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FUNCTIONAL PROPERTIES OF FACTOR Va SUBUNITS AFTER PROTEOLYTIC ALTERATIONS BY ACTIVATED PROTEIN C

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The two-subunit structure of the factor Va molecule is essential to its function in the prothrombinase complex. In the presence of phospholipids, the cleavage of the light chain of bovine factor Va by activated protein C proceeded at the same rate as the cleavage of the heavy chain. The limited proteolysis of factor Va is accompanied by a parallel loss of factor Va activity. Evidence that loss of activity was solely the result of the cleavage of the heavy chain, was obtained from reconstitution experiments utilizing cleaved and intact chains. The pseudo first-order rate constant of factor Va inactivation by activated protein C was found to be dependent on the amount of phospholipid-bound activated protein C and not on the amount of phospholipid-bound factor Va. However, phospholipids enhance the rate of proteolysis of the phospholipid-binding subunit, i.e. the light chain, and not the cleavage of the heavy chain. Cleavage of the heavy chain and as a consequence the inactivation of factor Va by activated protein C is mediated by phospholipid-bound light chain. After cleavage of the light chain, the 'two-subunit' structure, as well as the phospholipid-binding properties of factor Va were found to be conserved.

Introduction

Activated protein C, which is derived by limited proteolysis of the zymogen by thrombin, trypsin or factor X-converting protein from Russell's viper venom [1–3], exhibits anticoagulant activity. This anticoagulant effect is the result of the inactivation of factor Va and factor VIII : C by activated protein C [3–8].

Current knowledge about the molecular events that lead to the inactivation of factor Va by activated protein C has emerged from studies on factor Va degradation, utilizing SDS-polyacrylamide gel electrophoresis [3,7,8]. It was inferred that proteolysis of the 94-kDa subunit of factor Va (factor Va heavy chain) results in the inactivation of factor Va. Whether proteolysis of the 80-kDa subunit (factor Va light chain)affects the functional properties of factor Va is not known.

Phospholipids greatly enhance the rate of factor Va inactivation by activated protein C [2,3,8]. Activated protein C and factor Va bind to phospholipid with dissociation constants of approx. 10^{-5} M [9] and 10^{-8} M [10], respectively. However, it is an open question whether both proteins have to bind to phospholipid in order to obtain an efficient inactivation reaction [11]. Therefore, in view of the observations that the factor Va heavy chain is bound to phospholipid by means of factor Va light chain [10] and phospholipids stimulate the cleavage of factor Va heavy chain if factor Va light chain is present [3,8], the question has to be addressed as to the organization of the factor Va molecule on the phospholipid surface, when factor Va and especially factor Va light chain is processed by activated protein C.

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This study of the structural alterations in the subunits of factor Va as induced by activated protein C was undertaken to obtain better insight into the consequences as to the functional properties of factor Va and the inactivation process of factor Va. A preliminary account of this work has been published [12].

Materials and Methods

Materials. Russell's viper venom, ovalbumin and dioleoylphosphatidylcholine were from Sigma Chemical Co. The chromogenic substrate D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S2238) was partially donated by AB Kabi Diagnostica.

Protein preparations. Protein C was isolated from bovine plasma by a modification [3] of the method according to Stenflo [11]. Factor X-activator from Russell's viper venom (RVV-X) was purified as described by Schiffman et al [13]. Protein C was activated with RVV-X and purified as reported by Walker et al [3]. Activated protein C concentrations were calculated from the absorbance at 280 nm using $A^{1\%} = 13.7$ [1]. Bovine factor V and factor Va were prepared by a modification [14] of the method of Esmon [15]. Factor Va subunits and all other proteins used in this study were purified and quantitated as described previously [10].

Factor Va assay. A sample (10–100 μl) containing factor Va was incubated with factor Xa ($1.3 \cdot 10^{-11}$ M), phospholipid ($1.0 \cdot 10^{-5}$ M), and $CaCl_2$ ($1.0 \cdot 10^{-2}$ M) in 50 mM Tris, 100 mM NaCl, 0.5 mg ovalbumin/ml in a final volume of 0.9 ml for 5 min at 37°C in a plastic cuvette. The reaction was initiated by the addition of 0.1 ml of prothrombin ($2.0 \cdot 10^{-6}$ M). After 2 min, 1.0 ml buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mg of ovalbumin, 20 mM EDTA and 0.47 μmol S2238 was added to the reaction mixture. The amount of thrombin formed was calculated from the absorbance change as monitored with an Aminco DW-2 spectrophotometer operating in the dual wave-length mode ($\lambda_s = 405$ nm and $\lambda_r = 500$ nm), at 37°C. A factor Va preparation of which the molar concentration was determined as described previously [10], was used for the construction of standard curves.

