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Structural changes in factor VIIa induced by Ca^{2+} and tissue factor studied using circular dichroism spectroscopy

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Abstract

Factor VIIa (fVIIa) is composed of four discrete domains, a γ-carboxyglutamic acid (Gla)-containing domain, two epidermal growth factor (EGF)-like domains, and a serine protease domain, all of which appear to be involved, to different extents, in an optimal interaction with tissue factor (TF). All except the second EGF-like domain contain at least one Ca^{2+} binding site and many properties of fVIIa, e.g., TF and phospholipid binding and amidolytic activity, are Ca^{2+}-dependent. A CD study was performed to characterize and locate the conformational changes in fVIIa induced by Ca^{2+} and TF binding. In addition to intact fVIIa, derivatives lacking the Gla domain or the protease domain were used. Assignment of the Ca^{2+}-induced changes in the far-UV region of the fVIIa spectrum to the Gla domain could be made by comparing the CD spectra obtained with these fVIIa derivatives. The changes primarily appeared to reflect a Ca^{2+}-induced ordering of α-helices existing in the apo state of fVIIa. This was corroborated by models of the apo and Ca^{2+} forms of fVIIa constructed on the basis of known structures of homologous proteins. Far-UV spectra of the Gla domain of fVIIa, obtained as difference spectra between fVIIa derivatives, were very similar to those of isolated Gla peptides from other vitamin K-dependent plasma proteins. The near-UV CD spectrum of fVIIa was dominated by aromatic residues residing in the protease domain and specific bands affected by Ca^{2+} were indicative of tertiary structural alterations. The formation of a fVIIa:TF complex led to secondary structural changes that appeared to be restricted to the catalytic domain, possibly shedding light on the mechanism by which TF induces an enhancement of fVIIa catalytic activity.

Keywords: calcium binding; circular dichroism; factor VIIa; Gla domain; protein–protein interaction; serine protease domain; tissue factor

Factor VIIa (fVIIa) is one of several protein constituents involved in the blood coagulation cascade. In this process, fVIIa plays a key role in the initiation of the extrinsic pathway through binding to an integral membrane glycoprotein, tissue factor (TF). This association renders fVIIa able to activate factors IX and X. fVIIa is a multidomain protein consisting of an N-terminal γ-carboxyglutamic acid (Gla)-containing domain, two epidermal growth factor (EGF)-like domains, and a serine protease domain homologous with trypsin (Hagen et al., 1986). The fVIIa:TF complex has been studied extensively and the Gla, first EGF-like, and protease domains in fVIIa have been found to be involved in the interaction with TF (Sakai et al., 1990; Wildgoose et al., 1990, 1992; Ruf et al., 1991; Toomey et al., 1991; Clarke et al., 1992; Higashi et al., 1992, 1994; Kazama et al., 1993; Kumar & Fair, 1993; Petersen et al., 1994; Chang et al., 1995; Sabharwal et al., 1995). The recently published X-ray crystallographic structure of the fVIIa:TF complex corroborates the biochemical data and, in addition, identifies a small contribution from the second EGF-like domain (Banner et al., 1996). An important role for the Gla and second EGF-like domains in the optimal interaction with TF may be to support the structure of other regions in fVIIa in direct contact with TF (Wildgoose et al., 1992; Chang et al., 1995). The detailed three-dimensional structure of fVIIa has not yet been determined. Data obtained using X-ray and neutron scattering suggest an extended structure for fVIIa in solution (Ashton et al., 1995). This implies that the domains are ordered like pearls on a string, allowing physical interactions only between domains adjacent in the linear sequence. The struc-

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ture of the fVIIa:TF complex showed an extended but bent conformation of fVIIa in the complex (Banner et al., 1996). However, the lack of detailed structural information on fVIIa alone precludes deduction of the structural changes occurring in fVIIa in conjunction with TF binding.

A variety of approaches has been utilized to study Ca^{2+}-induced conformational changes in the vitamin K-dependent clotting factors, including fVIIa. It has been established that there are three different Ca^{2+} binding regions in fVIIa, including relatively high-affinity sites in the protease (Wildgoose et al., 1993) and the first EGF-like domains (Schiødt et al., 1992), and a heterogeneous set of sites of generally lower affinity in the Gla domain (Persson & Petersen, 1995). Together, these sites are responsible for an array of pivotal Ca^{2+}-induced changes in fVIIa underlying for instance the absolute Ca^{2+} requirement of the fVIIa:TF interaction at physiological protein concentrations.

CD can be used to monitor both the secondary and tertiary structure of a protein (Johnson, 1990). Thus, it is possible to study both local environmental changes around aromatic residues and more substantial changes of the overall backbone fold of the protein. CD measurements have been performed previously on vitamin K-dependent coagulation factors such as prothrombin and prothrombin fragment 1 (Bloom & Mann, 1978; Marsh et al., 1979; Nelsestuen et al., 1981), factor IX and factor X (Furie & Furie, 1976; Beals & Castellino, 1988), Gla-domainless factor X and protein C (Sugo et al., 1984), and the isolated Gla domains of factor IX (Vysotchin et al., 1993) and protein C (Colpitts & Castellino, 1994). We have employed CD spectroscopy in a systematic study in order to probe structural changes in fVIIa upon the binding of Ca^{2+} and upon forming a complex with TF. Changes in both the secondary and the tertiary structure were detected, and by employing derivatives of fVIIa lacking the Gla and protease domain, respectively, we were able to locate these changes to individual domains. Our results indicate that the Ca^{2+}-induced secondary structural changes were restricted to the Gla domain, whereas Ca^{2+}-induced tertiary structural changes were detected in the protease domain. The increased α-helical content of the Gla domain is substantial and seems mainly to be a stabilization of the Gla domain in an ordered conformation by Ca^{2+}. The secondary structural transitions in fVIIa associated with TF binding appeared to be located in the protease domain, conceivably related to the TF-induced enhancement of the catalytic activity of fVIIa.

