PROTHROMBIN-SEPHAROSE-PURIFIED FACTOR V AND ITS ROLE IN PROTHROMBIN CONVERSION

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ABSTRACT

Bovine Factor V isolated by the method of Esnouf and Jobin (1967) has been further purified by affinity chromatography through prothrombin-sepharose. Factor V bound quantitatively to the prothrombin-sepharose column. There was a 2-fold increase in the average specific activity (260,000 units/mg protein) of the Factor V recovered. Recovery of total Factor V activity and total protein was about 95% and 90% respectively. The effect of this Factor V preparation on the activation of prothrombin is described. Prothrombin was not activated by incubation with Factor V alone. When Factor V was added to a mixture of prothrombin, Factor Xa and calcium, the rate of prothrombin activation was about 110-fold greater than that of a mixture containing prothrombin, Factor Xa and calcium only. Inclusion of Factor V in a mixture containing prothrombin, Factor Xa, phospholipid and calcium increases the rate of prothrombin activation to 26,000-fold compared to that of activating prothrombin by Factor Xa and calcium only. Attempts to explain the quantitative effects of addition of the accessory components, Factor V, phospholipid and calcium, on the rate of thrombin formation are discussed.

INTRODUCTION

Factor V is a high molecular weight glycoprotein which circulates in plasma, congenital deficiency of this factor causes bleeding symptoms (Nolf, 1938). Although it is generally considered to be an active participant in the final stages of blood coagulation (Macfarlane, 1964; Davie & Ratnoff, 1964), little is known about the mechanism by which Factor V functions; however, it does appear that the protein must be proteolytically activated by thrombin (Colman, 1976; Prentice et al, 1967; Kandall et al, 1975) or some other protease (Colman, 1976; Smith & Hanahan, 1976) in order to become functional. There is little doubt that Factor V interacts with another protein (Factor X), phospholipids and calcium to form the prothrombinase complex or the prothrombin activator (Aoki et al, 1963) which attacks prothrombin with the release of thrombin for fibrin clot formation. In this report, partially purified Factor V isolated by the method of Esnouf and John (1967) has been further purified by affinity chromatography through prothrombin-sepharose and its function in prothrombin activation investigated.

MATERIALS AND METHODS

1. Purification of Bovine Factor V

Bovine Factor V isolated by the method of Esnouf and John (1967) was further purified by affinity chromatography through a column of prothrombin-sepharose. Sepharose was activated with cyanogen bromide by the method Cuatrecasas (1970). 20 ml of packed Sepharose 4B (Pharmacia [GB] Ltd) was added to an equal volume of distilled water and the pH adjusted to 10 with 5N sodium hydroxide solution. 2 g of cyanogen bromide, dissolved in about 10 ml of redistilled dimethylformamide was then added and the pH maintained at 10 with 5N sodium hydroxide. The temperature was kept below 20°C by the addition of ice chips in order to minimise the formation of side products. After about 15 minutes the mixture was filtered by suction on a sintered glass funnel and washed on the funnel with cold distilled water and then with about 1 litre of 0.2M sodium citrate/NaOH pH 6.9. Washing time was kept as short as possible (usually about 2 minutes).

The activated sepharose was quickly resuspended in 30 ml of citrate buffer containing 1 mg/ml of prothrombin. Reaction was allowed to proceed for about 6 hours at 4°C. The residual activated groups were then neutralized by filtering the sepharose and by washing the sepharose with 500 ml of 0.1 M glycine/NaOH pH 8.6 and resuspending in 30 ml of this buffer. This was left overnight with stirring and well washed on a sintered funnel with 50 mM Tris/HCl pH 8.0. The prothrombin-sepharose was stored in the latter buffer, ready for use.

