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Involvement of the hydrophobic stack residues 39–44 of factor VII_a in tissue factor interactions

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Abstract

Des(1–38) factor VII_a and des(1–44) factor VII_a were obtained by limited proteolysis. The binding of tissue factor to these factor VII_a-derivatives was assessed from its stimulation of the proteolytic activity on chromogenic oligopeptide substrates. Compared to native factor VII_a (K_{TF} = 0.6 ± 0.1 nM), Tissue factor binds to des(1–38) factor VII_a with a lower, but still significant affinity (K_{TF} = 4.8 ± 0.3 nM). The activity of des(1–44) factor VII_a was only slightly stimulated by TF (K_{TF} ~ 200 nM). Binding of TF depends critically on the presence of Ca^{2+} ions. Ca^{2+} ions stimulated the activity of factor VII_a/TF with an apparent K_{Ca} = 0.16 ± 0.02 mM. Factor VII_a in the absence of tissue factor was stimulated by Ca^{2+} with an apparent K_{Ca} = 0.05 ± 0.01 mM, and similar K_{Ca} values were obtained for the truncated derivatives of factor VII_a. Measurements of Ca^{2+}-induced changes in intrinsic protein fluorescence suggest a conformational change. The Ca^{2+} ion concentration at which this change occurred was higher for des(1–44) factor VII_a (apparent K_{Ca} = 0.14 mM) than for des(1–38)- and native factor VII_a (apparent K_{Ca} = 0.04 mM). The Tb^{3+} ion luminescence technique was used to further investigate the Ca^{2+} binding sites. Tb^{3+} ions bound with a lower affinity to des(1–44) factor VII_a than to des(1–38)-and native factor VII_a. The observed drastic decrease in affinity for tissue factor as a result of truncation of the 'hydrophobic stack' residues 39–44, suggest that this region of factor VII_a provides a structural determinant that together with other regions participates in tissue factor binding.

Key words: Factor VII; Tissue factor; Des(1–38) factor VII_a; Des(1–44) factor VII_a; Ca^{2+}-ion binding

1. Introduction

Factor VII is a vitamin K-dependent glycoprotein which participates in the extrinsic pathway of blood coagulation. The primary structure of factor VII [1] is homologous to that of other vitamin K-dependent coagulation proteins such as factors IX, X and protein C. The structure includes a N-terminal γ-carboxy-glutamic acid (Gla-) containing region, followed by two EGF-like domains and a C-terminal serine protease part. A well conserved 'stack' of hydrophobic residues is situated at the interface between the Gla-region and the first EGF-like domain. Factor VII is secreted as the one-chain zymogen form, and it is activated by limited proteolysis of its Arg^{152}–Ile^{153} peptide bond catalyzed by either factor X_a or factor IX_a.

The zymogen factor VII is essentially inactive, and even the activated protease, factor VII_a, possesses little activity in the absence of the cofactors, Ca^{2+} and tissue factor (TF). Thus it is only in the presence of TF and Ca^{2+}-ions that factor VII_a attains appreciable proteolytic activity towards simple peptide substrates [2] or towards its physiological substrates, factors IX and X [3].

Ca^{2+}-binding sites has been identified in the Gla-domain, the first EGF domain, and in the protease domain [4,5]. Binding of Ca^{2+}-ions to one or more of these sites appears to be essential for factor VII interaction with TF. The exact mechanism by which Ca^{2+}-ions affect the factor VII_a molecule and induce the structure required for its interaction with TF is, however, still a matter of debate, just as it has been difficult to identify the structural determinants of the molecule involved in the interaction with TF. Regions all over the factor VII_a molecule have been implicated as possible TF binding sites. The fact that the amidolytic activity of factor VII_a is stimulated by TF and Ca^{2+} [6] seems to indicate binding of these ligands to the catalytic domain, and substantiation of this interpretation is obtained by several independent observations [5,7–11]. TF interaction with the catalytic domain does, however, not exclude additional involvement of other regions, and the EGF domains of factor VIIa have been implicated as possible TF binding sites [12–14]. While it has long been known that γ-carboxylation of the factor VII Gla-domain is crucial for manifestation of full biologic activity, it is not until recently that the Gla-domain has been directly implicated in TF binding. Experiments conducted with Gla-domainless factor VII_a suggest that its binding to cell bound TF is markedly decreased [15]. Also measurements of TF/Ca^{2+}-induced stimulation of the activity with oligopeptide substrates suggest that this effect is strongly decreased [16]

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Abbreviations: Gla-, γ-carboxy-glutamic acid; EGF domain, epidermal growth factor-like domain; TF, tissue factor; (H-D-Ile-Pro-Arg-*p*NA), H-D-isoleycyl-L-prolyl-L-arginine-*p*-nitroanilide; tPA, tissue type plasminogen activator; uPA, urokinase-type plasminogen activator; PC, phosphatidyl choline; PS, phosphatidyl serine.