Iodination of factor Va subunits. Factor V was iodinated using Bolton-Hunter reagent [16]. ^{125}I-Factor V was activated with thrombin and the ^{125}I-labelled factor Va subunits were isolated as described previously [17].

Proteolysis of factor Va subunits by activated protein C. Factor Va light chain (6 μM) in 20 mM Tris, 50 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (100 nM) in the presence of 20 μM phospholipid for 10 min at 37°C. Upon complete conversion of factor Va light chain, activated protein C was quantitatively removed from the mixture by chromatography on a SP-Sephadex column (0.9 × 10 cm) and washing the column with the above buffer. Factor Va light chain proteolysis products, i.e. fragments with M_r 51 000 and M_r 32 000 were eluted from the column with 20 mM Tris, 200 mM NaCl, pH 7.5.

Factor Va heavy chain (7 μM) in 20 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (0.5 μM) for 90 min at 37°C. After complete conversion of factor Va heavy chain, the reaction mixture was applied to a QAE-Sephadex column (0.9 × 10 cm) equilibrated in the above buffer omitting Ca^{2+}. The factor Va heavy chain proteolysis products, i.e. fragments with M_r 72 000 and M_r 24 000, were eluted from the column with 20 mM Tris, 350 mM NaCl, pH 7.5.

Inactivation of factor Va. Factor Va (0.1 μM) in 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (2 nM) in the presence of varying amounts of phospholipid. After the reaction was initiated, samples were removed from the reaction mixture and assayed fro factor Va. For each time course the apparent first-order rate constant for the inactivation of factor Va was calculated from the slope of a plot of log factor Va activity versus time. The plots were linear until 60% of the factor Va was inactivated.

HPLC of factor Va subunits. Size-exclusion high-performance liquid chromatography was carried out on Spherogel TSK 3000 SW (7.5 × 600 mm) in 20 mM Tris, 700 mM NaCl, pH 7.5 at a flow rate of 1.0 ml/min. The instrument was a Beckmann Model 500 high-performance liquid chromatograph equipped with a Beckmannn Model 160 Absorbance Detector. A 280-nm filter was

used to detect the protein peaks. Samples (100 μl) were injected after a 30-min period of incubation at 37°C in the column buffer.

Protein-binding measurements. Binding of protein to phospholipid was measured as previously described [10]. Briefly, mixtures of large-volume vesicles and protein were incubated for 10 min at room temperature. Prior to and after centrifugation for 30 min at $30000 \times g$, aliquots were withdrawn and assayed for protein for determination of total protein concentration and the concentration of unbound protein, respectively.

Phospholipid preparations. Large-volume and sonicated phospholipid vesicles containing 80% (w/w) dioleoylphosphatidylcholine and 20% (w/w) dioleoylphosphatidylserine were prepared as described previously [10].

Results

Degradation of bovine factor Va by activated bovine protein C

Thrombin-activated factor V (unfractionated factor Va) consists of four polypeptides, e.g. factor Va light chain (M_r 80000), factor Va heavy chain (M_r 94000), a M_r 160000 fragment which is very rich in carbohydrate and a M_r 65000 fragment. Factor Va light chain is associated via calcium ions with factor Va heavy chain and together constitute active factor Va [14,15]. Identical activation fragments have been reported for thrombin-activated human factor V [17].

Unfractionated factor Va was incubated with activated protein C in the presence of phospholipids and the decrease in factor Va activity was compared with proteolytic events as monitored by SDS-polyacrylamide gel electrophoresis (Fig. 1). Factor Va activity decreased in parallel with the degradation of both factor Va heavy chain and factor Va light chain, giving rise to fragments ranging from M_r 72000 to M_r 24000.

The M_r 150000 and M_r 65000 fragments present in the unfractionated factor Va preparation are not visualized by staining with Coomassie Blue. As monitored by SDS-polyacrylamide gel electrophoresis and visualized by staining with periodic acid-Schiff's reagent, we found that these fragments are not degraded by activated protein C (data not shown).

