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Autoactivation of Human Recombinant Coagulation Factor VII

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ABSTRACT: Single-chain human recombinant factor VII produced by transfected baby hamster kidney cells was purified to homogeneity in the presence of benzamidine. The amidolytic activity of single-chain recombinant factor VII with a peptidylnitroanilide substrate, methoxycarbonyl-D-cyclohexanylglycyl-L-arginine-*p*-nitroanilide, was less than 1% of that obtained with factor VII_a. Purified single-chain recombinant factor VII spontaneously activated in the absence of inhibitor. The activation reaction was enhanced by at least 2 orders of magnitude in the presence of a positively charged surface, provided either as an anion-exchange matrix or as poly(D-lysine). The progress curve for factor VII_a generation was sigmoidal. Benzamidine inhibits recombinant factor VII_a activity and factor VII activation with identical inhibition constants (K_i) of 11 mM. In contrast, benzamidine inhibition of bovine factor X_a and bovine factor II_a was observed at K_i values equal to 0.3 and 0.5 mM, respectively. Bovine factors X_a and II_a are known activators of factor VII and the most likely contaminants of our recombinant factor VII preparations. Single-chain recombinant factor VII purified from cells cultured in the absence of bovine serum activated at the same rate as factor VII from cells cultured in the presence of bovine serum. This also excluded the possibility that the activation reaction was caused by contaminating bovine proteases. On the basis of these observations, we propose that factor VII is autoactivated in vitro in the presence of a positively charged surface.

Coagulation factor VII (coagulation FVII)[1] is a vitamin K dependent protein playing a key role in the extrinsic pathway of blood coagulation. The protein belongs to the family of serine proteases. In its activated form, $FVII_a$, the protease catalyzes the activation of two other vitamin K dependent coagulation factors of the serine protease family, FIX and FX. Ultimately, this leads to the formation of a fibrin clot (Davie et al., 1979). As reported by Berkner et al. (1986), FVII can be produced in high yields by transfected baby hamster kidney cells. rFVII is secreted into the cell culture medium in its single-chain form just as the native molecule when synthesized in vivo by liver cells (Wion et al., 1985). Whether or not single-chain FVII is a genuine zymogen has been a matter of controversy. Using the bovine plasma derived FVII, Zur et al. came to the conclusion that FVII as well as $FVII_a$ possesses enzymatic activity (Zur et al., 1982). This conclusion has recently been challenged in studies of the human molecule (Rao et al., 1986; Rao & Rapaport, 1988). The answer to this question is of importance for an understanding of the initiation of the extrinsic pathway of coagulation. However, the problem has been hard to solve because the physiological substrates, FIX and FX, back-activate FVII once they are activated. Physiologically the activation of FVII seems to occur as a result of FIX_a (Seligsohn et al., 1979) or FX_a generation (Rao et al., 1986); however, in vitro experiments have shown that activation of FVII can also be accomplished by $FXII_a$ (Broze & Majerus, 1980) or by FII_a (Radcliffe & Nemerson, 1975). During purification of the plasma-derived bovine protein (Radcliffe & Nemerson, 1975) and the human recombinant protein (Thim et al., 1988), FVII was activated into the two-chain form by hydrolysis of the Arg_{152}–Ile_{153} bond. In both cases, the activation occurred on an anion-exchange column. Radcliffe and Nemerson (1975) suggested that activation of the bovine protein could be due to contamination with another bovine coagulation enzyme. This could also be true for the recombinant protein since the cell culture medium contains bovine serum. To investigate this possibility, we purified single-chain rFVII from serum-free cell culture medium and compared the activation on the anion matrix of this purified protein to the activation of rFVII obtained from serum-containing cell culture medium. The effect on the activation reaction of various inhibitors was also tested. Furthermore, these preparations were used to study the kinetics of FVII activation. Finally, the rFVII preparations were used to answer the question whether rFVII relative to $rFVII_a$ possesses significant activity with a peptidylanilide substrate.

[1] Abbreviations: FVII, coagulation factor VII; rFVII, recombinant FVII; FIX, factor IX; FX, factor X; FII, factor II; FXII, factor XII; SBTI, soybean trypsin inhibitor; RVV, Russel's viper venom; CH_3OCO-D-CHA-Gly-Arg-*p*NA, methoxycarbonyl-D-cyclohexanylglycyl-L-arginine-*p*-nitroanilide hydroacetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Q FF, Q Sepharose Fast Flow.