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or absent [17] with Gla-less factor VII_a. Competition experiments, on the other hand, indicate that separate factor VII Gla-domains (residues 1–38 [6,18] or residues 1–49 [19]) were incapable of inhibiting the specific binding of factor VIIa to TF. Apparently the Gla-domain does not, in itself, contain the structural elements necessary for the formation of a stable binary factor VIIa:TF complex.

To further investigate this interaction we have studied two Gla-less derivatives, des(1–38) factor VII_a and des(1–43) factor VII_a, in the present work. We confirm that truncation of the entire Gla-region results in a strongly decreased TF binding, however, the results suggest that truncation of a slightly shorter N-terminal fragment, leaving the 'hydrophobic stack' region (residues 39–43) intact, provides a structure that partly preserves the Ca^{2+}-dependent interaction between TF and factor VII_a.

2. Materials and methods

2.1. Proteins

Recombinant human factor VII_a was purified from baby hamster kidney cell culture medium as described by Thim et al. [20]. Stock solutions containing 0.5–1 mg/ml of protein in buffer: 10 mM glycylglycine, 50 mM NaCl, 10 mM $CaCl_2$, pH 7.4 were stored at −20°C. Des(1–38) factor VII_a, kindly provided by Dr. T. Jørgensen (Novo Nordisk A/S, Denmark), was prepared as described by Sakai et al. [15]. Des(1–44) factor VII_a, kindly provided by Dr. E.M. Nicolaisen (Novo Nordisk A/S, Denmark), was prepared as described [21]. Ca^{2+} ion depleted factor VII_a preparations were obtained after two desalting steps: (i) gel filtration on a PD-10 column equilibrated with 10 mM glycylglycine, 0.1 M NaCl pH 6.5, essentially as described by the manufacturer (Pharmacia AB, Upsala, Sweden), and (ii) by ion-exchange using Chelex 100 (Bio-Rad, Richmond, CA). Approximately 20 mg chelex 100 was added to 3.5 ml (0.05 mg/ml, 1 μM) of gel-filtrated protein solution. Recombinant human tissue factor apoprotein, TF, (detergent solubilized) was obtained from Corvas International (San Diego, CA). Truncated recombinant human tissue factor TF_{1-218} was kindly provided by Dr W. Kisiel (Albuquerque, NM)

2.2. Chemicals

Substrate S-2288 (H-D-Ile-Pro-Arg-*p*NA) was from KabiVitrum (Stockholm, Sweden). $TbCl_3 \cdot 6H_2O$ (99.9% pure) was from Janssen Chimic (Brussels, Belgium) and $CaCl_2$ and all other chemicals were analytical grade either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

2.3. Protein fluorescence Ca^{2+} ion titration experiments

The change in intrinsic protein fluorescence induced by Ca^{2+} ion binding was measured as described before [4]. Titrations with Ca^{2+} ions were performed by adding series of small volumes (2–6 μl) of aqueous $CaCl_2$ (0, 5 mM or 0.5 M) to 1 ml of enzyme (1 μM) in a semimicro cuvette. The fluorescence emmision spectrum (Ex: 280 nm, Em: 310–400 nm) was measured after each addition. The Ca^{2+} ion concentration was in the range 0–1 mM. Fluorescence intensity, F, was taken as the area of the peak centred at 337 nm and the relative fluorescence change was calculated as $(F_0 - F)/F_0$, where F_0 was the intensity in the absence of Ca^{2+} ions.

2.4. Tb^{3+} ion phosphorescence experiments

Tb^{3+} ion phosphorescence experiments were performed as described before [4]. Spectra were recorded using a Perkin-Elmer LS-50 spectrofluorometer equipped with FLDM software. Excitation was provided by a pulsed Xenon light source with a width at half peak intensity of less than 10 μs. The background due to straylight and intrinsic protein fluorescence was eliminated by insertion of a 390 nm cut-off filter in the emission beam and by measurements of the phosphorescence intensity with a 50 μs delay. The protein-Tb^{3+} ion complex was excited at 285 nm (slit width 15 nm), and phosphorescence intensity (a.u.) was recorded in the range 420–640 nm (slit width 5 nm).

Titration was performed by adding series of small volumes (2 or 4 μl) of aqueous $TbCl_3$ (5 mM) to 1 ml of enzyme (1 μM), in the absence or presence of Ca^{2+} ions in a semimicro cuvette. A phosphorescence spectrum was measured after each addition. The Tb^{3+} ion concentration was in the range 0–100 μM. The integrated phosphorescence intensity, I, was in each case taken as the area under the 544 nm emission peak (535–560 nm).

