How the Linker Connecting the Two Kringles Influences Activation and Conformational Plasticity of Prothrombin

Nicola Pozzi, Zhiwei Chen and Enrico Di Cera

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104

Running title: Role of Lnk2 in Prothrombin Structure and Function

To whom correspondence should be addressed: Enrico Di Cera, Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA, Tel.: (314) 977-9201; Fax: (314) 977-9206; E-mail: enrico@slu.edu

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SUMMARY
A flexible linker (Lnk2) composed of 26 amino acids connects kringle-1 to kringle-2 in the coagulation factor prothrombin. Recent studies point to Lnk2 as a key determinant of the structure and function of this zymogen. Using a combination of mutagenesis, structural biology and single molecule spectroscopy we show how Lnk2 influences activation and conformational plasticity of prothrombin. Scrambling the sequence of Lnk2 is inconsequential on activation, and so is extension by as many as 22 residues. On the other hand, below a critical length of 15 residues, the rate of prothrombin activation increases (10-fold) in the absence of cofactor Va, and decreases (3-fold) in the presence of cofactor. Furthermore, activation by prothrombinase takes place without preference along the prethrombin-2 (cleavage at R271 first) or meizothrombin (cleavage at R320 first) pathways. Notably, these transitions in the rate and pathway of activation require the presence of phospholipids, pointing to an important physiological role for Lnk2 when prothrombin is anchored to the membrane. Two new crystal structures of prothrombin lacking 22 (ProTΔ146-167) or 14 (ProTΔ154-167) residues of Lnk2 document striking conformational rearrangements of domains located across this linker. FRET measurements of freely diffusing single molecules prove that these structural transitions are genuine properties of the zymogen in solution. These findings support a molecular model of prothrombin activation where Lnk2 presents the sites of cleavage at R271 and R320 to factor Xa in different orientations by pivoting the C-terminal kringle-2/protease domain pair on the N-terminal Gla domain/kringle-1 pair anchored to the membrane.

Prothrombin, or coagulation factor II, is a vitamin K-dependent zymogen abundantly present in the blood and comprising a Gla domain (residues 1-46), kringle-1 (residues 65-143), kringle-2 (residues 170-248), and the protease domain (residues 285-579). In the penultimate step of the coagulation cascade, prothrombin is converted to the mature protease thrombin by the prothrombinase complex composed of factor Xa, cofactor Va, Ca^{2+} and phospholipids (1). Activation is enhanced >2,000-fold by cofactor Va and phospholipids due to increased \( k_{\text{cat}} \) (1-4) and a significant portion of this effect comes from optimization of the conformation of factor Xa induced by cofactor Va (5,6). Conversion to thrombin involves cleavage at R271 and R320 along two alternative pathways, generating prethrombin-2 and meizothrombin, respectively. On synthetic membranes and in the absence of cofactor Va, prothrombin activation proceeds along the prethrombin-2 pathway (1). In the presence of cofactor Va, selection of the pathway is context dependent. On the surface of platelets (7,8), prothrombinase activates prothrombin along the prethrombin-2 pathway (8). On the membrane of red blood cells (9) and other prothrombotic surfaces enriched with phosphatidylerine such as circulating microparticles (10) and synthetic liposomes (11-13), prothrombinase activates prothrombin along the alternative meizothrombin pathway. How the pathway is selected at the molecular level remains an intriguing and hotly debated issue (12,14-17), although its relevance to blood physiology ultimately depends on whether meizothrombin or prethrombin-2 accumulate during prothrombin activation (2,8,15).
Notwithstanding decades of investigation, our understanding of the factors that control the rate and pathway of prothrombin activation is largely phenomenological rather than mechanistic and structure-based. For example, perturbations of the Gla domain affect the rate and switch the pathway of activation from meizothrombin to prethrombin-2 (3,12,18), but exactly how this domain anchored to the membrane communicates with the sites of cleavage at R271 and R320 located >80 Å away (19) has not been elucidated. Active site occupancy of prothrombin also switches the pathway of activation from meizothrombin to prethrombin-2 (20,21), but no structural explanation has been offered for this effect. Previous studies have dissected the role of the Gla domain (3,12,18,22), kringles (23-25) and protease domain (26-28) and have contributed to the formulation of numerous models of the prothrombinase-prothrombin complex (29-31). Unfortunately, these models have not benefited from recent information on the structure of prothrombin and its unanticipated conformational plasticity (19,32).