Experimental Procedures

Materials

Q and S Sepharose Fast Flow, CNBr-activated Sepharose 4B, PD-10 gel filtration columns, and low molecular weight

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standards for polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals. Soybean trypsin inhibitor (SBTI), benzamidine hydrochloride, Trizma base, and poly(D-lysine) (M_r 157 400) were from Sigma. Russel's viper venom was purchased from Wellcome. Chromogenic substrate for FX_a, methoxycarbonyl-D-cyclohexanylglycyl-L-arginine-*p*-nitroanilide hydroacetate (substrate FXa-1), was obtained from Nycomed A/S Norway. Bovine FII_a was generously provided by Dr. L. Sottrup-Jensen, Institute of Molecular Biology and Plant Physiology, University of Århus, Denmark. All other chemicals were of the highest purity commercially available.

Methods

FVII concentration was determined by an ELISA method. A monoclonal antibody to human FVII was used as catching antibody. A second monoclonal antibody conjugated with horseradish peroxidase was used as detecting antibody. Plasma-derived human $FVII_a$ was used as standard in the assay.[2]

HPLC. rFVII and FX were applied to a butylsilane wide-pore column (Vydac C4) and subjected to reversed-phase HPLC. The column was eluted with a gradient composed of 0.1% trifluoroacetic acid in water (solvent A) and 0.07% trifluoroacetic acid in acetonitrile (solvent B). With a flow rate of 1 mL/min, a gradient from 0 to 80% B was produced over 30 min.

SDS–PAGE. The analysis was performed essentially as described by Laemmli (1970). Gels were stained with Coomassie brilliant blue R 250. For quantification of the stained protein bands, an LKB Ultroscan XL laser densitometer was used. This apparatus was coupled to a Waters 840 expert integration system.

Western Blotting. The protein bands in the polyacrylamide gel were electrotransferred to a nitrocellulose membrane. The membrane was incubated with polyclonal rabbit anti-human $FVII_a$ antibodies (Dr. J. Selmer, NOVO Immunotechnology). Horseradish peroxidase conjugated goat anti-rabbit IgG was used as secondary antibody.

Cell Culture Medium. Serum containing cell culture medium included 1.5% bovine serum throughout the fermentation. Serum-free cell culture medium had the same initial serum concentration, but before collection of material for purification, the serum was replaced. The serum concentration in the serum-free medium was $\ll$0.05 ppm as judged by a sensitive ELISA against bovine IgG. The difference in serum concentration between the two media is thus more than a factor of 3×10^5.

Purification of Single-Chain rFVII. The cell culture medium was produced as described elsewhere (Berkner et al., 1986). The purification procedure used was essentially as previously described (Sakai et al., 1989). A Ca^{2+}-dependent monoclonal antibody column provides the key step of this procedure; 50 mM benzamidine was present throughout the purification. The single-chain rFVII was stored at −80 °C at a concentration >1 mg/mL in TBS (50 mM Tris/100 mM NaCl), pH 7.5, also containing 10 mM $CaCl_2$ and 50 mM benzamidine.

Purification of $rFVII_a$. The purification was performed as described previously (Thim et al., 1988).

Purification of Bovine FX. Bovine FX was purified from fresh bovine blood collected into 0.1 volume of 4% trisodium citrate, 50 mM benzamidine, and 30 mg/L aprotinin. The purification procedure was essentially as described by Hashimoto et al. (1985). The preparation contained no detectable contaminants as judged by reversed-phase HPLC and SDS–PAGE. It was stored at −20 °C in TBS, pH 7.4, with 1 mM benzamidine.

Activation of Bovine FX. Crude RVV was immobilized on CNBr-activated Sepharose 4B essentially according to the manufacturer's protocol. Five milligrams of purified bovine FX in a TBS buffer containing 10 mM $CaCl_2$ at pH 7.4 was allowed to rotate with 1 mL of gel in a stoppered plastic tube for 3 h at room temperature.

Activation Studies with Positively Charged Surfaces. Approximately 1 mg of purified single-chain rFVII was dialyzed against 1000 volumes of buffer A (50 mM Tris/100 mM NaCl, pH 8.6) containing the desired concentration of $CaCl_2$. The dialyzed solution was finally gel filtrated on a PD-10 column equilibrated in the same buffer. Incubation of the stoppered plastic tubes was carried out by end over end rotation. At time zero, the different activators and/or inhibitors were added. At the time indicated, 100-μL samples were withdrawn and boiled in reducing sample buffer for SDS–PAGE. The amount of $rFVII_a$ was determined by scanning of the Coomassie-stained gels with a laser densitometer. All experiments were carried out at room temperature.

