Two Forms of the Human Prothrombin Converting Enzyme (Active Factor X)[1] (35769)

D. L. Aronson and A. J. Mustafa
(Introduced by J. S. Finlayson)
Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

It is accepted that the *sine qua non* of prothrombin conversion to thrombin, physiologically, is the presence of a unique enzyme referred to as activated Factor X (X_a) or thrombokinase (TK). While this reaction will proceed in a solution containing only prothrombin and X_a, it is substantially accelerated by the presence of Ca^{2+}, phospholipid, and a plasma factor, Factor V (1, 2). However, Milstone, in testing a sample prepared in this laboratory, found that despite its ability to catalyze the conversion of prothrombin to thrombin, the catalytic activity of this particular preparation was not appreciably enhanced by Ca^{2+}, phospholipid, and Factor V.

This apparently discrepant phenomenon was investigated by the use of coagulation tests for X_a which served to differentiate X_a activity which was augmentable by the Factor V–Ca^{2+}–phospholipid system from that which was not. In the present studies these test systems were used in conjunction with chromatographic analyses of X_a carried out at two different stages of its conversion from Factor X.

Materials and Methods. Factor X and prothrombin were purified from human plasma by a previously described method (3). However, the Factor X was *not* subjected to preparative disc electrophoresis but, following chromatography on hydroxyapatite (Bio-Rad, Richmond, California), was dialyzed against H_2O and freeze dried.[2]

Three different test methods were used for the analysis of X_a. The first of these was a modification of Milstone's two reagent assay (1). To 0.5 ml of human prothrombin (3 mg/ml) was added 0.1 ml of the test sample, and the rate of thrombin formation was measured.[3] The rate of thrombin formation is a linear function of the amount of X_a present (4). A second method employed was that described by Macfarlane (5) (one-stage assay). To 0.1 ml of bovine Factor X-deficient plasma (Diagen, supplied by Diagnostic Reagents, Ltd., Thames, Oxon, England), was added 0.1 ml of 0.2% egg lecithin (Mann Research Laboratories, New York, N.Y.), followed by 0.1 ml of the test sample and 0.1 ml of 0.025 *M* $CaCl_2$. The clotting time was then determined. The third test method for X_a was a modification of Milstone's five-reagent assay (2). To 0.5 ml of prothrombin (30 μg/ml) were added 0.1 ml of 0.25% inosothin (soybean phospholipid, Associated Concentrates, Woodside, L.I., New York), 0.2 ml of a source of Factor V ($BaCO_3$ adsorbed beef serum diluted 1:100), 0.1 ml of test sample and 0.1 ml of 0.025 *M* $CaCl_2$. The amount of thrombin generated after 5 and 10 min of incubation was then measured. The two-reagent assay and the five-reagent assay were done at a room temperature of 22°, and the one-stage assay was done in a 37° water bath. The ability of X_a to hydrolyze tosyl-L-arginine methyl ester (TAME) was measured by incubating 0.1 ml of X_a with 5.0 ml of 0.01 *M* TAME in 0.15 *M* NaCl at the ambient temperature. The acid released was continuously titrated with 0.04

[1] A summary of these results was presented at the XIII International Congress of Haematology, Munich, 1970.

[2] This preparation also contains Factor IX and Factor VII, components which play a role in the activation of Factor X.

[3] For all assays tris(hydroxmethyl)aminomethane (Tris) buffered saline, 0.15 *M* NaCl–0.02 *M* Tris-Cl, was used as a solvent for all reagents.

