \text{ g}\cdot\text{litre}^{-1}$ bovine serum albumin (Sigma Chemical Co.).

Measurement of prothrombin conversion

The silicon slide was coated with a 20% DOPS / 80% DOPC lipid bilayer as described [8]. In order to minimize protein depletion by aspecific adsorption the cuvette was siliconized (Sigmacote, Sigma), and $0.5 \text{ g}\cdot\text{litre}^{-1}$ bovine serum albumin was added to the buffer. Factor Va (1-25 pM) was added to the cuvette and allowed to adsorb for various time intervals, before flushing of the cuvette with buffer. These low factor Va concentrations were determined by titration with factor Xa in a kinetic assay

[20]. During the experiments, the desorption of factor Va from the lipid bilayer is negligible [27, 8]. Then excess factor Xa was added (100 pM) and the assembly of prothrombinase was completed during 10 min of incubation. Finally, various concentrations of prothrombin were added and the rate of thrombin production was determined from measurements of thrombin concentration in serial 100 μ l samples taken from the cuvette [8].

FIG.1 The rotating disc and ellipsometer cuvette.

Rotating disc

Figure 1 presents a sketch of the rotating disc used in the present study. A cylinder made of polyethylene terephthalate (PETP or Arnite® from AKZO Plastics), can be rotated at the required speed by means of a motor with servo-controlled rotation speed (Maxon). The cylinder is placed outside the ellipsometer cuvette and contains two tiny magnets (Samarium-Cobalt, type BM 95100, Bakker Madava), rotating close to the backside cuvette wall. A silicon disc (N-100, Aurel GMBH) of 1.6 cm diameter was attached to another PETP cylinder, containing two magnets oriented perpendicular to the disc. The second cylinder fits into a thin, sharply edged, PETP ring mounted in a glass holder. In order to minimize friction, the sides of this cylinder were rounded. The movement of the silicon disc is guided by the sharp edge, protruding from the glass

holder. Rotation rates were measured with a Phywe (type DB 0034 Depex) stroboscope, and stable rotations could be obtained between 60 and 2000 rpm.

Analysis of data

The steady-state Navier-Stokes equation for the flow configuration of a disc, rotating at a constant angular velocity of $\omega \text{ rad}\cdot\text{s}^{-1}$ in a fluid with a kinematic viscosity (ratio of viscosity to density) of $\nu \text{ cm}^2\cdot\text{s}^{-1}$, can be solved analytically [12, 13]. This solution has the special feature that the steady-state velocity component perpendicular to the disc only depends on the distance y to the disc and not on the lateral (x, z) coordinates. For steady-state flow, quasi steady-state protein concentration profiles $C(y)$ will be established within a fraction of a second and the diffusion-convection equation is reduced to:

$$0 = -v(y) \frac{d}{dy} C(y) + D \frac{d^2}{dy^2} C(y) \quad (1a)$$

with the boundary conditions:

$$C(y \rightarrow \infty) = C_{\text{bulk}} \quad \text{and} \quad D \left[\frac{d}{dy} C(y) \right]_{y=0} = J[C(0), \Gamma] \quad (1b)$$

where $v(y)$ is the fluid velocity component in the y direction, D is the diffusion constant of the protein, C_{bulk} is the bulk concentration of protein and Γ the adsorbed protein mass per unit surface area. The second boundary condition expresses the fact that, because the disc is impermeable to the fluid, $v(y)$ will be zero at the surface and the rate of adsorption or conversion J must equal the diffusional flux $D \cdot dC/dy$ at the surface. Moreover, J is a function of the buffer protein concentration $C(0)$ at the surface and the surface coverage Γ .

Transport-limited adsorption and conversion- The maximal rate of adsorption occurs for high intrinsic adsorption rates, such that the solution at the surface is effectively depleted of protein ($C(0) \approx 0$). In that case [14], $v(y)$ is approximated by $v(y) = -ay^2$, with $a = 0.51 \cdot \omega^{3/2} \cdot \nu^{-1/2}$, and the protein concentration in the buffer by $C(y) = F(y) \cdot C_{\text{bulk}}$ with

$$F(y) = 0.62 D^{2/3} \cdot \nu^{-1/6} \cdot \omega^{1/2} \cdot \int_0^y \exp[-a \cdot (3D)^{-1} \cdot s^3] ds \quad (2)$$

The transport-limited rate of adsorption or conversion then equals:

$$J = D \left[\frac{d}{dy} C(y) \right]_{y=0} = 0.62 D^{2/3} \nu^{-1/6} \omega^{1/2} C_{\text{bulk}} = \Delta \cdot C_{\text{bulk}} \quad (3)$$

The effect of inclusion of higher order terms of the Taylor expansion in this approximation was estimated in ref. [28]. In the case of protein adsorptions, with diffusion constants D less than $10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$ and a kinematic viscosity of water at 20°C of $\nu = 0.010 \text{ cm}^2 \cdot \text{s}^{-1}$, these higher order terms contribute less than 1.5% to this expression.

Adsorption in the intermediate range- The general solution of equation (1) for a finite protein concentration $C(0)$ at the surface is given by:

$$C(y) = [C_{\text{bulk}} - C(0)] \cdot F(y) + C(0) \quad (4)$$

and the boundary condition (3) reduces to:

$$\begin{aligned} J &= D \left[\frac{d}{dy} C(y) \right]_{y=0} = 0.62 D^{2/3} \nu^{-1/6} \omega^{1/2} [C_{\text{bulk}} - C(0)] \\ &= \Delta [C_{\text{bulk}} - C(0)] \end{aligned} \quad (5)$$

The latter equation can be used to calculate $C(0)$ from the adsorption rate $J = d\Gamma/dt$.

RESULTS

The rotating disc used in the present study can simply be covered with another silicon disc, attached to a PETP cylinder (see Fig.1), which stick together by magnetic force. The thin fluid layer between the silicon surfaces prevents exposure of the surface to air, when the ensemble is removed from the cuvette. This possibility was used in the experiments on prothrombin conversion. The first step in these experiments, coating of the silicon surface with the lipid bilayer, was performed outside the ellipsometer in a separate vessel. Then the surface was covered with a second disc and transported to the ellipsometer cuvette. This precaution prevented lipid adsorption, and subsequent prothrombin conversion, on the cuvette walls.

FIG.2 Adsorption of prothrombin on the rotating disc (open circles) and on a fixed slide opposite a rotating stirrer (closed circles).

An example of adsorption of $1 \mu\text{g}\cdot\text{ml}^{-1}$ prothrombin, with a rotation velocity of $30 \text{ rad}\cdot\text{s}^{-1}$ on the rotating disc is presented in Fig.2, and compared with the result obtained for $2 \mu\text{g}\cdot\text{ml}^{-1}$ in the usual configuration of a fixed adsorbing slide in front of a rotating stirrer [2]. Apparently, the rotation of the adsorbing silicon surface does not increase experimental scatter. Both configurations cause effective mixing of the protein sample (5-25 ml) with buffer, and the first data point obtained after addition of protein to the cuvette did not deviate systematically from the adsorption curve. For the lower rotation velocities a sample volume of $200 \mu\text{l}$ was used.

Quantitative agreement between theory and experiment is shown for fibrinogen, lysozyme and prothrombin in the upper panel of Fig.3. Initial adsorption velocities of these proteins are transport-limited, and according to Equation (3), should satisfy the relation $d\Gamma/dt = \Delta \cdot C_{\text{bulk}}$, with $\Delta = 0.62 \cdot D^{2/3} \cdot \nu^{-1/6} \cdot \omega^{1/2}$, the fractional adsorption rate, that is, the adsorption rate expressed in $\mu\text{g}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$, for a buffer concentration $C_{\text{bulk}} = 1 \mu\text{g}\cdot\text{cm}^{-3}$. The initial adsorption velocities were determined as the time derivative in $t=0$ of an exponential fit on adsorbed protein mass up to 30% of its final equilibrium value. As shown in Fig.3, experiments were performed for 5 different rotation rates. A linear dependency of Δ on $\sqrt{\omega}$, predicted by theory, is indeed observed. The diffusion constants obtained from these data were $D = (10.9 \pm 0.5) \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ for lysozyme, $D = (6.2 \pm 0.2) \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ for prothrombin and $D = (2.1 \pm 0.1) \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ for fibrinogen, which is in agreement with the published values of $11.2 \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ [19], $6.2 \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ [29] and $2.0 \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$ [19], respectively. The lower panel of

Fig.3 presents similar measurements for factor Xa and factor Va, from which diffusion constants of respectively $D = (6.8 \pm 0.2) \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and $D = (5.3 \pm 0.1) \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ were obtained.

