Regulation of Activated Protein C by Protein S

**THE ROLE OF PHOSPHOLIPID IN FACTOR Va INACTIVATION***

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Protein S enhances the rate of Factor Va inactivation by activated Protein C (Walker, F. J. (1980) J. Biol. Chem. 255, 5521-5524). The activity of Protein S is saturable, appearing to interact stoichiometrically with activated Protein C. Diisopropylphosphate-modified activated Protein C reversed the effect of Protein S, further indicating that a Protein S-activated Protein C interaction is required for expression of the activity of Protein S. In the absence of phospholipid, Protein S had no effect on the rate of activated Protein C-catalyzed inactivation of Factor Va. The activity of Protein S was only expressed in the presence of phospholipid vesicles, where it appeared to increase the affinity of the inactivation system for phospholipid. Protein S had no effect upon the rate of Factor Va inactivation in the presence of saturating levels of phospholipid vesicles.

The effects of Protein S on the kinetics of Factor Va inactivation corresponded with its effect on the interaction between activated Protein C and phospholipid vesicles, measured by light scattering. In the presence of Protein S, the binding of activated Protein C to phospholipid vesicles was enhanced. Protein S had no effect upon the binding on the zymogen (Protein C to phospholipid vesicles).

In conclusion, the stimulatory effect of Protein S on the inactivation of Factor Va by activated Protein C can be attributed, in part, to the enhancement of the binding of activated Protein C to phospholipid vesicles.

Protein S is a vitamin K-dependent zymogen of a serine protease (1). When converted to its active form, activated Protein C, it is able to inhibit blood coagulation (2, 3) and possibly induced fibrinolysis (4, 5). Both Factor Va (2, 6) and Factor VIII (7) have been shown to be substrates for activated Protein C. From this observation it has been inferred that the primary mechanism by which activated Protein C inhibits blood coagulation is through the inactivation of Factors Va and VIII.

I have previously reported that the rate of inactivation of Factor Va by activated Protein C can be stimulated by another vitamin K-dependent protein, Protein S (8). Protein S had no effect upon Factor Va activity in the absence of activated Protein C, indicating that it is not a protease. In a Protein S-deficient plasma, the anticoagulant activity of activated Protein C was significantly reduced. When Protein S was added to the deficient plasma, the anticoagulant activity of activated Protein C was restored. This experiment provided the first evidence linking the anticoagulant activity of activated Protein C with its Factor Va inhibitory activity. Secondly, this was the first report that Protein S was required for the maximum expression of the anticoagulant activity of activated Protein C.

This paper reports that Protein S enhances the binding of activated Protein C to phospholipid vesicles resulting in an increased rate of Factor Va inactivation. The significance of this finding with regard to the regulation of coagulation is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Soybean trypsin inhibitor, quaternary aminooethyl Sephadex, blue dextran, and heparin were purchased from Sigma Chemical Co. Acrylamide was electrophoresis grade and was purchased from Eastman Chemical Co. Ultrogel 34 was purchased from LKB. Agarose-immobilized heparin and agarose-immobilized blue dextran were prepared by the cyanogen bromide method (9). All other reagents were of the highest grade commercially available.

**Preparation of Proteins**—All of the proteins used in this paper were prepared as previously described (8). Purity of the various proteins was ascertained in at least two acrylamide gel electrophoresis systems. Diisopropylphosphate-modified activated Protein C (DIP-activated Protein C) was prepared by incubating activated Protein C (0.4 mg/ml) with 5 mM diisopropylfluorophosphate in 0.1 M Tris-HCl (pH 7.5) at 37 for 2 h. The degree of modification was monitored by measuring the amidase activity during the course of the incubation. DIP-activated Protein C used in the paper was more that 90% inactivated by this method. Unreacted diisopropylfluorophosphate was removed by dialysis.

Protein was monitored by absorbance at 280 nm. The molecular weights and extinction coefficients used for all protein components were as follows: prothrombin, 72,000, E \(_{1\text{cm}}^{1%}\) 15.5, Xa, 45,000, E \(_{1\text{cm}}^{1%}\) 12.4 (10), activated Protein C, 56,000, E \(_{1\text{cm}}^{1%}\) 13.7 (11), Factor V, 300,000, E \(_{1\text{cm}}^{1%}\) 10.0, Factor Va, 180,000, E \(_{1\text{cm}}^{1%}\) 10.0 (12), Protein S, 64,000, E \(_{1\text{cm}}^{1%}\) 10.0 (13).