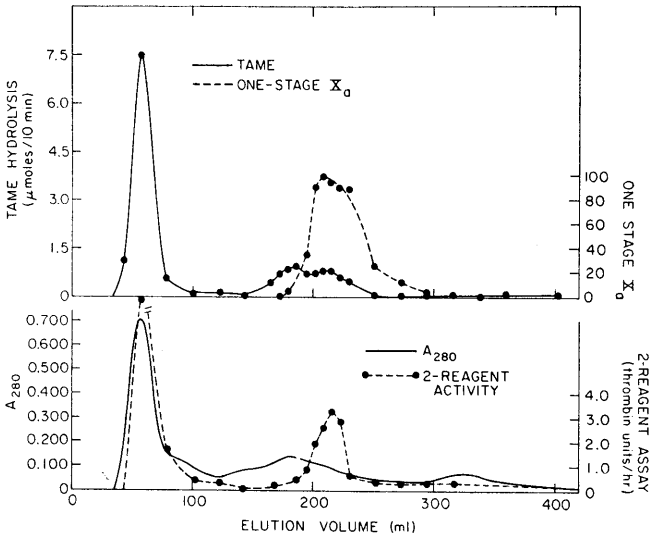

FIG. 1. Chromatographic location of X_a (TAME esterase, 2-reagent, and one-stage activities) after 25% sodium citrate activation. Chromatography was accomplished with a 2.2 × 35-cm column of DEAE-cellulose utilizing a linear salt gradient at constant pH (see text). One-stage activity is expressed as percentage of the activity of the peak fraction.

M NaOH using a Radiometer pH-stat so as to maintain the pH at 8.0. Results are expressed as microequivalents of acid released per 10 min.

DEAE-cellulose chromatography was done in columns of 2.2-cm diameter. These were either 8 or 35 cm high. In all cases a linear gradient from 0.15 *M* NaCl–0.02 *M* Tris-Cl to 0.50 *M* NaCl–0.066 *M* Tris-Cl (pH 7.4) was used with 250 ml of buffer in each of the starting reservoirs. When using the short columns, an initial wash of about 50 ml of the starting buffer was used prior to starting the gradient.

Results. Figure 1 is the chromatographic pattern found when a solution of activated Factor X was subjected to DEAE cellulose chromatography. The activation in this case was done in 25% sodium citrate; however, patterns of the same type have been obtained with tissue activated Factor X. Both the two-reagent assay and the measurement of TAME hydrolysis demonstrated at least two areas of enzymic activity. On the other hand, the one-stage assay showed significant activity only in the later-eluting chromatographic region.

When the activation of Factor X (10 mg/ml) in a 25% sodium citrate solution was followed by the same three test systems (Fig. 2), one observed a monotonic rise to a maximum level in the case of TAME hydrolysis and the two-reagent system. In striking contrast, however, was the one-stage assay in which X_a activity rose to a maximum level and then decayed. The maximum activity reached in the two-reagent system was 300 units of thrombin/ml/hr; the maximum TAME hydrolysis was 89.4 μmoles/10

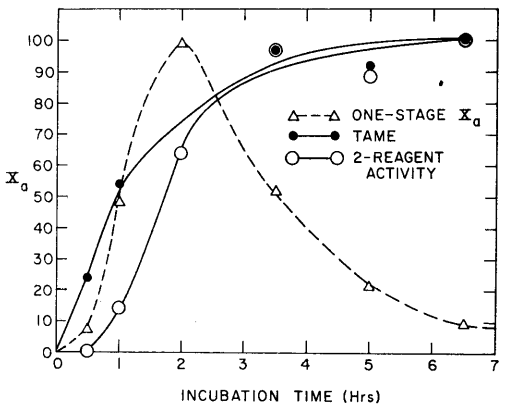

FIG. 2. Changes in the one-stage X_a assay, 2-reagent assay, and TAME esterase activity during incubation of Factor X (10 mg/ml) in 25% sodium citrate. The maximum value reached by each assay is defined as 100%.

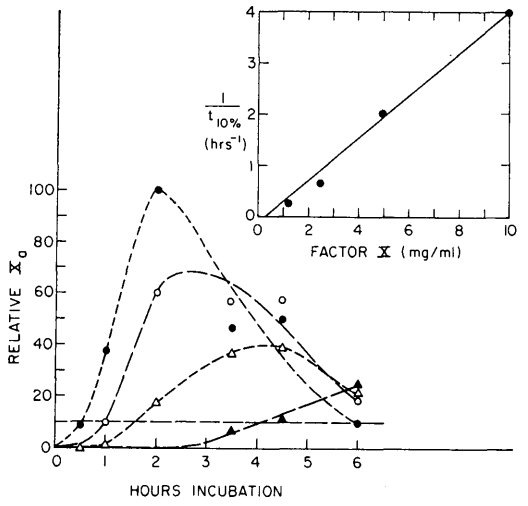

FIG. 3. The effect of Factor X concentration on the rate of formation and yield of X_a in 25% sodium citrate. Concentration of Factor X in the incubation mixture: (● - -) 10 mg/ml; (○ - -) 5 mg/ml; (△ - -) 2.5 mg/ml; (▲ - -) 1.25 mg/ml. The results are expressed as the percentage of the maximum activity attained in the 10 mg/ml sample. All tests were done at the same concentration of Factor X. The inset shows the linear relationship between rate of activation (t^{-1} to reach 10% activation) and the concentration of Factor X.

