Adsorption and Conversion of Prothrombin on a Rotating Disc

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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, such as the Michaelis-Menten equation, which actually measured overall conversion rates. However, the rotating disc is a classical example of a uniformly accessible surface and therefore was 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 lyszyme, prothrombin, and fibrinogen. After coverage of the disc with a 20% phosphatidylserine/80% phosphatidylocho-

line bilayer and preadsorption of factor Va, addition of excess factor Xa and prothrombin resulted in effective conversion of prothrombin. For high (10 fmol·cm⁻²) surface coverage of prothrombinase, the rate of conversion equals the transport limited adsorption rate of prothrombin. For low (0.1 to 0.5 fmol·cm⁻²) surface concentrations of prothrombinase, the conversion rate dropped below the transport limit and the intrinsic kinetic parameters could be estimated at $K_m = 7.1 \pm 1.2 \text{nM}$ and $k_{cat} = 25 \pm 1.0 \text{s}^{-1}$ (20°C). At these low surface activities of prothrombinase, the effect of the rotation rate (6 to 225 rad·s⁻¹) on prothrombin conversion could be explained by the rotation-rate dependent prothrombin transport. This indicates that the fluid shear rate has no drastic influence on the intrinsic kinetics of prothrombin conversion.© 1993 by The American Society of Hematology.

The influencing factors on the adsorption kinetics of proteins from solution have 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. However, usually 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. 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.

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, for instance, the protein-depleted boundary layer develops 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 rotating stirrer, the rotation rate is maximal at the site behind the center of the stirrer, but even behind the stirrer the adsorption rate drops in the downstream as well in the upstream direction, whereas a very pronounced reduction of the adsorption rate, up to fourfold, is observed at sites located directly above the upper edge of the stirrer.

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 and in similarly coated perfused capillaries. The latter system was introduced to study the activation of factor X by the lipid-bound tissue factor/factor VIIa complex. Because of the nonuniform 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.

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 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. Protein transport towards the surface 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 radially increasing shear rates. The rotating disc electrode has been extensively used in electrochemistry and was also used to study platelet adhesion, reaction kinetics of immobilized enzymes, and protein adsorption.

In the present study, a simple rotating disc was constructed that 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 Va and Xa, a prothrombin-converting surface was obtained. The paper discusses the influence of flow conditions on the adsorption kinetics of proteins from solution.
amount of prothrombinase present on the surface could be controlled by the concentration of factor Va and duration of the preadsorption. For high surface coverage with prothrombinase the prothrombin conversion was transport limited and corresponded to the transport-limited adsorption rate of prothrombin. For low and intermediate thrombin production, the conversion depended both on the transport rate of prothrombin and on intrinsic kinetics. A simple formula is presented that relates observed and intrinsic kinetics. This allows identification of intrinsic kinetics from measurements of prothrombin conversion rates as function of the prothrombin concentration at a fixed rotation rate. It appears that intrinsic kinetics conforms to the classical Michaelis-Menten scheme. Moreover, observed thrombin production rates as function of the rotation rate could be completely explained by intrinsic kinetics and transport conditions, indicating that the conversion of prothrombin is not affected by the shear rate.

MATERIALS AND METHODS

Proteins and phospholipids. Human lyophilized fibrinogen and lysozyme were purchased respectively from Kabi Diagnostica (Stockholm, Sweden) (grade L; clottability >90%) and Merck (Darmstadt, Germany) (chicken egg). Fresh solutions were prepared before each experiment and protein concentrations were determined by titration with factor Xa in a kinetic assay. During the experiments, the desorption of factor Va from the rotating disc has the special feature, that the velocity component of the flow perpendicular to the disc surface only depends on the distance to the surface and not on the lateral coordinates. This feature allows a considerable simplification of the three-dimensional convective-diffusion equations to one dimension, the axis perpendicular to the disc surface. This reduction not only implies that...
mass transfer to the disc surface is independent of the lateral coordinates, i.e., the rotating disc represents a uniform accessible surface, but also allows derivation of an explicit expression for the mass-transfer coefficient.\textsuperscript{29}

The limited transport capacity of convection and diffusion generally results in a decrease of the protein concentration $C_0$ near the adsorbing surface and two situations can be discerned (1) the transport-limited situation, where the intrinsic adsorption rate is huge compared with the transport limit; and (2) the intermediate situation with an intrinsic adsorption rate in the same range or smaller than the transport limit.