FIG.3 Upper part: The fractional adsorption rate constant Δ , as a function of rotation velocity, for lysozyme (upper curve), prothrombin (middle curve) and fibrinogen. Lower part: Similar plot for factor Xa (upper curve) and factor Va (lower curve). Mean values of 5 experiments, with SD, are indicated.

FIG.4 Adsorbed mass of lysozyme, as a function of time, measured at the center (○) and the edge (●) of the rotating disc. Mean results and standard deviations of 5 experiments are shown. A rotation rate of $78 \text{ rad}\cdot\text{s}^{-1}$, and $C_{\text{bulk}} = 1 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ were used.

FIG.5 Transport-limited (○) and inter-mediate-range (Δ) conversion of prothrombin. Mean values of 5 experiments and SD are indicated.

Uniform adsorption rates, independent of surface site and fluid shear rates, are shown for lysozyme in Fig.4. Values of Δ were $(15.9 \pm 0.6) \times 10^{-4} \text{ cm}\cdot\text{s}^{-1}$ and $(15.6 \pm 0.6) \times 10^{-4} \text{ cm}\cdot\text{s}^{-1}$ (mean $\pm$ SE), at the center and the edge of the slide respectively.

Transport-limited and intermediate-range conversion of prothrombin is shown in Fig.5. Transport-limited conversion was obtained by incubating the 20% DOPS / 80% DOPC bilayer for 10 min in 25 pM factor Va and 50 pM factor Xa. Then the cuvette was flushed and 20 nM prothrombin was added. Conversion rates were calculated from the concentration of thrombin in 100 μ l samples taken from the cuvette at 0, 1.5, 3 and 4.5 min after addition of prothrombin. Three, increasing, rotation rates were used in each experiment and corrections for the sample volumes taken from the cuvette and break-down of prothrombin were made. The value of $D = (6.2 \pm 0.3) \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$, calculated from these data, equals the value obtained from the adsorption experiments. For intermediate-range prothrombin conversion only 2 pM factor Va and 5 pM factor Xa was incubated for 5 min. From the rotation velocity of $78 \text{ rad} \cdot \text{s}^{-1}$, it can be calculated that in this case only $0.46 \text{ fmol} \cdot \text{cm}^{-2}$ of prothrombinase was present at the surface.

FIG.6 The rate of prothrombin conversion as a function of prothrombin concentration. Surface concentrations of prothrombinase were $0.14 \text{ fmol} \cdot \text{cm}^{-2}$ (o) and $0.42 \text{ fmole} \cdot \text{cm}^{-2}$ (●). Mean values of 5 experiments and SE are indicated. The dotted line indicates the calculated intrinsic curve, for prothrombin concentrations directly at the surface.

Fig.6 presents thrombin production on a surface with very low prothrombinase activities obtained by 3 min incubation at 1 pM factor Va (open circles) and 3 pM factor Va (closed circles), resulting in $0.14 \text{ fmol} \cdot \text{cm}^{-2}$ and $0.42 \text{ fmol} \cdot \text{cm}^{-2}$ adsorbed

factor Va. In both cases, an excess of 10 pM of factor Xa was used and the rotation velocity was 78 rad·s⁻¹. Fitting these curves to the Michaelis-Menten equation $v = k_{\text{cat}} \cdot E_0 \cdot C_{\text{bulk}} / (K_m + C_{\text{bulk}})$, and using the just quoted values of E_0 , the apparent values $K_m = 9.3 \pm 2.1$ nM, $k_{\text{cat}} = 25 \pm 1.9$ s⁻¹ and $K_m = 21 \pm 4.8$ nM, $k_{\text{cat}} = 30 \pm 2.4$ s⁻¹ were obtained (mean $\pm$ SE). In the intermediate range, the concentration $C(0)$ of prothrombin directly at the surface can be calculated from the conversion rate and the buffer concentration, using Equation (5). From the curve with the lowest prothrombinase activity, intrinsic values of $K_m = 8.6 \pm 2.0$ nM and $k_{\text{cat}} = 25 \pm 1.8$ s⁻¹ were thus found (dotted line).

DISCUSSION

Agreement between experiments and theory

The data in figs. 2 to 4 demonstrate that protein adsorption conforms to the theoretical predictions. The experimental range of rotation rates allows a variation of the transport-limited adsorption constant Δ by a factor of 6, as shown in Fig.3. For very high rotation velocities the laminar flow regime would collapse and the theoretical basis would be invalidated by turbulence. This is estimated to occur at Reynolds numbers (Re) of $6 \times 10^4 - 30 \times 10^4$ [14, 30]. With $Re = \omega \cdot r^2 \cdot \nu^{-1}$ and values of $r = 0.8$ cm for the disc radius and $\nu = 0.01$ cm²·s⁻¹ for the kinematic viscosity this corresponds to rotation velocities of $(1-5) \times 10^3$ rad·s⁻¹. Therefore, the system still operates in the laminar flow range and rotation velocities could be further increased by a factor of at least four.

The adsorption rates are sensitive to temperature. Using the relation $D_T = (T/293)(\eta_{293}/\eta_T)D_{293}$ between the diffusion constants D and viscosities η at absolute temperatures T and 293 °K [31], and inserting the values for $T = 293$ °K (20 °C) and $T = 310$ °K (37 °C), it is found that transport-limited adsorption at 37 °C proceeds 1.4 times faster than at 20 °C.

Advantages of the use of uniform accessible surfaces

For most proteins, adsorption kinetics do not conform to the classical Langmuir model with independent binding sites and a variety of binding isotherms is observed, especially in protein mixtures [32, 33, 34, 35]. Even for single protein adsorption, surface exclusion effects and molecular interaction will modify the adsorption isotherm when the surface coverage exceeds 30-50% of its maximal value [36]. The testing of hypothetical mechanisms for these altered adsorption kinetics, that is, an assumed expression for $J[C(0), \Gamma]$ in equation 1b, is greatly facilitated by reliable estimates of the protein concentration close to the surface. A similar situation exists

for surface-mediated protein conversion. The classical Michaelis-Menten scheme already provides non-linear boundary conditions for the conversion of proteins by surface-bound enzymes, and the situation may be further complicated by product inhibition, the presence of other proteins on the surface, etc. Again, testing of any hypothesis about the conversion kinetics is hampered if the concentration of protein adjacent to the surface cannot be directly estimated, and if the conversion at different sites does not proceed at identical rates.

For non-uniformly accessible surfaces, as mentioned in the Introduction, a complicated relation arises between the changing adsorption rate along the surface and the depletion of protein in the boundary layer. The concentration adjacent to a given site on the adsorbing surface depends on the adsorption rates of more upstream sites where the adsorption will mostly proceed faster. Analysis of intermediate adsorption kinetics then generally requires the numerical solution of the diffusion-convection equation, with the assumed expression for $J[C(0), \Gamma]$ in the boundary conditions [5]. The same is true for protein conversion at non-uniformly accessible surfaces [38]. The non-linear expressions for the conversion rate again necessitate numerical solution.

Equivalence with the unstirred layer model

The rotating disc configuration is formally equivalent with a classical unstirred-layer model, which describes the boundary layer as a thin ($\approx 5 \mu\text{m}$) layer of stagnant fluid, separating the adsorbing surface from a buffer solution with protein concentration C_{bulk} . Mass transfer through the boundary layer can only be effected by diffusion. As the unstirred layer is assumed to have uniform thickness d , transport conditions are also uniform and the surface is uniformly accessible. Comparison of equation (5) in the Methods with the corresponding equation for the unstirred layer model: $d\Gamma/dt = D[C_{\text{bulk}} - C(0)]/d$, shows the equivalence $1/d = 0.62 D^{-1/3} \nu^{-1/6} \omega^{1/2}$.