Results

Truncated variants of fVIIa

In this study, fVIIa and two derivatives thereof were used. In the N-terminally truncated variant, the first 38 amino acids had been removed by autoproteolytic cleavage, resulting in a fVIIa molecule lacking the N-terminal Gla domain [des(1–38)-fVIIa]. In the C-terminally truncated variant, virtually the entire protease domain had been removed by tryptic cleavage, giving a fVIIa fragment composed of the Gla and the two EGF-like domains (fVII-GlaEGF$_{NC}$). These two variants have been shown to retain the ability to interact tightly and specifically with TF (Kazama et al., 1993; Persson, 1996) and it is reasonable to assume that the remaining domains retain their native structures. Moreover, the binding of these fVIIa variants to TF is a Ca^{2+}-dependent process, as is the binding of intact fVIIa to TF (Persson & Petersen, 1995; E. Persson, unpubl. obs.). This collection of fVIIa fragments allows the investigation of specific structural changes in individual fVIIa domains.

CD spectra of fVIIa

Far-UV spectra of fVIIa in the presence and absence of Ca^{2+} are shown in Figure 1. The overall appearance of the spectra suggested a protein with a relatively high content of β-structure. The spectrum of Ca^{2+}-loaded fVIIa was different from the spectrum of Ca^{2+}-free fVIIa, indicating that some electronic or conformational changes had occurred in fVIIa as a result of the coordination of Ca^{2+}. The spectrum of Ca^{2+}-loaded fVIIa had a positive band at 188 nm ($\epsilon = 400$) and a negative band at 210 nm ($\epsilon = -880$). There was an increase in intensity and a red-shift of about 2 nm of the peak around 188 nm when Ca^{2+} ions bound to fVIIa. The CD signal in this wavelength region stems from π to π^* perpendicular electronical transitions and the red-shift indicated that part of the polypeptide chain of fVIIa shifted to a more apolar environment upon Ca^{2+} binding (Cascio & Wallace, 1995), although at this stage it could not be deduced in which of the Ca^{2+} binding regions this occurred. When estimating the amount of secondary structure from these two spectra using the variable-selection method (Manavalan & Johnson, 1987), the results indicated a small increase (from 13 to 17%) in α-helical structure when fVIIa coordinated Ca^{2+} (Table 1). This increase in α-helix content would correspond to a structural transition involving approximately 16 amino acid residues in fVIIa. Given that algorithms employed to extract secondary structural information from CD spectra have proven most useful for the α-helix motif, and because an increase of this structural motif appears to be the most relevant in this work, we have limited our later discussion to the calculated helical content.

The near-UV CD spectrum detects changes in the tertiary structure and aromatic residues, especially Trp, are known to contribute in this region (Freskgård et al., 1994). fVIIa had rather a low-intensity spectrum in the near-UV region (Fig. 2), indicating either that the aromatic residues were not in an asymmetric environment or that there was cancellation of different spectral bands from individual aromatic residues. The spectra

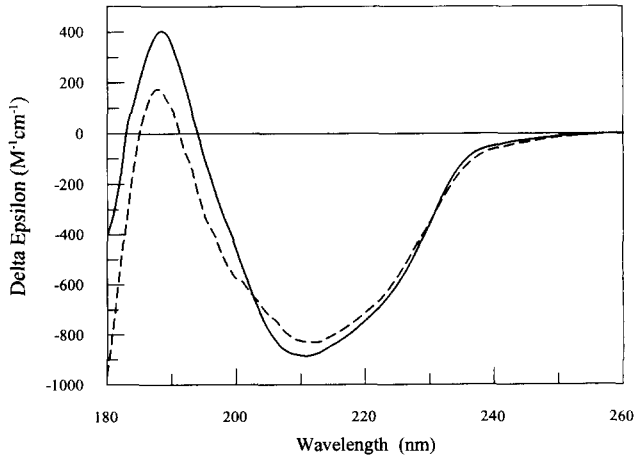

Fig. 1. Far-UV CD spectra of fVIIa in the absence of Ca^{2+} (dashed line) and in the presence of 5 mM Ca^{2+} (solid line).

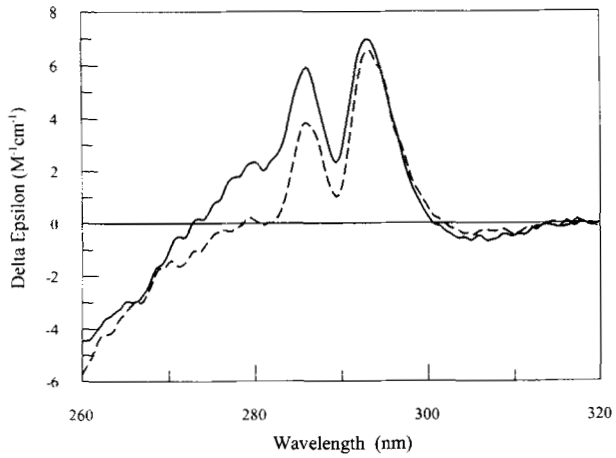

Fig. 2. Near-UV CD spectra of fVIIa in the absence of Ca^{2+} (dashed line) and in the presence of 5 mM Ca^{2+} (solid line).