Amidolytic Assay for $rFVII_a$-Catalyzed Activation of rFVII. The assay was performed as a two-step assay. The first step included incubation of 300 nM rFVII and various concentrations of $rFVII_a$ in 0.25 mM $CaCl_2$, 10 mM Tris, 100 mM NaCl, pH 8.0, and 1 μg/mL poly(D-lysine). The activation reaction was stopped at a fixed time by further addition of 5 mM $CaCl_2$. After addition of 0.3 mM CH_3OCO-D-CHA-Gly-Arg-*p*NA, the $rFVII_a$ generated was calculated by measuring the rate from the change in optical density at 405 nm (ΔOD_{405nm}).

Inhibition of Clotting Factors by Benzamidine. $rFVII_a$ (300 nM), bovine FX_a (5 nM), or bovine FIIa (3 nM) was incubated with benzamidine at various concentrations. The reaction was started by addition of CH_3OCO-D-CHA-Gly-Arg-*p*NA. At a fixed time, ΔOD_{405nm} was measured, and $1/\Delta OD_{405nm}$ versus inhibitor concentration was plotted. The inhibition constant (K_i) was determined from the intercept of the straight lines obtained at three different concentrations of CH_3OCO-D-CHA-Gly-Arg-*p*NA.

Kinetic Data Analysis. The model developed to describe autoactivation of coagulation FXII and prekallikrein (Tans et al., 1983, 1987) was applied to account for the time course of rFVII activation in the presence of poly(D-lysine). The most simple mechanism of FVII autoactivation is given by the second-order mechanism:

$$FVII + FVII_a \rightarrow 2FVII_a \quad (1)$$

The rate equation for $FVII_a$ generation is given by

$$d[FVII_a]/dt = k_1[FVII][FVII_a] \quad (2)$$

The final solution to this differential equation is given by (Tans et al., 1987)

$$\ln([FVII]_t/[FVII_a]_t) = \ln([FVII]_0/[FVII_a]_0) - k_1[FVII]_{tot}t \quad (3)$$

Here $[FVII]_{tot}$ denotes the total concentration of FVII plus $FVII_a$, whereas $[FVII]_t$, $[FVII_a]_t$ and $[FVII]_0$, $[FVII_a]_0$ are the respective concentrations of FVII and $FVII_a$ at time t and time zero. According to eq 3, a plot of $\ln([FVII]/[FVII_a])$ versus time (t) should give a straight line with a slope $\alpha = -k_1[FVII]_{tot}$. The intercept at $t = 0$ should be equal to the natural logarithm of the ratio $[FVII]_0/[FVII_a]_0$ present at the start of the activation reaction.

[2] T. Lund-Hansen, unpublished results.

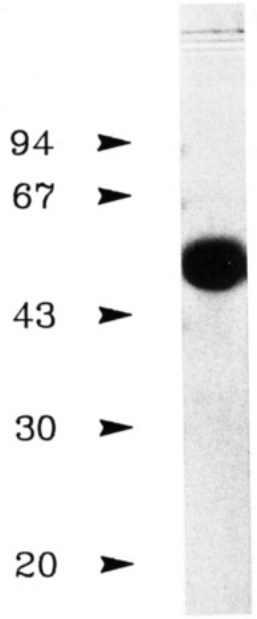

FIGURE 1: Reduced SDS–PAGE of single-chain recombinant FVII. rFVII (50 μg) was applied, and the gel was stained with Coomassie blue.

RESULTS

Purified Single-Chain rFVII. The preparation of rFVII from either serum-free or serum-containing cell culture medium resulted in a protein without detectable contamination of $rFVII_a$. The protein was visible as a single band (M_r 50000) on reduced SDS–PAGE gels stained with Coomassie blue (Figure 1). A single band with less than 1% two-chain impurities was also evident when 5 μg was applied to reduced SDS–PAGE and detected by the Western blotting technique using polyclonal antibodies raised against $rFVII_a$ (results not shown).

Spontaneous Conversion of Purified rFVII into $rFVII_a$. Spontaneous conversion of rFVII into the two-chain form ($rFVII_a$) at a slow rate was observed when the preparations were placed without inhibitors at room temperature for some days. This applied to the preparation obtained from serum-containing as well as that obtained from serum-free medium. The rate was strongly dependent on the buffer composition of the preparation. The half-life for the conversion of single-chain rFVII in 100 mM NaCl, 50 mM Tris, and 0.25 mM $CaCl_2$, pH 8.6 (buffer A), was about 100 h. When the preparations were placed at 4 °C at otherwise identical conditions, this half-life was prolonged 2-fold. The rate for the conversion was significantly reduced when Ca^{2+} was absent or when the concentration of this cation was increased to 5 mM. Buffer A was used throughout this study unless otherwise stated.