**Electrophoresis**—Sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli (14). Acrylamide gel electrophoresis in the absence of detergent was performed by the method of Davis (15). Gels were stained with Coomassie blue.

**Phospholipid Preparation**—Phospholipid was prepared from aceton-dried bovine brain by the method of Bligh and Dyer (16). Phospholipid vesicles were prepared by mixing the phospholipid in CHCl\(_3\) and then drying under nitrogen onto the walls of a glass tube. The lipid was dispersed into buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5) by sonication with a Branson bath sonicator for 4 h at room temperature. Following sonication, the lipids were filtered through a column of Ultrogel-22 (1.5 x 100 cm), and fractions (2.0 ml) were collected. Turbidity was monitored at 310 nm. A fraction from the trailing edge of the lipid peak was rechromatographed through the same column. It eluted as a single symmetrical peak which was used in the subsequent experiments. The phospholipid concentration was estimated by measuring organic phosphorous concentration by the methods of Chen et al. (17) and using a weight conversion factor of 25

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*The abbreviation used is: DIP, diisopropylphosphate.
Protein S. This indicates that Protein S must interact specifically to the rate in the absence of Protein S. In a similar experiment, the rate of Factor Va inactivation in the absence of Protein S was decreased from 12 μM by the addition of Protein S. The maximum rate of Factor Va inactivation in the presence of phospholipid was unchanged by the addition of Protein S. This may indicate that the main effect of Protein S is to enhance the interaction between activated Protein C and phospholipid vesicles.

In order to test the hypothesis that Protein S increased the binding of activated Protein C to phospholipid vesicles, I measured the interaction between phospholipid vesicles and activated Protein C by the light scattering method described by Nelsestuen and Lim (19). Confirming a previous report (20), I observed that both Protein S and Protein C have a higher affinity for phospholipid vesicles than activated Protein C (Table 1).

The addition of Protein S to the phospholipid prior to the addition of activated Protein C enhanced the affinity of activated Protein C for the lipid vesicles (Fig. 4). In the presence of Protein S, the observed dissociation constant for the interaction between activated Protein C and phospholipid vesicles decreased from 15 × 10^{-9} M to 1.4 × 10^{-8} M. The number of activated Protein C molecules bound to the lipid with the higher affinity was less than the number bound in the absence of Protein S. The maximum concentration of activated Protein C bound in the presence of 32 nM Protein S was 33 nM. This close agreement indicates that the interaction between the two proteins on the lipid surface is probably stoichiometric. Similar experiments were carried out with Protein C. Protein S had no effect upon the binding of Protein C to phospholipid vesicles. As an additional control the effect of prothrombin on the binding of activated Protein C to lipid vesicles was tested. Prothrombin had no effect upon the affinity of activated Protein C to lipid vesicles, but at high concentrations (10 μM) the number of molecules bound was reduced.

![Fig. 1. Titration of the Factor Va inactivation mixture with Protein (PS).](image-url)
Regulation of Activated Protein C by Protein S

The inactivation of Factor Va by activated Protein C is regulated by a number of factors. This paper reports on the relationship between two activators, phospholipid and Protein S. Protein S and activated Protein C bind to phospholipid vesicles (21). Binding is calcium dependent and presumed to be mediated through the γ-carboxyglutamic acid residues contained in each protein (13). In this paper I proposed that 1 mol of Protein S combines with 1 mol of activated Protein C on the surface of a phospholipid vesicle to form a Factor Va inactivation complex. The formation of this complex results in an increased rate of Factor Va inactivation. Evidence for the formation of this complex comes from kinetic and lipid binding studies. Titration of activated Protein C with Protein S in the presence of phospholipid indicated the formation of a tight one to one complex between these two proteins. The effects of phospholipid on the kinetics of Factor Va inactivation indicated that in the presence of Protein S the rate of inactivation was approximately 10 times more sensitive to the lipid concentration than in its absence. Protein-lipid binding experiments indicated that Protein S decreased the dissociation constant between activated Protein C and phospholipid vesicles by a factor of 10. The binding studies also indicated that a stoichiometric complex between Protein S and activated Protein C was responsible for the increased binding of Protein S on the rate of hydrolysis of the synthetic ester benzoyl-phenylalanyl-valyl-arginyl-p-nitroanilide (S2160) by activated Protein C. Since it was previously reported that the amidolytic activity was dependent upon the calcium concentration and ionic strength (22), these experiments were carried out over a wide range of calcium concentrations and ionic strengths. Under no conditions did I observe any effect of Protein S on the rate of hydrolysis.