The filtrate from the coupling reaction mixture was checked for any unbound protein by measuring its optical density. The coupling was always found to be quantitative. The rate of flow was 30 ml/hr and the void volume was about 9 ml. The column was then washed with the 50 mM ammonium chloride/Tris HCl, pH 7.5 and fractions of 3.5 ml were collected. After about two column volumes of buffer had passed through, the column was eluted with 500 mM ammonium chloride/50 mM Tris HCl pH 7.5 and again 3.5 ml fractions were collected.

All fractions were assayed for Factor V activity after their optical densities were measured at 280 nm using an ultraviolet spectrophotometer. The fractions containing Factor V activity were pooled and the total Factor V recovered was determined.

2. Assay of Factor V

To 0.1 ml of Factor V-deficient human plasma at 37°C was added 0.1 ml of the sample to be assayed, diluted in 20 mM Tris-HCl buffer pH 7.5, followed by 0.1 ml of a saline extract of brain, and finally 0.1 ml of 25 mM CaCl₂, and the clotting time was measured. A standard graph was obtained by recording the clotting times of fresh dilutions of citrated bovine plasma between 1:10, which was 30 sec., 1:100, which was 48 sec. and 1:1000 which was 76 sec.

The clotting time of the Factor V-deficient plasma in the absence of added Factor V was about 120 sec. A plot of the clotting time against dilution gave a straight line on a log-log paper. By definition 0.1 ml of citrated bovine plasma was said to contain 100 units of Factor V activity. The Factor V deficient human plasma was prepared from human blood which was collected into 0.1 volume of 0.1 M sodium oxalate, and the plasma obtained from this was incubated for 36 hr at 37°C and then stored at -20°C. The Factor V activity was in this way reduced to 0.1% of that of normal plasma. Though the amount of prothrombin in the plasma remained unchanged, that of the Factor X was

decreased to between 27% and 35% of normal plasma, which however was sufficient for the assay. The saline extract of brain was prepared as described by Biggs and Macfarlane (1962). The citrated bovine plasma was prepared from ox blood which was collected in 0.1 volume of 0.1 M trisodium citrate in a plastic container and then the blood was centrifuged to remove the formed elements. Solutions of Tris (Trisma Base) were adjusted to the required pH with N HCl, and the molarity of the buffer refers to the final concentration of Tris.

3. Preparation of proteins

Factor Xa was prepared from the method of Jesty and Esnouf (1973) by activating pool of purified Factor X prepared according to Esnouf et al (1973) with highly purified Russell's viper venom (RVV) coagulant protein isolated by the method of Williams and Esnouf (1962).

Prothrombin was prepared as described by Esnouf et al (1973) and rechromatographed on DEAE-sephadex A50.

4. Phospholipid stock solution

Phospholipid stock solution was obtained by dissolving 500 mg of inosithin in 10 ml diethyl ether, then emulsified with 10 ml 10 mM Tris/HCl pH 8.4 and the ether blown off with oxygen-free nitrogen at 37°C.

5. Activation of prothrombin

The activation of prothrombin was determined by using the esterase assay. Incubation mixtures in 50 mM Tris/HCl pH 8.0 buffer for the esterase assays of thrombin generated as a result of prothrombin activation were made up as follows: firstly activation by Factor V in the absence of calcium, (prothrombin, 200 µg/ml, Factor V, 6 µg/ml) and in the presence of calcium (prothrombin, 200 µg/ml, Factor V, 6 µg/ml, CaCl₂, 5 mM); activation by Factor Xa (prothrombin 200 µg/ml, Factor Xa 60 µg/ml); activation by Factor Xa and calcium (prothrombin 200 µg/ml, Factor Xa 3 µg/ml, CaCl₂ 5 mM); activation by an equimolar quantity of Factor Xa and prothrombin in the presence of calcium (prothrombin 200 µg/ml, Factor Xa 130 µg/ml, CaCl₂ 5 mM); activation by Factor Xa; calcium and phospholipid (prothrombin 200 µg/ml, Factor Xa 0.1 µg/ml, CaCl₂ 5 mM, phospholipid 300 µg/ml); activation by Factor Xa, calcium, Factor V (prothrombin 200 µg/ml, Factor Xa 0.1 µg/ml, CaCl₂ 5 mM, Factor V 6 µg/ml); activation of Factor Xa, calcium, phospholipid and Factor V (prothrombin 200 µg/ml, Factor Xa 10 ng/ml, CaCl₂ 5 mM, phospholipid 300 µg/ml, Factor V 6 µg/ml). In each experiment, the incubation mixture was kept at 37°C. At different intervals, aliquots were withdrawn for esterase assays.