2.5. Enzyme activity

Activity measurements were performed in 96 well microtiter plates. The absorbance at 405 nm, A_{405}, was measured on a MCC/340 photometer from Labsystems (Helsinki, Finland). Control experiments at the lowest and the highest Ca^{2+} ion concentration revealed a linear increase in A_{405} over 30 min after addition of enzyme.

The apparent dissociation constant (K_{TF}) for the binding of TF to factor VII_a was determined by measuring the amidolytic activity as a function of TF concentration at a fixed concentration of factor VII_a assuming one class of high affinity binding sites, and calculated essentially as described by Krishnaswamy [22]. Data analysis was performed using the nonlinear, least square regression program ENZFITTER (Biosoft, Cambridge, UK).

The apparent constant, K_{Ca}, for the Ca^{2+}-induced stimulation of factor VII_a amidolytic activity, v, was determined (see e.g. [23]) using Equation 1:

$$\frac{v - v_0}{v_0} = (\alpha - 1)\frac{[Ca^{2+}]}{K_{Ca} + [Ca^{2+}]} \quad (1)$$

where v_0 is the rate in the absence of Ca^{2+}, and α is the enhancement factor obtained at an infinite excess of Ca^{2+}.

3. Results

3.1. Enhancement of amidolytic activity by TF

The factor VII_a catalyzed cleavage of the substrate, D-Ile-Pro-Arg-*p*NA in the absence of Ca^{2+} and TF was measured. The activity was extremely low, although measurable even in the presence of 25 mM EDTA. At saturating concentrations Ca^{2+} this activity was enhanced by a factor 3–4, and a further 7- to 8-fold enhancement was observed in the presence of an excess of TF.

Several investigators have determined the dissociation constant, K_{TF}, for the interaction between factor VII_a and TF by measuring the increase in amidolytic activity induced by TF. A similar series of experiments is shown in Fig. 1A. The activity of intact factor $FVII_a$ was strongly enhanced by the addition of an approximately equivalent molar amount of TF. Analysis of the data in terms of a tight binding ligand ($n = 1$) using the approach outlined by Krisnaswamy [22] leads to a $K_{TF} = 0.6 \pm 0.1$ nM. When the same type of experiment was performed with the two des-Gla derivatives of factor VII_a the amount of TF required for enhancement was much higher. TF bound to des(1–38) factor VII_a with an apparent $K_{TF} = 4.8 \pm 0.3$ nM, whereas the stimulation by TF of the des(1–44) factor VII_a was nearly absent and suggested an apparent $K_{TF} \sim 200$ nM (Table 1). Measure-

