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Two different Ca^{2+} ion binding sites in factor VIIa and in des(1–38) factor VIIa

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The Ca^{2+} ion binding of factor VIIa and the derivative lacking the γ-carboxyglutamic acid domain, des(1–38) factor VIIa, was investigated using intrinsic protein fluorescence and Tb^{3+} ion phosphorescence methods. Binding of Ca^{2+} ions giving rise to a decrease in the intrinsic protein fluorescence (approximately 50% at saturating conditions) is seen with both proteins. Each of the saturation curves is in accordance with the formation of a 1:1 complex of factor VIIa-Ca^{2+} (K_D~30 μM) and des(1–38) factor VIIa-Ca^{2+} (K_D~40 μM)). Yet another Ca^{2+} ion binding site reveals itself in each protein in Tb^{3+} ion phosphorescence experiments. Ca^{2+} ion competition studies have showed 1:1 complexes (K_D's ~2 mM). The results are interpreted in terms of two different Ca^{2+} ion binding sites, one in the EGF-1 domain and one in the Gly-209–Gln-221 loop of the serine proteinase part.

Factor VIIa; des(1–38) factor VIIa; Ca^{2+} ion binding; Intrinsic protein fluorescence; Tb^{3+} ion phosphorescence

1. INTRODUCTION

Factor VII is a vitamin K-dependent glycoprotein which participates in the extrinsic pathway of blood coagulation. It is homologous to factor IX, factor X and protein C. The structure of these proteins includes an N-terminal γ-carboxyglutamic acid-containing region, followed by two EGF-like domains and a C-terminal serine proteinase part [1].

Factor VIIa consists of two peptide chains held together by a disulfide bridge, and results when the Arg-152–Ile-153 peptide bond of factor VII, its zymogen, is cleaved. The activity of factor VIIa is Ca^{2+} ion dependent. It is well known that γ-carboxyglutamic acid regions bind Ca^{2+} ions, and that they are involved in protein–phospholipid interactions. Recent results on factor IX, however, have shown that other important Ca^{2+} ion binding sites exist, one in the EGF-1 domain [2] and another one in the serine proteinase part [3]. The existence of an EGF-1 Ca^{2+} ion binding site has also been demonstrated in factor X [4] and in protein C [5,6]. Based on the homologies between factor VII and these coagulation proteins it seems reasonable to expect similar Ca^{2+} ion binding sites, perhaps of great importance, for the function of factor VIIa [3].

We have investigated the Ca^{2+} ion binding of factor VIIa and des(1–38) factor VIIa, a derivative of factor VIIa lacking the γ-carboxyglutamic acid domain, using protein fluorescence and Tb^{3+} ion phosphorescence, and report here evidence for two Ca^{2+} ion binding sites that are not present in the γ-carboxylglutamic acid domain of factor VIIa. It is suggested that one site is localized in the EGF-1 domain, whereas the other is in the serine proteinase part of factor VIIa.

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2. EXPERIMENTAL

2.1. *Proteins*

Recombinant human factor VII was purified from baby hamster kidney cell culture medium as described by Thim et al. [8]. Stock solutions contained 0.5–1 mg/ml of protein in buffer (10 mM glycylglycine, 50 mM NaCl, 10 mM $CaCl_2$, pH 7.4) and were stored at −20°C. Des-γ-carboxyglutamic acid factor VIIa (des(1–38) factor VIIa), kindly provided by Dr. T. Jørgensen (Novo Nordisk A/S, Denmark), was prepared as described by Sakai et al. [8]. Ca^{2+} ion-free preparations were obtained after two desalting steps: (i) in a PD-10 column equilibrated with buffer (10 mM glycylglycine, 0.1 M NaCl, pH 6.5) essentially as described by the manufacturer (Pharmacia AB, Uppsala, Sweden); and (ii) with chelex 100 (Bio-Rad, Richmond, CA) of which approximately 20 mg was used to prepare 3.5 ml (0.05 mg/ml, 1 μM) of enzyme.