The esterolytic activity of thrombin was measured by the method of Schwert and Takenaka (1955). 1 ml of 3 mM N- α -benzoyl arginine ethyl ester BAEE (Koch Light Laboratories Ltd, Colnbrook, England) in 50 mM Tris/HCl pH 8.0 was mixed with 0.1 ml of the incubated mixture and the volume made to 3.0 ml with 50 mM Tris/HCl pH 8.0 in 1 cm cuvette. The decrease in absorbance was measured at 253 nm at 25°C in a recording spectrophotometer (Gilford Instruments Laboratories, Ohio, USA). The rate of hydrolysis of the substrate by the incubation mixture was compared against control incubation mixtures which contained only Factor Xa in 50 mM Tris/HCl pH 8.0.

RESULTS

The purification of Factor V has been effected in this study by passing the protein through an affinity chromatography column of prothrombin-sepharose. The elution pattern of the Factor V through the prothrombin-sepharose column is shown in Figure 1. A broad protein peak (fraction 5 to 10), which contains very little Factor V activity

FIGURE 1
BEHAVIOUR OF FACTOR V THROUGH PROTHROMBIN SEPHAROSE COLUMN

(190 units/ml) was obtained when the column was washed with 50 mM ammonium chloride/50 mM Tris HCl, pH 7.5. On elution with 500 mM ammonium chloride/50 mM Tris HCl, pH 7.5, the column yielded a sharp elution peak (fraction 17 to 20) of protein. The protein peak has a specific activity of 330,000 units/mg protein when assayed for Factor V. Protein recovery amounted to 91.7% of the total protein loaded and the recovery of Factor V activity was found to be 95.0%. The average specific activity of the final Factor V pool was about 260,000 units/mg protein.

The specific activity of the Factor V obtained through this purification process was 2-fold greater than the Factor V isolated by the method of Esnouf and Jobin (1967) indicating that removal of non-Factor V components in the partially purified Factor V sample has taken place.

The results of the thrombin generated by the activation of prothrombin by Factor Xa singly, and with a combination of the other accessory factors like calcium, phospholipid and Factor V are shown in Figure 2 and 3. No thrombin was generated when prothrombin was incubated with Factor V in the absence or presence of calcium. When the prothrombin was incubated at 37°C with Factor Xa in the absence of calcium ions, a slow and progressive conversion of prothrombin to thrombin was observed, when the esterase activity of the product, thrombin, was measured (Figure 2a). It can be seen that the conversion was approaching a plateau phase or was virtually complete at about 300 minutes.

In activations including calcium, Factor Xa concentration was reduced to 1/20th of the above amount. The plateau phase was reached much earlier, at about 100 minutes as shown in Figure 2b. When calcium was included in the activation mixture of prothrombin and Factor Xa, the prothrombin was rapidly activated until about a third of the potential thrombin was formed (Figure 2b), after which thrombin production apparently ceased. When the Factor Xa concentration was doubled after reaching this plateau, only a very small amount of extra thrombin was generated. On the other hand, if an equimolar amount of Factor Xa and prothrombin was used, for the activation from the start, the rate of thrombin generation was enormous and the theoretical yield of thrombin could be achieved (Figure 2b).

When the experiment was carried out by including phospholipid in the incubation mixture containing, Factor Xa, calcium and prothrombin, the rate at which the plateau phase was reached, was much faster compared to the above. In this case, it took only about 20 minutes to reach the maximum esterolytic activity achieved by the thrombin generated under these conditions (Figure 2c).

