Proc. Nat. Acad. Sci. USA
Vol. 70, No. 5, pp. 1344–1348, May 1973

A Polypeptide Region of Bovine Prothrombin Specific for Binding to Phospholipids

(activation fragment/lipid-protein interaction/lipid-binding site/surface catalysis/factor X)

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Communicated by P. Roy Vagelos, February 20, 1973

ABSTRACT **The blood-clotting protein prothrombin can be converted to thrombin in free solution by the proteolytic enzyme, activated factor X. When prothrombin is bound to the surface of phospholipid vesicles, the rate of thrombin generation is increased more than 30-fold over the rate of unbound prothrombin. If the prothrombin activation process is terminated after a time interval in which less than 10% of the expected thrombin has been produced, two major products are found in the activation mixture. These products have been termed intermediate 1, a precursor of thrombin, and fragment 1. The conversion of intermediate 1 to thrombin is not accelerated by phospholipid nor can binding of this intermediate to phospholipid particles be demonstrated. In contrast, fragment 1, the other activation product derived from prothrombin, binds to phospholipid particles under the same conditions as prothrombin. On the basis of these observations, we propose that the prothrombin molecule contains a specific region of polypeptide chain for binding to phospholipid particles. This specific polypeptide region or lipid interaction site is part of the nonthrombin-forming activation fragment.**

The conversion of prothrombin to thrombin in the blood coagulation process is markedly accelerated in the presence of phospholipid vesicles (1–3). Both prothrombin (3, 4) and activated factor X (the enzyme catalyzing the proteolyses involved in thrombin formation) (5) bind to phospholipid particles. This binding requires divalent cations and is reversible by removal of the divalent cations. In contrast to prothrombin, the final active product of the activation process, thrombin, does not bind to phospholipid surfaces (3, 4).

Investigations into the prothrombin activation mechanism have demonstrated the existence of at least two intermediates between prothrombin and thrombin and two activation fragments (6–10). As the relationship of some of the products described in earlier investigations to those seen by sodium dodecyl sulfate–acrylamide gel electrophoresis (8–10) is not clearly established, no simple or universally accepted way of describing the activation intermediates and fragments exists. Intermediates are defined here as partial activation products that can be further activated to yield thrombin. These intermediates, which have been defined principally on the basis of the order of their appearance during prothrombin activation by use of Na dodecyl sulfate–acrylamide gel electrophoresis (7–10), are phenomenological intermediates and not necessarily kinetic intermediates in the activation process under all conditions. Activation fragments are, in contrast, those products that are not precursors of thrombin.

The phenomenological scheme (Owen, W. G., Esmon, C. T. & Jackson, C. M., manuscript in preparation)† is summarized as follows:

Prothrombin → Intermediate 1 + Fragment 1
Intermediate 1 → Intermediate 2 + Fragment 2
Intermediate 2 → Thrombin

Intermediate 1 and fragment 1 can be formed by the action of thrombin on prothrombin in both the presence and absence of factor Xa; however, factor Xa is required to form thrombin from prothrombin or either of the intermediates. Interaction between phospholipid vesicles and the activation products from partial activation of prothrombin with factor Xa were investigated in this study.

MATERIALS AND METHODS

Oleic acid (>99% purity), phospholipase D, bovine-serum albumin, Russell's viper (*Vipera russellii*) venom, and Tris·HCl were obtained from Sigma Chemical Co. DEAE-Sephadex was a product of Pharmacia, and Bio-Gel A 0.5 m was obtained from Bio Rad Laboratories. Dialysis tubing was a product of Union Carbide Corp., and silica gel GF-254 was a product of E. Merck Darmstadt. Preparative thin-layer chromatography (TLC) plates were obtained from Analtech Inc. All common laboratory chemicals were of analytical reagent grade and were obtained from major domestic supply houses. Bovine brain "cephalin" was prepared by the procedure of Folch (11).