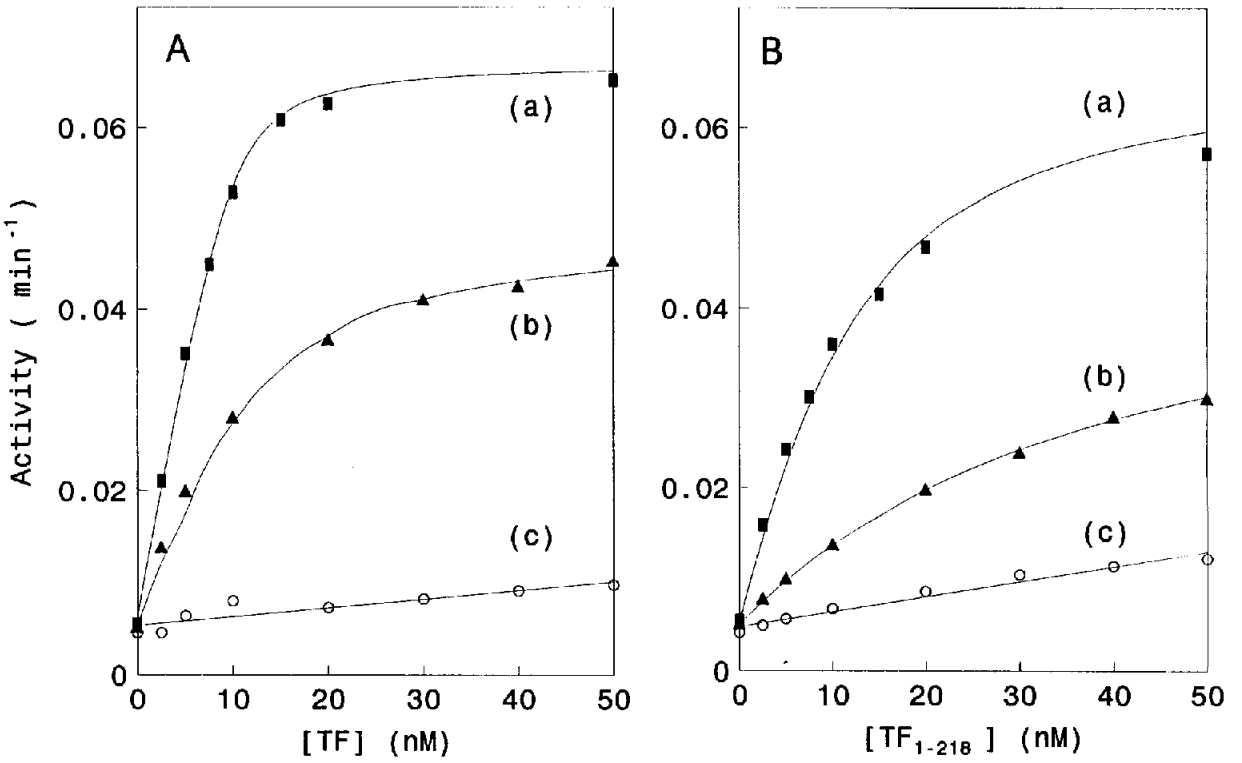

Fig. 1. Effect of TF on the amidolytic activity of factor VIIa derivatives. The activity was measured with 0.5 mM H-D-Ile-Pro-Arg-*p*NA as the substrate for factor VII_a (10 nM) at various concentrations of TF as indicated. Curve a, authentic factor VII_a; curve b, des(1–38) factor VII_a; curve c, des(1–44) factor VII_a. Panel A shows the results with detergent-solubilized full-length TF; and panel B the results with truncated TF_{1-218}. The activity was measured in TBS buffer pH 7.4 with 0.1% BSA and 5 mM $CaCl_2$ at 25°C.

ments with TF_{1-218} lacking the membrane spanning region (Fig. 1B) showed similar effects but the K_{TF} values obtained (Table 1) indicated a generally decreased binding of TF_{1-218} compared to full-length TF.

3.2. Effect of Ca^{2+} and on factor VII_a amidolytic activity in the presence of TF

The amidolytic activity of factor VII_a in the presence of TF was strongly enhanced by the addition of Ca^{2+} (Fig. 2A) with a maximum of approximately 20-fold stimulation. Apparently this effect was independent of the substrate (D-Ile-Pro-Arg-*p*NA) concentration applied. Analysis of the data shown in Fig. 2 according to Equation 1 yields an apparent K_{Ca} value for the Ca^{2+}-enhancement of 0.16 ± 0.02 mM. Approximately the same value (K_{Ca} = 0.13 ± 0.02 mM) was obtained for the Ca^{2+} stimulation of the amidolytic activity of the des(1–38) factor VII_a/TF complex (Fig. 2B).

3.3 Effect of Ca^{2+} on factor VII_a amidolytic activity in the absence of TF

In the absence of TF only approximately 3-fold maximal stimulation of the amidolytic activity of factor VII_a was obtained (Fig. 3). Using Equation 1 an apparent K_d = 0.05 ± 0.01 mM was obtained for factor VII_a. The effect of Ca^{2+} on the activity of des(1–38) factor VII_a and des(1–44) factor VII_a in the presence of TF was essentially as described for intact factor VII_a (results not shown).

3.4. Effect of Zn^{2+} on factor VII_a amidolytic activity

The activity of factor VII_a is strongly inhibited by Zn^{2+} ions [24]. Fig. 4 shows such inhibition of factor VII_a and the factor VII_a/TF complex activity in the presence of various fixed concentrations of Ca^{2+}. Factor VII_a was more sensitive to Zn^{2+} inhibition than was the factor VII_a/TF complex. It is seen that Ca^{2+} ions partially prevented Zn^{2+} inhibition.

Table 1
K_{TF} and K_{Ca} values for factor VII_a derivatives obtained from activity measurements

	K_{TF} (nM) TF_{1-263}*	K'_{TF} (nM) TF_{1-218}*	K_{Ca} (mM) TF_{1-263}#	K'_{Ca} (mM) no TP#
$FVII_a$	0.6 ± 0.1	6.0 ± 0.4	0.16 ± 0.1	0.05 ± 0.01
Des(1–38) $FVII_a$	4.8 ± 0.3	33.2 ± 0.4	0.10 ± 0.2	0.06 ± 0.02
Des(1–44) $FVII_a$	~200	~200	—	0.03 ± 0.01

*Results from Fig. 1; 50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA, pH 7.4, 25°C.
#Results from Figs. 2 and 3; 50 mM Tris-HCl, 100 mM NaCl, 0.1% BSA, pH 7.4, 25°C.

