# Identification of a Calcium Binding Site in the Protease Domain of Human Blood Coagulation Factor VII: Evidence for Its Role in Factor VII–Tissue Factor Interaction

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ABSTRACT: Previous studies have identified a putative calcium binding site involving two glutamic acid residues located in the protease domain of coagulation factor IX. Amino acid sequence homology considerations suggest that factor VII (FVII) possesses a similar site involving glutamic acid residues 210 and 220. In the present study, we have constructed site-specific mutants of human factor VII in which Glu-220 has been replaced with either a lysine (E220K FVII) or an alanine (E220A FVII). These mutants were indistinguishable from wild-type factor VII by SDS–PAGE but only possessed 0.1% the coagulant activity of factor VII. Incubation of E220K/E220A FVII with factor Xa resulted in a slower than normal activation rate which eventually yielded a two-chain factor VIIa molecule possessing a coagulant activity of ≈10% that of wild-type rFVIIa. Amidolytic activity measurements indicated that E220K/E220A FVIIa, unlike wild-type factor VIIa, possessed no measurable amidolytic activity toward the chromogenic substrate S-2288, even at high $CaCl_2$ concentrations. Addition of tissue factor apoprotein, however, induced the amidolytic activity of the mutant molecule to a level 30% of that observed for wild-type factor VIIa. This tissue factor dependent enhancement of E220K/E220A FVIIa amidolytic activity was calcium dependent and required a $CaCl_2$ concentration in excess of 5 mM for maximal rate enhancement. This was in sharp contrast to wild-type factor VIIa which required $CaCl_2$ levels of 0.5 mM for maximal enhancement of tissue factor dependent amidolytic activity. Competition binding experiments suggest that the decrease in amidolytic and coagulant activity observed in the factor VII mutants is a direct result of impaired tissue factor binding. Evidence for the participation of Glu-220 in calcium binding was obtained using the luminescent lanthanide $Tb^{3+}$. The binding of $Tb^{3+}$ to wild-type factor VIIa was described by a simple binding curve ($K_d$ = 9 μM). All binding monitored by this technique was independent of the factor VII–Gla domain and could be displaced by addition of $Ca^{2+}$ ($K_d$ = 1.5 mM). Substitution of Glu-220 with a Lys (E220K FVII) resulted in a 25-fold decrease in the affinity of the FVII molecule for $Tb^{3+}$. Our results are consistent with the existence of a low-affinity calcium binding site located in the protease domain of FVII and necessary for normal factor VII–tissue factor interaction.

Factor VII is a vitamin K dependent glycoprotein that is synthesized in the liver and participates in initiation of the extrinsic coagulation pathway. Like other vitamin K dependent coagulation proteins, factor VII is divided into several distinct structural domains. These include an N-terminal γ-carboxyglutamic acid (Gla)[1]-containing domain, two consecutive EGF-like domain, and a C-terminal serine protease domain (Hagen et al., 1986). Under normal conditions, factor VII circulates in the bloodstream mainly as a precursor to its activated form, factor VIIa. Upon vascular injury, however, factor VII forms a one-to-one complex in the presence of calcium ions with its cell surface cofactor tissue factor (TF). Once complexed to TF, the zymogen factor VII is rapidly converted to factor VIIa by cleavage of a single internal peptide bond located at $Arg^{152}$–$Ile^{153}$. Although a number of serine proteases including factor Xa (Bajaj et al., 1981; Radcliffe & Nemerson, 1976; Wildgoose & Kisiel, 1989), factor IXa (Masys et al., 1982; Wildgoose et al., 1989), factor XIIa (Kisiel et al., 1977; Broze & Majerus, 1981), thrombin (Broze & Majerus, 1980), and factor VIIa (Pedersen et al., 1989; Nakagaki et al., 1991) are capable of activating factor VII under in vitro conditions, the protease responsible for the in vivo activation of factor VII is unknown. Once activated, however, it is well-known that the factor VIIa–TF complex rapidly initiates blood coagulation by proteolytically activating its vitamin K dependent substrates factors IX and X, which eventually leads to thrombin formation and a fibrin clot.