In the transport-limited situation the protein in the solution near the disc surface is effectively depleted, i.e., the concentration $C_0$ of protein near the disc surface is essentially zero. The adsorption rate $J$ (mol cm$^{-2}$ s$^{-1}$) then can be expressed in terms of the rotation rate $\omega$ (rad/s) of the disc, the kinematic viscosity $\nu$ (cm$^2$ s$^{-1}$), the diffusion coefficient $D$ (cm$^2$ s$^{-1}$) of the protein and the solution concentration $C_{\text{bulk}}$ (mol cm$^{-3}$) of the protein:\textsuperscript{29}

$$J = 0.62D^2/\nu^{1/2}\omega^{1/2}C_{\text{bulk}} = \Delta C_{\text{bulk}}.$$  \hspace{1cm} (1)

The expression $\Delta = 0.62D^2/\nu^{1/2}\omega^{1/2}$ for the mass-transfer coefficient represents only a first order approximation and higher order terms have been calculated.\textsuperscript{29} In case of protein adsorptions, with diffusion constants below 10$^{-7}$ cm$^2$ s$^{-1}$ and a kinematic viscosity of water $\nu = 0.01$ cm$^2$ s$^{-1}$, these higher order corrections contribute less than 1.5% to the value of $\Delta$.

Thus, the transport-limited adsorption rate is equal to the mass-transfer coefficient $\Delta$ times the concentration in bulk solution. Moreover, the relation $\Delta = 0.62D^2/\nu^{1/2}\omega^{1/2}$ implies that the fractional adsorption rate $J/C_{\text{bulk}}$ is a linear function of $\omega^{1/2}$. Therefore, a plot of $J/C_{\text{bulk}}$ as function of $\omega^{1/2}$ gives meaningful information: a straight line through the origin indicates transport-limited adsorption and the slope of the line then allows calculation of the diffusion coefficient of the protein. In this situation, observed adsorption or conversion rates solely reflect the transport limit and no information on the intrinsic adsorption or conversion kinetics is obtained.

On the other hand, deviations from linearity indicate intermediate kinetics with incomplete protein depletion near the disc surface. In this situation, a quasi steady-state value of $C_0$ is established such that the intrinsic adsorption rate $J_{\text{int}}(C_0)$ (mol cm$^{-2}$ s$^{-1}$) is equal to the protein transfer from bulk solution to the disc surface:\textsuperscript{29}

$$J = \Delta(C_{\text{bulk}} - C_0) = J_{\text{int}}(C_0).$$  \hspace{1cm} (2)

Hence, for given $C_{\text{bulk}}$ and observed $J$ one can calculate $C_0$ from this equation and therefore the intrinsic kinetics $J_{\text{int}}(C_0)$ can be determined.

For enzymatic conversions conforming to Michaelis-Menten kinetics,

$$J_{\text{int}}(C_0) = V_{\text{max}}C_0/(C_0 + K_m),$$

with $V_{\text{max}}$ the maximal turnover rate (mol cm$^{-2}$ s$^{-1}$) and $K_m$ the Michaelis constant (mol cm$^{-3}$). Equation (2) represents a second-order equation in $C_0$ and allows an explicit solution:

$$J = \Delta(C_{\text{bulk}} - K_m + V_{\text{max}}/\Delta) - \sqrt{(C_{\text{bulk}} - K_m - V_{\text{max}}/\Delta)^2 + 4K_mC_{\text{bulk}}}/2.$$  \hspace{1cm} (3)

Nonlinear regression of this relation to measured conversion rates, with the BMDP subprogram PAR (Statistical Software Inc, Los Angeles, CA), was used to determine the intrinsic $V_{\text{max}}$ and $K_m$ of prothrombinase.

\section*{RESULTS}

Lipid adsorption on the cuvette walls results in prothrombin conversion apart from the conversion on the rotating disc. To avoid this complication, coating of the silicon surface was performed outside the ellipsometer in a separate vessel. The rotating disc used in the present study can simply be covered with another silicon disc, on a PETP cylinder (see Fig 1). Both cylinders stick together by magnetic force, and the thin fluid layer between the silicon surfaces prevents exposure of the lipid surface to air when the ensemble is removed from the cuvette. This possibility was used in the experiments on prothrombin conversion.