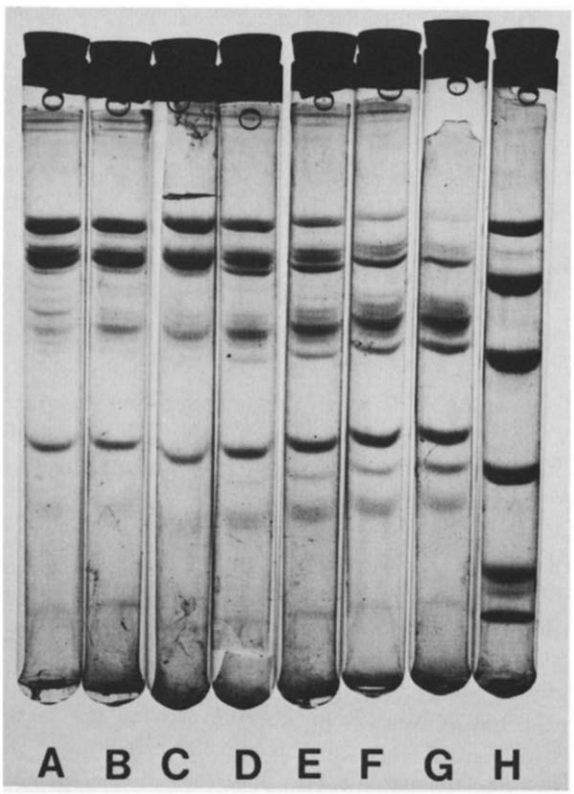

Fig. 1. Degradation of thrombin-activated factor V by activated protein C. Factor Va (1.0 μM) in 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5, phospholipid (100 μM) and activated protein C (20 nM) were incubated at 37°C. At intervals, aliquots were removed for factor Va assay and 10% SDS-polyacrylamide gel electrophoresis. The respective incubation times (min) and factor Va activities (%, in parentheses) were: A, 0 (100); B, 0.5 (95); C, 1 (90); D, 4 (45); E, 8 (24); F, 15 (9); J, 30 (0). Gel H, molecular weight standards (phosphorylase *b*, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 20100; α-lactalbumin 14400.

Isolated factor Va light chain and factor Va heavy chain were degraded by activated protein C to determine the origin of the degradation products. In accordance with the recently reported degradation pattern of human factor Va [8], we found that the fragments with M_r 51000 and M_r 32000 emerge from the doublet, factor Va light chain (M_r80000–78000). Factor Va heavy chain is converted into fragments with M_r 72000 and M_r 24000. A further degradation of the M_r 7200 fragment gave rise to a M_r 47000 and M_r 30000 fragment.

Effect of phospholipids on the rate of proteolysis of factor Va and its subunits

The rate of inactivation of factor Va by activated protein C is stimulated by negatively charged phospholipid [3,7,8]. However, the kinetics of factor Va inactivation are poorly understood.

As a first attempt to investigate this problem, we tried to correlate the apparent first-order rate constant of inactivation, k, with concentrations phospholipid-bound factor Va and/or phospholipid-bound activated protein C. Fig. 2 shows the effect of phospholipid concentration on the apparent first-order rate constant. It is demonstrated that the rate constant is proportional with the phospholipid concentration. The amount of phospholipid-bound activated protein C also increase proportionally with the phospholipid concentration. However, at 30 μM phospholipid nearly 100% of total added factor Va was bound. The binding experiments were performed at room temperature, while the kinetic studies were carried out at 37°C. However, the binding of factor Va did not change significantly in this temperature range. Apparently, under conditions of first-order kinetics, the rate constant, k, is independent of the amount of unbound factor Va and the substrate density at the phospholipid surface, but depends on the amount of phospholipid-bound activated protein C. However, inferences as to whether phospholipid-bound factor Va or unbound factor Va is the preferred substrate cannot be drawn.

To this end, we investigated the effect of phospholipids on the rate of proteolysis of the non-phospholipid-binding (factor Va heavy chain) and phospholipid-binding subunit (factor Va light chain), by activated protein C. The time course of cleavage of ^{125}I-labelled factor Va light chain and heavy chain by activated protein C in the absence and presence of phospholipids was monitored by measuring the radioactivity in respective regions of SDS-polyacrylamide gels corresponding to the position of factor Va heavy chain and factor Va light chain. Fig. 3A shows that the cleavage of factor Va light chain is stimulated by phospholipid, while the rate of conversion of the subunit that does not bind to phospholipid (factor Va heavy chain) is virtually unaffected by phospholipid (Fig. 3B). It is apparent that proteolysis by activated protein C is most effective when the substrate and activated protein C are bound to phospholipid. Moreover, it is interesting to note that in the absence of phospholipid the rate of degradation of factor Va light chain is higher than that of factor Va heavy chain.