The rate at which thrombin is formed from prothrombin when phospholipid is replaced by Factor V in the incubation mixture containing Factor Xa, calcium and prothrombin, appears to be lower compared to the above system. This time, the thrombin generation seemed to plateau off at about 30 minutes, instead (Figure 2d).

Finally, when the complete prothrombinase complex, consisting of Factor Xa, calcium, phospholipid and Factor V, was used to study the activation of the substrate, prothrombin, the product, thrombin was generated rapidly compared to what has been seen so far. The plateau phase begins as early as after 2 minutes of incubation, followed by a gradual and steady increase of esterolytic activity of thrombin, with time (Figure 3).

It is interesting to study the relative rates of prothrombin activation by Factor Xa with or without the accessory factors and Table 1 shows the results, calculated from the

FIGURE 2
ACTIVATION OF BOVINE PROTHROMBIN

Key:

..... Additional 3 µg/ml Xa added

by the serine protease, Factor Xa. The activation of prothrombin by Factor Xa alone is a slow process. When calcium was included, the rate of prothrombin conversion increases and about a third of the potential thrombin was generated. This may be attributed to the fact that the prothrombin was cleaved to prethrombin 1 and 2 only, with a build up occurring at the prethrombin 2 stage (Prowse et al 1976). When the Factor Xa concentration was doubled after the plateau is reached, only a very small amount of extra thrombin was generated. This apparent product inhibition could be due to the formation of prethrombin 1 by thrombin degradation of prothrombin and this prethrombin 1 then being a poor substrate for Factor Xa. The effect was only seen in the presence of calcium. Since the Fragment 1 region of prothrombin contains the calcium binding sites, the most likely mode for a calcium-dependent inhibition of prothrombin activation would appear to be a direct competition between Fragment 1 and prothrombin for Factor Xa, in the presence of calcium. These experiments apparently suggest that Fragment 1 inhibits the activation of prothrombin (Ong and Esnouf, 1979).

The acceleration due to phospholipid depends on the calcium-binding ability of the Fragment 1 region of prothrombin (Bull et al, 1972; Gitel et al, 1973). Activation in the presence of phospholipid differed qualitatively from that carried out with Factor Xa and calcium only in that no intermediate accumulated during the plateau phase of the activation (Prowse et al, 1976).

It may be suggested that the inclusion of phospholipid causes the reaction at the solid phase to be more significant and perhaps occurs to a greater extent since phospholipid promotes the aggregation of the clotting factors and hence enable closer contact of the factors to bring about the activation of prothrombin. It also seems probable that the accessory effect of the phospholipids, on blood coagulation is dependent on their ability to provide a catalytic surface in the aqueous medium for protein-protein interactions.

Acceleration in rate of prothrombin conversion was also observed with the inclusion of Factor V in the activator system. Factor V contribution to the rate increase by acting as a co-factor in the prothrombin activator complex.

The quantitative effects of addition of the accessory components, Factor V, phospholipid and calcium, on the rate of thrombin formation have also been measured in several laboratories (Bajaj et al, 1975; Esmon et al, 1974; Jobin & Esnouf, 1967; Seegers et al, 1972). These effects have been shown to be large where enhancements of several orders of magnitude being obtained with the addition of each accessory component (Esmon et al, 1974). These rate enhancement effects of the accessory components result from their action on the same basic pathway (Esmon & Jackson, 1974; Esmon et al, 1974), rather than on any of the possible independent parallel pathways. The separate and cumulative effects of each accessory component are shown in Table 1. The cumulative effect of the accessory factors increases the rate of thrombin formation by as much as 26,000 times compared to that observed with Factor Xa alone in the presence of calcium.