Bovine factor X was purified (12) and activated with the factor X coagulant protein from Russell's viper venom (13). Factor Xa was separated from the venom enzyme by chromatography on DEAE-Sephadex A50 (14).

Prothrombin was isolated from bovine plasma by a modification of the procedure of Cox and Hanahan (15). Intermediate 1 and fragment 1 were prepared by incubation of prothrombin with either thrombin or factor Xa. The isolated products prepared with either protease were indistinguishable by disc electrophoresis, Na dodecyl sulfate–gel electrophoresis, and aminoacid composition. The intermediates and fragments were isolated by chromatography on DEAE-Sephadex A50 or QAE-Sephadex Q50. All proteins were homogeneous as determined by sedimentation equilibrium

Abbreviations: factor Xa, factor X that has been activated by the factor X activating enzyme of Russell's viper venom; TLC, thin-layer chromatography.

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† This phenomenological scheme agrees completely with the scheme presented recently by Mann, K. G., Heldebrant, C. M., Fass, D. N., Bajaj, S. P. & Butkowski, R. J., 21st Annual Symposium on Blood, Wayne State University, Detroit, Mich.

and acrylamide gel electrophoresis at pH 9.5, with the system described by Ornstein (16) and Davis (17), and in Na dodecyl sulfate–acrylamide gel electrophoresis, with the system described by Laemmli (18). Dioleoyl lecithin was synthesized from oleic anhydride (19) and glycerophosphorylcholine (20) by the method of Cubero Robles and Van Den Berg (21). The lecithin was purified by chromatography on silicic acid (22) and Al_2O_3 (23). The product was homogeneous by TLC on silica gel G with $CHCl_3$–MeOH–H_2O 6.5:2.5:0.4 (v/v/v) solvent (24).

Dioleoylphosphatidylglycerol was synthesized from dioleoyl lecithin with phospholipase D (25) and isolated by preparative TLC with a solvent mixture of $CHCl_3$–MeOH–7 M NH_3 12:4.5:1 (v/v/v). The product was eluted from the silica gel with $CHCl_3$–MeOH–H_2O 10:20:1 (v/v/v). The final product chromatographed as a single spot on TLC with $CHCl_3$–MeOH–7 M NH_3 115:45:8 (v/v/v) as the developing system (26). The ratio of glycerol (27) to phosphate (28) in the synthetic dioleoylphosphatidylglycerol was 1.9:1 (theoretical 2:1).

Dioleoylphosphatidylethanolamine was also synthesized from dioleoyl lecithin with phospholipase D (25). This compound was isolated by preparative TLC with the solvent systems described above for phosphatidylglycerol. The final product was homogeneous by TLC.

Phospholipid concentrations were obtained from total phosphate determinations (28).

Protein concentrations were determined by the procedure of Lowry *et al.* (29), by absorbance at 280 nm, and by ninhydrin assay after alkaline hydrolysis (30, 31). Prothrombin and activation intermediates were assayed after conversion to thrombin, with Taipan snake venom and phospholipid (32). The thrombin was assayed by its ability to clot bovine fibrinogen prepared by the procedure of Straughn and Wagner (33). Fibrinogen was used as a 2 mg/ml solution in 10 mM Tris·HCl (pH 7.5). Thrombin activity was quantitatively defined from a calibration curve constructed with NIH standard thrombin, lot 3B.

Preparation of Phospholipid Dispersions. Dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol were dissolved in $CHCl_3$. The solutions were mixed such that the mole ratio of the phosphatidylglycerol to the phosphatidylcholine was 1:1. Phospholipid dispersions were prepared by addition of 1 ml of the appropriate buffer for the binding experiment to an ampoule containing 4 mg of phospholipid from which the $CHCl_3$ had been removed under a stream of N_2. The mixture was shaken on a Vortex mixer for several minutes to obtain a coarse suspension. A stream of nitrogen was bubbled through the suspension for 40 min before the ampoule was sealed under a positive nitrogen pressure. The solution in the sealed ampoule was dispersed with a Branson model 2 ultrasonic cleaning bath at 20°. A sonication time of 20–40 min was adequate to produce a visually clear dispersion. Under these conditions no degradation of the phospholipids could be detected by TLC with the previously described solvents.