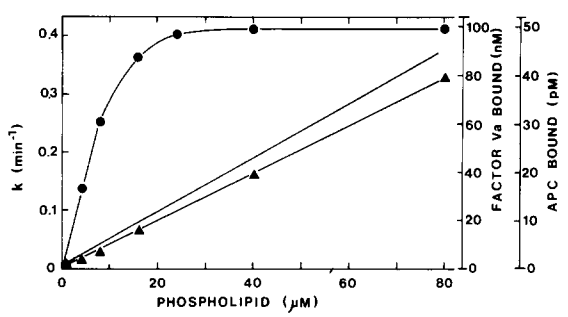

Fig. 2. Effect of phospholipid concentration on the pseudo first-order rate constant of factor Va inactivation by activated protein C (APC). The reaction was carried out as described in Materials and Methods. The rate constant, k (▲———▲), was determined from the slope of a plot of log factor Va concentration versus time. The concentration of phospholipid-bound factor Va (●———●) was determined from identical reaction mixtures but omitting activated protein C. The concentrations of phospholipid-bound activated protein C (———) were calculated from published binding parameters [9].

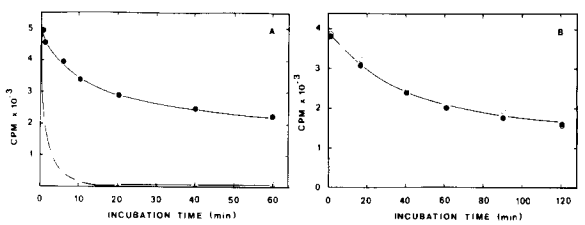

Fig. 3. Effect of phospholipid on the rate of proteolysis of factor Va heavy chain and factor Va light chain by activated protein C. Panel A, ^{125}I-factor Va light chain (1.8 μM, $6 \cdot 10^2$ cpm/nmol) in 20 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (45 nM) in the presence of 50 μM phospholipid (○———○) or in the absence of phospholipid (●———●). At intervals, aliquots were removed and electrophoresed on 10% SDS-polyacrylamide gels. The gels were sliced in 2 mm sections and counted for radioactivity. Plotted is the radioactivity corresponding to ^{125}I-factor Va light chain versus incubation time. Panel B, ^{125}I-factor Va heavy chain (0.2 μM, $4 \cdot 10^5$ cpm/nmol) was incubated with activated protein C (0.1 μM) in the presence (○———○) or absence (●———●) of phospholipid. Further experimental details as in panel A.

Phospholipid-binding properties of proteolytic altered factor Va light chain

Binding of factor Va to phospholipid is essential to the rate of inactivation of factor Va by activated protein C as it is essential to the function of factor Va in prothrombin activation. Since factor Va binding to phospholipid is mediated through factor Va light chain [10], it is of interest to investigate whether the phospholipid-binding properties of factor Va light chain are altered after factor Va light chain is degraded by activated protein C.

To this end, we performed a qualitative analysis of the phospholipid-binding properties of the factor Va light chain degradation products. Unfractionated degraded factor Va light chain was incubated with various amounts of large-volume vesicles. After centrifugation, the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. The amounts of M_r 51 000 and M_r 32 000 fragments were quantitated by densitometric scanning and plotted versus the phospholipid concentration (Fig. 4). It was demonstrated that both fragments bound equally well to phospholipid. The binding of both fragments could be reduced by adding calcium to the reaction mixture. We previously reported a similar effect of Ca^{2+} for factor Va light chain-phospholipid interaction [10]. It can be questioned whether the M_r 32 000 fragment arised from the M_r 51 000 fragment. However, both fragments are generated concomitant upon incubation of factor Va light chain with activated protein C. Prolonged incubation did not alter the M_r 32 000/M_r 51 000 fragments ratio.