Three flexible linkers connect the Gla domain to kringle-1 (Lnk1), the two kringles (Lnk2) and kringle-2 to the protease domain (Lnk3). Among these linkers, Lnk2 (residues 144-169) is unique insofar as it shares exon VII with kringle-2 in the longest coding region of the prothrombin gene (33) and, unlike Lnk1 and Lnk3, connects two adjacent domains of the zymogen not engaged in any intramolecular interaction. This feature presages a key role for Lnk2 in dictating the overall architecture of prothrombin. Movement of Lnk2 could trigger long-range perturbation of the conformation of the zymogen, far more pronounced than that produced by changes in Lnk1 and Lnk3 connecting domains in intramolecular contact. Lnk2 is long and non-helical (34) and should enable different arrangements of the N-terminal Gla domain/kringle-1 pair relative to the C-terminal kringle-2/protease domain pair (32). Indeed, it is easy to envision how prothrombin, anchored to the membrane via its Gla domain, may exploit the flexibility of Lnk2 to position the sites of cleavage at R271 in Lnk3 and R320 in the protease domain at different heights over the plane of the membrane for presentation to the active site of factor Xa in different order and orientation (see Figure 6 in the Discussion). Through this action, Lnk2 may contribute significantly to the rate and pathway of prothrombin activation.

The importance of Lnk2 is clearly established by previous studies (19,32), but exactly how this domain contributes to prothrombin function has not been established. Here we investigate the role of Lnk2 with deletions, insertions and scrambling of its sequence. We also present two new structures of prothrombin where Lnk2 lacks 22 (ProTΔ146-167) or 14 (ProTΔ154-167) of its 26 residues. These studies are complemented by measurements of prothrombin conformation in solution at the single molecule level. The combination of these approaches reveals how Lnk2 influences activation and conformational plasticity of prothrombin.