Like other reactions involving vitamin K dependent coagulation factors, the initiation of coagulation via factor VIIa–tissue factor is strictly dependent on the presence of calcium ions. Detailed calcium binding studies have been conducted on a number of vitamin K dependent coagulation factors including factor IX (Bajaj, 1982), factor X (Sugo et al., 1984; Monroe et al., 1991), and prothrombin (Pollock et al., 1988). Existing evidence suggests that the Gla domain of these vitamin K dependent coagulation factors contains several low-affinity calcium binding sites involved in the interaction of the protease with anionic cell-surface phospholipids. In addition to these low-affinity Gla-dependent sites, factor IX (Handford et al., 1990) and factor X (Monroe et al., 1991) also contain one high-affinity calcium binding site located in the EGF domain

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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and thought to be involved in the induction of calcium-dependent conformational changes. Recent studies indicate that factor IX also contains a second high-affinity site located in the protease domain and thought to be involved in the binding of factor IX to its cofactor, factor VIII (Bajaj et al., 1992). On the basis of sequence homologies to factor IX, trypsin, and pancreatic elastase, it was hypothesized that factor VII contains an analogous site involving factor VII residues 210–220. Using site-specific mutagenesis, we now demonstrate that factor VII contains a low-affinity calcium binding site located in the heavy chain of factor VII and that this site is important for the interaction of factor VII with its cell-surface cofactor, tissue factor.

## MATERIALS AND METHODS

Bovine serum albumin (fatty acid free), glycylglycine, vitamin K, and Tris-HCl were obtained from Sigma, St Louis, MO. Terbium(III) chloride hexahydrate was purchased from Janssen Chimic, Brussels, Belgium. Dulbecco's modified medium, minimum essential medium (Eagle's), and nonessential amino acids were purchased from Gibco, Uxbridge, England. Bovine brain phospholipids (Thrombofax) were purchased from Ortho, Raritan, NJ. The chromogenic substrates S-2288 (Ile-Pro-Arg-pNA) and S-2222 (Bz-Ile-Glu-Gly-Arg-pNA) were purchased from Kabi, Stockholm, Sweden. All other reagents were of the highest quality available from commercial sources.

*Proteins.* Recombinant human factor VIIa was purified from BHK cell culture medium as described (Thim et al., 1988). Recombinant S344A[2] factor VII was purified from BHK cell culture medium by affinity chromatography (Nakagaki et al., 1991). Gla-domainless factor VIIa (GD-VIIa) was prepared by prolonged incubation with EDTA (Sakai et al., 1990) and separated from intact factor VIIa by Mono Q fast protein liquid chromatography (Thim et al., 1988). Calcium-free preparations of factor VII were prepared by desalting on a Pharmacia PD-10 column followed by treatment with Chelex-100. Factor VII concentrations were determined using a solid-phase double-antibody ELISA assay kit obtained from Novo Nordisk, Bagsvaerd, Denmark (Lund Hansen et al., 1989). Human factor X was purified to homogeneity from plasmapheresis plasma essentially as described (Kondo & Kisiel, 1987). Factor X was activated by incubation with insolubilized RVV-X (Stern et al., 1984) and separated from residual factor X by Sephadex G-150 column chromatography (Wildgoose & Kisiel, 1989). Human tissue factor apoprotein was generously provided by Dr. Walter Kisiel, University of New Mexico, Albuquerque, NM. The tissue factor apoprotein was purified from human acetone brain powder by a combination of Triton X-100 extraction (Kondo & Kisiel, 1987) and immunoaffinity chromatography (Pedersen et al., 1990).

*Mutagenesis of Factor VII.* Factor VII mutations (E220A and E220K) were introduced into the cDNA of wild-type factor VII using a PCR strategy as described previously (Bjoern et al., 1991). Mutated cDNA fragments were subsequently inserted into a Zem219b expression vector (Petersen et al., 1990) from which the corresponding wild-type factor VII sequence had been removed. All mutated fragments were sequenced in entirety to confirm the individual point mutations and to avoid the possibility of PCR-induced sequence changes.

[2] Mutant factor VII is designated according to the notation described by Shapiro and Vallee (1989) in which the single-letter code for the original amino acid is followed by its position in the sequence and the single-letter code for the new amino acid.