An example of adsorption of 1 $\mu$g mL$^{-1}$ prothrombin, with a rotation velocity of 30 rad s$^{-1}$ on the rotating disc is presented in Fig 2, and compared with the result obtained for 0.5 $\mu$g mL$^{-1}$ in the usual configuration of a fixed adsorbing slide in front of a rotating stirrer.\textsuperscript{7} Apparently, the rota-
tion of the adsorbing silicon surface does not increase experimental scatter. Both configurations cause effective mixing of the protein sample (5 to 25 μL) 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, mixing problems were avoided by addition of protein in large (200 μL) volumes.

Quantitative agreement between theory and experiment is shown for fibrinogen, lysozyme, and prothrombin in Fig 3. Initial adsorption velocities of these proteins are transport-limited, and according to equation (1), should satisfy the relation dΓ/dt = Δ·C_{bulk}, with the mass transfer constant Δ = 0.62 · D^{0.3} · ν^{−1/6} · ω^{1/2}. The measured adsorption rates in Fig 3 are presented as fractional adsorption rates, that is, dΓ/dt (in μg·cm⁻²·s⁻¹) divided by the bulk concentration C_{bulk} (μg·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 five different rotation rates. A linear dependency of Δ on ω₀, predicted by theory, is indeed observed. The diffusion constants obtained from these data were D = (10.9 ± 0.5) × 10⁻⁷ cm²·s⁻¹ for lysozyme, D = (6.2 ± 0.2) × 10⁻⁷ cm²·s⁻¹ for prothrombin, and D = (2.1 ± 0.1) × 10⁻⁷ cm²·s⁻¹ for fibrinogen, which is in agreement with the published values of 11.2 × 10⁻⁷ cm²·s⁻¹, 6.2 × 10⁻⁷ cm²·s⁻¹, and 2.0 × 10⁻⁷ cm²·s⁻¹ respectively. Figure 3B presents similar measurements for factor Xa and factor Va, from which diffusion constants of respectively D = (6.8 ± 0.2) × 10⁻⁷ cm²·s⁻¹ and D = (5.3 ± 0.1) × 10⁻⁷ cm²·s⁻¹ were obtained.

Uniform adsorption rates, independent of surface site and fluid shear rates, are shown for lysozyme in Fig 4. Values of Δ were (15.9 ± 0.6) × 10⁻⁴ cm·s⁻¹ and (15.6 ± 0.6) × 10⁻⁴ cm·s⁻¹ (mean ± SE), at the center and the edge of the slide, respectively.

Transport-limited conversion of prothrombin is shown in Fig 5. To facilitate comparison to the transport-limited prothrombin adsorption rate, the conversion rate is presented as a fractional conversion rate Δ, that is the conversion rate (pmol·cm⁻²·s⁻¹) divided by the bulk concentration of prothrombin (pmol·cm⁻³). The prothrombin converting surface was prepared by incubating the 20%/80% DOPS/DOPC bilayer for 10 minutes in 25 pmol/L factor Va and 50 pmol/L factor Xa at a rotation rate of 78 rad·s⁻¹. Then the cuvette was flushed with fresh buffer to remove the factor Xa and factor Va from the solution and prothrombin conversion was started by addition of 20

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Fig 2. Adsorption of prothrombin on the rotating disc (upper curve) and on a fixed slide opposite a rotating stirrer. The silicon surface of the disc and slide were covered with a 40%/60% DOPS/DOPC bilayer and the prothrombin concentration was 15 nmol/L, and 7.5 nmol/L, respectively, for the experiment with the rotating disc and the slide in front of the stirrer. Experiments were performed at room temperature in Tris-HCl buffer (pH 7.5, 100 mmol/L NaCl and 3 mmol/L CaCl₂) containing 0.8 g·L⁻¹ BSA. The rotation rate of the disc was 30 rad·s⁻¹ and of the stirrer 200 rad·s⁻¹.