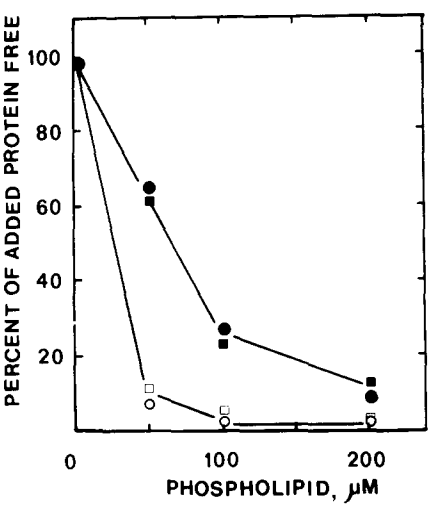

fig. 4. Phospholipid-binding properties of factor Va light chain degradation products. A mixture of the M_r 51 000 and M_r 32 000 fragments (2 μM) obtained after degradation of factor Va light chain by activated protein C was incubated with varying amounts of large volume vesicles in 50 mM Tris, 100 mM NaCl, pH 7.5 in the presence of 2 mM EDTA (open symbols) or 5 mM $CaCl_2$ (closed symbols). The amounts of unbound protein as a percentage of total added protein are plotted versus phospholipid concentration. ○——○ and ●——●, M_r 32 000 fragment; □——□ and ■——■, M_r 51 000 fragment.

Factor Va heavy chain-binding properties of proteolytic altered factor Va light chain

The divalent cation-mediated interaction between factor Va heavy chain and factor Va light chain is essential to the activity of factor Va [14,15]. Fig. 5 shows that factor Va activity, expressed as functional molar concentration [10], could be restored by incubation of equimolar amounts of factor Va light chain and factor Va heavy chain in the presence of Mn^{2+}. Titration of a fixed amount of factor Va heavy chain with unfractionated factor Va light chain degradation products, i.e. and equimolar mixture of M_r 51 000 and M_r 32 000 fragments, in the presence of Mn^{2+}, also resulted in the almost complete restoration of active factor Va (Fig. 5).

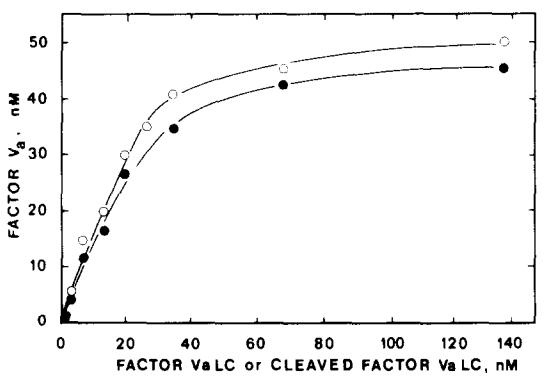

Fig. 5. Reconstitution of factor Va activity. Varying amounts of activated protein C-cleaved factor Va light chain (○——○) or intact factor Va light chain (●——●) were incubated with factor Va heavy chain (60 nM) in 50 mM Tris, 100 mM NaCl, 10 mM $MnCl_2$, pH 7.5 for 30 min at 37°C, Samples were taken and assayed for factor Va activity as described in Materials and Methods.

Factor Va activity could not be restored by titration of a fixed amount of factor Va light chain with unfractionated factor Va heavy chain degradation products, i.e. and equimolar mixture of M_r 72000 and M_r 24000 fragments (data not shown). To ascertain that the cleaved factor Va heavy chain preparation is not contaminated with activated protein C, we incubated 0.1 μM of factor Va in 20 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.5 with cleaved factor Va heavy chain (50 nM) in the presence of 50 μM phospholipid. Since no decrease in factor Va activity was observed, the presence of activated protein C could be ruled out.

Direct evidence that the factor Va light chain degradation products form a complex with factor Va heavy chain was obtained by size-exclusion HPLC. In the presence of EDTA, the elution times of factor Va heavy chain and Va light chain were 15.5 min and 19.5 min, respectively (Fig. 6A). The M_r 32000 and M_r 51000 factor Va light chain degradation fragments eluted together (elution time was 23.3 min) and close to the salt volume of the column, 24.0 min (Fig. 6C). The elution times of factor Va light chain and its fragments indicate a non-ideal size-exclusion retention mechanism. Apparently, protein-support surface interaction dominates in the chromatogram.

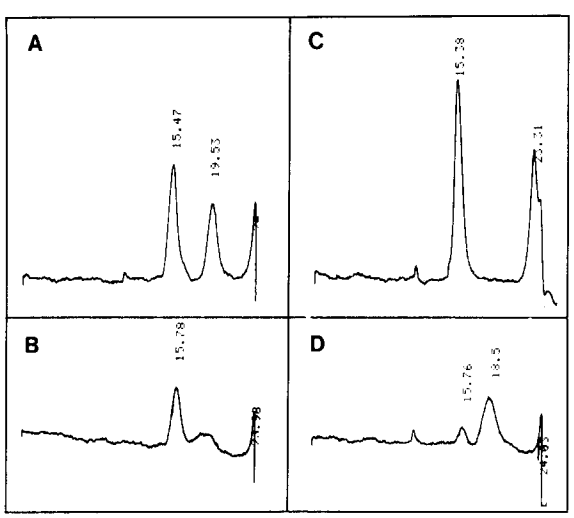

Fig. 6. Size-exclusion HPLC of factor Va subunits and restored factor Va. Elution diagrams for factor Va light chain (8 μg) and factor Va heavy chain (10 μg) in the presence of 2 mM EDTA (panel A) or 5 mM $MnCl_2$ (panel C) and for activated protein C-cleaved factor Va light chain (8 μg) and factor Va heavy chain (10 μg) in the presence of 2 mM EDTA (panel B) or 5 mM $MnCl_2$ (panel D). AUFS is 0.05.

