Prothrombin Contributes to the Assembly of the Factor Va-Factor Xa Complex at Phosphatidylserine-containing Phospholipid Membranes*

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The activation of prothrombin is catalyzed by prothrombinase, a complex of factor Xa and factor Va assembled on a negatively charged phospholipid membrane. We used a tubular flow reactor to identify the relative contributions of factor Va, prothrombin, and the negatively charged phosphatidylserine to the assembly of prothrombinase. Perfusion of phospholipid-coated capillaries with a mixture of factor Xa, factor Va, and prothrombin resulted in a steady-state rate of thrombin production that increased with (i) the phosphatidylserine content of the phospholipid bilayer, (ii) the factor Va concentration, and, most interestingly, (iii) the prothrombin concentration of the perfusion solution. Incorporation of 20 mol % phosphatidylethanolamine, a phospholipid with poor ability to promote prothrombinase activity, into a 5 mol % phosphatidylserine membrane also increased the steady-state rate of thrombin production. Direct measurements of the amount of prothrombinase in the flow reactor demonstrated that increased catalytic activities were the result of an increased steady-state amount of membrane-associated prothrombinase. Thus, similar turnover numbers of prothrombin activation (3100 min⁻¹) were calculated, irrespective of the phosphatidylserine content of the membrane. We established for membranes with low phosphatidylserine content (<10 mol%) a linear relationship between the prothrombinase activity and the arithmetical product of the factor Va concentration in the perfusion solution and the prothrombin concentration near the catalytic surface. Our results indicate that, in addition to factor Va, prothrombin also is essential to the assembly of prothrombinase at macroscopic surfaces with low phosphatidylserine content. The data further suggest that the prothrombin concentration near the surface, controlled by the prothrombinase activity and mass transfer, is an important regulator of the prothrombinase surface density.

Prothrombinase, the enzyme complex that converts prothrombin into thrombin, is composed of the serine protease factor Xa, the protein cofactor factor Va, and phospholipids (1, 2). Kinetic studies have indicated that the reversible protein-phospholipid and protein-protein interactions in the prothrombinase complex all contribute to the 10⁴-fold increase in the catalytic efficiency of factor Xa (3–6). It is generally accepted that the interaction of factor Xa with factor Va enhances the turnover number of factor Xa about 3000-fold (5), and that the interaction of prothrombin with the membrane is responsible for a dramatic decrease in the $K_m$ for prothrombin activation, namely from $\sim 90 \mu M$ in the absence of phospholipid (5) to about 3 $\mu M$ in the presence of a planar membrane (7, 8).

Negatively charged phospholipids are essential constituents of membranes that support prothrombin activation (9, 10). Optimum prothrombinase activity has been reported for membranes that contain at least 10 mol % phosphatidylserine (11–14). It is rationalized that this dependence is the result of the lower binding affinity of membranes with low phosphatidylserine content for the protein constituents of the enzyme complex (factor Xa and factor Va) as well as for its substrate prothrombin (15–17). Consequently, membranes with a low phosphatidylserine content require higher concentrations of fluid phase factor Va to incorporate the same amount of factor Xa into membrane-associated prothrombinase than membranes with a high phosphatidylserine content (11, 18, 19). The reported $K_{cat}$ values, therefore, do not always reflect the true catalytic ability (13, 14).

Because factor Va also interacts with phospholipid-bound prothrombin (20, 21), one cannot a priori exclude the possibility that prothrombin contributes to the stability of the prothrombinase complex. The consequence of such an effect is that increasing amounts of factor Xa will be incorporated into the prothrombinase complex with increasing prothrombin concentrations (22). It has been shown, though, that prothrombin does not contribute to the assembly of prothrombinase at membranes with 25 mol % phosphatidylserine, under conditions that were considered to be physiologically relevant with respect to the factor Va and factor Xa concentrations (19). However, no information is available about the role of prothrombin under conditions that are less optimal for prothrombinase assembly, e.g. low phosphatidylserine content and nonsaturating conditions with respect to reactant concentrations. Such a situation might prevail when coagulation takes place at the surface of activated platelets or other cells in flowing blood (23). Interestingly, a stabilizing effect of a substrate on its membrane-bound cofactor-enzyme complex has been reported, namely that of factor X on the tissue factor-factor VIIa complex (24).