Influence of transport limitation on the measurement of intrinsic adsorption rates

As demonstrated in the present study, the initial adsorption rates of proteins are often transport-limited. Using the relation $d\Gamma/dt = k_{\text{on}} \Gamma_{\text{max}} C(0)$ for the initial adsorption rate, it can be estimated from equation (5) that, for $\omega = 250 \text{ rad}\cdot\text{s}^{-1}$ and a typical protein with $\Gamma_{\text{max}} = 3 \text{ pmol}\cdot\text{cm}^{-2}$ and $D = 5 \times 10^{-7} \text{ cm}^2\cdot\text{s}^{-1}$, the transport conditions will lower the adsorption rate by less than 10% if the intrinsic adsorption constant k_{on} does not exceed $4 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$. For higher values of k_{on} , this influence will become larger but k_{on} may still be estimated from the apparent adsorption rate up to values of about $3 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$. For still higher values of k_{on} , the adsorption becomes completely transport-limited and k_{on} cannot further be estimated from the adsorption kinetics. The intermediate range thus extends between these boundary values of k_{on} .

Values of k_{on} for factor V, prothrombin and factor Xa, determined by stopped-flow light scattering on lipid vesicles containing about 20% phosphatidylserine, ranged from $2 \times 10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$ to $10^8 \text{ M}^{-1}\cdot\text{s}^{-1}$ [39, 40, 41]. This implies that the initial adsorption of these coagulation factors will be completely transport-limited, as was also observed for the other proteins in the present study. It must be anticipated that protein adsorption on macroscopic surfaces will often be transport-limited and examples to the contrary are indeed relatively scarce [1, 3].

Absence of shear effects on the conversion of prothrombin

The shear rate at the rotating disc surface increases linearly with the distance to the center of the disc. Measurements at the center of the disc at different rotation rates, allow the study of protein adsorption at low shear and variable adsorption rates. Also, possible effects of shear rate on protein adsorption could be detected by sampling more excentric sites on the disc, at constant rotation rate. It should be mentioned that, in contrast to the situation in laminar flow cells, higher shear rates at the rotating disc surface do not result in enhanced diffusional transport. Because of the uniform accessibility, the protein concentration will only depend on the distance to the surface and will not be influenced by lateral fluid transport. As the total conversion rate of prothrombin in the intermediate range conforms to the calculated surface coverage with Xa-Va complexes and the adsorption rate of prothrombin, it is concluded that neither the assembly of the prothrombinase complex, nor the conversion of prothrombin itself, is essentially altered by fluid shear rate.

GENERAL CONCLUSIONS

A rotating disc was adapted for ellipsometric determination of protein adsorption kinetics. This flow geometry provides a uniformly accessible surface and has a well founded theoretical basis. Local protein adsorption rates, as well as the overall assembly of prothrombinase and conversion of prothrombin, conformed to theory. The system allows determination of protein diffusion constants and, in the intermediate range, determination of the protein concentration in the solution directly adjacent to the surface. Specific mechanisms for surface-mediated protein conversion can thus directly be checked, as illustrated by confirmation of the Michaelis-Menten mechanism for prothrombin conversion and determination of the intrinsic values of K_m and k_{cat} . No effects of shear rate on the adsorption and conversion of proteins at the surface were observed.

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Chapter 6

Transport-limited Protein Adsorption on a Flat Surface Opposite a Rotating Stirrer

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SUMMARY

Protein adsorption was studied by ellipsometry in a configuration of a rotating stirrer opposite an adsorbing slide. The flow geometry of this system was treated as a vertical flow vortex opposite an infinite flat plate. An approximate solution for the flow field in the boundary layer was obtained. This solution allowed calculation of maximal, transport-limited, rates of protein adsorption. The resulting expression was compared with measurements of fibrinogen adsorption on a silicon slide. Quantitative agreement between theory and experiment was observed for the initial adsorption rate as function of the rotation velocity of the stirrer. A region on the slide was defined, upstream to the center of the stirrer, which is approximately uniformly accessible, that is, the protein adsorption rate is independent of position. In this region, the flow component directed towards the surface is site-independent, in spite of decreasing total flow and shear rates in the upstream direction. However, this uniform accessible area had a limited extension and, outside of it, the adsorption rates dropped sharply. This limitation, and the approximate nature of the theoretical basis, makes the flow configuration less suited for quantitative analysis of surface-mediated protein conversion than the rotating disc.

INTRODUCTION

The adsorption kinetics of prothrombin, albumin and fibrinogen have been studied by ellipsometry, for adsorption on planar phospholipid membranes opposite a rotating stirrer [1,2]. In these studies it was found that the initial adsorption rates behind the stirrer remained unchanged when the site of measurement was shifted some distance in the upstream direction. Apparently, this region was uniformly accessible, that is, the combined effects of convection and diffusion produced site-independent protein adsorption rates. As a result, the data were analyzed in terms of the classical unstirred layer model [2], a stagnant fluid layer of uniform thickness, separating the adsorbing surface from the well-stirred bulk solution. This model is formally equivalent [Chapter 5] with the adsorbing rotating disc, a uniformly accessible flow configuration for which both, the Navier-Stokes equations and the convection-diffusion equation for mass transfer towards the surface, can be rigorously solved [3].

Other studies compared locally measured adsorption rates of prothrombin with the total conversion rate of prothrombin on a phospholipid-coated area of the adsorbing slide [4, 5]. This conversion is effected by the so called prothrombinase complex of coagulation factors Xa and Va, adsorbed on the phospholipid bilayer. For sufficiently high prothrombinase activity on the bilayer, the conversion process becomes transport-limited, that is, the buffer solution directly adjacent to the surface is almost totally depleted of prothrombin, resulting in a maximal protein gradient in the boundary layer and a maximal rate of protein transport towards the surface. However, the average rate of prothrombin conversion, calculated as the total conversion rate divided by the phospholipid surface area, proved to be 2-3 times lower than the, also transport-limited, adsorption rate of prothrombin measured directly behind the stirrer.

This apparent discrepancy is investigated in the present analysis of transport-limited adsorption kinetics for the flow configuration of a rotating stirrer opposite a flat plate. A theoretical treatment of the diffusion-convection equation is presented, valid for a small region behind the stirrer. For this region, good agreement was obtained between predicted and measured transport-limited adsorption rates. The theory also predicts lower adsorption rates for sites outside this region, especially for sites located above the plane of the stirrer, which explains the mentioned discrepancy.

MATERIALS AND METHODS

Ellipsometry

Ellipsometry was performed as described earlier [1, 2]. This technique is based on the light polarization of a laser beam, reflected against a silicon slide in buffer solution. Altered polarization, due to protein adsorption on the slide, is measured from

the adjustments of two polarizer prisms, the polarizer P and the analyzer A, which are automatically rotated such that the final light intensity remains minimal (null-ellipsometry). The adsorbed mass of protein can be calculated with the Lorentz-Lorenz relation [1] which, for the optical constants of silicon, reduces to the simple relation $\Gamma(t) = 0.085\{P(t) - P_s\}$, with $\Gamma(t)$ the adsorbed protein mass in $\mu\text{g}\cdot\text{cm}^{-2}$ at time t , $P(t)$ the polarizer position in degrees at time t , and P_s the starting position of the polarizer [see Chapters 4 and 5]. Fibrinogen adsorption was measured in a cuvette, and with a flow configuration, as presented in Fig.1. Normally, the laser beam is reflected at a site directly opposite the rotating stirrer, but the cuvette can be positioned by micrometers such that the reflection site is changed in the horizontal (x) and the vertical (z) directions. The distance d , separating the stirrer from the plate, can also be changed by adjusting the position of the stirrer motor.