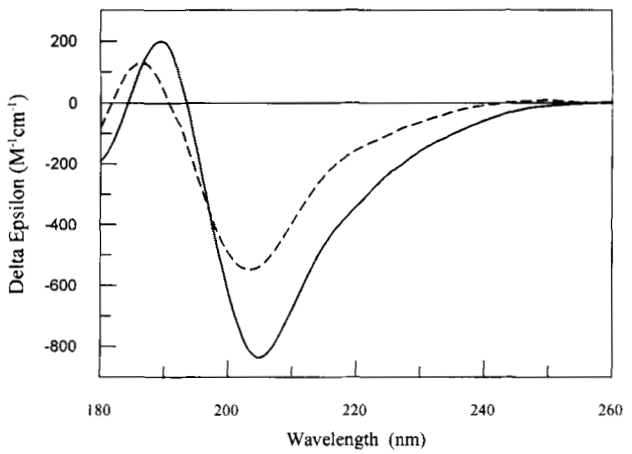

Fig. 3. Far-UV CD spectra of fVII-GlaEGF$_{NC}$ in the absence of Ca^{2+} (dashed line) and in the presence of 5 mM Ca^{2+} (solid line).

mainly displayed bands above 280 nm, suggesting that predominantly L_b transitions were involved. There were two prominent vibrational bands at 286 and 293 nm, respectively, presumably reflecting Trp residues in asymmetrical environments. fVIIa contains eight Trp residues, one located in the Gla domain and the remaining seven in the catalytic domain. When Ca^{2+} was removed, there was a small change in the near-UV spectrum of fVIIa (Fig. 2). The changes were most significant in the region between 270 and 290 nm, whereas the specific L_b transition at 293 nm was hardly affected by Ca^{2+} binding. This indicated that primarily Tyr residues sense the Ca^{2+}-induced structural changes.

CD spectra of truncated variants of fVIIa

Far-UV CD spectra of fVII-GlaEGF$_{NC}$ in the presence and absence of Ca^{2+} differed significantly (Fig. 3). Ca^{2+} red-shifted the spectrum in a similar way as seen with fVIIa, indicating a more compact and hydrophobic structure. In addition to a peak wavelength change from 187 nm to 190 nm upon Ca^{2+} binding, there were large differences between spectra above 200 nm, including the amplitude of the minimum. In the presence of Ca^{2+}, the spectrum had a minimum at 205 nm with a delta epsilon of −840, compared with a minimum at 203 nm and a delta epsilon of −550 in the absence of Ca^{2+}. In this region the spectra are similar to those published by Bloom and Mann (1978) for prothrombin fragment 1 (containing one Gla and one kringle domain). Analysis of the amount of α-helical secondary structure yielded an increase from 12 to 28% upon Ca^{2+} binding (Table 1). This corresponds to 26 residues entering α-helical structures, in reasonable agreement with the changes observed in fVIIa.

When the Gla domain had been eliminated from fVIIa, there were no detectable changes when Ca^{2+} ions were added to the solution, as seen in the far-UV CD spectra of des(1-38)-fVIIa (Fig. 4). This strongly indicated that the first 38 amino acid residues in the N-terminal Gla domain of fVIIa were responsible for the Ca^{2+} binding events that led to the changes observed in

Table 1. *Secondary structure content analysis of the different fVIIa derivatives*[a]

	CD analysis[b]		Model analysis[c]	
Variant	Ca^{2+}	apo	Ca^{2+}	apo
fVIIa	69 (17%)	53 (13%)	44	39
fVII-GlaEGF$_{NC}$	46 (28%)	20 (12%)	18	13
des(1-38)-fVIIa	51 (14%)	51 (14%)	39	39

[a] α-Helical content was calculated from the spectra and from polypeptide backbone angles in the fVIIa models. Numbers of residues in α-helices are given. In parentheses the values in percent of total residues derived from the CD spectra are shown.

[b] CD analysis using the variable-selection method (Manavalan & Johnson, 1987).

[c] Calculation of α-helical content according to Kabsch and Sander (1983).

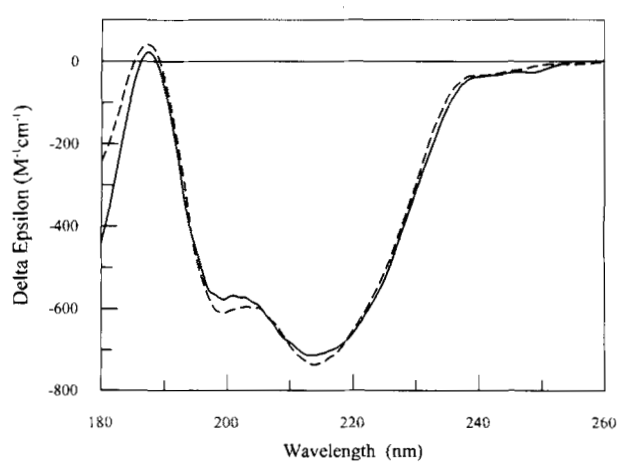

Fig. 4. Far-UV CD spectra of des(1-38)-fVIIa in the absence of Ca^{2+} (dashed line) and in the presence of 5 mM Ca^{2+} (solid line).

the far-UV CD spectra of fVIIa and fVII-GlaEGF$_{NC}$. Altogether, these results suggested that there was an increase in α-helical structure or an increased ordering of preformed α-helical elements on binding of Ca^{2+}, and these structural changes were induced in and/or by the Gla domain of fVIIa.

The near-UV CD spectra of the fVIIa variants in the presence of Ca^{2+} are compared in Figure 5. When the Gla domain had been removed from fVIIa, the CD signal increased in the range between 260 and 290 nm. On the other hand, if the protease domain was absent, all specific CD bands in the near-UV region disappeared. Thus, although it has been shown that aromatic residues in fVII-GlaEGF$_{NC}$ are in a more hydrophobic environment in the presence of Ca^{2+} (Persson & Petersen, 1995), the side chains are too flexible for the environment to be asymmetric. This clearly demonstrated that all near-UV CD bands emanated from the protease domain in fVIIa, and these bands were affected when Ca^{2+} ions bound to fVIIa (Fig. 2). Furthermore, it suggested that the Gla domain could influence the near-UV CD spectrum of fVIIa even though the Gla domain itself did not give rise to any specific CD bands.