In the present study, we used a tubular flow reactor to assess the contribution of prothrombin to the assembly of prothrombinase and the kinetics of prothrombin activation at low phosphatidylserine membranes. In earlier studies with prothrombinase in a tubular flow reactor, we demonstrated that the kinetics of prothrombinase assembly and the kinetics of thrombin production can be described adequately using a simple model for mass transport for immobilized enzymes (8).
fore, the tubular flow reactor may have some clear advantages over the experimental settings using unilamellar phospholipid vesicles. Firstly, the prothrombin concentration near the catalytic surface can be calculated easily from the flow conditions, the concentration of prothrombin in the bulk solution, and the thrombin production in the flow reactor. Secondly, the amount of prothrombinase in the flow reactor can be estimated readily from the amount of phospholipid-bound factor Xa. This paper reports in detail on the relative contributions of prothrombin, factor Va, and the phosphatidylserine content to the assembly of prothrombinase and their implications for the kinetics of prothrombin activation.

EXPERIMENTAL PROCEDURES

1,2-Dioleoyl-sn-glycero-3-phosphatidylserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), were purchased from Avanti Polar Lipids, Inc. S2238, the chromatographic substrate for thrombin, was obtained from Chromogenix (M¨olndal, Sweden). Bovine serum albumin (BSA) was isolated as described for bovine factor Xa (28). The molar concentration was determined by active site titration with E. hirae carminus venom (Sigma) by active site titration with p-nitrophenyl p’-guanidinobenzoate hydrochloride (26). Human prothrombin was purified as described (25), and the molar concentration was determined after complete activation with E. hirae carminus venom (Sigma) by active site titration with p-nitrophenyl p’-guanidinobenzoate hydrochloride (29). Small unilamellar vesicles of mixed phospholipids were prepared as described before (30). Throughout this paper, the mixed phospholipids are described with their mol % of the negatively charged phospholipid; the remainder of the phospholipid is DOPC.

The Flow Reactor—The capillary flow reactor used in this study has been described (8). Briefly, microcapillary tubes (length of 127 mm and inner diameter of 0.65 mm) obtained from Brand AG (Wertheim, Germany) were cleaned and coated with a bilayer of phospholipids. Thereafter, the phospholipid-coated capillaries were perfused with a reaction mixture containing factor Xa, factor Va, and prothrombin in Tris buffer (50 mM Tris-HCl, 175 mM NaCl, 5 mM CaCl2, pH 7.9, containing 0.5 mg of BSA/ml). Samples (60 μl) were collected at the outlet of the flow reactor into cuvettes containing 500 μl of 50 mM Tris-HCl, 175 mM NaCl, 20 mM EDTA, 0.5 mg of BSA/ml, pH 7.9, and assayed for thrombin.

Assembly of Prothrombinase from Perfusion Mixtures

The concentration of prothrombin in the bulk solution, and the phospholipid-bound factor Xa was determined by active site titration with E. hirae carminus venom (Sigma) by active site titration with p-nitrophenyl p’-guanidinobenzoate hydrochloride (29). Small unilamellar vesicles of mixed phospholipids were prepared as described before (30).

Throughout this paper, the mixed phospholipids are described with their mol % of the negatively charged phospholipid; the remainder of the phospholipid is DOPC.