MATERIALS AND METHODS

Reagents- Prothrombin cDNA wild-type (residues 1-579) modified to include an epitope for the HPC4 antibody at the C-terminal was cloned into a pDEST40 expression vector using the Gateway® cloning technology (Life Technologies, Carlsbad, CA). The constructs ProTΔ145-168, ProTΔ146-167, ProTΔ146-156, ProTΔ154-167, ProTΔ154-159, ProTins11, ProTins22, ProTscr146-167 carrying deletions, insertions and scrambling of various portions of Lnk2 (Figure 1), and mutants R320A and R271A in the wild-type and ProTΔ146-167 backgrounds were generated using the Quickchange Lightning kit (Agilent, Anta Clara, CA) and appropriate primers (Integrated DNA Technologies, Coralville, IA). After sequencing, the proteins were expressed in baby hamster kidney cells and purified by affinity chromatography, ion exchange chromatography, and size exclusion chromatography as described previously (32,35,36). SDS-PAGE and N-terminal sequencing verified homogeneity and chemical identity of final preparations. The level of γ-carboxylation was determined by alkaline hydrolysis coupled to amino acid analysis (37,38). Small unilamellar vesicles composed of phosphatidylcholine and phosphatidylserine in a 3:1 molar ratio were prepared by extrusion using 0.05 or 0.1 μm polycarbonate membranes (Avanti Polar Lipids, Alabaster, AL) and their size was confirmed by DLS. Human purified factor Va, factor Xa and the thrombin specific inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) were purchased from Hematologic Technologies (Essex Junction, VT). Protein concentrations were determined by reading at 280 nm with molar extinction coefficients adjusted based on the amino acid sequence. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).
Activation of Prothrombin- Prothrombin wild-type and mutants were buffer exchanged into 150 mM NaCl, 20 mM Tris, 5 mM CaCl₂ using a Zeba 7K MWCO desalting column. Prothrombin activation was monitored in two different ways, i.e., using a standard discontinuous assay (12,16) and a continuous assay analogous to the one developed for the analysis of protein C activation (39,40). In the discontinuous assay, prothrombin (0-3 µM) was activated by addition of factor Xa (0.1-5 nM) and phospholipids (20 µM), or factor Xa (10 pM), phospholipids (20 µM) and cofactor Va (10 nM). Aliquots were quenched after 0.5, 1, 2 and 5 min and active site generation was quantified in terms of the hydrolysis of the chromogenic substrate H-D-Phe-Pro-Arg-p-nitroanilide (FPF) specific for thrombin and meizothrombin. Initial velocities were converted to concentrations using a standard reference curve measured at the time of the experiment. The independent Michaelis-Menten parameters $k_{cat}$ and $k_{cat}/K_m$ were obtained by nonlinear fit of the experimental data collected in a SpectraMax i3x Multi-Mode Detection Platform using the software Origin 2015 (OriginLab Corporation, Northampton, MA). In the continuous assay, the real-time activation of prothrombin to thrombin was monitored by progress curves of substrate hydrolysis after slight modification of the method developed for the analysis of protein C activation by thrombin (39,40). Unlike the discontinuous assay, the continuous assay based on progress curves better captures the kinetics of activation, can be analyzed rigorously by integration of the underlying kinetic expressions and requires small amounts of reagents. The values of $k_{cat}/K_m$ or $k_{cat}$ are derived by using prothrombin concentrations below (Figure 2A) or above (Figure 2B-C) $K_m$. This feature is particularly useful in the case of reactions that affect mainly $k_{cat}$, as observed for the effect of cofactor Va on prothrombin activation (Figure 2B-E), or when $K_m$ is very large and prevents saturation of the enzyme with substrate, as observed when prothrombin activation is studied in the presence of factor Xa only (Figure 2A). Prothrombin (0.5-1.4 µM) activation was measured in the presence of 20 nM factor Xa (Figure 2A), 0.1 nM factor Xa and 20 µM phospholipids (Figure 2B), or 1 pM factor Xa, 20 µM phospholipids and 10 nM cofactor Va (Figure 2C), using 24 µM chromogenic substrate H-D-Phe-Pro-Phe-p-nitroanilide (FPF). Factor Xa has no appreciable activity toward FPF, but thrombin and meizothrombin cleave with $k_{cat}/K_m=0.13 \mu M^{-1}s^{-1}$ (41). The lack of product inhibition was the reason for choosing FPF in this assay over the analogous chromogenic substrate FPR. Data were collected on a SpectraMax i3x Multi-Mode Detection Platform and analyzed with Origin 2015 (OriginLab Corporation, Northampton, MA).

Electrophoretic Assays- The pathway of prothrombin activation was monitored by SDS-PAGE. Activation in the presence of 0.2 nM factor Xa, 20 µM phospholipids and 30 nM cofactor Va was carried out with 1.4 µM prothrombin dissolved in 150 mM NaCl, 20 mM Tris, 5 mM CaCl₂ and incubated at 25 °C for 5 min in the presence 60 µM DAPA, 20 µM phospholipids and 10 nM cofactor Va. Reactions involving all prothrombin constructs were performed at the same time to facilitate comparison. Following addition of factor Xa, samples (40 µL) were quenched at different time intervals with 10 µL NuPAGE LDS buffer containing β-mercaptoethanol as the reducing agent and 20 mM EDTA. Samples were loaded into 12% SDS-polyacrylamide gels or processed by NuPAGE Novex 4-12% Bis-Tris protein gels run with MES buffer. All gels were stained simultaneously with coomassie brilliant blue R-250 and analyzed by quantitative densitometry.

Activated Partial Thromboplastin Time (aPTT)- aPTT was measured using the TriniCLOT aPTT kit on a ST4 semi-automated coagulometer (Diagnostica Stago, Gennevilliers, France). Briefly, 50 µL of citrated prothrombin deficient plasma (Hematologic Technologies, Essex Junction, VT) was mixed with 50 µL of the desired prothrombin construct (0.1 mg/mL) and 50 µL of Reagent S in the appropriate cuvettes. The reaction was started by adding 50 µL of 20 mM CaCl₂.