Fig 3. (A) The fractional adsorption rate constant Δ, as a function of rotation velocity, for lysozyme (upper curve), prothrombin (middle curve), and fibrinogen (lower curve). (B) Similar plot for factor Xa (upper curve) and factor Va (lower curve). Mean values of five experiments, with SD, are indicated. For prothrombin factor Xa and factor Va adsorptions, the silicon surface was covered with a 40%/60% DOPS/DOPC bilayer and Tris-HCl buffer containing 0.5 g·L⁻¹ BSA was used. For fibrinogen and lysozyme the bare silicon surface was used and the albumin was omitted from the buffer. Temperature was 20° to 22°C.
**Fig 4.** Adsorbed mass of lysozyme as a function of time, measured at the center (upper curve) and the edge (lower curve) of the rotating disc. Mean values ± SD of five experiments are shown. Experimental conditions as in Fig 3. Lysozyme concentration was 1 μg·mL⁻¹ and the rotation rate was 78 rad·s⁻¹.

nmol/L prothrombin. Thrombin conversion rates were determined from the thrombin concentrations in four consecutive samples taken at 1.5-minute intervals and in each experiment, three increasing rotation rates were used. Corrections for the sample volumes taken from the cuvette and for prothrombin consumption were made. Figure 5 shows that the fractional conversion rate as function of ωⁿ/² presents a straight line through the origin indicating transport-limited conversion. This is confirmed by comparison with the prothrombin adsorption rates in Fig 3; identical slopes are found and, correspondingly, the diffusion coefficient D = (6.2 ± 0.3) × 10⁻⁷ cm²·s⁻¹ calculated from the data from Fig 5 is identical to the value obtained for the adsorption experiments.

**Fig 6.** Intermediate kinetics of prothrombin conversion. Mean values ± SE of five experiments are shown. (A) shows the prothrombin conversion rate as function of the prothrombin concentration for two surface concentrations of prothrombinase: 0.14 fmol·cm⁻² (△) and 0.42 fmol·cm⁻² (□). The solid lines represent the regression of equation (3) to the data. The bold upper curve indicates the calculated intrinsic kinetics (see text). Rotation rate of the disc was 78 rad·s⁻¹. (B) shows the dependence on the rotation rate of thrombin production by a disc surface containing 0.46 fmol·cm⁻² prothrombinase. Solution concentration of prothrombin was 20 nmol/L. The solid line represents the prediction [see equation (3)] of the conversion rate. Experimental conditions are as in Fig 5.

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of 0.14 and 0.42 fmol·cm⁻² in Fig 6A were obtained by 3 minutes of incubation with 1 and 3 pmol/L factor Va, respectively. Factor Va adsorption was stopped by flushing the cuvette with buffer and then 10 pmol/L factor Xa was added. After 10 minutes of incubation, prothrombin conversion was started by addition of prothrombin (2 to 64 nmol/L) and the conversion rate was estimated from the thrombin concentration in four consecutive samples taken from the cuvette at 1.5-minute intervals. Thrombin generation was linear, implying complete assembly of prothrombinase within 10 minutes. At a concentration of 10 pmol/L factor Xa, 5 to 10 times the dissociation constant for factor Va-factor Xa complex formation, the prothrombinase activity of lipid-bound factor Va is saturated and this was verified in control experiments with 25 pmol/L factor Xa.

Figure 6A shows that much higher prothrombin concentrations are required to saturate the conversion rate for 0.42 fmol·cm⁻² prothrombinase. This is confirmed by analysis of the uncorrected data with the Michaelis-Menten model, resulting in apparent $K_m$ values of 21.2 nmol/L for 0.42 fmol·cm⁻² versus 9.3 nmol/L for 0.14 fmol·cm⁻² prothrombinase. Despite this difference in apparent reaction kinetics, nearly identical intrinsic reaction kinetics are found by regression of equation (3) to these data: $k_{cat} = 24.2 ± 1.7$ s⁻¹, $K_m = 6.4 ± 1.6$ nmol/L for 0.14 fmol·cm⁻² and $k_{cat} = 24.8 ± 1.3$ s⁻¹, $K_m = 8.0 ± 1.8$ nmol/L for 0.42 fmol·cm⁻² prothrombinase. These differences are not significant and regression of equation (3) to both data sets simultaneously results in the values $k_{cat} = 24.6 ± 1.3$ s⁻¹, $K_m = 7.1 ± 1.2$ nmol/L. Thus, for high-surface activities, depletion of prothrombin near the surface, because of the transport limit, causes substantial differences between intrinsic and apparent conversion rates.