Lipid–Protein Binding Technique. Protein binding was determined by published methods (4, 5). In this investigation all studies were performed with a 0.9 × 30-cm column of Biogel Agarose A 0.5 m equilibrated with Tris·HCl buffer (pH 7.2) containing $CaCl_2$ at the concentration stated in the particular experiment. Samples of protein and phospholipid were mixed, and an aliquot of the mixture was applied to the column. Cochromatography of protein and phospholipid constitutes the criterion of binding. The particular conditions of each experiment are given in the corresponding figure legends. Phospholipid recoveries from the columns were 80–100%; and protein or activity recoveries were 60–90%. Both protein and biological activity were determined in the experiments with prothrombin, intermediate 1, and thrombin.

RESULTS

Effect of Phospholipids on the Rate of Thrombin Formation from Prothrombin and Intermediate 1. Prothrombin or intermediate 1 was incubated with the phospholipid dispersion and factor Xa at 28°. Aliquots of the reaction mixture were removed at intervals of several minutes and assayed for thrombin activity. The final concentrations of the various components in each experiment are given in the legends to the corresponding figures.

The reaction time courses for the generation of thrombin from prothrombin in the presence of various amounts of phospholipid are shown in Fig. 1. The effect of phospholipid on the formation of thrombin from intermediate 1 is shown in Fig. 2. Unlike prothrombin, the rate of conversion of intermediate 1 to thrombin is unaffected by phospholipid. No exceptions to these observations have been found with a wide range of both phospholipid and factor Xa concentrations.

Binding of Prothrombin, Intermediate 1, Fragment 1, and Thrombin to Phospholipid. The acceleration of prothrombin

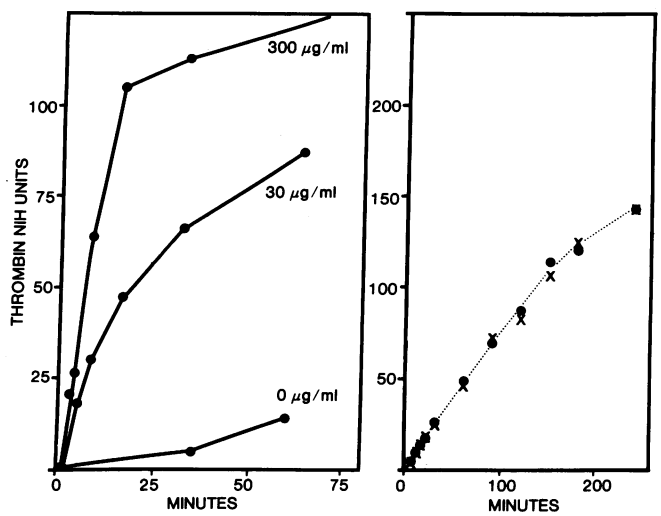

FIG. 1 (*left*). Effect of phospholipid on the rate of thrombin generation from prothrombin. Prothrombin (0.3 mg/ml) was incubated with factor Xa (40 μg/ml) and phospholipid (as specified in the figure). Phospholipid was an equimolar mixture of phosphatidylcholine and phosphatidylglycerol. The reactions were performed at 28° in a buffer containing 20 mM Tris·HCl (pH 7.5)–100 mM NaCl–10 mM $CaCl_2$. Thrombin activity, ●——●.

FIG. 2 (*right*). Rate of activation of intermediate 1 in the presence and absence of phospholipid. Intermediate 1 (0.3 mg/ml) was incubated with factor Xa (20 μg/ml). In the reaction mixture containing phospholipid, the concentration of the equimolar mixture of phosphatidylcholine and phosphatidylglycerol was 30 μg/ml. The reactions were performed at 28° in 20 mM Tris·HCl (pH 7.5)–100 mM NaCl–10 mM $CaCl_2$. Phospholipid present, ●; phospholipid absent, ×.