A third possible mechanism by which Protein S could enhance the rate of activated Protein C-catalyzed Factor Va inactivation would be to alter the proteolytic pathway of inactivation. Using sodium dodecyl sulfate acrylamide gel electrophoresis to monitor the proteolysis of Factor Va, I compared the inactivation of Factor Va by activated Protein C in the presence and absence of Protein S. No qualitative differences in the patterns of degradation products obtained in the presence and absence of Protein S were observed. The patterns were identical with those previously reported (2).

**Discussion**

**Table 1**

*Summary of protein-lipid binding data*

| Protein                 | Dissociation constant \(x \times 10^{-8} M\) | Bound/Free | Per cent bound in assay* |
|-------------------------|---------------------------------------------|------------|--------------------------|
| Protein C               | 23                                          |            |                          |
| Activated Protein C     | 15                                          | 0.5        | 23                       |
| Activated Protein C + Protein S | 1.4 | 3.5 | 78 |
| Protein S               | 5                                           | 1.0        | 50                       |
| Factor Va               | 0.35                                        | 14.3       | 94                       |

* Calculated using the concentration of protein and lipid used in the Factor Va inactivation assay.

An alternative hypothesis that is important to consider is that the enhancement of the rate of Factor Va inactivation is due to a change in the distribution of the amounts of free and bound Factor Va. The interaction between Factor Va and the phospholipid vesicles was measured by the light scattering method (Table I). The results are in close agreement with those reported by others (21) even though these experiments were carried out with different Factor Va and lipid preparations. The Factor Va binding data indicated that under the conditions used in the kinetic experiments most of the Factor Va molecules are bound to the lipid vesicles. Therefore, it is unlikely that changes in the distribution between bound and free Factor Va are responsible for the changes in the inactivation rates that were observed.

In addition to altering lipid binding properties of activated Protein C, it is possible that Protein S could change the catalytic properties of the enzyme. In order to ascertain if this was a possibility, I examined the effect of Protein S on the amidolytic properties of activated Protein C. Neither in the presence nor absence of phospholipid did I observe any effect of Protein S on the rate of hydrolysis of the synthetic ester N-benzoyl-phenylalanyl-valyl-arginyl-p-nitroanilide (S2160) by activated Protein C. Since it was previously reported that the amidolytic activity was dependent upon the calcium concentration and ionic strength (22), these experiments were carried out over a wide range of calcium concentrations and ionic strengths. Under no conditions did I observe any effect of Protein S on the rate of hydrolysis.

**Fig. 2 (left).** The effect of DIP-activated Protein C on the rate of Factor Va inactivation. The reaction was carried out as described under "Experimental Procedures" with a phospholipid concentration of 3.2 mg/ml (A) and with Protein S (10 nm) (△), Protein S (10 nm) and DIP-activated Protein C (60 nm) (●), and DIP-activated Protein C (60 nm) (○).

**Fig. 3 (center).** The effect of phospholipid on the rate of Factor Va inactivation. The reactions were carried out as described under "Experimental Procedures." Dissociation constants for activated Protein C were 1.4 \(\times 10^{-8} M\) in the presence of Protein S (32 μM) (○) and 15 \(\times 10^{-8} M\) in its absence (●).

**Fig. 4 (right).** The effect of Protein S on the binding of activated Protein C to phospholipid vesicles. Binding was measured by the light scattering method described under "Experimental Procedures." Dissociation constants for activated Protein C were 1.4 \(\times 10^{-8} M\) in the presence of Protein S (32 μM) (○) and 15 \(\times 10^{-8} M\) in its absence (●).
activated Protein C. The conclusion from these experiments is that Protein S increases the amount of activated Protein C bound to phospholipid vesicles and that the rate of Factor Va inactivation is proportional to the amount of activated Protein C bound to the lipid surface.