Figure 6B shows rotation-rate dependence of the prothrombin conversion rate at a fixed prothrombin concentration (20 nmol/L). The surface activity of prothrombinase was 0.46 fmol·cm⁻². Fitting equation (3) to the data results in intrinsic values for $k_{cat} = 24.0 ± 2.0$ s⁻¹ and $K_m = 10.6 ± 2.2$ nmol/L (solid line) not significantly different from the values obtained at the constant rotation rate of 78 rad·s⁻¹. Apparently the observed decrease of the prothrombin conversion rates with decreasing rotation rates is explained by the decrease in the mass-transfer coefficient $\Delta$, without major effects of the rotation rate on intrinsic conversion kinetics.

**DISCUSSION**

**Agreement between experiments and theory.** The data in Figs 3 and 4 show that protein adsorption conforms to the theoretical predictions. Figure 5 shows that this is also true for prothrombin conversion on DOPS/DOPC bilayers with an excess prothrombinase. The experimental range of rotation rates allows a variation of the mass-transfer coefficient $\Delta$ 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 invalid by turbulence. This is estimated to occur at Reynolds numbers ($Re$) of $6 \times 10^3$ to $30 \times 10^3$. With $Re = \omega \cdot r^2 \cdot \nu^{-2}$ 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 to 5) × $10^3$ rad·s⁻¹. Therefore, the system still operates in the laminar-flow regime and rotation velocities could be further increased by a factor of at least four.

The adsorption rates are sensitive to temperature. Using the relation $D_T = (T/293)(q/293/T)D_{293}$ between the diffusion constants $D$ and $T$ at absolute temperatures $T$ and $293°K$, and inserting the values for $T = 293°K$ (20°C) and $T = 318°K$ (37°C), it is found that transport-limited adsorption at 37°C proceeds 1.3 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. Even for single-protein adsorption, surface exclusion effects and molecular interaction will modify the adsorption isotherm when the surface coverage exceeds 30% to 50% of its maximal value. The testing of hypothetical mechanisms for these altered adsorption kinetics, that is, an assumed expression for the dependence of $J_{in}(C_{b0})$ in equation (2) on $C_{b0}$ and $\Gamma$, 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 nonlinear 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 nonuniformly 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. Thus, large variations of the transport rate limit along the surface may cause a substantial change of the mass-transfer coefficient during the adsorption and in such situations analysis of intermediate adsorption kinetics requires the numerical solution of the diffusion-convection equation. In principle, the same is true for protein conversion at nonuniformly accessible surfaces, although for low-surface activities and conversion rates below the transport limit, a simplified analysis, based on an overall averaged mass transfer, can be applied on steady-state conversion rates (see also below).

**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 (=5 μm) layer of stagnant fluid, separating the adsorbing surface from a buffer solution with protein concentration $C_{b0}$. Mass transfer through the boundary layer can only be affected 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 (2) in the Materials and Methods with the corresponding equation for the unstirred layer model: \( \frac{d\Gamma}{dt} = \frac{D(c_{\text{bulk}} - C_g)}{d} \), shows the equivalence 1/d = 0.62 \( D^{1/3} \cdot r^{1/6} \cdot \omega^{1/2} \).

**Influence of transport limitation on the measurement of intrinsic adsorption rates.** As shown in the present study, the initial adsorption rates of proteins are often transport-limited. Using the relation \( \frac{d\Gamma}{dt} = k_{\text{ads}} \Gamma_{\text{max}} C_g \) for the intrinsic initial adsorption rate, it can be estimated from equation (2) 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 \( k_{\text{on}} \) does not exceed 4 \( \times 10^8 \text{ mol/L} \cdot \text{s}^{-1} \). For higher values of \( k_{\text{on}} \), this influence will become larger but \( k_{\text{on}} \) may still be estimated from the apparent adsorption rate up to values of about 3 \( \times 10^9 \text{ mol/L} \cdot \text{s}^{-1} \). For still higher values of \( k_{\text{on}} \), the adsorption becomes completely transport-limited and \( k_{\text{on}} \) cannot further be estimated from the adsorption kinetics. Thus, the intermediate range extends between these boundary values of \( k_{\text{on}} \).

