The Preparation of Activated Factor X and its Action on Prothrombin

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The preparation of activated Factor X from reaction mixtures of bovine Factor X and Russell's-viper venom is described. The molecular weight of purified protein varies about a mean value of 40000; this variation is the result of at least two forms of Factor Xa. The action of activated Factor X, together with purified Factor V, was studied on purified prothrombin and the reaction products were isolated. In addition to thrombin, two other polypeptides with molecular weights of 16000 and 19500 were recovered.

In the preceding paper, Esnouf *et al.* (1973) described a method for the simultaneous preparation of Factor X and prothrombin from bovine plasma. In the present paper we describe the purification and some of the physical properties of activated Factor X (Factor Xa), prepared by the reaction of Factor X with the coagulant protein of Russell's-viper venom (Williams & Esnouf, 1962).

It has now been generally accepted that Factor Xa is the enzyme that converts prothrombin into thrombin (Papahadjopoulos & Hanahan, 1964; Barton *et al.*, 1967; Jobin & Esnouf, 1967; Baker & Seegers, 1967); although a considerable amount of work has been done on the chemistry of thrombin, little attention has been paid to the fate of the 'pro-piece', that is the non-thrombin residue of prothrombin, and in view of this we have fractionated prothrombin activation mixtures and have studied the reaction products.

Experimental

Materials

Columns and buffers. These were prepared as described in the preceding paper (Esnouf *et al.*, 1973).

Phospholipid emulsion. The phospholipid emulsion used in the reaction mixtures for the conversion of prothrombin was prepared from inosithin (Associated Concentrates Inc., Woodside, N.Y., U.S.A.), by the method of Jobin & Esnouf (1967).

Methods

Preparation of proteins. Factor X and prothrombin were prepared as described in the preceding paper (Esnouf *et al.*, 1973); Factor V was purified by the method of Esnouf & Jobin (1967). The coagulant protein from Russell's-viper venom was isolated as

described by Williams & Esnouf (1962), from dried venom generously given by Professor P. M. Dalal.

Preparation of Factor Xa. A solution containing 50–80 mg of Factor X (500 µg/ml) in 0.1 M-NaCl buffered with 15 mM-chloride-Tris, pH 7.5, was made 5 mM with respect to CaCl₂ and incubated at 37°C for 30 min with 5 µg of the coagulant protein of Russell's-viper venom/ml, isolated by the method of Williams & Esnouf (1962). The Factor X was activated fully in 30 min as judged by the esterolytic activity of the mixture. The reaction mixture was then dialysed against 5 litres of 0.1 M-CaCl₂ in 15 mM-chloride-Tris buffer, pH 7.5, for 5–6 h at 4°C, after which EDTA was added to the diffusate to give a final concentration of 0.1 mM and dialysis continued for a further 12 h, after which the mixture was chromatographed on a column of DEAE-Sephadex.

Gel electrophoresis. Disc-gel electrophoresis was carried out as described in the preceding paper (Esnouf *et al.*, 1973). Electrophoresis was also done in 5% polyacrylamide gels containing 1% sodium dodecyl sulphate (Weber & Osborn, 1969). In the present experiments the gels were stained with Coomassie Blue.

The stained gels were scanned with a micro-densitometer (Joyce, Loeb and Co. Ltd., Gateshead, Co. Durham, U.K.).

Clotting assays. Factor X was assayed by the method of Denson (1961), prothrombin as described by Jobin & Esnouf (1966), and Factor V, Factor Xa and the coagulant protein of Russell's-viper venom were assayed by the method of Jobin & Esnouf (1967).

Esterolytic activity. The esterolytic activities of Factor Xa and thrombin were measured spectrophotometrically (Schwert & Takenaka, 1955) at pH 8.0 with 1 mM-N- α -benzoylarginine ethyl ester as substrate. Measurements were made at 253 nm at 25°C (Esnouf *et al.*, 1973).

Physical methods. Specific-refractive-increment, partial-specific-volume and sedimentation-equilibrium experiments were done as described by Esnouf

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et al. (1973) except that in the sedimentation-equilibrium experiments with Factor Xa the rotor speed was 16220 rev./min and the solvent was 0.1 M-phosphate-sodium hydroxide buffer, pH 7.5, containing 10 mM-EDTA. In the experiments with the 'pro-pieces' the protein was dissolved in 0.15 M-NaCl buffered with 20 mM-Tris-chloride and centrifuged at 25980 rev./min. Thrombin was centrifuged at 12590 rev./min and was dissolved in 0.1 M-chloride-Tris buffer, pH 7.5.