Conversion of rFVII in the Presence of Q FF. Spontaneous conversion of rFVII into the two-chain form was strongly enhanced by Q FF. The reaction rate in the presence of a 5 mg/mL sample of this anion-exchange material was nearly 100 times faster than in its absence. Half-life, $t_{1/2}$, defined as the time in which 50% of the initial amount of single-chain FVII was converted into $FVII_a$, was determined to be approximately 43 min with the Q FF added (Figure 2). FVII purified from cell culture medium produced under serum-free conditions was activated at the same rate as FVII cultured and purified from a serum-containing medium.

Effect of Poly(D-lysine). In order to further characterize the stimulatory effect of Q FF, conversion in the presence of Sepharose 4B and CNBr–Sepharose 4B coupled with ethanolamine was also investigated. No enhancement was observed, suggesting that the polycation character of the Q FF matrix was necessary for obtaining the stimulatory effect (data not shown). Support for this idea was obtained by experiments with the soluble polycation poly(D-lysine). Spontaneous conversion of rFVII was strongly enhanced by this reagent. The observed enhancement of spontaneous conversion was the same at physiological pH (7.4) as that observed at pH 8.6 while it was abolished at an ionic strength of 0.5 M NaCl (data not shown). The effect of a negatively charged matrix capable of binding rFVII was also investigated. S Sepharose Fast Flow was used in these experiments and this matrix did not stimulate activation of rFVII in the pH interval 7–8.6. Binding to S-Sepharose was found at pH 7, but not at pH 8.6.

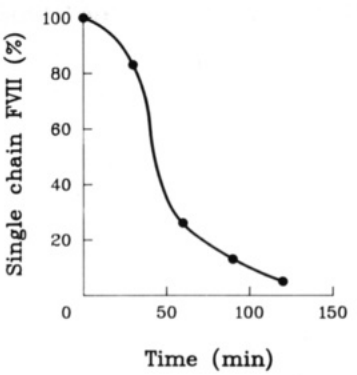

FIGURE 2: Conversion of FVII into $FVII_a$. rFVII (1 mg/mL) was incubated with 5 mg/mL (wet weight) Q Sepharose Fast Flow in buffer A. At the time intervals indicated, samples were withdrawn and subjected to SDS–PAGE under reducing conditions. The one-chain content in percent of total peak area was estimated from densitometer scans of Coomassie-stained gels.

Amidolytic Activity of $rFVII_a$ and rFVII Preparations in the Presence of 5 mM $CaCl_2$. A variety of peptidylnitroanilide substrates were tested for $rFVII_a$ activity. All were relatively poor substrates. The highest activity was obtained with the FX_a substrate, CH_3OCO-D-CHA-Gly-Arg-*p*NA. Figure 3A shows the activity as a function of substrate concentration with 300 nM $rFVII_a$ and with 300 nM rFVII. The activities of 1 nM FX_a and 3 nM FII_a are shown in Figure 3B for comparison. Whereas FX_a was saturable with substrate (K_m = 0.12 mM), saturation of $rFVII_a$ was not obtained at the concentration range 0–1.5 mM CH_3OCO-D-CHA-Gly-Arg-*p*NA. An apparent substrate inhibition observed at a concentration higher than approximately 1 mM substrate might occur as a result of substrate aggregation. Such aggregation was indicated by an opaque appearance of the reaction mixture. The activity of the rFVII preparation was 200-fold lower than that of the $rFVII_a$ preparation.

Enhancement of rFVII Activation by Substrate at 0.25 mM $CaCl_2$. Considerable nitroaniline production was observed when rFVII was added to a reaction mixture which contained 0.8 mM CH_3OCO-D-CHA-Gly-Arg-*p*NA in buffer A (Figure 4). In contrast to the progress curve obtained with $rFVII_a$, which was linear, the progress curve with rFVII was parabolic. Thus, nitroaniline production with the rFVII preparation might occur as a result of substrate-enhanced autoactivation. This idea was supported by the results shown in Figure 5. Reduced SDS–PAGE developed by immunoblotting revealed that in the presence of a high concentration of substrate, rFVII was approximately 90% converted into $rFVII_a$ during the assay, whereas in its absence no detectable cleavage occurred within 100 min. Maximal activation occurred at about 0.25 mM Ca^{2+} and about 1 mM substrate (results not shown). As indicated by the results shown in Figure 3A, no significant