X-ray studies- Crystallization of the prothrombin mutants ProTΔ146-167 and ProTΔ154-167 lacking 22 or 14 residues of Lnk2, respectively, was achieved at 20 °C by the vapor diffusion technique using an Art Robbins Instruments Phoenix liquid handling robot and mixing equal volumes (0.3 µL) of protein and reservoir solution (Table 1). Optimization of crystal growth was achieved by the hanging drop vapor diffusion method mixing 3 µL of protein (10 mg/ml) with equal volumes of reservoir solution. Crystals of ProTΔ146-167 were grown in less than two weeks in the presence of 10 mg/mL protein, 0.2 M Na₂HPO₄, 20% PEG3350. Crystals of ProTΔ154-167 were also grown in less than two weeks in the presence of 10 mg/mL protein, 0.1 M MES, 1.6 M
MgSO₄, pH 6.5. In both cases, crystals were cryoprotected prior to flash freezing in a solution of 25% glycerol from the original mother liquor. X-ray diffraction data were collected with a home source (Rigaku 1.2 kW MMX007 generator with VHF optics) Rigaku Raxis IV++ detector and were indexed, integrated, and scaled with the HKL2000 software package (42). Initially, the previous structure of Ca²⁺-free ProTΔ146–167 (19) solved at 2.8 Å resolution (PDB code 4NZQ) was used as search model using PHASER from the CCP4 suite (43), but no solution was found due to a different orientation of fragment-1 in the new structures. Then, the previous structure of Ca²⁺-bound ProTΔ146–167 (19) solved at 3.4 Å resolution (PDB code 4O03) was partitioned into fragment-1 (1-145) and prethrombin-1 (168-579) and the two separate portions were used as individual search models. Refinement and electron density generation were performed with REFMAC5 from the CCP4 suite, and 5% of the reflections were randomly selected as a test set for cross validation. Model building and analysis of the structures were conducted with COOT (44). Both structures were subject to a final round of refinement with PDB_REDO (45). TLS tensors modeling rigid-body anisotropic temperature factors were calculated and applied to the structure of ProTΔ154-167. Ramachandran plots were calculated using PROCHECK (46). Statistics for data collection and refinement are summarized in Table 1. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (accession codes 5EDK for the new structure of ProTΔ146-167 and 5EDM for ProTΔ154-167).

Single molecule measurements- Guided by structural information, residues S210 in kringle-2 and S101 in kringle-1 were mutated to Cys to generate the double mutant S101C/S210C in the ProTΔ146-167 background. Selective reaction of the unpaired cysteines was achieved by reducing the protein (12-14 µM) in 20 mM Tris, 350 mM NaCl, pH 7.4 at room temperature for 1 h in the dark in the presence of DTT at a molar ratio [SH]ₐ/[DTT]ₐ=1:1.4. After removal of excess DTT with G-25 desalting spin column, the solution sat for 1 h at room temperature in the dark before addition of 5-molar excess of Alexa Fluor-555 and Alexa Fluor-647 maleimide dyes (Molecular Probes). The labeling reaction was carried out for 2 h at room temperature. The monomeric protein free of unreacted dyes was purified on an analytical Superdex 200 column and the efficiency of derivatization (70-90%) was assessed by UV-Vis measurements. No incorporation of the fluorescent dyes was observed in the wild-type protein under the same conditions.