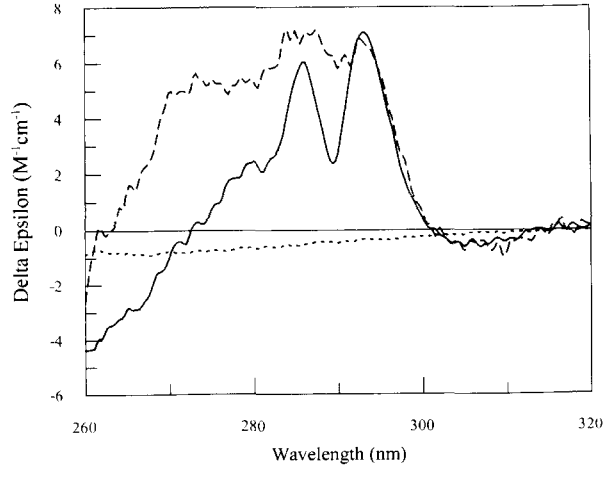

Fig. 5. Near-UV CD spectra of fVIIa (solid line), des(1-38)-fVIIa (dashed line), and fVII-GlaEGF$_{NC}$ (dotted line) in the presence of 5 mM Ca^{2+}.

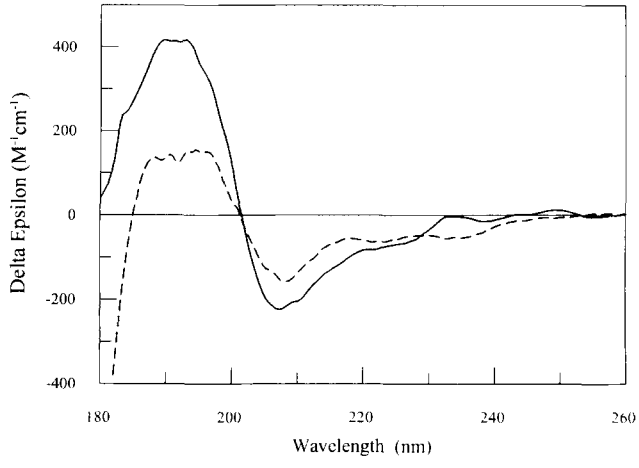

Fig. 6. Far-UV CD spectra of the apo (dashed line) and Ca^{2+} (solid line) forms of the fVIIa Gla domain (residues 1-38). The spectra were obtained as the difference between the fVIIa and des(1-38)-fVIIa spectra in the absence and presence of 5 mM Ca^{2+}, respectively.

Gla domain in fVIIa

The results obtained with des(1-38)-fVIIa (Fig. 4) implied that the Gla domain was responsible for the Ca^{2+}-induced changes in the far-UV CD spectrum of fVIIa (Fig. 1). If one subtracts the CD spectra of des(1-38)-fVIIa in the absence and presence of Ca^{2+} from the corresponding spectra of fVIIa, the resulting spectra would be those of the apo and Ca^{2+} forms of the Gla domain (residues 1-38), respectively (Fig. 6). A prerequisite for the validity of this maneuver is that the Gla domain should be an independent domain with no interactions with the rest of the structure or that such interactions, if present, should not affect the CD spectra. Interestingly, and in favor of these assumptions, the derived spectra of the fVIIa Gla domain were similar to those obtained with the isolated Gla domain of protein C (Colpitts & Castellino, 1994). They even resembled the spectra of the nonhomologous, vitamin K-dependent, 49-residue bone protein osteocalcin, containing three Gla residues, but no Gla domain (Atkinson et al., 1995). The CD spectrum of the Gla domain of fVIIa had a large positive peak centered around 189-194 nm and a minimum around 208 nm. Both bands were of higher intensity in the Ca^{2+}-loaded Gla domain, but the wavelengths at which they occurred were not shifted. The fact that no red-shift was observed upon Ca^{2+}-loading the Gla domain, but identical shifts were observed upon Ca^{2+} binding to fVIIa and fVII-GlaEGF$_{NC}$, suggested that the red-shift was caused by a structural change dependent on residues 39-144. This presumably involves Trp 41, the fluorescence of which is blue-shifted and quenched on Ca^{2+} binding to the Gla domain (Persson & Petersen, 1995).

Complex formation between fVIIa and soluble TF (sTF)

To investigate the structural changes accompanying fVIIa binding to TF, CD spectra of fVIIa, sTF, and the fVIIa:sTF complex were recorded. As expected, the far-UV CD spectra of sTF in the presence and absence of Ca^{2+} were identical (data not shown), indicating no specific binding sites for Ca^{2+} in sTF. The far-UV CD spectrum was almost identical to those obtained by Ruf and Edgington (1991) and by Stone et al. (1995), but significantly different from that reported by Kelley and coworkers (1995). The reason for this discrepancy is not known. The amounts of secondary structure calculated using the variable-selection method (Manavalan & Johnson, 1987) indicated a low amount of α-helix and a large amount of β-sheets, entirely compatible with the X-ray structure of sTF (Harlos et al., 1994; Muller et al., 1994). The CD spectra of free fVIIa and free sTF in the presence of Ca^{2+} were added in terms of molar epsilon values (based on the molar concentration of the particular protein) and compared with the molar epsilon values of the spectrum of the fVIIa:sTF mixture (Fig. 7A). The protein concentration in the co-incubation was high enough to ensure that a vast majority would exist in the complex form and this was also the case when fVII-GlaEGF$_{NC}$ and des(1-38)-fVIIa were coincubated with sTF (Persson, 1996; E. Persson, unpubl. obs.). Interestingly, there was a blue-shift of the maximum around 188 nm and