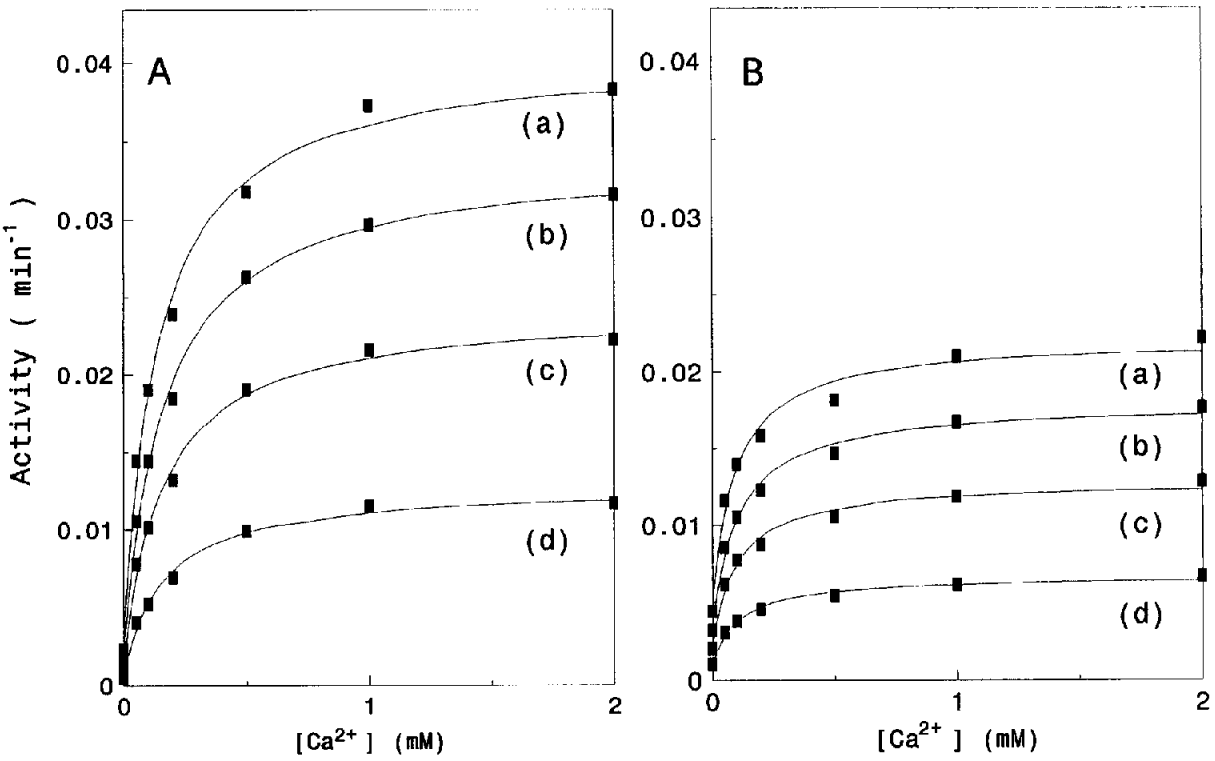

Fig. 2. Effect of Ca^{2+} ions on the amidolytic activity of factor VI_a derivatives in complex with TF. The reaction mixture contained: 10 nM factor VII_a, 16 nM TF and the peptidyl substrate H-D-Ile-Pro-Arg-*p*NA at concentrations of 0.4 mM (curve a); 0.3 mM (curve b); 0.2 mM (curve c); and 0.1 mM (curve d). Other conditions were as described in Fig. 1. Panel A shows the results obtained with authentic factor VII_a, and panel B sohws the results with des(1–38) factor VII_a.

3.5. *Fluorescence measurements*

Binding of Ca^{2+} to intact factor VII_a induces a considerable quenching of the intrinsic protein fluorescence. The tryptophan residue in position 41 may contribute to this effect and the effects of Ca^{2+} on the fluorescence properties of the two des-Gla factor VII_a forms were therefore investigated. Table 2 summarizes the results of these studies. Binding of Ca^{2+} to intact factor VII_a and des(1–38) factor VII_a results in quenching of nearly half the intrinsic fluorescence, whereas addition to des(1–44) factor VII_a of a saturating excess of Ca^{2+} results in much less quenching. The apparent K_{Ca} obtained for intact-and des(1–38) factor VII_a (0.04 mM and 0.03 mM, respectively) was similar to the value obtained from the kinetic measurements on the amidolytic activity in the absence of TF.

As shown in Table 2 also Zn^{2+} resulted in a concentration dependent quenching of the intrinsic fluorescence. In this case the relative changes were approximally the same with des(1–44) factor VII_a and factor VII_a.