Förster Resonance Energy Transfer (FRET) measurements of freely diffusing single molecules were performed with a confocal microscope MicroTime 200 (PicoQuant, Berlin, Germany). The donor and acceptor dyes were excited with a ps pulsed diode laser at 532 and 638 nm, respectively. To achieve pulsed interleaved excitation (47), the 532 nm laser was electronically delayed 25 ns relative to the 638 nm laser (48,49). A dual band dichroic mirror reflecting 532 nm and 638 nm guided the light to a high numerical aperture apochromatic objective (60x, N.A. 1.2, water immersion, Olympus) that focused the light to a confocal volume of 1.0 fl for excitation at 532 nm and detection at 575 nm. Fluorescence from excited molecules was collected with the same objective and focused onto a 50 µm diameter pinhole. The donor and acceptor emission were separated via a dichroic long pass filter with a dividing edge at 620 nm. Suited bandpass filters were inserted to eliminate the respective excitation wavelength and minimize spectral crosstalk. The fluorescence was detected with two avalanche photodiodes (SPAD) using Time-Correlated Single Photon Counting with the TimeHarp 200 board. Data was stored in the Time-Tagged Time Resolved Mode. Measurements were performed 25 µm deep in the solution with a total acquisition time of 1 h and repeated fresh up to four times on each protein sample (50 pM) in 20 mM Tris, 145 mM NaCl, 5 mM CaCl₂, 0.01% Tween 20,  pH 7.4. Signals from single molecules were observed as bursts of fluorescence. Appropriate correction for direct excitation of the acceptor at the donor excitation wavelength, leakage of the donor in the acceptor channel and the instrumental γ factor was calculated using a mixture of double stranded DNA models with known FRET efficiency (E) and stoichiometry (S) labeled with dyes AF555 and AF647 (50). Integration time was set to 0.5 ms and bursts with more than 35 counts were included in the analysis. Only molecules with a stoichiometry in the range 0.25-0.75 were considered in the final analysis and their distribution was fit to Gaussian curves using Origin 2015 (OriginLab Corporation, Northampton, MA). The number of independent Gaussians was determined according to the corrected Akaike information criterion (AICc). Data recording and
RESULTS
Lnk2 in human prothrombin is composed of 26 amino acids and is particularly rich in Gln, Pro, Ser and Val residues (Figure 1) that favor non-helical and flexible conformations, consistent with recent X-ray studies (32). Sequence alignment among 29 different species reveals high variability in length, from 20 residues in the fugu to 32 residues in dogs. Amino acid composition varies widely among species and only a handful of conserved residues cluster in the proximal portion of Lnk2 (residues 144-154), upstream of the site of cleavage at R155 targeted by thrombin and factor Xa in the absence of cofactor Va (19,51). The distal portion (residues 156-169) contains a single conserved residue, P164. The only documented naturally occurring mutation of Lnk2 is the E157K substitution in prothrombin Canberra associated with mild bleeding (52). Taken together, these observations indicate that a viable Lnk2 likely requires a length of 20-30 amino acids but little conservation of sequence. We therefore investigated the role of sequence and length of Lnk2 with substitutions, deletions and insertions.

To establish the role of any conserved residue within Lnk2, we scrambled the entire sequence 146-167 containing the site of cleavage at R155. The scrambled construct ProTscr146-167 shows no appreciable perturbation of the rate of prothrombin activation by factor Xa in the presence of Ca\(^{2+}\) and phospholipids, with or without cofactor Va (Table 2, Figure 2B-E). The specific sequence of residues 146-167 of Lnk2 is largely inconsequential on function. This observation should be considered in the context of our recent analysis of the prothrombin construct ProTΔ146-167 carrying a deletion of residues 146-167 in Lnk2 (19). Notable for ProTΔ146-167 is the 10-fold increase in the rate of activation in the absence of cofactor Va, and the decrease (3-fold) in the rate of activation in the presence of cofactor (Table 2). Deletion of a smaller portion of Lnk2, i.e., residues 154-159 in the ProTΔ154-159 construct, was also reported previously (19) and has no significant effect on the rates of activation by factor Xa in the presence of Ca\(^{2+}\) and phospholipids, with or without cofactor Va (Table 2). Given the drastic functional differences between ProTΔ146-167 and ProTΔ154-159, we constructed selective deletions of the proximal (residues 146-156) and distal (residues 154-167) portions of Lnk2 encompassing the site of cleavage at R155. Notwithstanding the different degree of conservation between the two portions, much higher for the proximal region (Figure 1), the two constructs ProTΔ146-154 and ProTΔ154-167 feature rates of activation by factor Xa in the presence of Ca\(^{2+}\) and phospholipids, with or without cofactor Va, that scale with the length of the linker (Table 2, Figure 2B-E). The observed rates are intermediate between those of wild-type and ProTΔ146-167. Removal of 24 of the 26 residues of Lnk2 in the construct ProTΔ145-168 produces functional properties very similar to those of ProTΔ146-167 (Table 2, Figure 2B-E). On the other hand, when the length of Lnk2 is increased by partial or full addition of the scrambled sequence of ProTscr146-167 in ProTins11 and ProTins22, the functional properties are practically identical to wild-type (Table 2, Figure 2B-E).