*Expression of Mutant FVII.* Baby hamster kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Subconfluent cells were transfected with the factor VII expression plasmid by the calcium phosphate procedure (Graham & van der Eb, 1973). Two days posttransfection, cells were trypsinized and diluted into selective medium containing 250 nM methotrexate. After 12–14 days, individual colonies were screened for factor VII production by an immunofilter assay as described (Busby et al., 1988) and grown individually for protein analysis and purification.

*Purification of Mutant FVII.* Preparations of recombinant E220K and E220A factor VII were purified by immunoaffinity chromatography essentially as described (Wildgoose et al., 1990a). Prior to chromatography, culture supernatants (2 L) were centrifuged to remove potential cell debris. The medium was subsequently made 20 mM in $CaCl_2$ and 10 mM in benzamidine and allowed to sit at 4 °C for 15–30 min. The calcified medium was subsequently applied (1.5 mL/min) to a calcium-dependent anti-factor VII monoclonal antibody–Sepharose column equilibrated with 10 mM Tris-HCl (pH 7.5)/150 mM NaCl/10 mM $CaCl_2$. Following sample application, the column was washed with 2.0 M NaCl/10 mM Tris-HCl (pH 8.0)/10 mM $CaCl_2$ at a flow rate of 1.5 mL/min in order to elute nonspecifically bound protein. Mutant factor VII preparations were subsequently eluted from the antibody column with 40 mM EDTA/10 mM Tris (pH 7.5)/150 mM NaCl. The EDTA-eluted factor VII fractions were pooled on the basis of factor VII antigen concentration as determined by ELISA. The pooled material was concentrated by ultrafiltration (Amicon YM-10 membrane) and dialyzed against 50 mM Tris-HCl/100 mM NaCl (pH 7.4). Mutant factor VII preparations were routinely assayed for γ-carboxyglutamic acid content and found to be fully γ-carboxylated.

*Activation of Mutant Factor VII.* Mutant factor VII preparations were activated by incubation with soluble factor Xa in the presence of calcium and phospholipids essentially as described (Wildgoose et al., 1989). Activation reactions were performed at 37 °C using a 1:100 weight ratio of factor Xa to factor VIIa in a buffer containing 0.1 M NaCl/0.05 M Tris/5 mM $CaCl_2$ and 10% bovine phospholipids (Thrombofax), pH 7.4. Following a 3-h incubation period, factor VIIa was removed from contaminating factor Xa and phospholipids by immunopurification on a calcium-dependent anti-factor VII monoclonal antibody column as described earlier. The purified factor VIIa preparations routinely contained greater than 95% factor VIIa as judged by SDS–PAGE. No contaminating factor Xa was observed in the purified factor VIIa preparations as judged by the inability of concentrated factor VIIa preparations to cleave the factor Xa specific chromogenic substrate S-2222.

*Factor VIIa Amidolytic Activity Assays.* The effect of calcium on the intrinsic amidolytic activity of wild-type and mutant factor VII/VIIa was measured essentially as described previously (Wildgoose et al., 1992). In this assay system, the chromogenic substrate S-2288 (2.5 mM) was added to a 96-well microtiter plate containing either mutant or wild-type factor VIIa (50 nM) along with varying concentrations of $CaCl_2$ (0–10 mM). Following the addition of chromogenic substrate, the increase in absorbance at 405 nm was measured using a Titertek microtiter plate reader. An essentially identical assay system was also used to measure the effect of calcium on the tissue factor stimulated amidolytic activity of mutant and wild-type factor VIIa. In this one-stage amidolytic

assay, mutant or wild-type factor VIIa (5 nM) was incubated with varying concentrations of calcium chloride (0–10 mM), tissue factor apoprotein (5 nM), and S-2288 (2.5 mM). The increase in absorbance at 405 nm was subsequently determined as described above. A variation of this tissue factor stimulated amidolytic assay system was also used to measure the ability of amidolytically inactive single-chain factor VII mutants to compete for tissue factor apoprotein. These assays were conducted essentially as described above using 2.5 mM S-2288, 5 mM $CaCl_2$, 10 nM tissue factor apoprotein, and 20 nM wild-type factor VIIa, along with varying concentrations of inactive mutant factor VII (0–60 nM).