3.6. *Tb^{3+} phosphorescence measurements*

The terbium ion luminescence technique has often been used to investigate Ca^{2+} ion binding to proteins. Since the effective radius of the Tb^{3+} ion is the same as that of Ca^{2+} the two ions are often interchangeable in the protein lattice. Tb^{3+} may then serve as a reporter molecule of the Ca^{2+} binding site due to absorbtion of energy from tryptophanes in close vicinity to the bound Tb^{3+}, followed by phosphorescence emission that can be observed at 489, 544, 585 and 621 nm. Tb^{3+} ions did bind each of the three forms of factor VII_a and produced strongly enhanced phosphorescence emission intensities. Measurements of the phosphorescence as a function of the Tb^{3+} concentration was used to calculate the apparent K_{Tb} value. The results are listed in Table 2. As previously described [4], Tb^{3+} ions binds to intact factor VII_a and the des(1–38) form with similar affinities, however, the affinity for des(1–44) factor VII_a was significantly decreased.

Table 2
K_{Tb} and K_{Ca} values for factor VII_a derivatives obtained by phosphorescence/fluorescence measurements

	K_{Ca} (μM)	K_{Zn} (μM)	K_{Tb} (μM)
$FVII_a$	40	10	9
Des(1–38) $FVII_a$	30	n.d.	8
Des(1–44) $FVII_a$	142	15	30

10 mM glycylglycine, 100 mM NaCl, pH 6.5, 25°C.

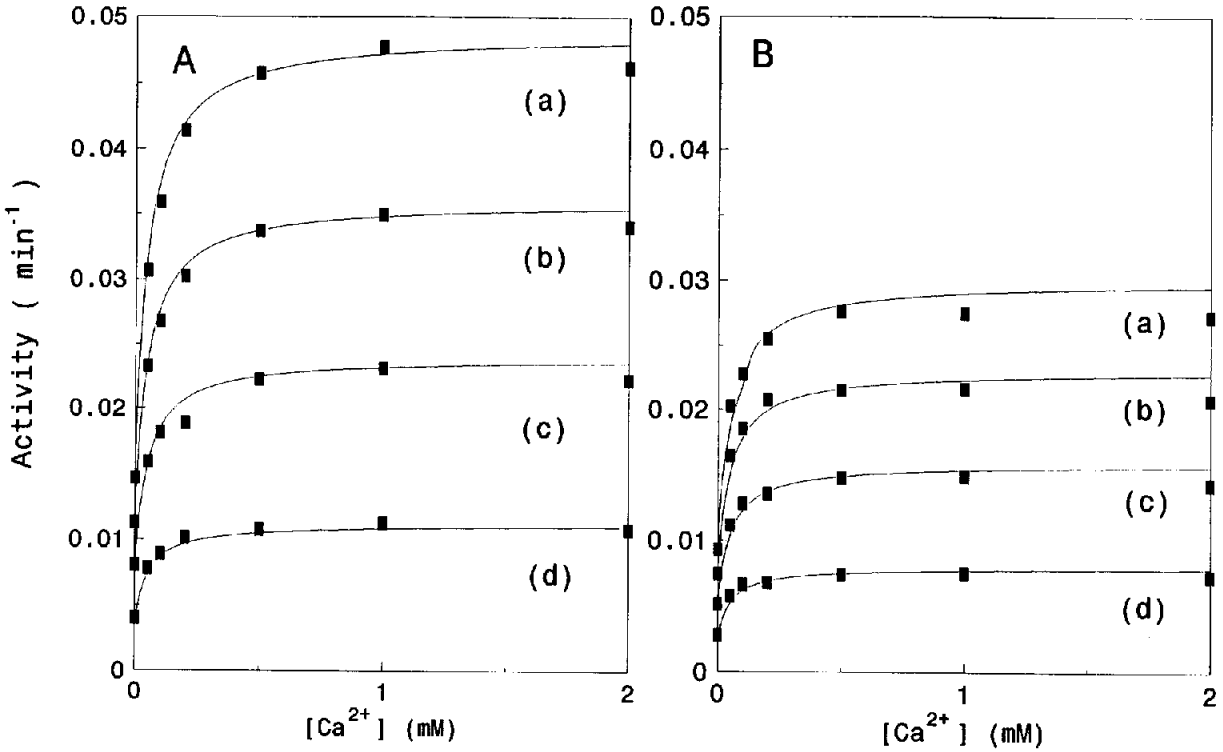

Fig. 3. Effect of Ca^{2+} ions on the amidolytic activity of factor VI_a derivatives. The reaction mixture contained: 300 nM factor VII_a, and the peptidyl substrate H-D-Ile-Pro-Arg-*p*NA at concentrations of 0.4 mM (curve a); 0.3 mM (curve b); 0.2 mM (curve c); and 0.1 mM (curve d). Other conditions were as described in Fig. 1. Panel A shows the results obtained with authentic factor VII_a, and panel B sohws the results with des(1–44) factor VII_a.