Altogether, these findings indicate that the length of Lnk2 and not its composition influences the rate of prothrombin activation (Figure 3). As the length of Lnk2 decreases, the value of \(k_{cat}/K_m\) for prothrombin activation increases up to 10-fold in the absence of cofactor Va and decreases up to 3-fold in the presence of cofactor (Figure 3). The dependence is sigmoidal in both cases, with a transition centered on a critical length of 15 residues. Interestingly, the documented range of 20-32 residues for Lnk2 found in 29 different species falls in an asymptotic region of the profiles. The length of Lnk2 was optimized during evolution to maximize the effect of cofactor Va on prothrombin activation and the variability in length of Lnk2 observed among species occurs in a range where perturbation of function is minimal. Notably, the dependence of the rate of activation on the length of Lnk2 (Figure 3) is observed in the presence of phospholipids (Figure 2B-E) but not when prothrombin is free in solution (Figure 2A). This result is consistent with previous mutagenesis studies on prothrombin derivatives lacking the entire fragment 1 comprising residues 1-155 (3,24,53).

Lnk2 also controls the pathway of prothrombin activation. Activation of prothrombin to thrombin requires cleavage at two sites, R271 and R320, along two mutually exclusive pathways producing the intermediates prethrombin-2.
absence of cofactor Va (1,12,16) because of a more favorable orientation and accessibility of R271 to proteolysis (12). In the presence of cofactor Va in the prothrombinase complex, the choice of the pathway becomes context dependent. On the surface of platelets (7,8), activation proceeds along the prethrombin-2 pathway because cleavage at R271 is preferred in 95% of the cases over cleavage at R320 (7). On non-platelet surfaces such as red blood cells (9) or synthetic phospholipids (1,11,12,16), prothrombin activation proceeds along the meizothrombin pathway. The relative accessibility of R271 and R320 is in this case controversial. Measurements carried out under nearly identical solution conditions by three different groups have reported that the sites are either equally accessible (12), or R320 is 4-fold (7) or 20-fold (16) more accessible than R271. The prothrombinase complex activates prothrombin along the meizothrombin pathway even when the length of Lnk2 is extended by 22 residues (Figure 4). Two distinguishing features of this pathway are the rapid and transient accumulation of F1.2.A (the fragment of prothrombin containing all domains but the catalytic B chain) and the lack of a significant amount of prethrombin-2. In contrast, reduction of the length of Lnk2 below 15 residues causes activation to proceed without appreciable preference between the meizothrombin and prethrombin-2 pathways, with both intermediates clearly detectable after 10 min (Figure 4). Densitometric analysis of the intermediates indicates that the integrity of Lnk2 is critical for selecting the meizothrombin pathway and that preference for this pathway is lost when the length of Lnk2 drops below 15 residues.

The change in preferential cleavage at R320 vs R271 below a critical length of Lnk2 is directly demonstrated by constructs carrying Ala substitutions of R271 or R320 in the wild-type and ProTΔ146-167 backgrounds. The conversion of prothrombin to meizothrombin (cleavage at R320 in constructs carrying the R271A mutation) or prethrombin-2 (cleavage at R271 in constructs carrying the R320A mutation) was measured directly in view of previous conflicting results (7,12,16). Consistent with the findings of Wood et al. (7), cleavage at R320 in the construct carrying the R217A mutation is preferred 5-fold over cleavage at R271 in the construct carrying the R320A mutation (data not shown). On the other hand, cleavage at R320 or R271 takes place with comparable rates in constructs carrying the R271A or R320A mutation in the ProTΔ146-167 background (Figure 4).

The relevance of these findings extends to blood physiology because scrambling, extending or deleting Lnk2 affects the aPTT in ways consistent with the biochemical data (Figure 5). Notably, addition of ProTΔ145-168, ProTΔ146-167 and ProTΔ154-167 to prothrombin deficient plasma prolongs the activated partial thromboplastin time (aPTT) 1.5-1.7-fold, indicating significant inhibition of the intrinsic pathway of the coagulation cascade leading to prothrombin activation. The effect is comparable to the defect in thrombin generation documented in patients with hemophilia A or treated with anticoagulants such as heparin. The prolongation of aPTT observed upon deletion of Lnk2 may be due to disruption of preferential activation of prothrombin along the meizothrombin pathway and resulting accumulation of the inactive intermediate prethrombin-2.