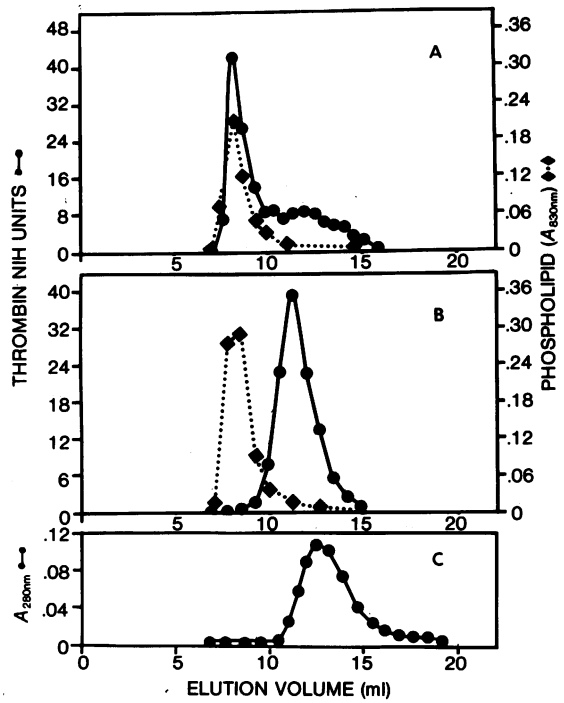

FIG. 3. Binding of prothrombin to phospholipid. (*A*) Equal volumes of prothrombin (1.2 mg/ml) and phosphatidylcholine/phosphatidylglycerol (4 mg/ml) in 40 mM Tris·HCl (pH 7.2)–70 mM NaCl–20 mM $CaCl_2$–0.02% NaN_3 were mixed and allowed to equilibrate for 30 min. A sample of this mixture (0.25 ml) was applied to the Biogel A 0.5 column, and 0.5-ml fractions were collected from the column. Thrombin activity, ●——●; phospholipid, ◆···◆. (*B*) All conditions were the same as in (*A*) except the buffer contained no $CaCl_2$ and, instead, contained 1.5 mM EDTA. Thrombin activity, ●——●; phospholipid, ◆···◆. (*C*) Prothrombin alone, 0.25 ml of a 1.2 mg/ml solution was applied to the column, which was equilibrated with 40 mM Tris·HCl (pH 7.2)–70 mM NaCl–5 mM $CaCl_2$–0.02% NaN_3. Absorbance at 280 nm, ●——●.

activation as a consequence of its binding to phospholipid particle surfaces has been suggested (1, 4). The absence of an effect of phospholipid on the rate of conversion of intermediate 1 that has been demonstrated here could be explained if, in contrast to prothrombin, intermediate 1 did not bind to the phospholipid. Loss of binding ability during some stage in the activation was suggested from the observation that the final product, thrombin, does not bind to phospholipid (3, 4). The possibility that the capacity of prothrombin to bind to phospholipid vesicles disappears upon its conversion to intermediate 1 was tested by direct binding studies. Fig. 3*A* demonstrates the binding of prothrombin to vesicles prepared from an equimolar mixture of dioleoyl lecithin and dioleoylphosphatidylglycerol. These data confirm and extend the observations previously reported by others that prothrombin binds to phospholipids (3, 4). The particular phospholipid mixture that has phosphatidylglycerol as the acidic lipid has not heretofore been used to our knowledge, thus necessitating its examination. Its ability to function was predicted, however, on the basis of earlier work (34–37). Fig. 3*B* confirms the Ca^{2+} ion requirement for binding, and Fig. 3*C* demonstrates that the cochromatography of prothrombin with phospholipid is not an artifact of prothrombin aggregation in the presence of Ca^{2+} ions.