4. Discussion

4.1. The 'hydrophobic stack'

It has been shown that binding of TF to factor VII_a in the presence of Ca^{2+} induces a pronounced enhancement of the activity with peptidyl substrates [25], and this [5,16,22,26–28] as well as other methods [29,30] have been used for the assessment of the dissociation constant, K_{TF}. Our results (Table 1) on intact $FVII_a$ intreaction with detergent solubilized TF (K_{TF} = 0.6 ± 0.1 nM) and with TF_{1-218} (K_{TF} = 6.0 ± 0.4 nM) agrees reasonably well with previous values [22,27]. Much lower values (K_{TF} = 7–27 pM) have, however, been obtained in recent studies with TF in (PC/PS) phospholipid vesicles [27,30] thus indicating that membrane constituents may contribute to stabilization of the complex.

In agreement with previous results the present study also shows that truncation of the N-terminal Gla-domain strongly affects the interaction between factor VII_a and TF as assessed by the amidolytic activity. Some studies showed, however, that truncation of the Gla-domain resulted in a complete loss of the TF-dependent stimulation of activity [17], whereas others observed that some augmentation was still preserved in des-Gla factor VII_a [16]. Chymotryptic cleavage [17], and also cleavage by cathepsin G [21], result primarily in generation of the des(1–44) factor VII_a form, whereas auto-cleavage primarily results in the formation of the des(1–38) form [16]. Previous studies did not consider the possiblity of distinct functional properties of the two des-Gla forms, which may, according to our present findings, at least partly explain the controversy. Thus the present study confirms that truncation of the Gla-(1–44)region results in a strongly decreased TF binding, however, it also suggest that truncation which leaves the 'hydrophobic stack' region (residues 39–43) intact provides a structural determinant which partly preserves the Ca^{2+}-dependent interaction between TF and factor VII_a. The interaction between TF and des(1–44) factor VII_a, but not des(1–38) factor VII_a, has been investigated in a recent study [27]. The results suggesed that Gla domain depletion produced a 10- to 1000-fold decrease in binding of factor VII_a to TF, and that the most pronounced effect was obtained with lipidated TF. Our present results agree with the general conclusion, however, the effect that we observe with TF_{1-218} is somewhat higher than the 10-fold decrease in binding observed by these authors with a similar TF preparation.

4.2. Effect of Ca^{2+}-ions on factor VII_a's ability to bind to TF

The Ca^{2+} ion-dependency of the amidolytic activity of factor VII_a was significantly affected by TF (Table 2). A semiquantative explanation for this effect is possible in

terms of a model where the enzymatically active form is represented by the factor $VII_a/TF/Ca^{2+}$ complex, and where this complex can dissociate to either an 'inactive' Ca^{2+}-free- or an 'inactive' TF-free factor VII_a derivative. A simplified model of TF/Ca^{2+} stimulated activity and the corresponding rate equation (Equation 2) is shown below:

$$E\cdot Ca^{2+} + TF \overset{K_{TF}}{\rightleftarrows} E\cdot TF\cdot Ca^{2+} \overset{K_{Ca}}{\leftrightarrows} E\cdot TF + Ca^{2+}$$
$$\downarrow$$
$$S \rightarrow P$$

$$v = \frac{k \times e[S]}{1 + \frac{K_{Ca}}{[Ca^{2+}]} + \frac{K_{TF}}{[TF]}} \quad (2)$$

This model, which considers the presence only of the three predominant enzyme species, the factor VII_a/Ca^{2+} complex, the factor $VII_a/TF/Ca^{2+}$ complex and the factor VII_a/TF complex, accounts for the decrease in apparent K_{Ca} (K_{Ca}') with progressive dissociation of the activator (TF).

Our observation that TF affects K_{Ca} is at variance with recent results reported by Butenas et al. [28] who measured a K_{Ca} of 0.27 mM in the absence as well as the presence of TF. Also in contrast to these authors we did not observe a significant effect on K_{Ca} of the substrate concentration applied (Figs. 2 and 3). The difference may be attributed to the difference in substrate used.