Fibrinogen

Human lyophilized fibrinogen was purchased from Kabi Diagnostica (grade L; clottability > 90%). Fresh solutions in deionized water (Millipore, Milli-Q3 system) were prepared before each experiment and protein concentrations were determined by spectrophotometry (Cobas Bio System, Hoffmann-La Roche) at 280 nm with $E_{1\%,1\text{cm}}^{280} = 13.6$ [6]. Silicon slides (N-100, Aurel GMBH) were pretreated with chromic acid as described [5]. Under these circumstances, fibrinogen has a very high binding affinity for the silicon surface, with a maximal surface concentration Γ_{max} of about $1 \mu\text{g}\cdot\text{cm}^{-2}$ and a dissociation constant K_d of less than 1 pM. This facilitates the measurement of initial adsorption rates which were obtained from exponential fits on the data points up to a surface coverage of $0.3 \mu\text{g}\cdot\text{cm}^{-2}$.

Calculation of the steady-state flow field

A solution of the flow equation is obtained in the classical approximation of boundary layer effects, assuming a frictionless flow in the bulk solution and limitation of the viscosity effects to a thin fluid layer adjacent to the plate. This approximation is based on relatively high and low Reynolds numbers (ratio of inertial to viscosity effects) in respectively the bulk and in the boundary layer. The flow field of a stirrer, rotating in the horizontal x,y -plane and with its center situated at the origin, is approximated by a single vortex with vortex strength Γ , defined as the integral of the flow velocity over a full circle. For frictionless flow, Γ is a constant and thus must be equal to its value at the circumference of the stirrer: the velocity ωR times the circumference $2\pi R$, or $\Gamma = 2\pi\omega R^2$, where R is the stirrer radius and ω the rotation velocity in $\text{rad}\cdot\text{s}^{-1}$. The configuration of a stirrer separated a distance d from the plate, is modelled by two infinitely long vortexes parallel to the z -axis, with their centers

situated at $(x = 0, y = a)$ and $(x = 0, y = -a)$ with $a = R + d$, and rotating in opposite directions.

FIG.1 The ellipsometer cuvette and flow configuration. Upper picture: cuvette with stirrer opposite the silicon slide and reflecting laser beam from ellipsometer. Lower picture: view from above.

The resulting flow field will be identical to a single vortex at distance a of an infinite flat surface. This is a consequence of the existence of a unique solution of the flow equation, satisfying the condition of surface-parallel flow in the plane of symmetry between the vortices, and with vortex strength $\Gamma = 2\pi\omega R^2$. For a single vortex situated at the origin, the velocity components u and v , respectively in the x -direction parallel to the surface and in the y -direction perpendicular to the surface, can be described by a stream function $\Psi = -(\Gamma/2\pi) \ln\{(x^2+y^2)^{1/2}\}$, with $u = \delta\Psi/\delta y$ and $v = -\delta\Psi/\delta x$ [7].

Substitution of $y \rightarrow y+a$ and $y \rightarrow y-a$ and addition of the two stream functions with opposite signs, in order to account for opposite rotation directions, gives the following flow components:

$$u(x,y) = \omega R^2 \left[\frac{y+a}{x^2+(y+a)^2} - \frac{y-a}{x^2+(y-a)^2} \right] \quad (1a)$$

$$v(x,y) = \omega R^2 \left[\frac{x}{x^2+(y+a)^2} - \frac{x}{x^2+(y-a)^2} \right] \quad (1b)$$

For $y=0$, the component v vanishes and the bulk flow equals:

$$u(x,0) = u_1(x) = 2\omega R^2/[a(1 + x^2/a^2)] \quad (2)$$

In the classical treatment of Von Kármán of the boundary layer it is assumed that this velocity is reached at a small distance $\delta(x)$ from the surface, while closer to the surface the viscosity becomes dominant and the flow velocity becomes zero at the surface, the so called no slip condition, which has been confirmed experimentally. The flow $u(x,y)$ in the boundary layer is then approximated by:

$$u(x,y) = u_1(x) \sin \left[\frac{\pi}{2} y/\delta(x) \right] \quad (3)$$

It is easily verified that this expression indeed vanishes for $y=0$ and becomes equal to $u_1(x)$ for $y=\delta(x)$. The boundary layer thickness $\delta(x)$ is determined by the method of Von Kármán [8], using the momentum integral equation for the boundary layer, which follows from the conservation of momentum in a steady-state flow profile for each volume element in the boundary layer [7]:

$$\frac{d}{dx} \left[\int_0^{\delta(x)} u(u_1-u)dy \right] + \frac{du_1}{dx} \int_0^{\delta(x)} (u_1-u)dy = \nu \left[\frac{\partial}{\partial y} u(x,y) \right]_{y=0} \quad (4)$$

where ν is the kinematic viscosity (the ratio of viscosity to density) of the fluid. Inserting expressions (2) and (3) into equation (4), the integrations can be performed with the following result:

$$\delta(x) = \left[\frac{\pi^2 \nu a^2 F(x/a)}{(4-\pi)\omega R^2} \right]^{(1/2)} \quad (5)$$

$$\text{with } F(x/a) = [1 + (x/a)^2]^{9.32} \int_{-\infty}^{x/a} (1+s^2)^{-8.32} ds$$

Close to the surface ($y \ll \delta(x)$) expression (3) can be approximated by $u(x,y) = (\pi/2) u_1(x) (y/\delta(x))$. Using the incompressibility condition $\delta u/\delta x + \delta v/\delta y = 0$, one then obtains $v(x,y) = -(\pi/4) \frac{d}{dx} [u_1(x)/\delta(x)] y^2$. Inserting the expressions (2) and (5) one obtains:

$$v(x,y) = -(1-\pi/4)^{1/2} \nu^{-1/2} \omega^{3/2} (R/a)^3 \frac{dG(x/a)}{d(x/a)} y^2 \quad (6)$$

$$\text{with } G(x/a) = \{1 + (x/a)^2\}^{-1} F(x/a)^{-1/2}.$$

The function $G(x/a)$ determines the x -dependency of this expression and, as shown in Fig.2, this term can be approximated in the interval $-1.1 < x/a < -0.3$ by the straight line $1.77 x + 2.8$. The differential will then contribute a factor of 1.77 and expression (6) reduces to:

$$v(y) = -0.82 \nu^{-1/2} \omega^{3/2} (R/a)^3 y^2. \quad (7)$$

Apparently, the flow component towards the surface does not depend on x in the interval $-1.1 < x/a < -0.3$, which means that the surface is uniformly accessible in this region.

Calculation of the maximal steady-state mass transfer towards the surface

The quantity of protein in the boundary layer is very small compared to the capacity for adsorption of the slide and the transport capacity of diffusion towards the slide. As a result, a quasi steady-state concentration profile $C(y)$ of a protein in the boundary layer will be established within a fraction of a second after addition of protein to the cuvette [2]. $C(y)$ can be obtained from the diffusion-convection equation with the time-derivative in the lefthand side approximated by zero:

$$0 = -v(y) \frac{d}{dy} C(y) + D \frac{d^2}{dy^2} C(y) \quad (8)$$

where D is the diffusion constant of the protein.

The steady state adsorption rate will be maximal when the concentration gradient at the surface is maximal, that is, when the fluid directly adjacent to the surface is almost totally depleted of protein. In that case, equation (8) must be solved with the boundary conditions $C(y=0) = 0$ and $C(y \rightarrow \infty) = C_{\text{bulk}}$, with C_{bulk} the concentration of protein in the bulk solution. For the special case of $v(y) = -ky^2$, equation (8) can be solved [3] with the solution:

$$C(y) = \frac{3^{2/3}}{\Gamma(1/3)} k^{1/3} \cdot D^{-1/3} \cdot C_{\text{bulk}} \int_0^y \exp\left(-\frac{k}{3D} s^3\right) ds$$

with a value of the gamma function $\Gamma(1/3) = 2.679$.

As a result, one obtains for the maximal adsorption velocity V_{max} :

$$V_{\text{max}} = D \left[\frac{d}{dy} C(y) \right]_{y=0} = 0.776 k^{1/3} \cdot D^{2/3} \cdot C_{\text{bulk}}.$$

Or, inserting the value of $k = 0.82\nu^{1/2} \cdot \omega^{3/2} (R/a)^3$ from equation (7):

$$V_{\text{max}} = 0.73 \frac{R}{R+d} \nu^{-1/6} \cdot \omega^{1/2} \cdot D^{2/3} \cdot C_{\text{bulk}} = \Delta \cdot C_{\text{bulk}} \quad (9)$$

FIG.2 The function $G(x/a)$ with the region $-1.1 < x/a < -0.3$, in which the derivative can be approximated by a straight line.