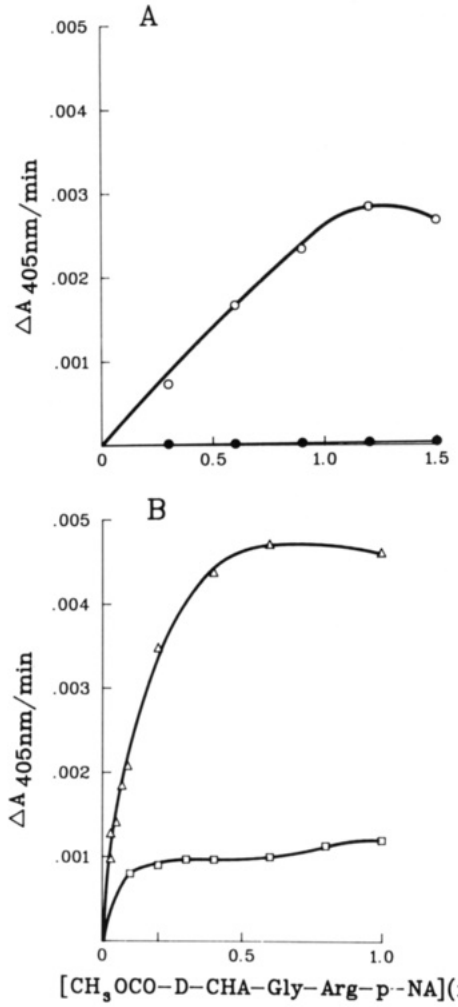

FIGURE 3: Amidolytic activity of $FVII_a$, FVII, FX_a, and FII_a with CH_3OCO-D-CHA-Gly-Arg-*p*NA. Various concentrations of chromogenic substrate were added to a reaction mixture containing 50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 8.6, and (A) 300 nM $rFVII_a$ (○) and 300 nM rFVII (●) or (B) 3 nM FII_a (□) and 1 nM FX_a (△).

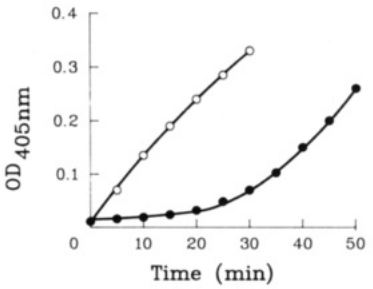

FIGURE 4: Progress curve for FVII- and $FVII_a$-induced nitroaniline generation in the presence of CH_3OCO-D-CHA-Gly-Arg-*p*NA and 0.25 mM $CaCl_2$. Additions: 0.8 mM CH_3OCO-D-CHA-Gly-Arg-*p*NA, 100 mM NaCl, 50 mM Tris-HCl, 0.25 mM $CaCl_2$, pH 8.6, and 300 nM $FVII_a$ (○) or 300 nM FVII (●).

activation took place in the presence of 5 mM $CaCl_2$. This property was utilized to develop a two-step assay applied in the following to study the activation kinetics in further detail.

Activation Kinetics. Activation was initiated by the addition of rFVII and poly(D-lysine) to a reaction mixture containing poly(D-lysine) and 0.25 mM $CaCl_2$. After incubation, the activation was stopped by further addition of 5 mM $CaCl_2$, and the amount of $rFVII_a$ was determined after subsequent addition of CH_3OCO-D-CHA-Gly-Arg-*p*NA and measurement of amidolytic activity. The activity was assumed to