FIG. 1. Thrombin generation in a tubular flow reactor. Capillaries coated with a phospholipid membrane containing 5 mol % DOPS-95 mol % DOPC (●) and 25 mol % DOPS-75 mol % DOPC (○) were perfused with Tris buffer containing factor Xa (2 pm), factor Va (50 pm), and prothrombin (100 nm). The perfusion was performed at a flow rate of 30 μl/min (wall shear rate of 20 s⁻¹) at 37 °C. The rate of thrombin production is plotted versus the perfusion time.

revealed that the amount of factor Xa bound to the membrane but not complexed with factor Va was negligible. Capillaries with 10 mol % DOPS/90 mol % DOPS were perfused with factor Xa (2 pm) and prothrombin (200 nm) in the absence of factor Va. The total amount of factor Xa in the capillary at the end of the perfusion did not differ significantly from the amount that was present in the fluid phase of the capillary.

Kinetics for the Transport-limited Prothrombin Conversion—The rate of conversion of prothrombin to thrombin by membrane-bound prothrombinase equals the rate at which prothrombin can be supplied from the bulk solution to the catalytic surface (32):

\[ V = \Delta(C_b - C_i) \]  

(Eq. 1)

where \( \Delta \) is the mass transfer coefficient, \( C_b \) is the prothrombin concentration near the catalytic surface, the mass transfer coefficient is a function of the geometry of the capillary, the volumetric flow rate, the kinematic viscosity of the fluid, and the diffusion coefficient of the protein. For a flow rate of 30 μl/min and the capillary (length 12.7 cm and inner diameter of 0.065 cm) used in this study, the mass transfer coefficient, \( \Delta_b \), for prothrombin equals 0.01 cm²/min (cf. Ref. 8).

The maximum rate of prothrombin activation,

\[ V_{max} = \Delta C_b \]  

(Eq. 2)

is thus obtained when the membrane-bound enzymatic activity causes a total depletion of the prothrombin concentration near the surface (\( C_b = 0 \)). For intermediate situations when the rate of prothrombin activation is smaller than the prothrombin mass transfer rate, the prothrombin concentration at the capillary wall can be calculated from Equation 1:

\[ C_b = C_i - \frac{V}{\Delta_b} \]  

(Eq. 3)

RESULTS

The Assembly of Prothrombinase from Perfusion Mixtures Containing Factor Xa, Factor Va, and Prothrombin—Phospholipid-coated capillaries were perfused (30 μl/min) with Tris buffer containing 2 pm factor Xa, 50 pm factor Va, and 100 nm prothrombin. Fig. 1 shows that the rate of thrombin production increased until a steady state was reached of 0.2 pmol/min for the membrane containing 5 mol % DOPS and a maximum of 1 pmol/min for the membrane containing 25 mol % DOPS. Measurement of the amount of factor Xa bound to the phospholipid bilayer in the flow reactor at the end of the perfusion experiments showed that 0.06 fmol and 0.66 fmol of prothrombinase were assembled at the 5 mol % and 25 mol % DOPS membranes, respectively. The turnover numbers of prothrombin activation are thus 2970 min⁻¹ and 1500 min⁻¹, respectively.

1 The abbreviations used are: DOPS, 1,2-dioleoyl-sn-glycero-3-phosphatidylserine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; BSA, bovine serum albumin.

2 The amidolytic assay does not discriminate between thrombin, meizothrombin, and meizothrombin-des-fragment I. Previous work from our laboratory (31) has shown that under the conditions used, the major amidolytic activity is thrombin. Therefore, throughout this paper, the amidolytic activity is referred to as thrombin activity.
The steady-state rate of thrombin formation as a function of the DOPS content of the membrane. Capillaries with membranes of varying DOPS content were perfused as described for Fig. 1. The lower panel gives the steady-state rate of thrombin production (●) and the amount of prothrombinase in the capillary at the time the steady state of thrombin production was obtained (○) as a function of the DOPS content of the membrane. The upper panel gives the turnover number of prothrombin activation calculated from the rate of thrombin production and prothrombinase concentration.

As pointed out under “Experimental Procedures,” the steady-state rate of thrombin production under the conditions of the experiment cannot exceed 1 pmol/min (Equation 2). Therefore, it is reasonable to assume that in the latter case the transport-limited supply of prothrombin causes the lower turnover number. Interestingly, the turnover number measured for the 5 mol% DOPS membrane is close to the transport rate limit of 1 pmol/min. The results obtained for 25 mol% DOPS membrane is close to the turn-off previously reported for 25 mol% phosphatidylserine membranes (8).