RESULTS

The fractional adsorption rate constant Δ was calculated from the transport-limited adsorption rate of fibrinogen $d\Gamma/dt$ and the buffer concentration C_{bulk} , as $\Delta = (d\Gamma/dt)/C_{\text{bulk}}$. Fig.3 presents the dependence of Δ on the square root of the angular velocity ($\sqrt{\omega}$), which according to Equation (9) should be linear. The straight line in Fig.3 is the theoretical curve, obtained from Equation (9) by inserting the values $R = 0.5$ cm and $d = 0.12$ cm for the flow geometry, $\nu = 0.01$ cm²·s⁻¹ for the kinematic viscosity of water at 20 °C, and $D = 2.0 \times 10^{-7}$ cm²·s⁻¹ for the diffusion constant of fibrinogen [6]. A close agreement between theoretical and experimental results is observed.

This is also true for the influence of the distance d on adsorption rates, as shown in Fig.4. Only a weak effect is observed and this also follows immediately from Equation (9), because the stirrer radius R is large compared to the distance d , and the factor $R/(R+d)$ will thus deviate little from unity.

The existence of an upstream region with uniform adsorption rates follows from Fig.5.

FIG.3 The fractional adsorption constant Δ of fibrinogen as a function of the square root of the angular rotation velocity ($\sqrt{\omega}$). The theoretical curve is also presented.

FIG.4 Effect of the distance d , separating the stirrer from the flat plate, on the fractional adsorption constant Δ of fibrinogen. The theoretical curve is also presented.

FIG.5 Effect of the lateral location x of the site of measurement on the fractional adsorption constant Δ of fibrinogen.

However, the data do not completely fit the theory in this case. For an upstream site with $x/a = -1$, adsorption rates are already somewhat lower, whereas theory predicts uniform adsorption up to $x/a = -1.1$. This could reflect a boundary effect, because the

measurements at $x/a = -1$ were performed close to the edge of the slide, while the theory is based on an infinite flat surface. Although, as expected, the adsorption rates become lower downstream, the data from this region cannot be compared with theory. Physically realistic solutions require a positive value of the derivative of $G(x/a)$, in contrast to the downstream situation shown in Fig.2, where the derivative becomes negative. So the approximations obviously only hold in the upstream part of the slide, that is for negative x values. This is a consequence of the fact that the boundary layer approximation is only valid in the region with increasing flow velocity [7].

Drastic lowering of the adsorption rates on sites located above the stirrer is shown in Fig.6. The laser beam has a diameter of 0.2 cm, so the lowest vertical position at which the measurements could be performed was at $z = 0.15$ cm. The stirrer height was 0.3 cm and Fig. 6 shows a decrease of the adsorption rate by a factor of about 3 for a site located two times higher.

FIG.6 Effect of the vertical location z of the site of measurement on the fractional adsorption constant Δ of fibrinogen.

DISCUSSION

In spite of the approximations needed for the calculation of the flow field, the present study demonstrates a satisfactory agreement between theoretically predicted and measured adsorption rates. The existence of a uniformly accessible zone implies that the measured protein adsorption rates are not influenced by the upstream events,

in contrast to the situation for a flow cell. It also allows the analysis of adsorption kinetics in terms of a simple unstirred-layer model [2].

For a stirrer diameter R large compared to the distance d separating the stirrer from the adsorbing plate, the factor $R/(R+d)$ in equation (9) approaches unity and the maximal adsorption rate becomes equal to $0.73 \cdot \nu^{1/6} \cdot \omega^{1/2} \cdot D^{2/3} \cdot C_{\text{bulk}}$. This is close to the value of $0.62 \cdot \nu^{1/6} \cdot \omega^{1/2} \cdot D^{2/3} \cdot C_{\text{bulk}}$, obtained for the maximal adsorption rate on a rotating disc [cf. Chapter 5]. From the expression for the flow component parallel to the surface (see "Materials and Methods") $u(x,y) = (\pi/2)u_1(x)y/\delta(x)$ it follows that the shear rate at the surface is $\gamma = [\delta u/\delta y]_{y=0} = (\pi/2)u_1(x)/\delta(x) \text{ s}^{-1}$. Inserting expressions (2) and (5) for $u_1(x)$ and $\delta(x)$ and the values of $R = 0.5 \text{ cm}$, $d = 0.1 \text{ cm}$ and $\nu = 0.01 \text{ cm}^2 \cdot \text{s}^{-1}$, one finds that for rotation velocities of ω between 10 and 300 $\text{rad} \cdot \text{s}^{-1}$ the shear rates at $x = 0$ will increase from about 200 s^{-1} to 8000 s^{-1} . The latter value is somewhat higher than reported for experiments in laminar flow cells, for which γ usually remains below 4000 s^{-1} [9, 10] and the transport-limited adsorption rates of proteins reported in these studies also are a factor of 2-4 lower than obtained in the present study. Apparently, the flow component directed towards the adsorbing surface in the uniform accessible area, although very small compared to the parallel component, causes very effective protein transport. It is remarkable that for such widely differing flow systems as a rotating disc, a stirrer opposite a plate and a laminar flow cell, transport-limited adsorption rates of the same order of magnitude are obtained.

The main advantage of the configuration of a rotating stirrer opposite the adsorbing surface is experimental convenience. The adsorbing slide can easily be manipulated, for instance be covered with a 100% phosphatidylserine bilayer with the Langmuir-Blodgett technique [1, 2, 4]. A wide range of rotation rates is obviously more easily obtained than for an adsorbing disc, allowing ellipsometry [see Chapter 5]. Disadvantages of the system are the less solid theoretical basis and the fact that only a small part of the surface is uniformly accessible.

The latter limitation was demonstrated by measurement of prothrombin conversion on a small area of 0.8 cm wide and 0.6 cm high (2 times the stirrer height) covered with a 20% dioleoyl-phosphatidylserine (DOPS) / 80% dioleoyl-phosphatidylcholine (DOPC) phospholipid mixture. The surface was situated with its center at $x=0$ and excess prothrombinase activity was preadsorbed in order to make the conversion process transport-limited. A stirrer with radius $R = 0.5 \text{ cm}$, separated a distance of 1.2 mm from the surface, was positioned with its center also at $x=0$. The rotation velocity was 250 $\text{rad} \cdot \text{s}^{-1}$. Inserting these values into Equation (9), and using also values of $D(\text{prothrombin}) = 6.2 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and $\nu = 0.010 \text{ cm}^2 \cdot \text{s}^{-1}$ [see Chapter 5], a value of $\Delta = 15 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ is obtained. However, a value of $\Delta = 5.2 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ was calculated from the overall conversion of prothrombin [5]. Apparently, the

lower adsorption rates downstream and above the stirrer, made the average transport-limited adsorption rate for the whole surface three times lower than the adsorption rate directly behind the stirrer. This limitation could be overcome by covering only an upstream region with $-1.1(R+d) < x < -0.3(R+d)$ of the slide with phospholipids and using a stirrer with a vertical height of 1 cm. The width of the slide should be larger than indicated in Fig.1, in order to prevent boundary effects as indicated by Fig.5. However this would introduce experimental complications and spoil the main advantage of the system, its simplicity.

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Chapter 7

Summary and Concluding Remarks

SUMMARY AND CONCLUDING REMARKS

The prothrombinase complex consists of coagulation factors Xa and Va which, in the presence of calcium ions, are bound to a phospholipid surface. It functions as an enzyme, catalysing the formation of thrombin from prothrombin. Thrombin plays a central role in blood coagulation and stops bleeding in a number of ways. In order to make thrombin formation an efficient process, a surface is necessary that not only binds the factors Xa and Va, but also prothrombin, the substrate of the reaction. Prothrombin is provided from the solution and adsorbs onto the surface containing the prothrombinase complex. It is subsequently transported to this complex by lateral diffusion. At a successful collision with the enzyme, prothrombin will be converted. The surface functions as a funnel for this reaction. Since it restricts movement to two dimensions, the prothrombin molecules bound to the surface have a much higher chance of encountering the complex than prothrombin molecules in free solution. Up to a certain limit, a larger empty space surrounding the complex, that is a bigger funnel, will increase the possibility of collision. This model is called the bound-substrate model.