Results

Preparation of Factor Xa

The chromatographic separation of Factor Xa from the dialysed activation mixture is shown in Fig. 1. The major component was Factor Xa, which was eluted at a slightly higher salt concentration than unchanged Factor X. A small amount of coagulant activity was also eluted at 0.15 M-NaCl; this was the venom coagulant protein.

If the concentration of Factor X in the activation mixture was greater than 500 µg/ml, then during activation a precipitate invariably formed, which resulted in a low recovery of Factor Xa.

Precipitation also occurred if the EDTA was added directly to the activation mixture to remove the Ca^{2+} ions; if, however, the Ca^{2+} was not removed before the activation mixture was chromatographed the separation of the components was impaired.

The Factor Xa prepared by this method moved as

a single band on polyacrylamide-gel electrophoresis (Fig. 2). If, however, the incubation of the Factor X and the venom protein was followed by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels (Fig. 3) it was found that the Factor Xa formed initially was slowly degraded to a smaller molecule. This change in size was accompanied by some loss in the specific esterase activity of the activation mixture and a disproportionate fall in coagulant activity. The gradual appearance of a precipitate, which occurs during the activation of Factor Xa in solvents of low concentration (0.02 M), may well be associated with the formation of this second form of Factor Xa.

The variation in the size of the Factor Xa molecule was also detected in sedimentation-equilibrium experiments. The M_w of several different preparations ranged from 34000 to 48000, but the majority lay in the region of 40000–42000. Factor Xa, like Factor X, shows a tendency to aggregate. Factor Xa appears to contain less carbohydrate than Factor X as judged from the higher partial specific volume of 0.721 ml/g compared with 0.714 for Factor X and an increased specific refractive increment of 0.180 ml/g compared with 0.176 for Factor X. The specific extinction coefficient based on three different preparations is 9.42, which is slightly lower than the value of 9.62 obtained for Factor X (Esnouf *et al.*, 1973).

Action of Factor Xa on prothrombin

Activated Factor X is a serine protease (Leveson & Esnouf, 1969), but there has so far been little

Fig. 1. Chromatographic separation of Factor Xa from a dialysed activation mixture on a column (1.9 cm × 20 cm) of DEAE-Sephadex A-50

The dialysed activation mixture was applied to the column at a flow rate of 45 ml/h; the protein was eluted with a 1-litre gradient (----) of 0.1–0.6 M-NaCl buffered with 15 mM-chloride-Tris, pH 7.5. The specific activity of the enzyme (○) was measured by using *N*-α-benzoylarginine ethyl ester as substrate. —, E_{280} .

Fig. 2. Disc gel electrophoresis (Davis, 1964) of activated Factor Xa on 7% polyacrylamide gel

For details see the text.

investigation into its activity on its natural substrate prothrombin. In view of this we incubated prothrombin with a mixture of Factor Xa, Factor V and phospholipid until no further prothrombin remained. The phospholipid was then removed by centrifugation and the mixture was subjected to ion-exchange chromatography.

Solutions of prothrombin (600–800 μ g/ml) in 80mM-chloride-Tris buffer, pH 7.5, were incubated at 37°C in the presence of 5mM-CaCl₂, inosithin emulsion (0.35mg/ml), Factor V (approx. 1mg/ml) and Factor Xa (0.25 μ g/ml). After 45 min 0.1 vol. of 0.1M-EDTA-sodium hydroxide buffer, pH 7.5, was added and the mixture centrifuged for 30 min at 120000g to remove the phospholipid and bound Factor V. The supernatant was then chromatographed on a column of CM-Sephadex C-50. The protein that was not retained by the column was frozen at -20°C until required for further chromatography. The proteins that were eluted from this column (Fig. 4) were a small amount of unconverted prothrombin and a major peak of thrombin, which was identified by both its coagulant activity on fibrinogen and its esterolytic activity.

The protein that did not bind to the CM-Sephadex was applied to a column of DEAE-Sephadex A-50, and the column was eluted with a chloride gradient. Two major components were recovered (Fig. 5), neither of which had any coagulant activity and which must have arisen from the non-thrombin portion of prothrombin ('pro-piece'). The summation of the extinction units in the two fractions and in the thrombin from the C-50 column accounted for over 80% of the prothrombin in the initial activation mixture; apart from a small amount of unconverted prothrombin eluted from the CM-Sephadex no other

reaction products could be detected. This result suggests that prothrombin is cleaved in at least three places by Factor Xa; two breaks are involved in the formation of the 'pro-pieces' and thrombin, and an additional break also occurs within the thrombin molecule giving rise to the two thrombin chains (Magnusson, 1971).