Another interesting feature of the flow experiment with the 5 mol% DOPS membrane is that, in spite of a continuous perfusion with a mixture of 2 pm factor Xa, 50 pm factor Va, and 100 nm prothrombin, not more than 0.06 fmol of prothrombinase could be assembled. Based on reported binding parameters for the factor Va-factor Xa complex (cf. Ref. 13), we estimated that the binding capacity of the 5 mol% DOPS membrane is at least 10^4-fold higher. It is apparent that an equilibrium was established between membrane-associated prothrombinase and the reactants in the perfusion solution.

The steady-state rate of thrombin production as a function of the DOPS content of the membrane—Capillaries with phospholipid bilayers that contained varying mol% DOPS (2–10 mol%) were perfused with factor Xa (2 pm), factor Va (50 pm), and prothrombin (100 nm). The perfusion was continued until a steady-state rate of thrombin production was reached. At the end of the perfusion, we measured the amount of prothrombinase present in the capillary. Fig. 2 shows the relationship between the rate of thrombin production and the amount of prothrombinase as a function of the DOPS content of the membrane. It is apparent that both the steady-state rate of thrombin production and the amount of prothrombinase increased equally with the DOPS content of the membrane. As a result, the turnover number (3100 min^-1) did not change with the DOPS content of the membrane. We note that in all experiments the steady-state rate of thrombin production was below the transport-limited rate of 1 pmol/min. The results obtained so far indicate that if the thrombin production reaches a steady state, the amount of prothrombinase in the flow reactor is in equilibrium with the reactants in the perfusion solution. It is concluded that under these conditions the thrombin production is proportional to the amount of prothrombinase assembled at the phospholipid-coated capillary wall.

To examine the contribution of factor Va to the assembly of an equilibrium concentration of prothrombinase in the flow reactor, phospholipid (5 mol% DOPS)-coated capillaries were perfused with Tris buffer containing 2 pm factor Xa, 100 nm prothrombin, and varying concentrations of factor Va (0.01–2 nm) until a steady-state rate of thrombin production was reached. The steady-state rate of thrombin production increased with the factor Va concentration in the perfusion solution until a maximum of 0.9 pmol/min (Fig. 3). This value is close to the transport rate limit of 1 pmol of thrombin/min.

The steady-state rate of thrombin production increased with increasing prothrombin concentrations (50, 100, and 200 nm). The steady-state rate of thrombin production increased with increasing prothrombin concentrations in the perfusion solution and with increasing DOPS content of the membrane (Table I). It is important to note that the observed rates of thrombin production were well below the transport-limited rate of thrombin conversion. The increase of the steady-state rates of thrombin production with increasing prothrombin concentration and increasing DOPS content was due to an increased assembly of prothrombinase (Table I). It appeared that the turnover numbers calculated from the rate of thrombin production and the prothrombinase content of the flow reactor did not vary significantly with the prothrombin concentration and the DOPS content of the membrane. As a matter of fact, the turnover numbers thus obtained were close to the values reported earlier (8) for membranes with 25 mol% phosphatidylserine.

The steady-state rate of thrombin production as a function of the prothrombin and factor Va concentration—We already...
Effect of prothrombin and lipid composition on the assembly and catalytic activity of prothrombinase