Values of \( k_{\text{on}} \) for factor V, prothrombin, and factor Xa, determined by stopped-flow light scattering on lipid vesicles containing about 20% phosphatidylserine, ranged from 2 \( \times 10^5 \text{ mol/L} \cdot \text{s}^{-1} \) to 10\(^6\) \text{ mol/L} \cdot \text{s}^{-1} \). This implies that the initial adsorption of these coagulation factors will be completely transport-limited and \( k_{\text{on}} \) cannot further be estimated from the adsorption kinetics. Thus, the intermediate range extends between these boundary values of \( k_{\text{on}} \).

**Prothrombin conversion on the rotating disc surface.** The values \( k_{\text{off}} = 25 \text{ s}^{-1} \) and \( K_m = 7.1 \text{ nmol/L} \) for the intrinsic conversion rate of prothrombin found in this study are in a reasonably good agreement with the values \( k_{\text{off}} = 33 \text{ s}^{-1} \) and \( K_m = 5.8 \text{ nmol/L} \) presented in our previous report on prothrombin conversion at macroscopic surfaces.

In this study we also found a much higher value for \( K_m \) (170 nmol/L) on lipid vesicles. This marked difference in prothrombin conversion kinetics between planar bilayers and lipid vesicles was explained by analyzing the fluxes of prothrombin to the prothrombinase complex in both situations. It appears that on planar bilayers, the bimolecular rate constant \( k_{\text{off}}/K_m \) exceeds the maximal collisionally limited solution-mediated transport rate of prothrombin to the prothrombinase complex by a factor 3. With the low surface concentration (0.31 fmol \cdot cm\(^{-2}\)) of prothrombinase used in the study, the large (\( \approx 1 \mu^2 \)) lipid area surrounding the prothrombinase apparently collects prothrombin efficiently from the solution and transfers the prothrombin by lateral diffusion on the lipid membrane.

On lipid vesicles the lipid area surrounding prothrombinase is much smaller and the supply of prothrombin is then limited by the adsorption rate to the vesicle, which is proportional to the prothrombin concentration. Given the high value of \( k_{\text{off}} \), this transport easily becomes rate limiting, resulting in an increase of the apparent value of \( K_m \). The values \( K_m = 170 \text{ and } K_m = 42 \text{ nmol/L} \) found for small (20 to 30 nm diameter) and large (60 to 80 nm) vesicles correspond to values of the adsorption-rate constants approximately equal to 5% of the Smoluchowski collision limit.

Equations (1 through 3) offer a sound physical basis for the separation of the effect of the transport limit from intrinsic kinetics. In the earlier study we used a vertical slide in front of a stirrer rotating on the bottom of the cuvette, which is not uniformly accessible with about a fourfold lower adsorption rate at the upper edge of the lipid surface compared with the site behind the center of stirrer. This obviously presents a complication for application of equation (2). Therefore, the average mass transfer coefficient \( D_{\text{avg}} \), determined from transport-limited prothrombin conversion with an excess prothrombinase on the surface, was used for the correction of apparent to intrinsic kinetics. Ellipsometric measurements of prothrombin adsorption were performed at a site about halfway between the upper edge of the stirrer and the upper limit of the lipid-covered area on the slide, where the local mass-transfer coefficient was equal to \( D_{\text{avg}} \). Although this approach lacks theoretical foundation for nonuniformly accessible surfaces and may involve error, for the low prothrombinase activities at the lipid surface this error only represents a second order correction as is apparent from the good agreement between both studies.

**Absence of shear-rate effects on protein adsorption and conversion.** The shear rate at the rotating disc increases linearly with the distance to the center of the disc and in this could present a disadvantage if shear would affect protein adsorption or conversion. As shown in Fig 4, protein adsorption rates measured at the center of the disc (low-shear rate) were not different from the adsorption rates at the edge of the disc (high-shear rate). In experiments of prothrombin conversion the catalytic activity of the entire disc surface is measured, although most surface area is present in the high-shear region. Therefore, a possible shear effect was studied by variation of the rotation rate, ie, the average shear rate. Figure 6 shows that the observed thrombin generation conforms to equation (3) with intrinsic reaction parameters not dependent on the rotation rate and it is concluded that shear has no drastic influence on prothrombin conversion.

**REFERENCES**


23. Fujikawa K, Legaz ME, Davie EW: Bovine factor X(1) and X(2) (Stuart factor) mechanism of activation by a protein from Russell’s viper venom. Biochemistry 11:4892, 1972


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