To test further the idea that prothrombin was cleaved only into thrombin and the two 'pro-pieces', we estimated the molecular weight of each of the separate components, by equilibrium sedimentation. In these experiments the partial specific volume of the individual component was taken as 0.72ml/g. The M_z of 'pro-piece I', that is the protein eluted first from the DEAE-Sephadex column was 16000, and the M_z of 'pro-piece II' was 19500. The M_z of thrombin measured on the protein eluted from the CM-Sephadex was 35000. Summation of these three molecular weights gives a value of 70500, which is in good agreement with the value of 70300 obtained for prothrombin by Esnouf *et al.* (1973), a result which is consistent with the idea that only these three derivatives could be formed from a single prothrombin molecule.

We next investigated the effect of the addition of each 'pro-piece' on the rate of activation of prothrombin. An activation mixture containing 0.1ml of 'pro-piece' (1mg/ml) or buffer, 25 μ l of Factor Xa (5 μ g/ml), 25 μ l of Factor V (10 μ g/ml), 0.1ml of inosithin emulsion (3.5mg/ml), 25 μ l of CaCl₂ (0.025M) and 50mM-Tris-chloride buffer, pH 8.0, to a total of 0.5ml was prepared. Prothrombin (0.5ml, 600 μ g/ml) was added and the activation was carried out at 37°C. At intervals samples (0.1ml) were withdrawn for the measurement of the esterolytic activity of the mixtures for the assay of thrombin. Control experiments were carried out to correct for the slight esterase activity of the Factor Xa. The results (Fig. 6) show that whereas 'pro-piece I' by itself had little effect on the initial rate of thrombin formation, 'pro-piece II' had a considerable effect. The implication of this observation will be discussed below. Since one of the products of this reaction, thrombin, is a proteolytic enzyme an experiment was carried out to test its effect on prothrombin. It was found that prothrombin was rapidly degraded by a dilute thrombin solution (4 μ g/ml). This degradation was detected by disc-gel electrophoresis (Fig. 7). Attempts to generate thrombin from this degraded prothrombin were unsuccessful.

The similarity between these gels and those obtained with prothrombin solution that had been stored in the cold for 3 weeks at pH 8.0 was noteworthy (Fig. 7d), and even after 30 min at 37°C slight degradation was noticed. These observations suggest that the instability of the preparations were due to trace contamination with thrombin. The enzymic nature of prothrombin degradation was also indicated

Fig. 3. Electrophoresis of Factor X, at various times during activation, on sodium dodecyl sulphate–polyacrylamide gels

Factor X (500 μg/ml) in 0.1M-NaCl in 15mM-chloride-Tris, pH 7.5, was incubated at 37°C with the venom coagulant protein (2 μg/ml) in the presence of 5mM-CaCl₂. After 3h of incubation the activation mixture was stored at 4°C until the 20h samples were taken. At the times shown samples were removed and added to 8M-urea containing 1% mercaptoethanol and 1% sodium dodecyl sulphate. The reduced and denatured protein was then subjected to electrophoresis by the method of Weber & Osborn (1969). In addition samples were removed for the assay of both the esterolytic and coagulant activities (see the Experimental section).

in a study of the stability of prothrombin at different pH values (Fig. 8). The curve obtained bears a close resemblance to the pH optimum of thrombin. The

two extreme points at pH 4.0 and 10.0 were low, owing to the precipitation and the chemical degradation of the protein,

Fig. 4. Chromatography of prothrombin, after activation with Factor *Xa*, Factor *V*, phospholipid and Ca^{2+} ions (see the text), on a column ($3.7\text{ cm} \times 37\text{ cm}$) of CM-Sephadex C-50

The column was prepared in 0.1 M-chloride-Tris buffer, pH 7.5, and eluted with a 2-litre gradient (----) of 0–0.5 M-NaCl buffered with 0.1 M-chloride-Tris buffer, pH 7.5, at a flow rate of 100 ml/h. The specific activity ($\circ$) was measured by using *N*- α -benzoylarginine ethyl ester as substrate. —, E_{280} . The fractions containing thrombin ($\uparrow\uparrow$) were pooled.