| Phospholipid composition | Prothrombin concentration | Rate of thrombin production | Membrane-associated prothrombinase | Turnover rate |
|--------------------------|---------------------------|----------------------------|------------------------------------|--------------|
|                          | nm                        | pmol/min                   | mol                                | min^-1       |
| PSPC 5:95                | 50                        | 0.09                       | 0.024                              | 3700         |
|                          | 100                       | 0.19                       | 0.055                              | 3400         |
|                          | 200                       | 0.31                       | 0.11                               | 2800         |
| PSPC 6:94                | 50                        | 0.10                       | 0.026                              | 3800         |
|                          | 100                       | 0.45                       | 0.09                               | 3000         |
|                          | 200                       | 0.59                       | 0.21                               | 2800         |
| PSPC 7:93                | 50                        | 0.13                       | 0.033                              | 3900         |
|                          | 100                       | 0.36                       | 0.14                               | 2600         |
|                          | 200                       | 0.85                       | 0.28                               | 3000         |
| PSPC 8:92                | 50                        | 0.25                       | 0.1                                | 2500         |
|                          | 100                       | 0.46                       | 0.14                               | 3200         |
|                          | 200                       | 0.90                       | 0.31                               | 2900         |
| PSPC 10:90               | 100                       | 0.87                       | 0.29                               | 3000         |
|                          | 200                       | 1.44                       | 0.45                               | 3200         |

*PSPC, phosphatidylserine/phosphatidylcholine.

The Effect of Phosphatidylethanolamine on the Assembly of Prothrombinase—Natural membranes contain relatively large amounts of phosphatidylethanolamine (DOPE), a phospholipid that by itself does not stimulate prothrombin activation, but that might modulate the procoagulant activity of phosphatidylserine (33). To investigate the contribution of DOPE to the kinetics of thrombin production, we incorporated 20 mol %
DOPE in the phospholipid bilayer of the flow reactor that contained 5 mol % DOPS and perfused the capillary with 2 pM factor Xa, 50 nM factor Va, and varying prothrombin concentrations (50–100-200 nM). Fig. 7 gives the steady-state thrombin production in capillaries coated with membranes of 5 mol % DOPS-95 mol %DOPC, 5 mol %DOPS-20 mol %DOPE-75 mol % DOPC, and 25 mol % DOPS-75 mol % DOPC as a function of the prothrombin concentration in the perfusion solution. As expected, the lowest steady-state rate of thrombin production was observed with 5 mol % DOPS membranes. The thrombin production at membranes containing 25 mol % DOPS was close to the transport-limited rate as predicted by mass transfer coefficient and prothrombin concentration. Incorporation of 20 mol % of DOPE in the 5 mol % DOPS membrane apparently resulted in an increase of the steady-state rate of thrombin production. The rate of prothrombin activation became indistinguishable from those seen with 25 mol % DOPS. Membranes that contained only DOPE as the anionic phospholipid, e.g. 20 mol % DOPE/80 mol % DOPC, did not support prothrombin activation.

DISCUSSION

The relative contributions of protein-phospholipid interactions and protein-protein interactions to the overall stability of the prothrombinase complex and their consequences for the catalytic efficiency of the enzyme have been studied extensively (14, 34, 35 and references therein). The experimental design with a flow reactor, as presented here, enables, however, a straightforward study of prothrombinase assembly and kinetics of prothrombin activation. The clear advantage over a system with small unilamellar phospholipid vesicles is the readily controlled delivery of the individual components of the prothrombinase complex, factor Xa and factor Va, and the substrate prothrombin to the planar (macroscopic) phospholipid bilayer and, most importantly, the possibility of a direct determination of the amount of prothrombinase assembled on the macroscopic surface.

Initially, we wanted to determine the kinetics of prothrombin activation by known amounts of assembled prothrombinase as a function of the DOPS content of the membrane as described previously (8). However, pilot experiments revealed that perfusion of capillaries with mixtures of factor Va and factor Xa, in case the membrane contained a low DOPS content, resulted in surprisingly low and unstable levels of membrane-associated prothrombinase. In contrast, when prothrombin was added to the factor Xa-factor Va mixtures, increasing amounts of thrombin appeared at the outlet of the capillary until a steady-state was reached. We hypothesized that, with decreasing affinity of factor Va and factor Xa for DOPS membranes, prothrombin by virtue of its affinity for both factor Va and factor Xa significantly promotes the stability of the prothrombinase complex (cf. Refs. 15-17 and 36).