**DISCUSSION**

The factors controlling the rate and pathway of prothrombin activation have been investigated for decades (1,2,54-56). Previous studies have pointed out the importance of the context and composition of the membrane in determining the rate and pathway of prothrombin activation (1,3,11,12,18), implying a long-range communication between the Gla domain anchored to the membrane and the sites of cleavage at R271 and R320 located >80 Å away. The results presented here support Lnk2 as a key structural conduit for this long-range communication under conditions relevant to physiology. Scrambling most of Lnk2 is inconsequential on function, and so is its extension by as many as 22 residues. Reducing Lnk2 below a critical length of 15 residues increases the rate of activation (up to 10-fold) in the absence of cofactor Va and decreases it (up to 3-fold) in the presence of cofactor. Furthermore, below the critical length of 15 residues, prothrombinase no longer cleaves prothrombin preferentially at R320 over R271. All these effects depend on the presence of phospholipids, which lends support to the physiological relevance of Lnk2 in prothrombin activation.

It is of interest to cast the role of Lnk2 in the context of structural information. The current structure of ProTΔ146-167 (19) has documented a
contorted architecture of the zymogen with domains not vertically aligned (Figure 6A). Overall, prothrombin can be pictured as two rigid bodies, the Gla domain/kringle-1 pair on the N terminus and the kringle-2/protease domain pair on the C-terminus, whose relative arrangement is dictated by the flexibility of Lnk2 connecting the two kringles. When the Gla domain is anchored to the membrane as proposed by Furie (57) and Tajkhorshid (58), the rest of the molecule assumes a bent conformation with the site of cleavage at R320 positioned 45 Å over the plane of the phospholipids (Figure 6D). FRET studies have reported that this plane is 60-70 Å away from a fluorophore conjugated to the active site of factor Xa (59-61). Hence, engagement of the site of cleavage at R320 by factor Xa would require a substantial conformational rearrangement of factor Xa and/or prothrombin upon assembly of the enzyme-substrate complex. Furthermore, access to the R271 site of cleavage initiating the prethrombin-2 pathway would require an even more drastic rearrangement. R271 is in a disordered region of Lnk3. T274, the closest residue to R271 detected in the density map, sits only 21 Å above the membrane in an orientation predicting non-optimal cleavage of R271 by factor Xa. The lack of preferential cleavage by prothrombinase at R271 and R320 in the ProTΔ146-167 mutant is difficult to reconcile with the architecture of prothrombin currently documented by X-ray crystallography (19). Two new structures address this issue directly and reveal the importance of Lnk2 in the mechanism of activation.

A new structure of ProTΔ146-167, solved at 3.2 Å resolution, portrays the two rigid portions of the zymogen in a relative arrangement that is flipped almost 180° relative to the original structure (Figures 6C and 7A). The kringle-2/protease domain pairs in two structures align with an rmsd of only 0.56 Å, but the Gla domain/kringle-1 pair in the new structure of proTΔ146-167 flips on the opposite side of the main vertical axis of the molecule (Figure 7A) producing an overall architecture that is slightly more elongated (92 vs 85 Å). When this new conformation is docked on a phospholipid surface (Figure 6F), residues T274 and R320 flip their position and sit respectively 70 and 60 Å above the plane of the membrane, well within the reach of the active site of factor Xa. Just four of the 26 residues of Lnk2 in the ProTΔ146-167 mutant enable enough flexibility to present R271 and R320 to the active site of factor Xa in drastically different orientations. The four residues of Lnk2 in ProTΔ146-167 act as a molecular switch between α-helix (Figure 8A) and β-strand (Figure 8C) configurations and broker a sharp conformational rearrangement of kringle-1 (Figure 7A). The remarkable flexibility of Lnk2 documented by X-ray crystallography was investigated further with measurements in solution to rule out any potential artifact due to crystal packing. Single molecule FRET measurements with probes attached at residues 101 and 210 across Lnk2 in the ProTΔ146-167 background were carried out to complement recent luminescence resonance energy transfer (LRET) measurements between the same two residues in full length prothrombin (32). Experiments were performed with pulsed interleaved excitation, which reports the status of both donor and acceptor fluorophores by sorting molecules on the basis of relative donor:acceptor stoichiometry (S) and apparent FRET efficiency (E). The ability of single molecule FRET to detect and resolve dynamic behavior within a single or multiple populations enabled identification of two distinct and comparable populations for ProTΔ146-167 (Figure 7B), with inter-probe distances of 51 Å (low energy transfer) and 36 Å (high energy transfer). These values are consistent with the Cα-Cα distances between residues 101 and 210 in the two conformations of ProTΔ146-167 documented by X-ray crystallography (Figure 7A) and are also very similar to the distances of 54 Å and 34 Å measured by LRET between probes attached at residues 101 and 210 in full length prothrombin (32). Hence, the conformational plasticity of Lnk2 detected by X-ray crystallography in the ProTΔ146-167 mutant is highly relevant to physiology. The X-ray structural data are consistent with solution measurements of the ProTΔ146-167 mutant by single molecule FRET and of full length prothrombin by LRET (32).