Intermediate 1 was found not to bind to the phospholipid vesicles, and it appears that this inability to bind can account for the failure to observe an effect of phospholipid on thrombin formation from intermediate 1. The results of the binding experiment are shown in Fig. 4*A*. As an additional control, thrombin–phospholipid binding was also investigated with the phosphatidylglycerol and phosphatidylcholine mixture. In agreement with the results obtained with other phospholipids (3, 4), no binding could be demonstrated (Fig. 4*B*).

It appears from these data that the absence of an effect of phospholipid on the rate of conversion of intermediate 1 to thrombin is related to the conversion of prothrombin from a protein capable of binding phospholipid to a species that has lost this capacity.

On the basis of the phenomenological mechanism described earlier, the above observations suggest that the fragment produced from prothrombin concomitant with intermediate 1 might be responsible for the binding of prothrombin to the lipid surface. The results of the fragment 1–phospholipid binding experiments are shown in Fig. 5*A*. The appropriate control for a Ca^{2+}-free buffer is shown in Fig. 5*B*, and the demonstration that cochromatography of fragment 1 and phospholipid is not due to aggregation of fragment 1 in the presence of Ca^{2+} is shown in Fig. 5*C*. Fragment 2, the other activation fragment, does not bind to phospholipid vesicles (data not shown).

Two additional considerations are required to establish the general significance of these results. First, binding of prothrombin and fragment 1 to phospholipid should occur not only at the optimum Ca^{2+} concentration for the activation process, i.e., 10 mM (10), but should be demonstrable at concentrations comparable to those of plasma, i.e., 2 mM (38). Table 1 demonstrates the protein-binding relationships for a range of Ca^{2+} concentrations. Second, in view of the earlier work that demonstrated that a variety of phospholipids function in the clotting process, it was necessary to establish

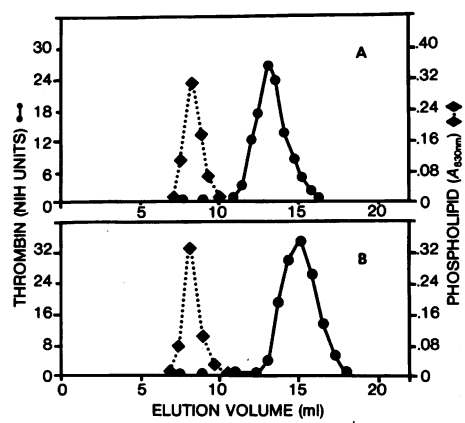

FIG. 4. Neither intermediate 1 nor thrombin binds to phospholipid. (*A*) Equal volumes of intermediate 1 (1.5 mg/ml) and phosphatidylcholine/phosphatidylglycerol (4 mg/ml) were mixed and allowed to equilibrate for 30 min. 0.25 ml was applied to the Biogel column. The sample and column were equilibrated in 40 mM Tris·HCl (pH 7.2)–70 mM NaCl–10 mM $CaCl_2$–0.02% NaN_3; 0.5-ml fractions were collected. Thrombin activity, ●——●; phospholipid, ◆···◆. (*B*) Equal volumes of thrombin (0.4 mg/ml) and phosphatidylcholine/phosphatidylglycerol (4 mg/ml) were mixed, and 0.25 ml was applied to the column. All other conditions were the same as in (*A*). Thrombin activity, ●——●; phospholipid, ◆···◆.

that binding occurs with vesicles prepared from other types of phospholipids. Table 1 contains the data obtained from experiments on the binding of prothrombin and fragment 1 to mixtures of synthetic phosphatidylethanolamine and phosphatidylglycerol and to a preparation of crude bovine brain "cephalin."