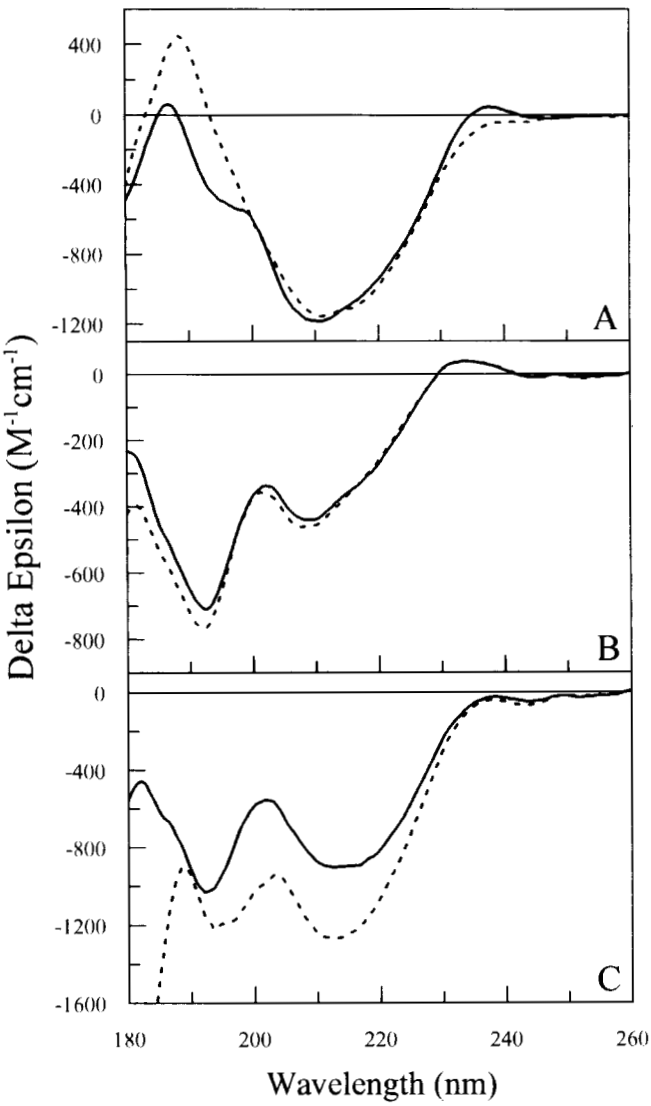

Fig. 7. Far-UV CD spectra of complexes between sTF and different fVIIa derivatives. The fVIIa derivatives are fVIIa (**A**), fVII-GlaEGF$_{NC}$ (**B**), and des(1-38)-fVIIa (**C**), respectively. CD spectra of the complexes (solid line) and the sum of the spectra of the individual proteins (dashed line) are shown. All solutions contained 5 mM Ca^{2+}.

the minimum around 210 nm when the fVIIa:sTF complex formed. This was opposite to what we found when Ca^{2+} ions coordinated to fVIIa. As pointed out earlier, this shift presumably involves Trp 41 and indicates a conformational change in the region connecting the Gla and first EGF-like domains. If the additional spectrum of free fVII-GlaEGFNC and free sTF was compared to the spectrum of the equimolar mixture, i.e., the complex of the two proteins, no significant changes in the CD signal were recorded except for a small difference in the region 180–195 nm. This suggested that no large structural changes took place when the fVII-GlaEGF$_{NC}$:sTF complex formed (Fig. 7B). On the other hand, when des(1-38) fVIIa and sTF were mixed, the sum of the spectra of the free proteins and the spectrum of the complex were different (Fig. 7C). The blue-shifts of the wavelengths of ellipticity minimum and maximum were similar to those observed with the fVIIa:sTF mixture. Thus, the association of sTF with fVIIa derivatives containing the protease domain yielded changes in the far-UV CD spectrum. Whether this CD change was due to alterations in the fVIIa and/or sTF structure could not be elucidated from these measurements, but the data strongly suggested structural changes in the catalytic domain of fVIIa upon association with sTF.

Discussion

The homologous and structurally very similar vitamin K-dependent proteins factors VII, IX, X, and protein C are composed of at least four individual folding domains. The N-terminal Gla domain is composed mainly of α-helix structure. Following consecutively are two EGF-like domains, each approximately 35 residues long, which contain mostly β-structure and are stabilized by three disulfide bonds. The C-terminal part of fVIIa contains the protease domain (which can be divided into two subdomains), homologous to members of the serine protease superfamily and made up mostly of β-structure (Fig. 8). The Gla, first EGF-like, and protease domains of fVIIa all harbor one or more Ca^{2+} binding sites. The sites in the first EGF-like and protease domains are saturated at plasma concentration of Ca^{2+}, but some of the Ca^{2+} binding sites in the Gla domain are only partly saturated. Ca^{2+} ions may regulate fVIIa and fVIIa:TF activity under certain conditions such as those developing in platelet aggregates (Owen et al., 1995), but otherwise it is reasonable to consider Ca^{2+} ions a constant structural determinant of the protein. Because Ca^{2+} binding is important for the conformation and stability of many proteins, structural aspects of Ca^{2+} binding to fVIIa is of great importance for the understanding of its physiological function. The Ca^{2+}-induced structural changes in the vitamin K-dependent plasma proteins have been monitored with a variety of techniques, most commonly using antibodies directed against Ca^{2+}-induced epitopes or measurements of intrinsic protein fluorescence. The availability of antibodies directed against corresponding regions in these proteins and the identical positioning of certain strategic Trp residues make comparisons of the Ca^{2+}-dependent properties and structures feasible. As might be expected from the spatial distribution of Ca^{2+} binding sites, Ca^{2+}-induced structural changes have been found to occur throughout the protein molecules, and the different clotting factors appear to be affected virtually identically by Ca^{2+}. This CD study was undertaken with the aim to obtain additional complementary information regarding the Ca^{2+} (and TF) effects on fVIIa structure.