*Terbium Phosphorescence Experiments.* The binding of terbium to wild-type factor VIIa, zymogen factor VII, E220K FVII, and Gla-domainless FVIIa was assessed by phosphorescence spectroscopy essentially as described previously (De Jersey et al., 1980; Schiødt et al., 1992). Terbium binding experiments were conducted at 25 °C using calcium-free preparations of enzyme dissolved in a 0.1 M Na/Cl 10 mM glycylglycine, pH 6.5. Terbium titrations were performed by sequential addition of 2–3-μL aliquots of concentrated aqueous $TbCl_3$ (5 mM) to semimicrocuvettes containing the various factor VII preparations. Phosphorescence spectra were recorded on a Perkin Elmer LS-50 spectrofluorometer equipped with FLDM software and a 390-nm filter. All phosphorescence experiments (excitation at 285 nm) were conducted with a 15-nm excitation slit and a 5-nm emission slit. Phosphorescence emission was measured after a cutoff delay of 0.05 ms to avoid interference from protein fluorescence and stray light. Emission spectra were recorded using a 535–560-nm observation range. Following the addition of terbium, intensities were monitored as peak areas centered at 544 nm.

Calcium competition experiments were conducted essentially as described above, with the exception that various fixed concentrations of $Ca^{2+}$ (0–4 mM) were added prior to titration with terbium. An apparent binding constant for terbium at each calcium concentration was determined, and a $K_D$ for calcium binding was subsequently obtained by plotting this parameter as a function of the $CaCl_2$ concentration.

*SDS–PAGE.* SDS–polyacrylamide slab gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) using 12.5% polyacrylamide separating gels. Following electrophoresis, the proteins were visualized by staining with Coomassie Brilliant Blue.

*Coagulation Assays.* The coagulant activities of E220K and E220A factor VIIa preparations were determined in a one-stage coagulation assay using hereditary factor VII deficient plasma (<1% antigen) and human brain thromboplastin (Wildgoose et al., 1990a). Clotting times were compared to a standard curve constructed with varying dilutions of normal pooled plasma.

## RESULTS

*Purification of Mutant Factor VII Forms.* The E220K and E220A factor VII mutants were immunopurified from the culture supernatants of BHK cells stably transfected with plasmids containing either the E220K or the E220A factor VII sequence. The purified proteins were indistinguishable from wild-type recombinant factor VII as judged by SDS–PAGE under reducing and nonreducing conditions. Incubation of the two mutants with factor Xa, calcium, and phospholipid resulted in proteolytic activation, albeit at a slower rate than that observed using wild-type factor VII (data not shown), to a two-chain form that migrated with the same mobility as wild-type factor VIIa.

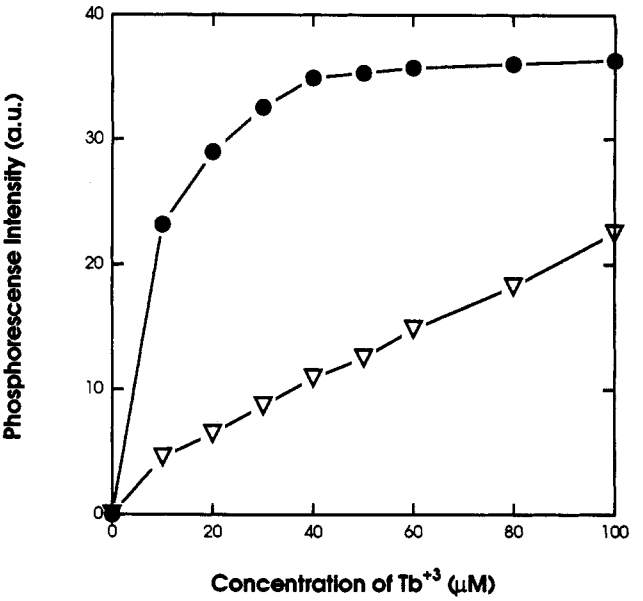


FIGURE 1: Binding of terbium to wild-type and E220K factor VII. The binding of terbium to either wild-type factor VIIa (●) or E220K factor VII (▽) was assessed by terbium phosphorescence spectrometry as described under Materials and Methods.