DISCUSSION

The ability of phospholipid to increase the rate of thrombin formation from prothrombin appears to arise as a direct consequence of the binding of prothrombin to phospholipid particles. Two primary types of evidence for this conclusion have been presented: (*i*) the loss of the accelerating effect of phospholipid on the conversion of intermediate 1 to thrombin concomitant with the loss in ability of intermediate 1 to bind to phospholipid, and (*ii*) the direct demonstration that fragment 1, a nonthrombin-forming activation product, binds to phospholipid under the same conditions as prothrombin. On the basis of these observations, we suggest that binding of prothrombin to phospholipid occurs as a consequence of the binding capacity of a specific portion of the prothrombin polypeptide chain.

TABLE 1. *Protein binding to phospholipid vesicles**

Protein	Phospholipid†	$CaCl_2$ (mM)	Protein bound‡
Prothrombin	PG/PC	0	0
		2	103
		5	97
		10	153
		20	200
	PG/PE	5	83
	Cephalin (PS/PE)	5	330
Intermediate 1	PG/PC	5	<1
		10	<1
	PG/PE	5	<1
Fragment 1	PG/PC	0	0
		2	111
		5	61
		10	158
	PG/PE	5	131
	Cephalin (PS/PE)	5	300
Thrombin	PG/PC	10	0

* All binding experiments were performed at 23–25°. Equal volumes of protein and phospholipid (4 mg/ml) were mixed and permitted to equilibrate for 30 min. A sample of the particular mixture (0.25 ml) was then applied to the Biogel A 0.5 m column (0.9 × 30 cm), and 0.5-ml fractions were collected. The phospholipid type and $CaCl_2$ concentration is given in the table; zero $CaCl_2$ is 1.5 mM EDTA. Prothrombin was used at a concentration of 1.2 mg/ml, intermediate 1 at 1.5 mg/ml, and thrombin at 0.4 mg/ml. The Biogel columns were equilibrated with 40 mM Tris·HCl (pH 7.2)–70 mM NaCl–0.02% NaN_3. Fragment 1 was used at a concentration of 1.5 mg/ml, and the Biogel and protein were equilibrated with 4 mM Tris·HCl (pH 7.2)–106 mM NaCl–0.02% NaN_3.

‡ Binding is expressed as NIH units of thrombin per μmol of phospholipid for prothrombin, intermediate 1, and thrombin. For fragment 1, the binding is μg of protein per μmol of phospholipid.

† *PC*, phosphatidylcholine; *PG*, phosphatidylglycerol; *PS*, phosphatidylserine; *PE*, phosphatidylethanolamine.

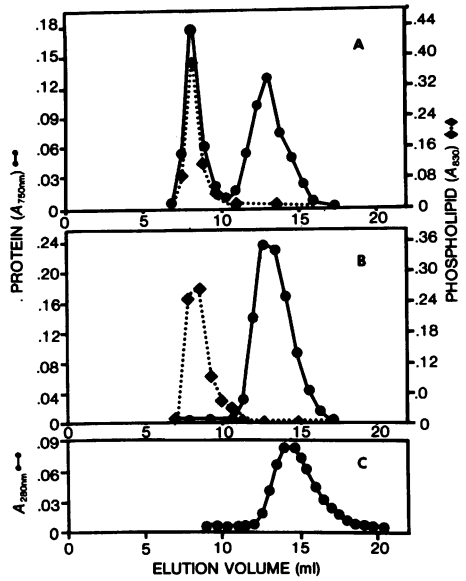

FIG. 5. Binding of fragment 1 to phospholipid. (*A*) Equal volumes of fragment 1 (1.5 mg/ml) and phosphatidylcholine/phosphatidylglycerol (4 mg/ml) were mixed and allowed to equilibrate for 30 min. 0.25 ml was applied to the Biogel column. The sample and column were equilibrated with 4 mM Tris·HCl (pH 7.2)–106 mM NaCl–2 mM $CaCl_2$–0.02% NaN_3. Protein (absorbance at 750 nm), ●——●; phospholipid, ◆···◆. (*B*) All conditions were the same as in (*A*) except the equilibration buffer contained no $CaCl_2$ and, instead, contained 1.5 mM EDTA. Protein (absorbance at 750 nm), ●——●; phospholipid, ◆···◆. (*C*) Fragment 1 (0.25 ml) in 4 mM Tris·HCl (pH 7.2)–106 mM NaCl–5 mM $CaCl_2$–0.02% NaN_3 was applied to the column, which was equilibrated with the same buffer. Protein (absorbance at 280 nm), ●—— ●.