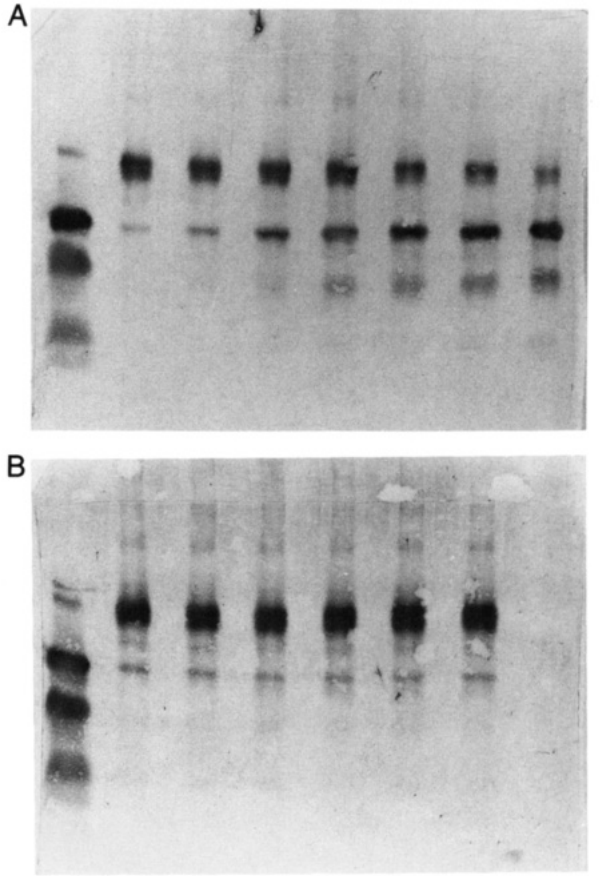

FIGURE 5: Generation of $FVII_a$ in the presence of CH_3OCO-D-CHA-Gly-Arg-*p*NA and 0.25 mM $CaCl_2$. The reaction conditions were as described in Figure 4. Samples were withdrawn at the time intervals indicated and applied to SDS–PAGE. The two-chain content was visualized by Western blotting as described under Experimental Procedures. Panel A shows the results with substrate added. Panel B shows the control experiment with omission of substrate.

Table I: Benzamidine Inhibition of Amidolytic Activity[a]

	K_i (mM)
FII_a	0.5
$FVII_a$	11
FX_a	0.3
autoactivation	11

[a] Experiments were performed in 100 mM NaCl, 50 mM Tris, and 0.25 mM $CaCl_2$, pH 8.6, 25 °C.

monitor rFVII activation, and the time course of this reaction is shown in Figure 6A. The progress curve was clearly sigmoidal, and the activation was considerably enhanced by the addition of small amounts of $rFVII_a$ (Figure 6A). The characteristics of this process resembled the autoactivation reaction observed when FXII (Tans et al., 1983) or prekallikrein (Tans et al., 1987) was incubated with sulfatides or dextran sulfate. The results were therefore analyzed according to the model proposed by Tans et al. (1987) (see Experimental Procedures) and plotted as a second-order semilogarithmic plot (Figure 6B).

Inhibition by Benzamidine and SBTI. The impact of the protease inhibitor benzamidine on activation in the presence of Q FF was investigated in Figure 7, which shows a plot of the one- to two-chain conversion as a function of benzamidine concentration. The benzamidine inhibition of FII_a, $rFVII_a$, and FX_a obtained from the kinetics with chromogenic substrate was determined for comparison (Figure 8A–C). All three proteases are competitively inhibited by benzamidine. The K_i values estimated from the intercept of plots obtained at three

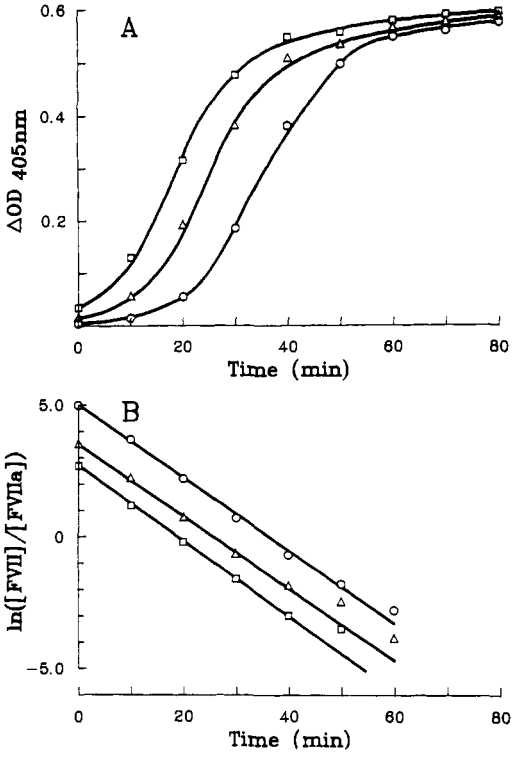

FIGURE 6: Generation of $rFVII_a$ amidolytic activity in the presence of poly(D-lysine). (A) The reaction mixture contained 300 nM rFVII and 1 μg/mL poly(D-lysine) in 100 mM NaCl, 50 mM Tris-HCl, and 0.25 mM $CaCl_2$, pH 8.6. Activation was initiated by addition of poly(D-lysine) and $FVII_a$, and the reaction was stopped at the time indicated by addition of 5 mM $CaCl_2$. $rFVII_a$ amidolytic activity was then measured in the presence of 0.3 mM CH_3-OCO-D-CHA-Gly-Arg-*p*NA. Poly(D-lysine)-induced autoactivation with 300 nM purified single-chain rFVII preparation added (○) and with 3 nM (△) and 10 nM (□) of the rFVII preparation replaced by a $rFVII_a$ preparation. (B) Second-order semilogarithmic plot of the data obtained in (A). The ratio $[FVII]/[FVII_a]$ was determined as described under Experimental Procedures.