2.2. *Chemicals*

The buffer was 10 mM glycylglycine, 0.1 M NaCl, pH 6.5. Salts: $TbCl_3 \cdot 6H_2O$ 99.9% pure was from Janssen Chimie (Brussels, Belgium), $CaCl_2$ and all other chemicals were analytical grade, either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

2.3. *Tb^{3+} ion phosphorescence titration experiments*

The Tb^{3+} ion luminescence technique has often been used to investigate Ca^{2+} ion binding to proteins (for reviews, see [9,10]). The technique takes advantage of the ability of Tb^{3+} ions to bind at the same sites as Ca^{2+} ions. These ions have similar effective ionic radii. When a Tb^{3+} ion is bound in a protein it is possible to displace it with a Ca^{2+} ion, and thus to gain information on the binding constants of both

ions. A measure of bound Tb^{3+} ions is obtained from their phosphorescence. The protein–Tb^{3+} ion complex is excited by light of a wavelength (around 280 nm) at which absorption of the Tb^{3+} ions is negligible, but the aromatic amino acids, mainly the tryptophans, absorb. Most of the absorbed excitation energy of the tryptophan residues give rise to intrinsic protein fluorescence. The tryptophans in close vicinity to a bound Tb^{3+} ion are also deactivated by non-radiative energy transfer of the Förster type. Phosphorescence corresponding to transition from $^5D_4$ to various sublevels of ^7F in the Tb^{3+} ion is then observed at 489, 544, 585 and 621 nM (Fig. 1).

All experiments were performed at pH 6.5, 25°C. Spectra were recorded in 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 then 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 mm) (Fig. 1).

Titrations were performed by adding a 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 semi-micro 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 of the 544 nm emission peak (535–560 nm).

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

The change of intrinsic protein fluorescence induced by Ca^{2+} ion binding was measured. In these experiments no time delay and no filters in the emission beam were used. Excitation wavelength was at 280 nm, the spectral bandwidth of both slits were 5 nm. The emission spectra were taken in the range 310–400 nm and the maximum intensity emission wavelength λ_{max}, was found at 337 nm. A marked decrease in the intensity occurred, but no change of λ_{max} was observed when Ca^{2+} ions were added. 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 semi-micro cuvette. The fluorescence spectrum was measured after each addition. The Ca^{2+} ion concentration was in the range 0 –1 mM. The fluorescence intensity, F, was taken as the area of the peak centred at 337 nm, and the relative fluorescence changes were calculated as $(F_0-F)/F_0$, where F_0 is that in the absence of Ca^{2+} ions.

3. RESULTS AND DISCUSSION

3.1. *Tb^{3+} ion phosphorescence titration experiments*

Phosphorescence spectra of the factor VIIa–Tb^{3+} ion complex and of aqueous Tb^{3+} ions are shown in Fig. 1. Emission from free Tb^{3+} ions in the relevant concentration range was negligible, but as seen from Fig. 1 the Tb^{3+} ion phosphorescence was strongly enhanced when Tb^{3+} ions were bound to factor VIIa. The dependence of the Tb^{3+} ion concentration on the phosphorescence was investigated. The integrated intensities of factor VIIa–Tb^{3+} ion complex phosphorescence, I, is plotted against the Tb^{3+} ion concentration in the presence and absence of Ca^{2+} ions in Fig. 2. Also shown are the curves fitted to the data according to the expression of a simple binding curve (Eqn. 1):

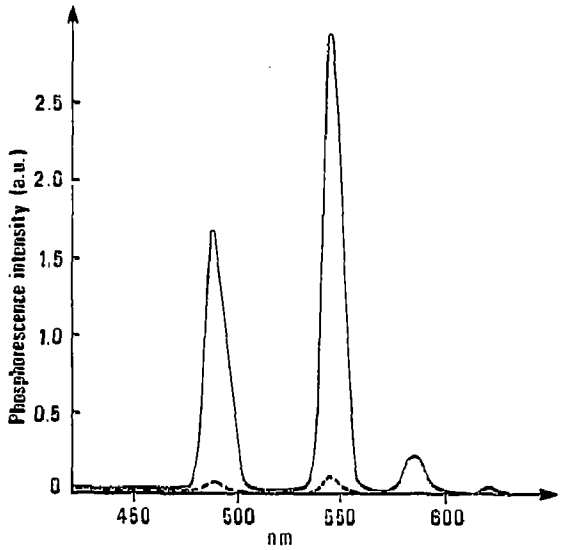

Fig. 1. Phosphorescence spectra of the factor VIIa–Tb^{3+} ion complex and of aqueous Tb^{3+} ions. Excitation at 285 nm was provided by a pulsed xenon light source and the phosphorescence intensity emitted was measured after a delay of 50 μs in the range shown. (–) Factor VIIa–Tb^{3+} ion complex (Tb^{3+} ion, 10 μM; factor VIIa, 1 μM). (----) Aqueous Tb^{3+} ion, 10 μM. Experimental conditions: 25°C. Buffer: glycylglycine 10 mM, NaCl 0.1 M, pH 6.5.

$$I = \frac{I_{max}}{1 + \frac{K_{Tb}}{[Tb^{3+}]}} \quad (1)$$

where I is the intensity, I_{max} is the theoretical limit value at saturating Tb^{3+} ion conditions and K_{Tb} is the dissociation constant of an 1:1 Tb^{3+} ion–enzyme complex.