Only fVIIa and fVII-GlaEGF$_{NC}$, i.e., the Gla-containing derivatives of fVIIa, exhibited Ca^{2+}-induced changes in the far-UV CD spectrum. The increase in α-helical structure was larger in fVII-GlaEGF$_{NC}$ than in fVIIa, but, considering the uncertainties in the calculations, the transitions in the fragment most likely correspond to those occurring in the GlaEGF region of intact fVIIa (Table 1). In the wavelength interval available for comparison (200–250 nm), the spectra of fVIIa and fVII-GlaEGF$_{NC}$ resemble those of prothrombin and prothrombin fragment 1, respectively, and the increase in α-helix in prothrombin fragment 1 could likewise account for the changes seen in prothrombin (Bloom & Mann, 1978; Marsh et al., 1979; Nelsestuen et al., 1981). The spectrum of des(1-38)-fVIIa was barely sensitive to Ca^{2+}, a finding in agreement with previous analy-

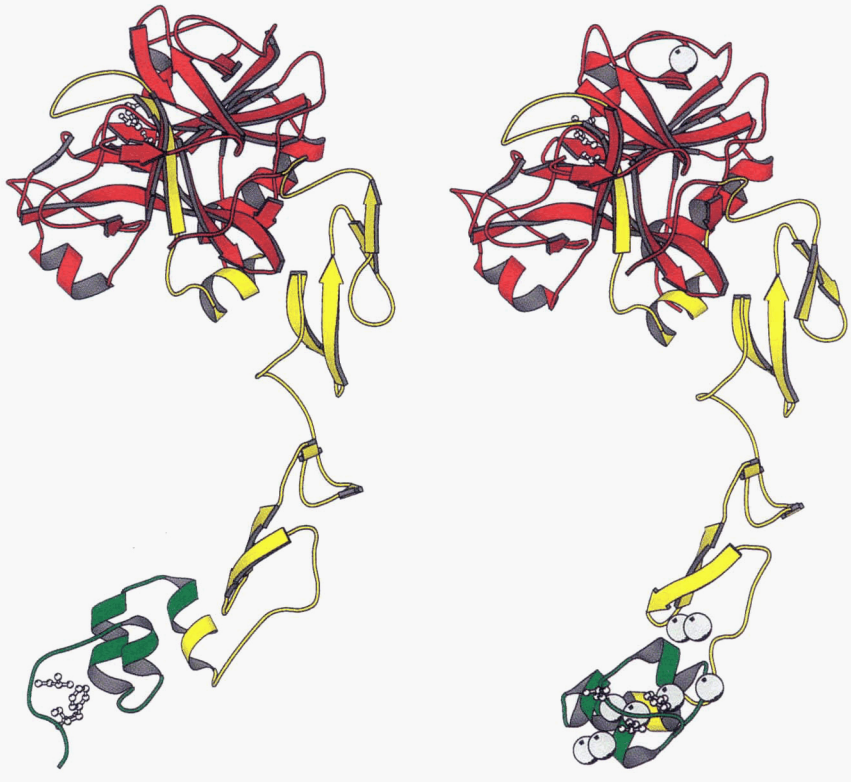

Fig. 8. Models of the three-dimensional structures of the apo (left) and Ca^{2+}-loaded (right) forms of fVIIa. The coloring of the backbone trace denotes the various fVIIa derivatives used in this study [des(1–38)-fVIIa (yellow and red), fVII-GlaEGF$_{NC}$ (green and yellow), and fVIIa (green, yellow, and red)]. The side chains of the active-site residues (His 193, Asp 242, and Ser 344) and the hydrophobic residues in the Gla domain presumably involved in phospholipid binding (Phe 4, Leu 5, and Leu 8) are indicated. Coordinated Ca^{2+} ions are represented by grey balls. The figure was generated using MOLSCRIPT (Kraulis, 1991).

sis of Gla-domainless factor X and Gla-domainless protein C (Sugo et al., 1984). Thus, our results suggest that the Ca^{2+}-induced changes in the secondary structure of fVIIa are restricted to the Gla domain and they are corroborated by data obtained with homologous proteins. Our data also imply that Ca^{2+}-induced alterations in other parts of the fVIIa molecule, such as the protease domain, are of a relatively subtle and local tertiary structural nature, detectable only in the near-UV region. Nevertheless, these transitions in the protease domain are presumably essential for TF binding because earlier work in our laboratory points toward a role for the Ca^{2+} binding site in the catalytic domain in cofactor binding (Wildgoose et al., 1993; Persson & Petersen, 1995). Models of the apo and Ca^{2+} forms of fVIIa, which are based on NMR and X-ray structures of (among other proteins) vitamin K-dependent prothrombin and factor X/Xa, support the conclusion that changes in secondary structure are limited to the Gla domain (Fig. 8). Calculations of the amount of α-helix in the two models (Kabsch & Sander, 1983) identify a Ca^{2+}-induced increase only in the Gla domain, albeit smaller than that estimated from the CD spectra (Table 1). The difference might reflect the effect of Ca^{2+} on structural dynamics, which is picked up in the CD analysis. It should be pointed out that these models do not attempt to mimic the global, quaternary structure of the four domains. The relative orientation of domains is speculative except when multiple close contacts are defined, such as between the second EGF-like and the protease domains. An extended structure of fVIIa has been inferred from X-ray and neutron scattering data (Ashton et al., 1995), but unpublished observations in our laboratory indicate a possible interaction between the Gla and protease domains.

The apparent Ca^{2+}-induced increase in α-helical content of the Gla domain may be deceptive because the CD spectral changes to a large extent appear to represent ordering of preformed helical elements. This interpretation is based on X-ray crystallographic and NMR spectroscopic structures of prothrombin fragment 1, factor IX, and factor X (Soriano-Garcia et al., 1992; Brandstetter et al., 1995; Freedman et al., 1995; Sunnerhagen et al., 1995), as well as calorimetric studies of protein C (Medved et al., 1995). They all suggest a more ordered, rigid, and compact structure of the Gla domain in the Ca^{2+}-loaded form. It has been demonstrated very elegantly with the regulatory domain of troponin C, that, although CD studies showed a Ca^{2+}-induced increase in negative ellipticity, suggesting an increased helical content (Li et al., 1994), NMR studies of the apo and Ca^{2+} states revealed no difference in helical content but merely a spatial reorientation of one out of five helices (Gagné et al., 1994). Other factors, such as the dynamical motion of the protein and the length of the helices also affect the ellipticity (Hirst & Brooks, 1994). In addition, previous theoretical calculations (Manning et al., 1988; Manning & Woody, 1989; Woody, 1994) and experimental studies (Vuillemier et al., 1993; Freskgård et al., 1994) have shown that aromatic amino acids, especially Trp residues, can make significant contributions not only in the near-UV region, but also in the far-UV region.