min/0.1-ml sample.

Figure 3 shows the effect of the concentration of Factor X on the rise and fall of one-stage X_a activity. Various levels of Factor X were incubated at 23° in 25% sodium citrate. After incubation for the desired length of time, aliquots were taken and diluted prior to assay. Thus all tests were done at a final dilution representing the same concentration of Factor X. Results are expressed in terms of the percentage of the maximum X_a activity attained in the sample incubated at a Factor X concentration of 10 mg/ml, the highest level investigated. As shown, the time at which the maximum X_a was attained, as well as the maximum level attained, was a function of the initial Factor X concentration. The inset in Fig. 3 is a plot of the concentration of Factor X versus the inverse of the time to reach a level of 10% of maximum X_a and demonstrates a linear relationship between the Factor X concentration and the activation rate.

In the light of these results, a preparation of Factor X at a concentration of 10 mg/ml was activated in a 25% sodium citrate solution. Aliquots were desalted by gel filtration on G-25 Sephadex and chromatographed on DEAE-cellulose (a) when there was maximum one-stage activity (after 3 hr of incubation); and (b) when the one-stage activity had fallen to 20% of the peak value (after 8.5 hr). After 3 hr, the sample had X_a activity in the later-eluting region by TAME hydrolysis, one-stage assay, and two-reagent assay (Fig. 4). The early peak already contained some two-reagent X_a activity and TAME activity. The sample chromatographed after 8.5 hr activation had almost no measurable enzymic activity in the later-eluting region, and almost all the A_{280} had moved to the breakthrough peak along with the two-reagent activity (Fig. 4). The small amount of one-stage activity seen here was probably due to a small amount of thrombin formed in the incubation mixtures. The relative recovery of A_{280}, two-reagent activity, and TAME activity were the same at the different times; however, the recovery, after chromatography, of the one-stage activity

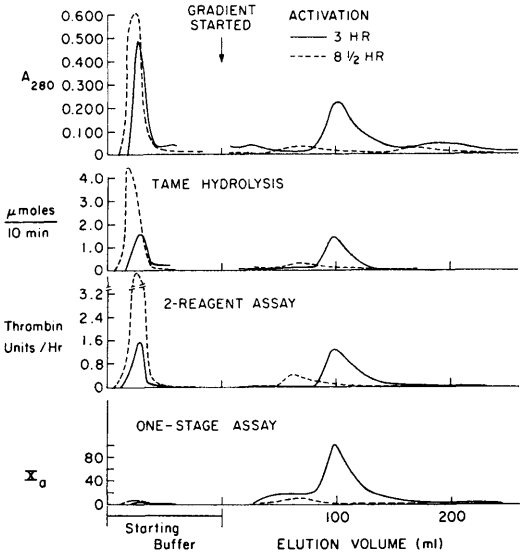

FIG. 4. Chromatographic distribution of X_a after 3 hr and 8.5 hr of activation in 25% sodium citrate. The samples were chromatographed on 2.2 × 8-cm columns of DEAE-cellulose with a linear gradient (see text).

TABLE I. Generation of Thrombin by Early-Eluting and Late-Eluting X_a in the Presence of Factor V, Ca^{2+}, and Phospholipid.[a]

	Dilution of X_a stock solutions[b]		
	1:10	1:100	1:1250
Early-eluting X_a	65.6[c]	124.6	—
Late-eluting X_a	20.9	22.0	41.8

[a] Incubation mixtures consisted of 0.5 ml of prothrombin, 0.2 ml of Factor V, 0.1 ml of Ca^{2+}, 0.1 ml of phospholipid, and 0.1 ml of test sample. After 5 min, 0.1 ml was added to 0.4 ml of fibrinogen and the clotting time was determined.