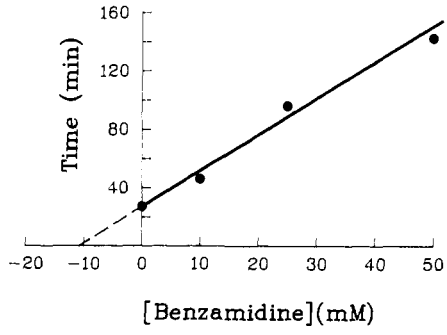

FIGURE 7: Benzamidine inhibition of the FVII conversion induced by anion-exchange material. The reaction mixture contained 20 μM rFVII and 5 mg/mL Q Sepharose Fast Flow. The generation of $rFVII_a$ at the benzamidine concentrations indicated was followed by SDS–PAGE and gel scanning as described under Experimental Procedures. The time for 20% activation was plotted as a function of benzamidine concentration.

different substrate concentrations are listed in Table I. SBTI was a potent inhibitor of bovine FX_a (K_i = 0.03 μM). In contrast, SBTI did not significantly inhibit activation in the presence of Q FF nor did it inhibit the amidolytic activity of $rFVII_a$.

Discussion

The present study describes the purification of human single-chain rFVII from serum-containing as well as serum-free cell culture medium. A slow spontaneous conversion of rFVII

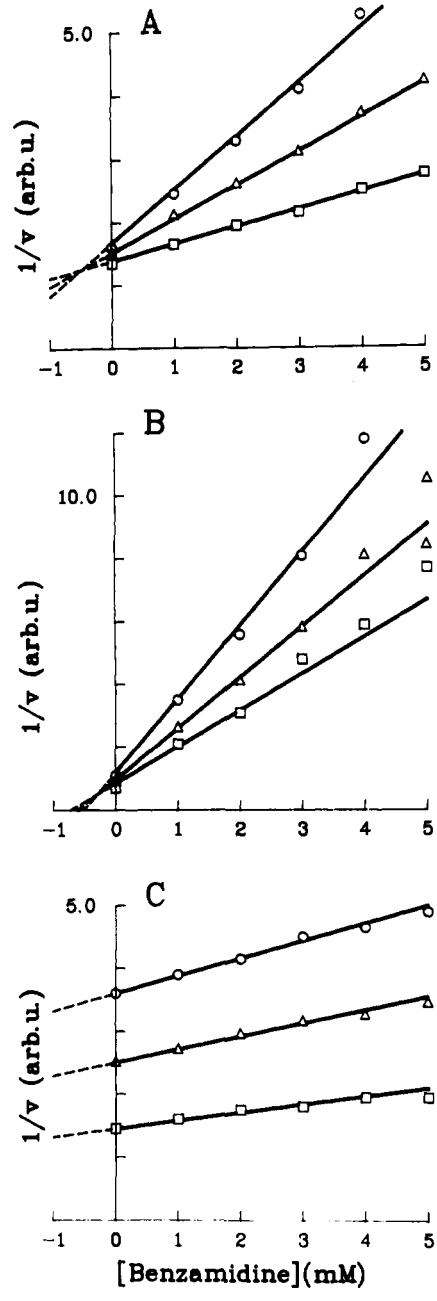

FIGURE 8: Benzamidine inhibition of the amidolytic activity of FII_a, FX_a, and $rFVII_a$. Reciprocal amidolytic activity as a function of the benzamidine concentration obtained in the presence of various fixed concentrations of CH_3OCO-D-CHA-Gly-Arg-*p*NA: (○) 0.2 mM; (△) 0.3 mM; (□) 0.6 mM. (A) 3 nM FII_a; (B) 5 nM FX_a; (C) 300 nM $FVII_a$.

to $rFVII_a$ was observed for both preparation types. The rate of this activation was strongly enhanced when the rFVII preparations were exposed to a suitable positively charged surface. Profound activation enhancement was observed with an anion-exchange resin (Q FF), poly(D-lysine), and aggregates of CH_3OCO-D-CHA-Gly-Arg-*p*NA. In contrast, the activation was not enhanced by a surface of high negative charge density such as a cation-exchange column (S Sepharose Fast Flow). This was true also under conditions where FVII bound to this matrix. The progress curve for the poly(D-lysine)-enhanced activation was clearly sigmoidal. Addition of trace amounts of $rFVII_a$ caused a significant augmentation of the activation reaction (Figure 6). This suggested that $rFVII_a$ generation was caused by autoactivation, so that the zymogen, rFVII, was cleaved by its own enzymatically active form,