This complicates the interpretation of far-UV CD spectra, particularly when the actual spectrum is of low intensity (Freskgård et al., 1994). As stated earlier, the Gla domain appears to be responsible for the Ca^{2+}-induced CD changes of fVIIa. The N-terminal 38 residues in fVIIa contain three Phe residues, but no Trp or Tyr residues. In addition, there are no specific CD bands in the near-UV region of fVII-GlaEGF$_{NC}$, i.e., no asymmetric environments around aromatic residues, which argues against aromatic contributions from the Gla domain to the far-UV CD spectrum of fVIIa. Based on these two facts, it is reasonable to assign the CD changes in the far-UV region when Ca^{2+} ions coordinate to fVIIa to secondary structure alterations in the Gla domain, including spatial reorientation of structural elements present in the apo form of fVIIa.

The spectra of the Gla domain of fVIIa shown in Figure 6 were derived as difference spectra between fVIIa and des(1-38)-fVIIa. Thus, they possibly contain contributions from interactions between the Gla domain and other domains in fVIIa, interactions absent in des(1-38)-fVIIa. Evidence of Ca^{2+}-dependent interactions between the Gla and first EGF-like domains in factors IX and X (Vysotchin et al., 1993; Medved et al., 1994; Valcarce et al., 1994) and between the Gla and protease domains in protein C (Medved et al., 1995) have been reported. However, the derived spectra of the fVIIa Gla domain are very similar to those obtained with peptides consisting of residues 1-48 of protein C (Colpitts & Castellino, 1994) and residues 1-45 of factor IX (Vysotchin et al., 1993). This strongly suggests that the differences observed between the spectra of the Ca^{2+}-free and Ca^{2+}-loaded forms of the Gla domain of fVIIa primarily reflects Ca^{2+}-induced structural changes within the Gla domain itself. Indeed, a similar increase in secondary structure content has been observed for the isolated Gla peptides from factor IX and protein C. These secondary structural changes, which have been demonstrated to turn the entire structure of the Gla domain inside out, and which presumably occur also in fVIIa, bring about the exposure of phospholipid-interactive side chains (Christiansen et al., 1995; Freedman et al., 1995; Sunnerhagen et al., 1995) and may be important for the binding of fVIIa to TF (Fig. 8). Ca^{2+} fixes the orientation of helices and stabilizes the structure and without the bound metal ions the structure may resemble an early folding intermediate with properties of a molten globule state.

The structural changes in factor X upon zymogen-to-enzyme conversion are subtle as judged by CD (Furie & Furie, 1976), but the two structures are clearly discernable using antibodies (Keyt et al., 1982; Persson et al., 1991). The changes brought about by activation of fVII are most likely very similar, making the conformational changes induced by TF binding seem larger than those accompanying cleavage of the Arg 152-Ile 153 peptide bond in fVII. The multipoint interaction between fVIIa and TF involves contacts between several domains in fVIIa and both domains of TF (Martin et al., 1995; Banner et al., 1996). The complexation of fVIIa with TF brings about an increase in the catalytic activity of fVIIa easily measurable using small substrates (Higashi et al., 1992) and may also establish the proper distance between the active site of fVIIa and the membrane surface. The structural transitions underlying the activity enhancement are obscure, and they will remain elusive even with a known structure of the fVIIa:TF complex (Banner et al., 1996) as long as a detailed three-dimensional structure of free fVIIa is not available. However, the crystal structure of fVIIa:TF identified a loop comprising fVIIa residues 307-329 possibly involved in the activation. The demonstration of changes in the far-UV spectra upon fVIIa:sTF and des(1-38) fVIIa:sTF complex formations, but not upon the association of fVII-GlaEGF$_{NC}$ and sTF, suggests that conformational changes upon fVIIa:sTF complex formation require the presence of the protease domain. We prefer to interpret this as TF-induced transitions in the catalytic part of fVIIa, leading to optimal positioning of the active-site residues, exposure of the substrate binding pocket, and increased activity.

In summary, our results lead us to believe that fVIIa undergoes significant structural changes in the Gla domain upon Ca^{2+} binding. These alterations are crucial for the interaction with TF and the lipid bilayer in which TF is embedded. Although our data are supportive of α-helix formation, the possibility of mere reorientation and restraint of pre-existing helices can not be ruled out. The latter is supported by data from structure determinations of homologous proteins, and Ca^{2+} ions most likely induce a combined effect. Another distinct structural transition in fVIIa is induced by TF and occurs predominantly in the protease domain, bringing about an activity enhancement. The precise changes that fVIIa undergoes when it associates with TF are currently under investigation in our laboratory, helping us understand how and why fVIIa binds TF.

Materials and methods

Materials

Chelex™ 100 was from Bio-Rad, Richmond, CA, and used to remove traces of metal ions from all buffers before use. Isopropyl-β-D thiogalactopyranoside, lysozyme, reduced and oxidized glutathione were from Sigma, St. Louis, MO. Pefabloc SC was from Pentapharm, Basil, Switzerland, guanidinium chloride from Fluka, Buchs, Switzerland, and urea from Merck, Darmstadt, Germany. All other chemicals were of reagent grade or better.