The rate increase observed in the presence of the accessory prothrombin activator components may be accounted for by several possible reasons. Firstly, a change in the catalytic efficiency of the proteinase, Factor Xa, as a result of its interaction with phospholipid, Factor V and calcium ions, or secondly, due to its binding to phospholipid and or Factor V and calcium ions may contribute to the observations seen in these experiments. Nelsestuen (1978) and Mayer et al (1983) found that protein-membrane interactions in the presence of calcium plays an important part in increasing the affinity of the

Table 1: Initial Rates of Prothrombin Activation Measured By The Esterase Method

ACTIVATOR SYSTEM	ESTERASE ACTIVITY/MIN	RELATIVE RATE
Prothrombin, Factor Xa	0.048	1
Prothrombin, Factor Xa, Ca ²⁺	0.074	31
Prothrombin, Factor Xa, Ca ²⁺ , Phospholipid	0.43	5379 (173.6)
Prothrombin, Factor Xa, Ca ²⁺ , Factor V	0.27	3407 (109.8)
Prothrombin, Factor Xa, Ca ²⁺ , Phospholipid, Factor V	6.60	808929 (26473)

Values in parenthesis are relative to Prothrombin, Factor Xa and Calcium system.

proceeding graphs obtained under different conditions as described. A tangent was drawn from the initial part of each of the graphs in Figure 2 and 3, and the esterase activity per unit time was estimated in each case. The relative rates of prothrombin activation was calculated with reference to the rate of thrombin formation in the case where only Factor Xa was present. The specific interaction of each of the accessory components in turn, and their separate and collective effects on the rate of thrombin generation from prothrombin can thus be judged from their relative rates.

It can be seen that, the rate of prothrombin activation is greatly enhanced by the presence of the accessory factors. However, Factor V by itself does not activate prothrombin at all. Factor V contributes to a lesser extent when compared to phospholipid in increasing the rate of prothrombin conversion by Factor Xa to thrombin. The ratio of the system containing phospholipid to that containing Factor V was found to be 1.6:1. The most remarkable rate enhancement was achieved when the completed prothrombinase complex, Factor Xa, calcium, phospholipid and Factor V, was used to activate prothrombin and represents an average of some 26,000-fold increase in activity when compared to the mixture where calcium was present as the only accessory factor.

DISCUSSION

The fact that prothrombin-sepharose has been used to purify Factor V, indicates that prothrombin is able to bind Factor V. As such, it suggests that Factor V is an important accessory component of the prothrombin activator which converts prothrombin to thrombin. Our other study (unpublished data) also indicates that the binding of Factor V to prothrombin is in the Fragment 2 region and not the Fragment 1 region of the prothrombin molecule.

The activation study on prothrombin reveals that Factor V has no enzymatic activity, contrary to earlier beliefs and by itself it has no effect on prothrombin. It acts merely as a co-factor in prothrombin activation. The activation of prothrombin is brought about

binding site of the clotting factors. In addition, protein-protein interaction also contributes to the above. Nelsestuen (1978) concluded that the prothrombinase complex can be viewed kinetically as a dissociable three-component enzyme (Factor Xa, Factor V and phospholipid) acting on the soluble substrate, prothrombin. Alternatively, the rate enhancements may result simply by greatly increased local reactant concentrations that occur in the vicinity of the phospholipid surface due to their adsorption to the surface and to the juxtaposition of enzyme and substrate that is achieved by the binding of both of these components to Factor V. Obviously, these mechanisms are not mutually exclusive and any or a combination or all of them may operate simultaneously.

There is one report that Factor Xa catalytic efficiency toward para-toluenesulphonyl-L-arginine methyl ester (TAME), is increased by Factor V and phospholipid in the presence of calcium ions (Colman, 1970). This suggests that the proteolytic efficiency of Factor Xa may also be affected by Factor V. No evidence from prothrombin activation experiments has been reported that bears directly on the additional possibility that a peptide bond in prothrombin is more susceptible to hydrolysis by Factor Xa when bound to Factor V. Currently work along this direction is being pursued in our laboratory.

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