The demonstration that fragment 1 binds to phospholipid after cleavage from prothrombin further suggests that the lipid interaction site(s) is determined by the primary structure of this polypeptide fragment and is not related to structural features that require a complete, intact prothrombin molecule. The possibility that the association constant for binding of fragment 1 to phospholipid is quantitatively altered when fragment 1 is part of the prothrombin polypeptide chain cannot be ruled out by the type of binding studies done to date. The recent report by Lux *et al.* (39) on a lipid-binding fragment from apo high density lipoprotein (ApoLP-Gln-II) and the description of a highly apolar polypeptide from cytochrome b_5 (40, 41) suggests that specific lipid-binding regions of polypeptide chains may be a relatively general phenomenon.

Aminoacid analysis of fragment 1 (Owen, Esmon, and Jackson, manuscript in preparation) indicates a relatively high proportion of both acidic residues and apolar residues. The actual mole ratios of (Glu + Asp)/(Val + Ile + Leu + Tyr + Phe)/(Lys + His + Arg) are found to be 1.8/1.6/1. The high acidic residue composition is not surprising in view of the Ca^{2+} requirement for binding; the relatively high apolar residue content suggests that more than electrostatic interactions may be involved.

The chemical mechanism responsible for the increase in

rate of thrombin formation from prothrombin when prothrombin is bound to a lipid surface is unknown. The recent proposal by Stenn and Blout (9) that thrombin generation from prothrombin can occur by two separate reaction paths suggests one possible explanation. Attempts in this laboratory to obtain evidence for an alternative reaction path have not been successful, although this result may reflect only the inability to perform a truly unambiguous experiment. It is also possible that the order in which the peptide bonds in prothrombin are cleaved differs when prothrombin is bound to lipid surface. If this is so, the kinetic mechanism will differ from the phenomenological mechanism, which is based primarily on mass balance. Another possibility is that local concentration and orientation effects may be responsible for the increased rate when the proteins are adsorbed to the phospholipid surface. Unfortunately, this last possibility may be very hard to test in view of the limited techniques now known for following the prothrombin activation process.

Bovine factor X, the zymogen of the protease responsible for prothrombin activation, is made up of two disulfide-linked polypeptide chains (42). The heavy chain of factor X, which is the chain containing the active site in factor Xa (43), possesses an aminoacid composition strikingly similar to that of thrombin (42). The light chain of factor X has an aminoacid composition similar to that of fragment 1 (Jackson, unpublished observations). Since factor Xa binds to phospholipid (5), it is possible that the light chain of factor X may be involved in lipid binding.

Prothrombin isolated from cows fed the vitamin K antagonist, dicoumarol, is activated extremely slowly in comparison to prothrombin from normal animals (44, 45). However, with nonphysiological activators, the thrombin formed from this "abnormal prothrombin" is indistinguishable in its catalytic activity from the "normal" thrombin (45). A recent report by Nelsestuen and Suttie (46) has demonstrated a striking difference in Ca^{2+} binding between the two prothrombins, whereas no other chemical differences could be found (47). In view of the Ca^{2+} requirement for binding of prothrombin and fragment 1 to lipid and the lipid effect on prothrombin activation rate described here, it becomes interesting to ask if vitamin K action, prothrombin activation rate, calcium binding to prothrombin, and lipid acceleration of prothrombin activation are related phenomena. If this is found to be so, the chemical function of vitamin K may be more readily determinable.

We thank Drs. George Drysdale, P. Roy Vagelos, and David Silbert for their many helpful discussions and comments. This work was supported by the National Heart and Lung Institute, HL-12820-03, HL-14147-01-02, and HL-52357-01.

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