Chapter 2 describes a series of experiments demonstrating the role of surface area around the complex. A spherical vesicle surface of 20 nm diameter has little space surrounding a prothrombinase complex and needs a high concentration of free prothrombin to achieve a half maximal conversion rate ($K_m = 170$ nM). Prothrombinase on vesicles of 80 nm diameter already converts prothrombin more easily ($K_m = 40$ nM), while the prothrombinase complex bound to a macroscopic surface, being surrounded by lots of space, needs only 6 nM of prothrombin for half-maximal production of prothrombin. The theory of Smoluchovsky allowed us to calculate how many collisions take place between substrate molecules and vesicles of a certain diameter in free solution. From this it is concluded that nearly all successful collisions, with a prothrombinase containing vesicle result in conversion. It means that the rate of conversion of prothrombin in vesicle suspensions is not determined by the conversion capacity of the prothrombinase complex, but by the frequency of collisions of a vesicle with free prothrombin. It also implicates that the concentration of bound prothrombin on a prothrombinase containing vesicle is immeasurably low, since all supply is almost instantaneously converted. This was not the case on a macroscopic surface. We chose the adsorption rate of prothrombin such that it exceeded the conversion velocity. The collisional frequency of free prothrombin with bound prothrombinase was also calculated. It appeared that prothrombin conversion from free solution, i.e. without prior binding to the surface, could not explain the measured conversion rate. The question whether the prothrombinase complex converts bound prothrombin or prothrombin from free solution has been a matter of dispute in the literature for more than fifteen years, and now it seems to be answered.

Chapter 3 describes the prelude to prothrombin conversion. Prothrombinase consists of two proteins that have to assemble into a complex in order to enable fast prothrombin conversion. One protein, factor Xa, is the actual enzyme. The maximal conversion rate achieved by this enzyme (the k_{cat}) is increased by a factor of 1000 after formation of a complex with the other protein, the cofactor protein factor Va. This assembly of factor Xa and factor Va occurs again on a phospholipid surface. A certain quantity of factor Va was bound to a macroscopic phospholipid surface and prothrombin was added. Thrombin formation does not occur at that moment because factor Xa is still lacking. After addition of factor Xa, thrombin started to be generated at a rate which increased as more factor Xa complexed with factor Va. From this increase of the thrombin generation rate, the velocity of complex formation could be determined. Similar experiments in vesicle suspensions revealed that the size of the surface surrounding the factor Va molecule, determined the assembly rate. As in Chapter 2 the surface functioned as a kind of funnel which collected free factor Xa from the solution and optimized its transport to bound factor Va. Moreover, these experiments showed that the concentration of factor Xa in free solution, necessary for saturating half the amount of the lipid-bound factor Va is only 1 pM. This value is 10 to 100 fold less than reported in the literature.

The differences between experiments carried out in vesicle suspensions and those carried out on macroscopic surfaces, could entirely be explained by differences in the conditions of transport to a vesicle and a planar layer. In the literature, these discrepancies are often attributed to differences in binding affinities of proteins for macroscopic layers and vesicles. In order to explain them, various causes are mentioned. Vesicles are spherical, and the molecules in such a spherical surface have a different orientation. In addition, the inner and the outer monolayer of a vesicle could contain concentrations of specific phospholipids, differing from the concentrations in the overall mixture, which could influence the binding affinities of the proteins. Moreover, the techniques used to measure binding differ for vesicles and planar layers. It would therefore be useful to be able to measure on both kinds of surfaces using the same technique. In this thesis, ellipsometry was employed to measure binding on a planar bilayer. This is an optical technique, where light is reflected by a silicon plate covered with a phospholipid membrane to which proteins adhere. When protein is added to a cuvette containing such a plate, the binding of the protein to this surface can be followed in time using this technique. The rate of adsorption of proteins to the surface is dependent on the concentration of protein in the buffer, the diffusion constant of the protein, and the flow conditions. The latter are regulated by a rotating stirrer in front of the macroscopic surface. When binding of various concentrations of a certain protein is followed in time, the initial adsorption rate will be linearly dependent on the buffer concentration of protein. This phenomenon was used in the experiments described in Chapter 4. The constant describing the relationship between the concentration of a protein and its adsorption rate, can be determined by addition of

a known amount of protein to the buffer. If in addition to the protein, vesicles are present in the buffer as well, the amount of free protein will decrease as a consequence of their binding to the vesicles. Since the relation between adsorption rate and free concentration is known, measurement of that rate allows the free concentration to be calculated. Because the total added concentration of protein is also known, the amount of protein bound to the vesicles can be determined. The concentration of protein necessary for occupying half of the vesicle surface (K_d) can be calculated after repetition of the experiment with various concentrations of protein. Chapter 4 demonstrates that, using this technique, the dissociation constant of factor Xa and prothrombin on vesicles, prepared from a mixture of 20% dioleoyl-phosphatidylserine and 80% dioleoyl-phosphatidylcholine could be measured. The values found hardly deviated from the values determined on a planar bilayer. It proved that the transport conditions, and not some changed binding properties, are the explanation for the differences between vesicles and planar bilayers, as found in Chapters 2 and 3.

Conversion of prothrombin present on a macroscopic surface, requires transport of prothrombin to the planar layer. Normal diffusion of proteins in a fluid is not sufficient to get measurable rates of thrombin formation. Therefore an artificially produced flow is necessary, for continuously mixing of the fluid. There are several techniques to realise this. A laminar flow profile, i.e. a fluid flowing through a tube, is closest to the physiological reality of blood flowing through a vessel. But this has a disadvantage. The concentration of the protein decreases downstream by conversion of the protein by enzyme bound to the wall. Since the rate of transport of proteins to the vessel wall is dependent on the protein concentration, the rate of transport to the wall becomes non-uniform. An adsorbing protein causes comparable problems because, due to depletion, its concentration also decreases downstream. Correlating enzyme activity with the free substrate concentration thus becomes a difficult task. Furthermore, the amount of enzyme present on the surface cannot be accurately controlled, which obscures the relation between surface activity and bound enzyme concentration. A solution to this problem is described in Chapter 5. A rotating disc in a fluid produces a flow profile with special properties. The component of the flow directed towards the surface is only dependent on the distance from the surface, and independent of the location of the site. As a consequence every site on the surface receives exactly the same amount of protein and we have a so called uniformly accessible surface. Protein transport is only dependent on the diffusion constant, the concentration of the protein, and the rotation velocity of the disc. A rotating disc was constructed running sufficiently smoothless to enable ellipsometric measurement of proteins adsorption. In this way it proved possible to determine the diffusion constants of factors Va and Xa, prothrombin, fibrinogen, and lysozyme. These being known, it was possible to adsorb a low quantity of factor Va on the surface, which could subsequently be saturated with factor Xa. In this way, a known quantity of prothrombinase was assembled on the surface, to which various concentrations of

prothrombin could be added. The K_m and k_{cat} of prothrombin conversion in this set-up could be measured and resulted in similar values as found in the experiments described in Chapter 2. It could furthermore be proven that by variation of the surface concentration of enzyme, the conversion rate changed from being enzyme-limited to transport-limited.

Only in the experiments of Chapter 5 a rotating disc was used as uniform accessible surface. The experiments described in the other chapters were carried out using a rotating stirrer in front of a macroscopic surface. Chapter 6 shows that a part of the surface opposite the stirrer can be considered as approximately uniform accessible. However, ellipsometric measurements of adsorption rates in areas not directly opposite the stirrer but above or next to it, showed that the transport velocity decreases considerably in those areas. The transport velocity inside the uniform accessible surface area turned out to be dependent on the stirring velocity and the diffusion constant of the protein, as was the case for the rotating disc. The experiments of the Chapters 2, 3 and 4, for that matter, were carried out with a surface only barely larger than this area. Still it is preferable to perform such experiments on a rotating disc.