*Binding of Terbium to Wild-Type and Mutant Factor VII.* Terbium has previously been used as a luminescent probe to characterize calcium binding sites in numerous proteins (Martin & Richardson, 1979) including factor VII (Schiødt et al., 1992). Due to the ionic characteristics of terbium, it will mimic calcium binding, and upon excitation of the complex in the near-ultraviolet region, energy transfer will occur from neighboring tyrosine and tryptophan residues which will result in a strong enhancement of terbium luminescence. In agreement with earlier studies (Schiødt et al., 1992), we found that terbium bound, at a single site, to both wild-type factor VIIa and zymogen factor VII with a $K_d$ of 9 μM (Figure 1). This binding was independent of the Gla domain, as assessed by the binding of terbium to Gla-domainless factor VIIa, and could be inhibited by the addition of calcium ions with a $K_i$ of 1.4 mM. These results suggest that the observed $Tb^{3+}$ binding is occurring at one of the two Gla-independent calcium binding sites that we have previously identified using equilibrium dialysis techniques (Monroe et al., 1992). On the basis of homologies to factor IX (Bajaj et al., 1992), these binding sites are thought to be located in the factor VII EGF 1 domain and the factor VII protease domain (residues 210–220). To directly test this hypothesis, we compared the binding of terbium to wild-type factor VIIa and E220K factor VII. We found that substitution of glutamic acid residue 220 with lysine virtually abolished all terbium binding (Figure 1). These results strongly suggest that factor VII contains a calcium binding site located in the protease domain involving glutamic acid residue 220.

*Coagulant and Amidolytic Activity of Mutant and Wild-Type VIIa.* To investigate the physiologic significance of the putative calcium binding site involving glutamic acid residue 220, we initially measured the coagulant activity of E220K factor VII/VIIa. The zymogen E220K factor VII possessed a specific coagulant activity corresponding to ≈0.1% that of wild-type zymogen factor VII. In contrast, the activated form of this mutant possessed a specific coagulant activity corresponding to ≈10% that of wild-type factor VIIa. To more directly investigate the role of calcium on E220K factor VIIa activity, we also measured the effect of calcium on the amidolytic activity of wild-type and mutant factor VIIa. In

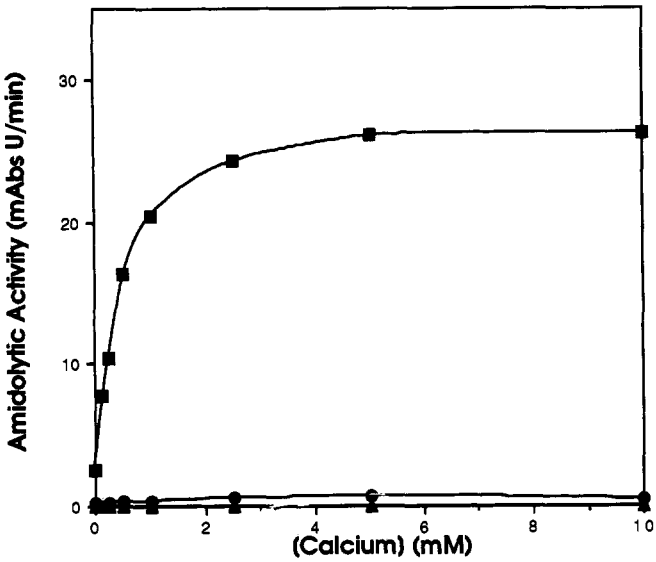


FIGURE 2: Effect of calcium on the amidolytic activity of wild-type factor VIIa (■), E220A factor VIIa (●), and E220K factor VIIa (▲). The amidolytic activity of wild-type (50 nM) or mutant factor VIIa (50 nM) was determined in the presence of varying concentrations of calcium chloride (0–5 mM) and the chromogenic substrate S-2288 (2 mM).