4.3. Binding of Ca^{2+}, Zn^{2+} and Tb^{3+} to des Gla-factor VII_a

The apparent K_{Ca} obtained from activity measurements in the absence of TF was found to be 0.05 mM close to the value obtained from fluorescence measurements [4,31], which was suggested to reflect binding of Ca^{2+} ions to a site in the EGF-1 domain [4,32]. The present work shows that Ca^{2+} ion binding also affects the intrinsic fluorescence of des(1–44) factor VII_a. Since neither the EGF-1 nor the EGF-2 domain contain tryptophanes, this suggests that residues in the serine protease part work as monitors of Ca^{2+}-ion binding, and that Trp^{41} is likely to play a minor role for such Ca^{2+}-induced fluorescence changes in the intact molecule. The results seem to suggest that Ca^{2+}-ion binding to the EGF-1 domain induces a conformational change in the protein with implications for the catalytic properties of the serine protease part. The results also agree with previous suggestions that Ca^{2+} binding appears to be a prerequisite to effective TF binding.

The stimulatory effect of Ca^{2+}-ions, and the antagonistic, inhibitory effect of Zn^{2+}-ions on the amidolytic activity of factor VII_a appears to be rather unique. We have tested a number of other proteases including trypsin, chymotrypsin, pancreatic elastase, leukocyte elastase, cathepsin G, thrombin, factor X_a, factor XII_a, factor XI_a, activated protein C, plasmin, tPA and uPA for similar effects, but found only rather weak effects of these ions on the activity (results not shown). Histidines are prominent ligands in Zn^{2+}-ion coordination in zinc binding proteins whereas Ca^{2+}-ion usually coordinates with carboxyl oxygens or main chain carbonyl oxygens, and in order to account for the apparent antagonistic character of these divalent ions, it is therefore most likely that the two binding sites are distinct but possibly affecting the same local environment.

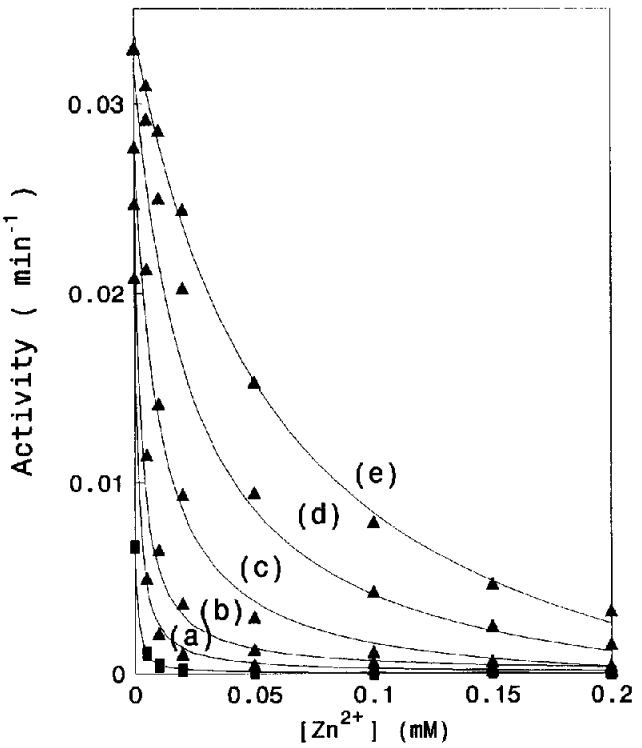

Fig. 4. Effect of Zn^{2+} ions on the amidolytic activity of factor VI_a. The reaction mixture contained: 300 nM factor VII_a, and 0.4 mM H-D-Ile-Pro-Arg-*p*NA and $CaCl_2$ at concentrations of 0.1 mM (curve a); 0.25 mM (curve b); 0.5 mM (curve c); 2.0 mM (curve d) and 5.0 mM (curve e). The Zn^{2+} ion inhibition in the absence of Ca^{2+} ions is shown for comparison (filled squares). The reaction was performed in 50 mM Tris-HCl, 100 mM NaCl 0.01% Tween 80, pH 7.4 at 25°C.

We have previously proposed that the Ca^{2+}-binding site monitored by Tb^{3+} phosphorescence should be attributed to the Gly^{209}–Gln^{221} loop of the protease part [4]. In accordance with this proposal we found that the des(1–44) factor VII_a still able to bind to Tb^{3+} ions. This allows us to exclude Trp^{41} from being involved in non-radiative energy transfer to Tb^{3+}. Homology considerations based on the amino acid sequences of trypsin and pancreatic elastase points to Trp^{284} as the most likely candidate for such an interaction with Tb^{3+}. As indicated by comparative measurements of the two des-Gla forms (Table 2) apparently the binding of Ca^{2+} monitored by intrinsic fluorescence, and also the binding of Tb^{3+} monitored by phosphorescence, decreases upon removal of

the hydrophobic stack residues 39–44. We have no obvious explanation for this phenomenon, however, it is possible that residues 39–44 contribute to the stabilization of the overall structure of the protein in the presence of Ca^{2+}.

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