Proteins

Human recombinant fVIIa was expressed and purified as described previously (Thim et al., 1988). Des(1-38)-fVIIa and the fVII-GlaEGF$_{NC}$ fragment (residues 1-144 and 248-266) were isolated according to published procedures (Sakai et al., 1990; Persson & Petersen, 1995). sTF (residues 1-219) was expressed in *Escherichia coli*, harvested, and refolded as described by Stone et al. (1995) with the following modifications: sTF was expressed in BL21 cells using the pET system (Novagen, Abingdon, UK). When the absorbance at 660 nm reached a value of 0.6-1.0, isopropyl-β-D-thiogalactopyranoside was added to induce sTF transcription. The cell paste was suspended and lysed in 50 mM Tris, pH 8.0, containing 2 mM EDTA, 2 mg/mL lysozyme, and 1 mM Pefabloc SC. We employed a different purification procedure. After refolding, sTF was dialyzed against 20 mM Tris, pH 8.0, 20 mM NaCl, and applied to a 35-mL column of Q Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. The protein was eluted with a linear NaCl gradient from 20 to 300 mM over 100 min at a flow rate of 5 mL/min. The fractions containing sTF were pooled, dialyzed against 50 mM Tris, pH 8.0, containing 10 mM $CaCl_2$, and applied to a 300-mL column of fVIIa-

Sepharose (fVIIa coupled to CNBr activated Sepharose (Pharmacia Biotech, Uppsala, Sweden) at a density of 5 mg/mL). sTF was eluted with 30 mM EDTA in Tris buffer and concentrated using a CentriPrep 10 (Amicon, Beverly, MA). The following molar extinction coefficients at 280 nm and molecular weights were used: fVIIa 69,500 M^{-1} cm^{-1} and 50,000; sTF 32,750 M^{-1} cm^{-1} and 25,000; des(1-38)-fVIIa 69,500 M^{-1} cm^{-1} and 42,320; fVII-GlaEGF$_{NC}$ 13,050 M^{-1} cm^{-1} and 18,630.

CD measurements

All samples were thawed and prepared immediately before the measurements. The fVIIa variants (1.2–1.4 mg/mL) were treated with 0.1 mg/mL Chelex™ 100 for about 15 min and passed through a 0.45-μm filter (Millipore, Bedford, MA) to remove any aggregated protein (and Chelex). The proteins were then passed through a NAP 10 column (Pharmacia Biotech, Uppsala, Sweden) to change the buffer to 10 mM borate, pH 7.5. EDTA (final concentration 10 mM) was added to samples used for Ca^{2+}-free measurements in the near-UV region, but not to those to be used in the far-UV region. $CaCl_2$, final concentration 5 mM, was added prior to acquiring CD spectra of Ca^{2+}-loaded proteins. The final protein concentration was 0.5–0.7 mg/mL (or approximately 14 μM fVIIa, 13 μM des(1-38)-fVIIa, and 34 μM fVII-GlaEGF$_{NC}$, respectively). In the studies of various fVIIa:sTF complexes, the fVIIa derivatives and sTF were treated individually, as described above, and equimolar amounts were mixed prior to analysis. The protein concentrations in the mixtures were: fVIIa (10 μM):sTF (10 μM), des(1-38)-fVIIa (9 μM):sTF (9 μM), and fVII-GlaEGF$_{NC}$ (17 μM):sTF (17 μM).

Each CD spectrum was recorded both on a Mark 5 and on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France), employing constant N_2 flushing. The instruments were calibrated using 10-camphorsulfonic acid at 290 nm (Hennessey & Johnson, 1982). Each CD spectrum represents the average of three scans obtained by collecting data at 0.5-nm intervals with an integration time of 2 s. In the far-UV region, a 0.1-mm path length was used, whereas it was 5 mm in the near-UV region. After baseline subtraction, a small degree (20–30 Savitsky Golay) of numerical smoothing was applied. The observed delta absorbance values were converted to molar epsilon values on the basis of the molar concentration of the particular protein to facilitate direct comparison of free proteins and protein–protein complex.

Secondary structure prediction from CD spectra

The variable-selection method of Johnson and co-workers (Manavalan & Johnson, 1987; Toumadje et al., 1992) was used according to their instructions. The analysis was performed on the spectra between 180 and 260 nm to acquire enough information to determine the amounts of the various forms of secondary structure. Only the results obtained for α-helix structure were used in this study because this structural element is the one most accurately predicted and also the most relevant form of secondary structure in this study.

Homology modeling

Structures of the separate domains of fVIIa were built based on molecular homology modeling using Modeler implemented in the modeling package Quanta (Molecular Simulations, Inc., San Diego, CA). The models of the apo form of the Gla and first EGF-like domains were based on the NMR structure of the corresponding part of bovine factor X (Sunnerhagen et al., 1995). Sequence alignment followed by a Modeler session and energy minimization resulted in a model of the two first domains in fVIIa. The Ca^{2+} form of the Gla domain was derived using the X-ray structure of bovine prothrombin fragment 1 (Soriano-Garcia et al., 1992; entry code 2PF2) and the procedure described above. The Ca^{2+} structure of the first EGF-like domain was obtained by placing a Ca^{2+} ion in position followed by energy minimization (Selander-Sunnerhagen et al., 1992). The models of the second EGF-like and protease domains were based on the X-ray structures of bovine factor Xa (Padmanabhan et al., 1993; entry code 1HCG), the bovine trypsin Ca^{2+} complex (Chambers & Stroud, 1979; entry code 4PTP), and bovine ϵ-thrombin (Brandstetter et al., 1992; entry code 1ETR). The sequence of the second EGF-like and protease domains of fVIIa was aligned to the sequences of the three structures and a fVIIa structure was generated by Modeler. The Ca^{2+} ion in the protease domain was placed according to its position in the trypsin structure. Energy minimization of the apo and Ca^{2+} structures resulted in the final structures. The two parts of the fVIIa structure (Gla-EGF1 and EGF2-protease) were then adjoined by simply putting them on top of each other without any attempts to predict the relative orientation of the two building blocks. The α-helical contents of the fVIIa models were calculated using the algorithm of Kabsch and Sander (1983).

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