$rFVII_a$. Several lines of evidence supported this conclusion. The progress curves for $rFVII_a$ generation were well described by the kinetic model for autoactivation developed by Tans et al. (1987) as evidenced by the fit to a straight line when the data were plotted in a second-order semilogarithmic plot (Figure 6B). Support for the autoactivation model was also obtained when the fraction of $rFVII_a$ present initially was varied. In accordance with the model, parallel plots were obtained in this case. At excess poly(D-lysine) (5 μg/mL), the slope of the second-order semilogarithmic plot was also proportional to $[FVII]_{tot}$ as predicted by eq 3 (results not shown). A second-order rate constant for the $FVII_a$-catalyzed cleavage of FVII, $k = 7 \times 10^3$ M^{-1} s^{-1}, was estimated from these plots. This was within the same range as described for the sulfatide-dependent autoactivation of FXII (Tans et al., 1983) and prekallikrein (Tans et al., 1987). Further, the autoactivation mechanism was supported by inhibition experiments. The inhibition of the activation reaction by benzamidine was described by an inhibition constant $K_i = 11$ mM, which was identical with the K_i obtained for benzamidine inhibition of the amidolytic activity of $rFVII_a$. Since the affinity of benzamidine for FII_a and FX_a was much higher (K_i = 0.5 and 0.3 mM, respectively), these serine proteases were not likely to be involved in the activation process. This was also indicated by the fact that SBTI, which strongly inhibited FX_a ($K_i = 0.03$ μM), did not inhibit poly(D-lysine)-dependent rFVII activation. Finally, the involvement of plasma-derived contaminants in the activation reaction was excluded by the fact that the rFVII preparation obtained from serum-free cell culture medium was characterized by the same activation properties as the one prepared from serum-containing medium. The activation was decreased 2-fold at 4 °C compared to 20 °C. Thus, the autoactivation seems to be distinct from the plasma phenomenon described as cold-promoted activation of FVII (Gjønnaes, 1972).

The question whether FVII is a genuine proenzyme with no intrinsic enzymatic activity has been a matter of controversy. Technically, it has been difficult to assess the activity with the natural substrates, FIX and FX, because the products of the respective activation reactions cause back-activation of FVII. Recently, it has been shown that it was possible to block back-activation reactions with antithrombin III and heparin (Rao & Rapaport, 1988) or by using an inactive FIX variant molecule (Rao & Rapaport, 1986). Such studies suggested that FVII possessed little or no enzymatic activity. The present study supports these observations. We found that the amidolytic activity of rFVII is approximately 0.6% of the activity of $rFVII_a$. In contrast to this observation, Zur and Nemerson (1978) reported a profound esterolytic activity of bovine FVII. However, in a later study of the clot activity, the bovine zymogen was estimated to be less than 1% active as compared to bovine $FVII_a$ (Zur et al., 1982).

The activation of FVII appears to be a crucial step in the extrinsic pathway of coagulation in vivo. Apparently, the activation occurs very rapidly in the presence of lipidated tissue factor and FX_a (Rao & Rapaport, 1988) whereas the second-order rate constant for the autoactivation reaction $k = 7 \times 10^3$ M^{-1} s^{-1} is rather small. In vivo, $FVII_a$ generation is therefore most likely to occur via FX_a-catalyzed activation. It should be emphasized, however, that the physiological equivalence to poly(D-lysine) is not known and that the presence of a suitable procoagulant surface may enhance autoactivation further. It should be pointed out that the autoactivation reaction in the presence of poly(D-lysine) occurred at similar rates at pH 7.4 and 8.6.

Some indication of an autoactivation of FVII under physiological conditions exists. Using human bladder carcinoma cells, Sakai et al. (1989) observed a slow activation reaction when the cells were incubated with FVII. This activation reaction was not inhibited by antibodies against human clotting factors II, IX, X, and XII and bovine FX, and in the presence of 10 mM benzamidine, the activation was only partly inhibited (Sakai et al., 1989), although on a speculative basis one could hypothesize that an autoactivation reaction might play a role for maintaining a low steady-state level of $FVII_a$.

It is notable that the initiators of the extrinsic as well as the intrinsic coagulation cascade all undergo autoactivation in the presence of charged surfaces. An antagonistic function in vivo might be indicated by the observation that a positively charged surface is required for FVII autoactivation, whereas a negatively charged surface stimulates FXII and prekallikrein autoactivation.

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