The results shown in Fig. 2 are in accordance with a simple 1:1 binding of Tb^{3+} ion to the factor VIIa. Several Ca^{2+} ion binding sites of factor VIIa were expected and yet only one Tb^{3+} ion binding site revealed itself in the phosphorescence experiment. It seems reasonable to assume that this Tb^{3+} ion binding site is the only one in sufficiently close proximity to an aromatic amino acid side chain to allow for a Förster energy tranfer.

Ca^{2+} ions are expected to inhibit the Tb^{3+} ion phosphorescence competitively. If so, this results in an increase in the observed dissociation constant, K_{obs}, where K_{obs} is related to the dissociation contants, K_{Tb} and K_{Ca} as follows (Eqn. 2):

$$K_{obs} = K_{Tb}\,(1 + \frac{[Ca^{2+}]}{K_{Ca}}). \quad (2)$$

A plot of K_{obs} as a function of the Ca^{2+} ion concentration is shown in Fig. 2 (insert). The plot is linear, as expected from Eqn. 2, showing that Tb^{3+} ions and Ca^{2+} ions are indeed competing for the same site. The values of the individual dissociation constants obtained from the experimental data, according to Eqn. 1 and 2, are given in Table I.

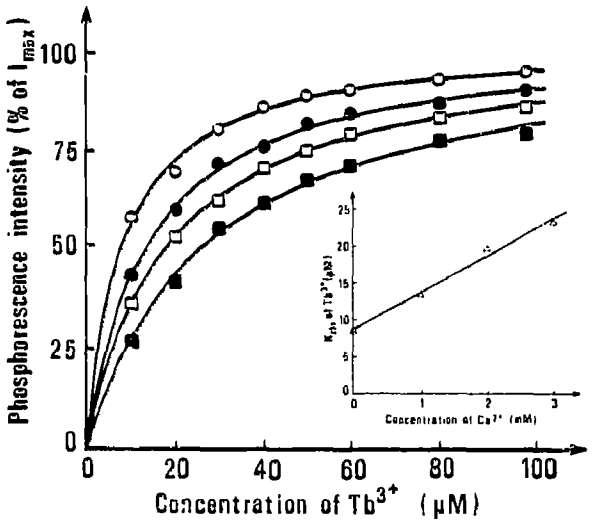

Fig. 2. Factor VIIa-Tb^{3+} ion complex phosphorescence. The dependencies of the Tb^{3+} ion concentration on the phosphorescence in the presence and absence of competing Ca^{2+} ions are shown. The phosphorescence intensities were obtained from the area under the 544 nm emission peak (Fig. 1). The lines illustrated represent the best fit of Eqn. 1 (see text) to the experimental data, using a non-linear regression analysis program. The intensities are given in the units % of I_{max}. Experimental conditions (○) 0, (●) 1 mM; (□) 2 mM, (■) 3 mM concentration of Ca^{2+} ions; FVIIa 1 μM; buffer glycylglycine 10 mM, NaCl 0.1 M, pH 6.5, 25°C. Insert: plot of K_{obs} vs. the concentration of Ca^{2+} ions. Values for K_{obs} were obtained from the fits of Eqn. 1. The same experiments, but on des(1–38) factor VIIa, showed essentially identical results.