The complex between Protein S and activated Protein C appeared to form only in the presence of phospholipid. Protein S had no effect upon either the gel filtration or sedimentation equilibrium properties of activated Protein C (data not shown). These experiments indicated that any physical interaction between activated Protein C and Protein S would have a dissociation constant greater than 0.01 mM. Since the proteins express their activities in the nanomolar concentration range, any soluble phase interaction between the two proteins would not affect the rate of Factor Va inactivation. This conclusion was confirmed by the observation that Protein S had no effect upon the rate of Factor Va inactivation in the absence of phospholipid vesicles.

A number of proteases of the coagulation cascade are derived from vitamin K-dependent proteins. Except for thrombin, all of these proteases retain the γ-carboxyglutamic acid residues upon activation and require the presence of protein S for their full expression of activity (23). In recent years much attention has been given to the properties of Factor Va, the cofactor protein in the activation of prothrombin by the protease Factor Xa (12, 21, 24–26). Binding and kinetic studies have indicated that Factor Xa and Factor Va form a tight complex on the surface of phospholipid vesicles (26). This immobilized enzyme complex, the prothrombinase complex, converts prothrombin to thrombin. The rate of prothrombin activation is approximately 100,000 times faster than that catalyzed by Factor Xa alone. Though prothrombin can bind to phospholipid vesicles, compared to the high affinity of the prothrombinase complex for phospholipid vesicles it can be considered to be a soluble substrate.

The Factor Va inactivation complex is similar in several respects to the prothrombinase complex. First, both of the complexes bind to phospholipid vesicles with a higher affinity than the individual protein components. Second, Protein S and activated Protein C, like Factor Va and Factor Xa, form stoichiometric complexes on the lipid surface. Finally, the lipid-bound enzyme complexes have much more activity toward protein substrates than do the soluble enzymes. Therefore, the reactions catalyzed by each of the enzyme complexes takes place on the surface of a lipid vesicle.

The Factor Va inactivation complex is not entirely analogous with the prothrombinase complex. The most important difference is the nature of the substrate. As mentioned, for the prothrombinase complex the substrate, prothrombin, can be considered to be soluble. This is not the case for the Factor Va inactivation complex. Factor Va, the substrate in this reaction, binds tighter to lipid vesicles than does the inactivation complex so in the reaction mixture it is predominantly bound to the lipid surface. So in this system it is not entirely clear whether the substrate for the inactivation complex is either soluble Factor Va or lipid-bound Factor Va. Though this is an open question, several previously reported experiments tend to indicate that soluble Factor Va may be the preferred substrate for the complex.

As previously mentioned, kinetic experiments indicate that Factor Xa increased the binding of Factor Va to the lipid surface by a factor of 1000 (26). Therefore, in the presence of Factor Xa the amount of soluble Factor Va is reduced by approximately that same amount. I previously reported that Factor Xa can inhibit the inactivation of Factor Va by activated Protein C (2). At the time I interpreted this to mean that Factor Xa and activated Protein C could bind to a similar site on the Factor Va molecule. In a subsequent study, I reported that phospholipid was required in order for Factor Xa to protect Factor Va (27). I now propose that the inhibition of the inactivation of Factor Va by Factor Xa was due to the removal of Factor Va from solution, thereby reducing the amount of substrate available for the inactivation complex.

This model is under further investigation in my laboratory.

The proposal that solution phase Factor Va is the substrate for activated Protein C-Protein S-lipid complex has some interesting implications for the regulatory function of this complex in the coagulation cascade. This hypothesis predicts that only soluble Factor Va molecules are inactivated by the complex. Those bound to Factor Xa in the prothrombinase complex which would be generated at the site of a wound would be protected from inactivation and would catalyze the production of thrombin leading to rapid clot formation. Away from the site of the wound, Factor Va molecules that are not involved in prothrombin activation would tend to be inactivated by the insoluble Factor Va inactivation complex. In this manner, clot formation could be localized by a mechanism that would tend to inactivate an active factor at a site distal from site of thrombin generation.

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