[b] Stock solutions of early- and late-eluting X_a were prepared by diluting to a two-reagent activity of 7 units of thrombin/hr/ml.

[c] Clotting time (sec).

was less in the 8.5 hr sample.

Evidence for the biochemical and physiologic significance of the two different forms of X_a was provided by the following experiment Preparations of the early- and late-eluting X_a were diluted to the same two-reagent ac tivity. The activities of these stock solutions were then measured in the five-reagent assay, which provides a quantitative measurement of the extent to which the catalysis of prothrombin conversion by the test sample of X_a is enhanced by the cofactors Ca^{2+}, phospholipid, and Factor V (2). There was more than a 125-fold difference in the activities of early- and late-eluting X_a in this test, with prothrombin conversion by late-eluting X_a manifesting far more acceleration by the cofactors (Table I).

Discussion. The relationship of the rate of the activation to the Factor X content of the activation mixture (Fig. 3) is consistent with the finding of Papahadjopoulos *et al.* (6) that X_a may play a role in the activation of Factor X. The preparations of Factor X used above are known to contain both Factor IX and Factor VII in approximately the same relative amounts found in plasma, *i.e.*, there has been no purification of Factor X relative to Factor IX and Factor VII. It is possible that one or both of these coagulation components could be responsible for the activation of Factor X in sodium citrate solutions. The dependence of the rate of activation on the Factor X concentration rules out a dissociation mechanism as the rate controlling step in this system and is consistent with earlier work on the activation of prothrombin in high concentrations of $(NH_4)_2SO_4$ (7). Papahadjopoulos *et al.* (6) discuss other factors involved in this type of activation.

The present study has demonstrated two types of human X_a which differ in their chromatographic properties and their ability to interact with Factor V in the presence of calcium and phospholipid. Milstone (8) has recently found a variant of bovine X_a which seems to have the same characteristics as the early-eluting human X_a described in the present work. This variant apparently accumulated during storage; however, Milstone found that one method of *deliberately* producing it was by treating X_a with chymotrypsin. His finding suggests that the variant is a proteolytic derivative of the one-stage active form of X_a (8). It is in complete agreement with the work presented here in which the late-eluting human X_a underwent conversion to the early-eluting form.

It is of considerable interest that another clotting factor, prothrombin (Factor II), which has some structural similarity to Factor X (3), undergoes conversion to several consecutive proenzyme derivatives prior to achieving enzyme activity (9). Factor X, on the other hand, exhibits multiple inactive forms (10), and at least two *active* forms (demonstrating, in this respect, similarity to the various molecular species of chymotrypsin and chymotrypsinogen). The form of X_a that would seem to be of greater physiologic importance (*i.e.*, the one whose activity can be modulated by cofactors) can be thought of as an intermediate in the activation scheme. That is, after its initial production by a proteolytic alteration of Factor X, it undergoes a functional (partial) inactivation by virtue of being converted, presumably proteolytically, to a form relatively unresponsive to the cofactors Ca^{2+}, phospholipid, and Factor V. This unresponsiveness of the second (*i.e.*, early-eluting) form of X_a was manifested by its low activity in the five-reagent assay (Table I) and apparently accounts for its lack of one-stage activity (Figs.

1–4). Whether this second form of X_a plays a quantitatively significant role *in vivo* is unknown. However, in this context Stadtman (11) has characterized one class of regulatory enzymes as those which catalyze identical reactions but which differ from each other with respect to cofactor specificity.

Summary. Chromatographic analysis and concomitant coagulation measurements have revealed two forms of active Factor X (X_a). Both have the ability to convert prothrombin to thrombin. Measurements at different stages during the activation of human Factor X demonstrated that the first X_a formed is eluted from DEAE-cellulose at relatively high ionic strength. Its enzymic activity is greatly enhanced by Factor V in the presence of calcium and phospholipid. Upon further incubation, this material is converted to a second form of X_a which is eluted at lower ionic strength and which shows little acceleration of prothrombin conversion in the presence of calcium, phospholipid, and Factor V.

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