In the presence of Mn^{2+}, factor Va heavy chain and factor Va light chain form a complex with an elution time of 15.8 min (Fig. 6B). Factor Va activity eluted with the protein peak. The elution time of the complex (M_r 174000) also showed non-ideal size-exclusion behaviour as was observed for factor Va light chain.

A mixture of equimolar amounts of factor Va heavy chain and cleaved factor Va light chain preincubated in the presence of Mn^{2+}, eluted from the column as a major protein peak, which contained factor Va activity. The elution time is 18.5 min. The minor protein peak, elution time of 15.8 min, contained less then 1% of the total recovered factor Va activity (Fig. 6D).

Discussion

Single chain bovine factor V (M_r 330000) is converted by thrombin into factor Va, a two-subunit protein, i.e. a M_r 94000 component associated via calcium ions with a M_r 80000 component, and activation peptides with M_r 150000 and M_r 65000. As a consequence of this action of thrombin, the pro-cofactor acquires factor Xa and prothrombin binding sites, while the phospholipid-binding properties are only slightly changed [10,17]. Because of these interactions, factor Va is essential to the assembly and function of the prothrombinase complex.

However, the structure-function relationship between factor Va subunits and the other components of the prothrombinase complex has not yet been elucidated. Activated protein C has been used to probe the functions of the individual subunits [3,7,8]. These studies showed that factor Va activity decreased in parallel with the degradation of the M_r 94000 component (factor Va heavy chain).

Information as to the consequences of the cleavage of factor Va light chain by activated protein C was not obtained, because the light chain of factor Va was partially [3] or not significantly [8] cleaved. That is, the cleavage of the heavy chain proceeded faster as compared to the cleavage of the light chain of factor Va. This is in contrast with our findings, where cleavage of the

light chain proceeded at the same rate as the cleavage of the heavy chain (Fig. 1). It was noted by Walker et al. [3] that the differences in relative rates of proteolysis of the heavy and light chain was reduced in the presence of phospholipid. In view of the effect of the phospholipid concentration on the pseudo first-order rate constant of inactivation of factor Va and the phospholipid-binding properties of factor Va and activated protein C (Fig. 2), the notion arises that the differences in rates of light chain proteolysis as compared to the rate of proteolysis of the heavy chain might be due to the extent of the solution phase inactivation of factor Va.

Direct evidence that inactivation of factor Va by activated protein C is solely the result of cleavage of the heavy chain was provided by the reconstitution experiments (Fig. 5). Whether or not cleavage of the heavy chain results in the loss of factor Xa and/or prothrombin binding to factor Va, is an open question. Preliminary data showed that the M_r 72000 fragment, derived from the heavy chain by activated protein C, interacts via calcium ions with phospholipid-bound factor Va light chain. Whether such a complex binds factor Xa and or prothrombin is the subject of further investigation.

As to the cleavage of the light chain, it is of interest to see that this proteolytic event does not effect the functional properties of factor Va (Fig. 5). We clearly demonstrated that the degradation products of the light chain bind to phospholipid (Fig. 4) and form a complex with the heavy chain (Fig. 6). It is apparent that both features are essential to the function of factor Va in the prothrombinase complex.

As to the stimulatory effect of phospholipid on the rate of inactivation of factor Va, the two-subunit structure appears to be important. Although the rate of inactivation seems to depend on the amount of phospholipid-bound activated protein C (Fig. 2), binding of the substrate to phospholipid is required (Fig. 3). Proteolysis of factor Va heavy chain results in the inactivation of factor Va. The factor Va light chain-mediated binding of the heavy chain to phospholipid is of paramount importance for a rapid cleavage by activated protein C. Therefore the conservation of the phospholipid and heavy chain binding properties of factor Va light chain after cleavage, is essential to the stimulatory effect of phospholipids on the inactivation of factor Va by activated protein C.

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