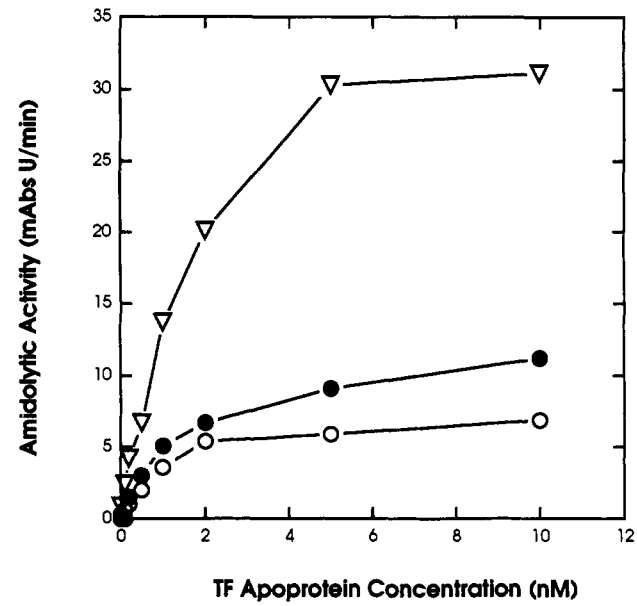


FIGURE 3: Effect of tissue factor apoprotein on the amidolytic activity of wild-type factor VIIa (▽), E220A factor VIIa (○), and E220K factor VIIa (●). The amidolytic activity of wild-type factor VIIa or mutant factor VIIa was determined in the presence of tissue factor apoprotein (0–10 mM), $CaCl_2$ (5 mM), and the chromogenic substrate S-2288 (2 mM).

agreement with earlier studies (Pedersen et al., 1991), we found that wild-type factor VIIa possessed an intrinsic amidolytic activity toward the chromogenic substrate S-2288 which progressively increased upon addition of calcium chloride until saturation was obtained at around 1–2 mM calcium (Figure 2). In contrast, no amidolytic activity could be detected with the E220K factor VIIa mutant, even in the presence of calcium chloride concentrations in excess of 10 mM (Figure 2). Since the coagulation assay indicated that the E220K factor VIIa mutant possessed ≈10% coagulant activity, it was of interest to assess the ability of tissue factor apoprotein to stimulate the amidolytic activity of E220K factor VIIa. We found that addition of an equimolar ratio of tissue factor apoprotein induced the amidolytic activity of E220K factor VIIa to a level ≈30% of that observed using wild-type factor VIIa (Figure 3). This tissue factor dependent enhancement of E220K factor VIIa amidolytic activity was calcium dependent, and required a calcium chloride concentration in excess of 5 mM for maximal rate enhancement. This was in sharp contrast to wild-type factor VIIa which required a $CaCl_2$ level of only 0.5 mM for full enhancement of tissue factor dependent amidolytic activity (data not shown).

In order to test the possibility that the observed activity resulted from our choice of changing a glutamic acid residue to lysine, we also produced a second factor VII mutant in which we mutated glutamic acid residue 220 to an alanine (E220A factor VII). This was done to circumvent the possibility that the lysine residue in the E220K mutant was taking the place of calcium and forming a salt bridge with adjacent residues, and thus producing the observed coagulant and tissue factor stimulated amidolytic activities. This was found not to be the case since E220A factor VIIa also possessed a coagulant activity corresponding to approximately 10% of wild-type factor VIIa. In agreement with our earlier finding, we found that E220A factor VIIa possessed no intrinsic amidolytic activity even at calcium chloride concentrations in excess of 5 mM (Figure 2). Once again, addition of an equimolar ratio of tissue factor apoprotein did lead to an increase in amidolytic activity, corresponding to ≈20% of that observed using wild-type factor VIIa (Figure 3).

*Analysis of Mutant Factor VII Binding to Tissue Factor.* To determine whether the decrease in coagulant and tissue factor dependent amidolytic activity observed with the mutant factor VIIa molecules was a direct result of impaired tissue factor binding, we conducted a series of competition experiments to assess the binding of E220K/E220A factor VII to tissue factor apoprotein. Since the single-chain form of the E220K/E220A factor VII mutant possessed virtually no amidolytic activity, it was possible to assess the tissue factor binding properties of this mutant by measuring its ability to inhibit the tissue factor dependent stimulation of wild-type factor VIIa amidolytic activity. As a positive control, we initially tested the effect of the zymogen form of a factor VII mutant in which the active-site Ser has been replaced with an Ala (S344A factor VII). As expected, S344A factor VII, which binds to tissue factor apoprotein in a normal manner but lacks amidolytic activity (Nakagaki et al., 1991), inhibited the tissue factor stimulated amidolytic activity of wild-type factor VIIa in a dose-dependent manner (Figure 4). The E220K/E220A factor VII mutants were less efficient inhibitors of tissue factor stimulated amidolytic activity, inhibiting this reaction with an affinity of ≈30% that of S344A factor VII (Figure 4).