To verify our notion, we examined the assembly of prothrombinase at membranes with varying DOPS content from perfusion solutions that contained factor Xa (2 pM), factor Va (50 nM), and varying concentrations of prothrombin. We observed in all perfusion experiments that the thrombin production in the flow reactor reached a steady-state rate well below the transport limit. From direct prothrombinase measurements we learned that the amount of membrane-associated prothrombinase in the flow reactor dramatically decreased with the DOPS content of the membrane. Increasing prothrombin concentrations in the perfusion solution, however, did increase the membrane-bound amount of prothrombinase. Although the turnover values, calculated from the steady-state rate of thrombin production and the corresponding amount of prothrombinase (Fig. 2 and Table I), varied between 2500 and 3900 min⁻¹, it is reasonable to conclude that no gross differences were observed for the catalytic activities of the prothrombinase assembled at the membranes that varied in DOPS content from 2 to 10 mol % and prothrombin concentrations that varied between 50 and 200 nM. The turnover numbers obtained here are close to the previously reported kcat value of 3600 min⁻¹ for 25 mol % phosphatidylserine membranes (8).

The observation that similar kinetics of thrombin production were found with a limited steady-state amount of membrane-associated prothrombinase indicates that the prothrombinase complex remains assembled only at the membrane of the flow reactor when it is fully occupied with its substrate prothrombin. That is, prothrombinase that is not acting on its substrate will dissociate from the membrane. Consequently, under these conditions, the kinetic parameter K_{nv} defined as the prothrombinase density at membranes with varying DOPS on the arithmetical product of the factor Va concentration in the perfusion solution and the prothrombin concentration near the surface. Capillaries with 2, 5, and 10 mol % DOPS membranes were perfused with 2 pM factor Xa, 5-100 pM factor Va, and 50-200 nM prothrombin. The amounts of prothrombinase at the steady-state phase of thrombin production and the prothrombin concentration near the surface were calculated from the steady-state rates of thrombin production and prothrombin concentration in the perfusion solution, respectively. Further details are given in the text.
bin concentration that is required to obtain half-saturation of the enzyme, loses its meaning.

Regarding the relative contributions of factor Va and prothrombin to the stability of the complex, we present evidence that with a constant amount of factor Xa in the perfusion solution, the amount of membrane-associated prothrombinase is proportional to the arithmetical product of the factor Va concentration in the perfusion solution and the prothrombin concentration close to the catalytic surface (Fig. 5). The proportionality constant of this relationship, which probably reflects the protein-protein and protein-phospholipid affinities, decreases 2000 fold when the DOPS content of the membrane increases from 2 to 10 mol%. Only a 10-fold difference was seen between 5 mol% and 10 mol% DOPS membranes.

Prothrombinase-catalyzed conversion of prothrombin into thrombin is a multistep reaction rather than a single product (thrombin) reaction. It has been reported (37) that the prothrombin concentration and the composition of the phospholipid membrane has an effect on the relative amounts of thrombin and meizothrombin formed. That is, higher prothrombin concentration increases 2000 fold when the DOPS content of the membrane increases from 2 to 10 mol%. Only a 10-fold difference was seen between 5 mol% and 10 mol% DOPS membranes.

Accordingly, phosphatidylethanolamine is an important regulator of the procoagulant activity in membranes with low phosphatidylserine content. It is apparent that only for low DOPS membranes with limiting amounts of factor Va and prothrombin, DOPE contributes to the assembly of prothrombinase which in turn results in an increased catalytic efficiency of prothrombin activation.

In summary, studies on the prothrombinase assembly from a perfusion solution to planar phospholipid membranes that contained varying mol% DOPS have provided evidence that the steady-state prothrombinase surface density is a function of the prothrombin concentration. Because the prothrombin concentration near the surface is controlled by convection and diffusion and the catalytic activity of that surface, prothrombinase seems to regulate its assembly via the prothrombin concentration near the surface.

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