It will be clear from this thesis that substrate conversion by enzymes bound to macroscopic surfaces differs from conversion in free solution. The influence of flow and lateral diffusion on this process could largely explain the differences found. This has consequences for the description of *in vivo* reality and the experiments should be repeated under more physiological conditions. As mentioned before, blood coagulation occurs on a vessel wall, and not on a rotating disc or a flat plate opposite a stirrer. It further occurs in the human body, and with human instead of bovine proteins. It finally does also not happen in 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 3 mM $CaCl_2$ and 0.5 g/l of albumin, but in whole blood containing all coagulation proteins. The conclusions from this thesis can therefore not be unconditionally projected onto the physiological reality. The chosen experimental conditions are nevertheless justifiable. Purified systems *in vitro* provide the opportunity to develop useful models, something that is not yet possible in the presence of the complete plasma content. In order to be able to say something about the flow conditions at the vessel wall, it is imperative to choose an experimental set-up that allows for the right techniques of measurement and keeps the data interpretable. This implies concessions to physiology. The ultimate aim of these experiments, a description of prothrombin conversion *in vivo*, has still to be achieved.

Hoofdstuk 7

Samenvatting en Slotopmerkingen

SAMENVATTING EN SLOTOPMERKINGEN

Het protrombinase complex bestaat uit de bloedstollingsfactoren factor Xa en factor Va die in aanwezigheid van calcium gebonden zitten op een phospholipide-oppervlak. Het is een enzym dat de vorming van trombine uit protrombine katalyseert. Trombine speelt een centrale rol in de bloedstolling. Het helpt op verschillende fronten een bloeding te stoppen. Om de vorming van trombine efficiënt te doen verlopen is een oppervlak nodig dat niet alleen factor Xa en Va bindt, maar dat ook het substraat protrombine adsorbeert. Protrombine wordt aangeleverd vanuit de oplossing en zal op het oppervlak, waarop het protrombinase complex zit, adsorberen en vervolgens over het oppervlak naar het complex toe getransporteerd worden via laterale diffusie. Bij het complex aangekomen zal het na een succesvolle ontmoeting worden omgezet. Het oppervlak fungeert hierbij als een trechter. De op het oppervlak gebonden protrombine-moleculen hebben door hun 2-dimensionale beweging veel meer kans om het complex te ontmoeten dan protrombine-moleculen die nog in vrije oplossing zijn. Hoe groter de lege ruimte rondom het complex - dus hoe groter de doorsnede van de trechter - des te groter deze invang-kans. Dit model is bekend onder de naam bound-substrate model.

Hoofdstuk 2 beschrijft een serie experimenten die de rol van die hoeveelheid oppervlak rondom het complex laten zien. Een bolvormig vesicle-oppervlak met een diameter van 20 nm biedt weinig ruimte rondom een protrombinase-complex en heeft een hoge vrije protrombine-concentratie nodig om op halfmaximale omzettingssnelheid te komen ($K_m = 170$ nM). Protrombinase op vesicles met een doorsnede van 80 nm komt al sneller op gang ($K_m = 40$ nM), terwijl het protrombinase complex gebonden op een macroscopisch oppervlak, met als het ware een zee van ruimte rondom ieder complex, al genoeg neemt met nog geen 6 nM protrombine. Met behulp van de theorie van Smoluchovsky kon worden uitgerekend hoeveel botsingen van substraat in vrije oplossing er plaats vinden met een vesicle van een bepaalde doorsnede. Hieruit volgde de conclusie dat vrijwel iedere succesvolle botsing met een protrombinase-bevattende vesicle resulteert in een omzetting. Dit betekent dat in vesicle-suspensies de omzettingssnelheid van protrombine niet bepaald wordt door de omzettingscapaciteit van het protrombinase-complex maar door de botsingsfrequentie van het vesicle met vrij protrombine. Ook houdt dit in dat de gebonden concentratie protrombine op een protrombinase-bevattend vesicle onmeetbaar laag is. Alles wat wordt aangevoerd wordt immers bijna onmiddellijk omgezet. Op een macroscopisch oppervlak was dit niet het geval. De aanvoersnelheid van protrombine was hier zo gekozen dat die de omzettingscapaciteit te boven ging. Ook is er uitgerekend hoe groot de botsingsfrequentie was van vrij protrombine met gebonden protrombinase. Hieruit bleek dat omzetting vanuit vrije oplossing, dus zonder voorafgaande binding aan het oppervlak van protrombine, de gemeten

snelheden van omzetting niet konden verklaren. De vraag of het gebonden protrombine of het protrombine uit de oplossing door het protrombinasecomplex wordt omgezet is al meer dan 15 jaar een twistpunt in de literatuur en lijkt door deze experimenten definitief beantwoord.

Hoofdstuk 3 beschrijft hetgeen er voorafgaat aan de protrombine-omzetting. Protrombinase bestaat uit twee eiwitten die eerst een complex moeten vormen voordat een snelle omzetting plaats kan vinden. Het ene eiwit, factor Xa, is het eigenlijke enzym, de maximale omzettingssnelheid van protrombine (de k_{cat}) die dit enzym haalt wordt met ongeveer een factor 1000 vergroot als het enzym met factor Va, de co-factor complexeert. Die assemblage van die twee eiwitten, factor Xa en factor Va, gebeurt ook op een phospholipide-opervlak. Er werd een hoeveelheid factor Va op een macroscopisch oppervlak gebonden en vervolgens werd er een hoeveelheid protrombine bij gezet. Op dat moment zal er nog geen trombine gevormd worden aangezien het noodzakelijke factor Xa nog niet aanwezig is. Na toevoeging van Xa kwam er een trombinevormingssnelheid op gang die toenam naarmate er meer factor Xa met factor Va assembleerde. Uit die toename in trombinevormingssnelheid kon de snelheid van complexvorming worden bepaald. Soortgelijke experimenten in vesicle-suspensies wezen uit dat de grootte van het oppervlak rondom het molecuul Va bepalend was voor de assemblage-snelheid. Ook hier speelde het oppervlak de rol van een trechter die het vrije factor Xa uit de oplossing verzamelde en het transport van factor Xa naar het gebonden Va optimaliseerde. Bovendien wezen de experimenten uit dat de concentratie factor Xa in vrije oplossing benodigd om de helft van het factor Va te complexeren slechts 1 pM is. Dat is 10 tot 100 keer minder dan eerder werd gepubliceerd.

De genoemde verschillen tussen experimenten uitgevoerd in vesicle-suspensies en die op macroscopische oppervlakken konden volledig worden verklaard uit verschillen in transportcondities naar een vesicle in vergelijking met een vlakke laag. In de literatuur zijn deze discrepanties vaak toegeschreven aan verschillen tussen bindingsaffiniteiten van eiwitten aan een macroscopische laag en aan een vesicle. Allerlei oorzaken worden genoemd om deze verschillen te verklaren. Vesicles zijn rond, en de moleculen in zo'n bol oppervlak hebben een andere oriëntatie. Bovendien bestaan de phospholipide-mengsels waarmee de vesicles zijn gemaakt uit verschillende moleculen zodat de binnenste en buitenste schil van een vesicle verschillende concentraties van die moleculen zouden kunnen bevatten waarmee ook de bindingsaffiniteit van de vesicles beïnvloed wordt. De technieken waarmee de bindingen zijn gemeten verschillen ook voor metingen op vesicles en vlakke lagen. Het zou daarom nuttig zijn om met dezelfde techniek metingen te verrichten op beide soorten oppervlakken. In dit proefschrift is gebruik gemaakt van ellipsometrie om binding te meten op een vlakke laag. Dit is een optische techniek waarbij licht wordt gereflecteerd tegen een silicium plaatje dat bedekt is met een phospholipide-membraan waaraan eiwitten hechten. Als een eiwit wordt toegevoegd aan een cuvet met daarin