## DISCUSSION

Previous studies, using a combination of 3D-modeling and synthetic peptides, have identified a high-affinity calcium binding site located in the protease domain of blood coagulation factor IX (Bajaj et al., 1992). On the basis of sequence homology to factor IX, it has been suggested that factor VII possesses an analogous calcium binding site involving glutamic acid residues 210 and 220 (Bajaj et al., 1992). In the present study, we have constructed, expressed, and purified a mutant form of factor VII in which glutamic acid residue 220 has been changed to lysine. To determine the role of Glu-220 with regard to calcium binding, we initially compared the binding of terbium to wild-type and mutant factor VII. Terbium is well suited for this purpose as it possesses an ionic radius similar to that of calcium and upon binding to a protein

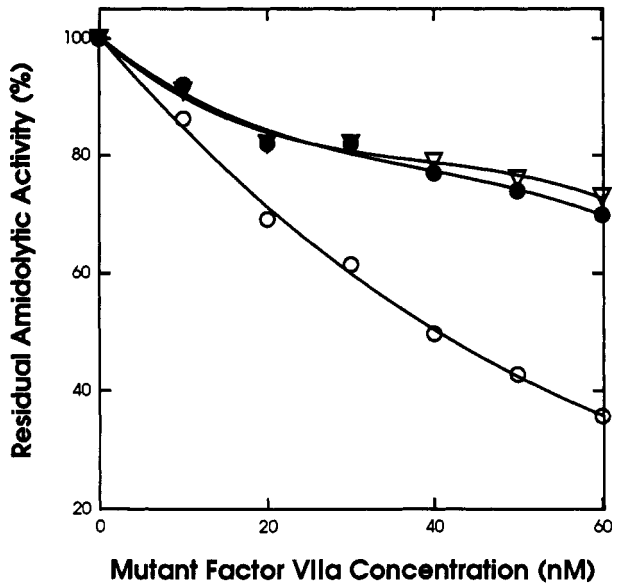


FIGURE 4: Effect of E220A factor VII, E220K factor VII, and S344A factor VIIa on the tissue factor dependent enhancement of wild-type factor VIIa amidolytic activity. The amidolytic activity of 20 nM wild-type factor VIIa and 10 nM tissue factor apoprotein was measured in the presence of 5 mM $CaCl_2$ and varying concentrations (0–60 nM) of either E220A factor VII (▽), E220K factor VII (●), or S344A factor VIIa (○) along with the chromogenic substrate S-2288 (2 mM). The residual amidolytic activity is defined as the amidolytic activity in the absence of mutant factor VIIa divided by the activity in the presence of mutant factor VIIa.

is capable of phosphorescence enhancement by energy transfer from aromatic side chains such as tryptophan which lie immediate to the binding site (De Jersey & Martin, 1980). Our experiments indicated that wild-type factor VIIa contains one such terbium binding site which is independent of the factor VII Gla domain and that terbium binding to this site can be inhibited by the addition of $Ca^{2+}$ with a $K_i$ of 1.5 mM. Consistent with the existence of a calcium binding site involving glutamic acid residue 220, we found that substitution of Glu-220 with lysine virtually abolished the ability of factor VII to bind terbium. In contrast to the homologous high-affinity calcium binding site located in the factor IX molecule (Bajaj et al., 1992), our $Ca^{2+}$ competition binding data suggest that this is a low-affinity site with a $K_D$ in the millimolar range. Of course we cannot discount the possibility that our observed affinity results from the fact that all $Ca^{2+}$ competition experiments were conducted at pH 6.5 so as to avoid terbium precipitation. Nevertheless, the existence of a low-affinity calcium binding site in the protease domain of factor VII agrees well with our observation that Gla-domainless factor VIIa contains one low-affinity and one high-affinity calcium binding site (Monroe et al., 1992).