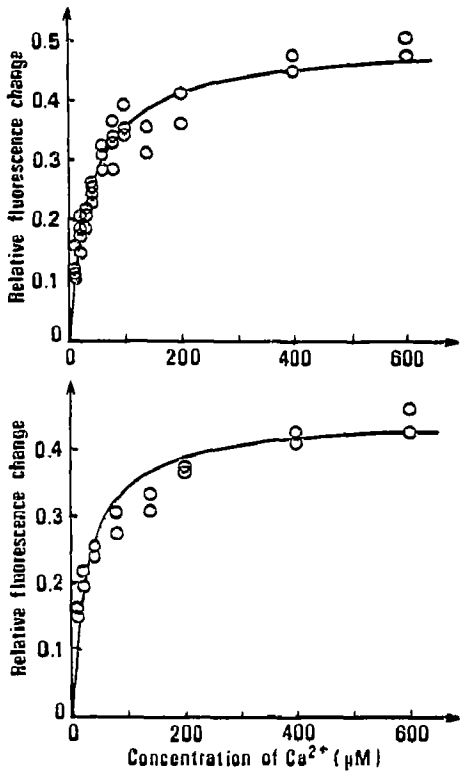

Fig. 3. Relative change of the intrinsic protein fluorescence plotted as a function of the concentration of Ca^{2+} ions. The lines illustrated are each obtained from a fit to a simple Ca^{2+} ion binding expression (equivalent to Eqn. 1), using a non-linear regression analysis program. (Upper figure) Results on binding of Ca^{2+} ions to factor VIIa. (Lower figure) Results on binding of Ca^{2+} ions to the derivative des(1–38) factor VIIa. Experimental conditions: enzymes 1 μM; buffer, glycylglycine 10 mM, NaCl 0.1 M, pH 6.5, 25°C.

The binding of Tb^{3+} and Ca^{2+} ions to des(1–38) factor VIIa was also studied. The experimental results (Table I) for this derivative of the enzyme were in essence identical to the wild-type factor VIIa (Fig. 2). Thus the Tb^{3+} and Ca^{2+} ion binding site observed is not a γ-carboxyglutamic acid-dependent site. This means that it is localized either in an EGF-domain or in the serine proteinase part of the molecule.

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

A decrease in the intrinsic protein fluorescence of factor VIIa and of des(1–38) factor VIIa was observed when Ca^{2+} ions were added. The concentration dependencies of this phenomenon are shown in Fig. 3. Each curve is in accordance with the formation of a 1:1 Ca^{2+} ion–enzyme complex (Eqn. 1). The resulting values of the dissociation constants are given in Table I. The relative fluorescence change obtained at saturation of this Ca^{2+} ion binding site of factor VIIa, as well as that of des(1–38)factor VIIa, amounts to approximately 50%, apparently gross changes of the proteins occur. Interestingly, both enzymes show values of the dissociation constant of this site of approximately 50-times less than those of the site giving rise to Tb^{3+} ion phosphorescence. We therefore conclude that two different Ca^{2+} ion binding sites are present in the C-terminal Leu-39–Pro-406 part of factor VIIa.

The observations reported here are in good agreement with results on factor IX [2,3] where two different Ca^{2+} ion binding sites not dependent on the γ-carboxyglutamic acid part of the molecule were demonstrated. One site was in the EGF-1 domain and one in the serine proteinase part, and the most tight binding was the site in the EGF-1 domain. Further, Ca^{2+} ion binding to the

Table I

Dissociation constants of the enzyme–ion complexes

Type of experiment	Enzyme	K_{Tb} (mM)	K_{Ca} (mM)
Phosphorescence	factor VIIa	0.009 ± 0.001	1.8 ± 0.2
	des(1–38) factor VIIa	0.008 ± 0.001	3.0 ± 0.8
Fluorescence	factor VIIa	-	0.04 ± 0.003
	des(1–38) factor VIIa	-	0.03 ± 0.005

EGF-1 site of protein C (K_D = 0.1 mM [5] and K_D = 44 – 61 μM [6]) induces an intrinsic protein fluorescence change [6]. A K_D = 9 μM of the EGF-1 site of factor X has been reported [4]. Based on these results, and the homologies of the proteins, we find it highly likely that the Ca^{2+} ion binding site of factor VIIa that gives rise to the fluorescence change is an EGF-1 binding site. Also, the K_D values obtained point in this direction.

A Ca^{2+} ion binding site in the proteinase part of factor IX, similar to sites in trypsin and in pancreatic elastase [11,12], has been assigned to the loop Gly-234–Gln-246 (K_D = 500 μM), and the corresponding loop Gly-209–Gln-221 in factor VIIa has been hypothesized to bind one Ca^{2+} ion [3]. Our results provide experimental evidence for a Ca^{2+} ion binding site which is less tight than the presumed EGF-1 site. We suggest that the Ca^{2+} ion binding site observed in the Tb^{3+} ion phosphorescence experiment is localized in the serine proteinase part of factor VIIa in the loop Gly-209–Gln-221.

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