een macroscopisch oppervlak kan met behulp van deze techniek de binding in de tijd worden gevolgd. De snelheid van adsorptie van eiwitten aan het oppervlak is afhankelijk van de concentratie eiwit in de buffer, de diffusieconstante van het eiwit en de stromingscondities. De laatste worden geregeld met een draaiende roerboon tegenover het macroscopisch oppervlak. Als de binding in de tijd gevolgd wordt bij verschillende concentraties van een bepaald eiwit onder verder dezelfde condities, zal de initiële adsorptiesnelheid lineair afhankelijk zijn van de bufferconcentratie eiwit. Van dit fenomeen is gebruik gemaakt in de experimenten beschreven in hoofdstuk 4. Als er een bekende hoeveelheid eiwit aan de buffer wordt toegevoegd kan een constante worden bepaald die de relatie aangeeft tussen de concentratie eiwit en de adsorptiesnelheid. Als er nu behalve eiwit ook vesicles in de buffer aanwezig is zal de hoeveelheid vrij eiwit dalen tengevolge van binding aan de vesicles. Nu is de vrije concentratie eiwit niet bekend, maar omdat de relatie tussen adsorptiesnelheid en vrije concentratie dat wel is, kan toch door meting van die snelheid de vrije concentratie worden berekend. Doordat de totale toegevoegde concentratie ook bekend is kan de hoeveelheid aan de vesicles gebonden eiwit worden bepaald. Door dit bij verschillende eiwitconcentraties te herhalen kan de concentratie eiwit worden berekend die nodig is om de helft van het vesicleoppervlak te bezetten (K_d). Dit is in hoofdstuk 4 gelukt, er is komen vast te staan dat de bindingsconstante van factor Xa en protrombine op vesicles bestaande uit een mengsel van 20% dioleoylphosphatidylserine en 80% dioleoylphosphatidylcholine nauwelijks afwijkt van de waarde gemeten op een vlakke laag. Het zijn dus inderdaad de transportcondities en niet de veranderde bindingseigenschappen die de verschillen in resultaten tussen vesicles en vlakke lagen, zoals gevonden in hoofdstuk 2 en 3, verklaren.

Om protrombine te activeren door een op een macroscopisch oppervlak aanwezige hoeveelheid protrombinase is er een transport van protrombine naar de vlakke membraan nodig. Om meetbare trombinevormingssnelheden te kunnen meten is de normale diffusie van eiwitten in een vloeistof niet toereikend. Er zal dus kunstmatig een stroming in gang moeten worden gezet die de vloeistof voortdurend mengt. Hiervoor zijn verschillende oplossingen mogelijk. Een laminair flowprofiel, d.w.z. een vloeistof die door een buisje gepompt wordt, komt het dichtst bij de fysiologische werkelijkheid van bloed dat door een vat stroomt. Hieraan kleeft echter een nadeel. Aangezien de transportsnelheid van eiwitten naar de wand van het vat afhankelijk is van de concentratie zal niet iedere plaats op de wand dezelfde transportsnelheid ontmoeten. Immers, de concentratie eiwit neemt stroomafwaarts, door omzetting van het eiwit door enzym wat op de wand zit, af. Een adsorberend eiwit geeft soortgelijke problemen, stroomafwaarts neemt de concentratie door depletie af. Dit maakt het lastig om de enzymactiviteit te correleren aan de concentratie vrij substraat. Ook de hoeveelheid op het oppervlak aanwezig enzym valt niet nauwkeurig te doseren zodat de relatie tussen oppervlakte-activiteit en gebonden enzymconcentratie niet helder is. Een oplossing voor dit probleem werd gevonden in

hoofdstuk 5. Een draaiende schijf in een vloeistof veroorzaakt een flowprofiel met bijzondere eigenschappen. De component van de flow die naar een plaats op het oppervlak toe gericht is, is enkel afhankelijk van de afstand tot het oppervlak en niet van die plaats. Dit heeft tot gevolg dat iedere plaats op het oppervlak exact evenveel eiwit uit de oplossing ontvangt. Het eiwittransport is dan alleen nog afhankelijk van de diffusieconstante en concentratie van het eiwit en de rotatiesnelheid van de schijf. Er werd een draaiend schijfje vervaardigd dat voldoende trillingsvrij is om ellipsometrische metingen mogelijk te maken, zodat de adsorptiesnelheden van eiwitten daarna konden worden gemeten. Op deze manier was het mogelijk de diffusieconstanten van factor Va, Xa, protrombine, fibrinogeen en lysozym te bepalen. Als die bekend zijn geeft dat de mogelijkheid een uitgebalanceerde hoeveelheid factor Va op het oppervlak te zetten die vervolgens kon worden verzadigd met factor Xa. Op deze manier werd een bekende hoeveelheid protrombinase op het oppervlak gezet waaraan verschillende concentraties protrombine konden worden toegevoegd. De K_m en k_{cat} van protrombine omzetting kon worden gemeten in deze opstelling, waarbij vergelijkbare waarden werden gevonden als beschreven in hoofdstuk 2. Bovendien kon worden aangetoond dat door variatie in de oppervlakte-concentratie enzym de omzettingssnelheid van enzym-gelimiteerde omzetting veranderde in transport-gelimiteerde omzetting. Hoofdstuk 5 is het enige hoofdstuk waar gewerkt werd met een draaiende schijf, het enige uniform toegankelijke oppervlak. De experimenten beschreven in de overige hoofdstukken werden uitgevoerd met een draaiende roerboon tegenover een macroscopisch oppervlak. Hoofdstuk 6 laat zien dat een fors gedeelte van het oppervlak tegenover een roerboon nog redelijk uniform toegankelijk is. Ellipsometrische metingen van de adsorptiesnelheid op een gebied dat niet recht tegenover de draaiende boon maar erboven of ernaast zijn gedaan lieten echter zien dat de transportsnelheid snel afneemt buiten dit gebied. Binnen het uniform toegankelijke oppervlak bleek de transportsnelheid net als bij de draaiende schijf afhankelijk te zijn van de draaisnelheid van de boon en de diffusieconstante van het eiwit. De proeven beschreven in de hoofdstukken 2-4 zijn overigens gedaan met een oppervlak dat qua grootte het omschreven gebied nauwelijks te buiten ging. Toch verdient het de voorkeur in de toekomst soortgelijke experimenten te verrichten met behulp van de draaiende schijf.

Het moge duidelijk zijn uit dit proefschrift dat substraat omzetting door een enzym dat gebonden zit aan een macroscopisch oppervlak verschilt van omzetting door ongebonden enzym. De invloed van stromingscondities op dit proces en het transport via laterale diffusie verklaren voor een groot deel de gevonden verschillen. Dat dit consequenties heeft voor de beschrijving van de *in vivo* realiteit is zeer waarschijnlijk. Het ligt dan ook voor de hand om het een en ander in meer fysiologische omstandigheden te verifiëren. Bloedstolling geschiedt immers op de wand van een bloedvat en niet op een draaiende schijf of een vlak plaatje tegenover een roerboon. Bovendien geschiedt het in het menselijk lichaam niet met eiwitten uit

het bloed van koeien maar met humaan eiwit. Ook gebeurt het niet in 0.05 M Tris-HCL buffer met 0.1 M NaCl en 3 mM Calcium en 0.5 g-liter albumine maar in vol bloed met alle stollingseiwitten aanwezig. Daarom zijn de conclusies uit dit proefschrift niet zondermeer toepasbaar op de werkelijke fysiologie en dienen slagen om de arm te worden genomen. Toch zijn de gekozen condities verdedigbaar. Een gezuiverd systeem in vitro geeft de mogelijkheid een model te ontwikkelen dat in aanwezigheid van de complete plasma-inhoud niet mogelijk is. Om iets te kunnen zeggen over de stromingscondities aan de wand van een bloedvat zal de proefopstelling moeten worden gekozen die de meettechnieken toelaat en de data interpreteerbaar houdt. Daarom moeten concessies worden gedaan aan de fysiologie. Het hogere doel achter deze proeven, een beschrijving geven van protrombine omzetting in vivo, is daarmee niet bereikt.

Curriculum Vitae

Peter Giesen is geboren op 8 februari 1963 in Tilburg. In 1981 rondde hij de VWO-opleiding in Etten-Leur af. In hetzelfde jaar begon hij de studie geneeskunde aan de Rijksuniversiteit Limburg te Maastricht. In november 1987 kreeg hij het artsendiploma en in januari 1988 begon hij als Onderzoeker In Opleiding in dienst van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO) en verbonden aan de Rijksuniversiteit Limburg de studie die tot dit proefschrift heeft geleid.