To determine the functional importance of this putative calcium binding site, we also measured the activity of E220K factor VIIa in a series of amidolytic and coagulant assays. Although E220K factor VIIa contained no intrinsic amidolytic activity, addition of tissue factor apoprotein did induce an amidolytic activity corresponding to ≈30% of that observed using wild-type factor VIIa. Consistent with impaired calcium binding, this tissue factor dependent induction of E220K factor VIIa amidolytic activity required 5–10-fold higher concentrations of calcium than are needed when using wild-type factor VIIa. In agreement with a decreased tissue factor stimulated amidolytic activity, we also found that E220K factor VIIa possessed a decreased coagulant activity corresponding to 5–10% of normal. Interestingly, the single-chain form of this mutant possessed an even lower specific coagulant activity, corresponding to ≈0.1% that of an equal preparation of single-chain factor VII. This suggests that this calcium binding site plays a role in the activation of factor VII to VIIa, a speculation supported by our observation that the activation of E220K factor VII by factor Xa is significantly reduced in comparison to normal factor VII. To rule out the possibility that our choice of mutation was accounting for the observed differences, we also produced and tested a factor VII mutant in which glutamic acid residue 220 was changed to an alanine. Mutagenesis of Glu-220 to an alanine resulted in a mutant factor VII molecule possessing essentially identical coagulant and amidolytic characteristics. In all likelihood, the lack of coagulant activity observed in the E220A factor VIIa molecule is a direct result of impaired tissue factor binding. This hypothesis is supported by our observation that the E220K factor VIIa mutant is incapable of competing with wild-type factor VIIa for binding to tissue factor apoprotein.

Thus, our current findings not only confirm the existence of a calcium binding site located in the heavy chain of factor VII but also suggest a role for this site in the binding of factor VII to its cofactor, tissue factor. These latter results support our previous findings (Wildgoose et al., 1990b), as well as those of others (O'Brien et al., 1991), that the protease domain of factor VII is crucial in factor VII–TF interactions. Our present data appear to be at variance with a previous report, using chimeric proteins consisting of portions of factor VII and factor IX, that the high-affinity tissue factor recognition determinant is located in the EGF-like domains of factor VII (Toomey et al., 1991). Precisely why our results differ from those of Toomey et al. is unclear, but may be related to that fact that those experiments were conducted using versions of factor VII that were only 70% γ-carboxylated. In this regard, it is well-known that the factor VII Gla domain is crucial for the tight binding of factor VII to tissue factor apoprotein (Sakai et al., 1990). Our present data, however, are in accordance with previous predictions (Bajaj et al., 1992) that factor VII residues 210–220 are part of a surface loop common to several coagulation proteases and thought to be involved in protease–cofactor interactions. Previous observations using a combination of antibodies and peptides have implicated the analogous regions of factor IXa (Bajaj et al., 1985) and factor Xa (Chattopadhyay & Fair, 1989) in binding to their respective cofactors, factor VIII and factor V. Although our results indicate that mutagenesis of Glu-220 affects factor VII–tissue factor interactions, it is not clear whether this residue is involved in direct physical contact or whether it provides some calcium-dependent structural integrity necessary for normal tissue factor binding. The idea of a direct involvement of residue 220 in this interaction is rather unlikely since peptides spanning factor VII residues 206–218 have no effect on factor VII–tissue factor binding (Wildgoose et al., 1990b). A more likely scenario, however, is that calcium binding to residues 210–220 is a prerequisite for the exposure of adjoining tissue factor binding epitopes such as residues 195–206 (Wildgoose et al., 1990b). In this regard, we find that a monoclonal antibody (PW-4) produced against a peptide spanning residues 195–206 binds to factor VIIa in a calcium-dependent manner, requiring millimolar concentrations of $CaCl_2$ for maximal binding (Wildgoose et al., 1992). Preliminary results indicate that this antibody also binds to E220K factor VIIa but with a much reduced affinity (unpublished observations).

Collectively our results support the existence of a low-affinity calcium binding site located in the protease domain of factor VII and involved in calcium-dependent conformational

changes necessary for the binding of factor VII to tissue factor apoprotein. The exact role of other calcium binding sites such as those located in the factor VII EGF domain is unclear, and is currently under investigation.

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