Fig. 5. Chromatography, on a column ($2.5\text{ cm} \times 32\text{ cm}$) of DEAE-Sephadex A-50, of proteins not retained by the column shown in Fig. 4

The column was prepared in 0.1 M-chloride-Tris buffer, pH 7.5. The bound protein was eluted with a 1-litre gradient (----) of 0–0.5 M-NaCl buffered with 0.1 M-chloride-Tris buffer, pH 7.5, at a flow rate of 60 ml/h. The protein was detected by measuring the E_{280} of 10 ml fractions (—). The fractions containing the 'pro-pieces' were pooled as indicated ($\uparrow\uparrow$).

Fig. 6. Effect of 'pro-pieces' on the rate of thrombin formation

The composition of the incubation mixture is described in the text. At the times indicated, samples were withdrawn and assayed for esterase activity against *N*- α -benzoylarginine ethyl ester. The specific activities refer to the amount of original prothrombin. The mixture ($\circ$) is the control. 'Pro-piece I' was added to ($\blacktriangle$) and 'pro-piece II' to ($\bullet$). The mixture ($\blacksquare$) contained both 'pro-pieces'.

Fig. 7. Disc gel electrophoresis (Davis, 1964) of bovine prothrombin

For details see the text. (a) Freshly prepared; (b) after 30 min at 37°C, pH 7.5; (c) as (b), but with thrombin (4 μ g/ml) added; (d) as (a), but after storage for 21 days at 0°C at pH 8.0.

Fig. 8. Effect of pH on the degradation of prothrombin

The prothrombin was dialysed against 0.1 M-glycine-sodium hydroxide buffer (pH 9 and 10), 0.1 M-phosphate-sodium hydroxide buffer (pH 6-8), or 0.1 M-acetate-sodium hydroxide buffer (pH 4 and 5). The various protein solutions were stored for 21 days at 0°C after which they were subjected to electrophoresis (Davis, 1964). The gels were stained and scanned with the microdensitometer. The residual prothrombin was estimated from the area under the peaks.

Discussion

The action of coagulant protein of Russell's-viper venom on Factor X appears to be one of progressive proteolysis, as is suggested by the range of molecular weights obtained for different preparation of Factor Xa. A similar progressive degradation of human Factor X was observed by Aronson & Mustafa (1971), and Milstone *et al.* (1971) have found that chymotrypsin degrades bovine thrombokinase in such a way that its coagulant activity is lost before its estero-lytic activity. The activation of the Factor X by the venom involves cleavages of both the heavy and the light chain of the zymogen; this is in contrast to the relatively minor changes caused by the activation of Factor X by the intrinsic system (Fujikawa *et al.*, 1971). Other forms of Factor Xa have been reported in which more than 50% of the zymogen is removed (Seegers *et al.*, 1963). All these forms of Factor Xa, although differing widely in their molecular weights, appear to have similar biological specificity. This phenomenon is not confined to Factor Xa. Rosenberg & Waugh (1970) and Mann *et al.* (1971a,b) showed that bovine thrombin can exist in multiple forms, arising from either genetically different prothrombins or as the result of a continuous sequence of enzymic cleavages. It remains to be seen, however, how many are formed during coagulation *in vivo*.

A further aspect of the conversion of prothrombin is the role of the non-thrombin residue of prothrombin. It has been suggested that this residue gives rise to a molecule of Factor Xa (Seegers *et al.*, 1967;

Magnusson, 1971) or a second molecule of thrombin (Seegers *et al.*, 1970). Neither of the 'pro-pieces' formed under the conditions described in this paper had any detectable coagulant activity on either prothrombin or fibrinogen. However, the possibility that these 'pro-pieces' were originally formed as enzymes and have been subsequently degraded by thrombin or Factor Xa cannot be excluded. Since we did not run sodium dodecyl sulphate-polyacrylamide gels of the reaction mixtures we cannot say when the 'pro-pieces' were formed during the course of the conversion of prothrombin into thrombin. It seems likely, from the results of Mann *et al.* (1971*a,b*), that the activation of prothrombin proceeds by the removal of one of the 'pro-pieces', followed by the further cleavage of the prothrombin molecule to give thrombin and a second 'pro-piece'.

In contrast with the results of Mann *et al.* (1971*a,b*) we obtained only three products from prothrombin, but this may be because they carried out incubations for up to 140 h, compared with 45 min in our experiments.

One of the most interesting properties of 'pro-piece II' is its inhibitory action on the conversion of prothrombin. It is tempting to suggest that this residue contains the binding site of the original prothrombin molecule for the other components of the prothrombin-converting complex, and as such should be a useful reagent for investigating the mechanism of prothrombin conversion.

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