Further insight on the role of Lnk2 comes from the structure of ProTΔ154-167, solved at 2.2 Å resolution, that reveals the architecture of prothrombin lacking only 14 residues in unprecedented detail (Figure 6B). In this structure, the kringle-2/protease domain pair assumes the same conformation seen in the two structures of proTΔ146-167 (Figures 6A and 6C), but the Gla domain/kringle-1 pair features a drastic reorientation. Kringle-1 rotates 3° anticlockwise and moves almost 40 Å downward relative to its position in proTΔ146-167 (Figure 6A), taking the Gla domain with it (Figure 8E). As a result, the
overall conformation of ProTΔ154-167 stretches up to 100 Å and shows an almost perfect alignment of its two rigid domains (Figure 6B). The overall shape of ProTΔ154-167 is stabilized by Lnk2 whose 12 residues are all visible in the density map and engage kringle-1 in several H-bonding interactions (Figure 8B). Lnk2 assumes a β-strand configuration as in the new structure of ProTΔ146-167 (Figure 8C), supporting a key role for the first two residues of the linker in triggering alternative conformations (Figure 8A,C). The trigger likely works in the wild-type as well. In fact, the Cα-Cα distance between residues 101 and 210 in the structure of ProTΔ154-167 is 34 Å and practically identical to one of the two distances measured by LRET between probes attached at residues 101 and 210 in full length prothrombin (32). This makes the high resolution structure of ProTΔ154-167 highly relevant to physiology, just as the structures of ProTΔ146-167. When the conformation of ProTΔ154-167 is docked on a phospholipid surface (Figure 6E), both T274 and R320 move up above the plane of the membrane to 55 and 65 Å, respectively, again within the range of the predicted position of the active site of factor Xa (59-61). Unfortunately, the site of cleavage at R271 remains too disordered to detect in the structure of ProTΔ154-167, notwithstanding the high resolution.

The structures shown in Figure 6 offer unprecedented snapshots of the conformational plasticity of prothrombin, with Lnk2 controlling the relative orientation of the two rigid kringle-2/protease domain and Gla domain/kringle-1 pairs. Once anchored to the membrane via the Gla domain, prothrombin utilizes the flexible Lnk2 as a pivot point to orient the kringle-2/protease domain pair containing the sites of cleavage at R271 and R320. Through this action, Lnk2 adjusts the height of R271 and R320 over the plane of the membrane and present them to the active site of factor Xa in the order and orientation necessary to define rate and pathway of activation. This scenario also provides context for the effect of membranes and cofactor Va.

A final comment should be made about the overall conformation of Lnk2 in the three structures of prothrombin discussed in this study (Figure 6). The arrangement of the N-terminal Gla domain/kringle-1 pair relative to the C-terminal kringle-2/protease pair changes from being distorted when Lnk2 is only 4 residue long in ProTΔ146-167 (Figure 6A,C) to almost linear when Lnk2 is 12 residue long in ProTΔ154-167 (Figure 6B). However, Lnk2 never assumes a conformation promoting full extension of prothrombin along the vertical axis. The rate and pathway of prothrombin activation are perturbed when the length of Lnk2 drops below the critical threshold of 15 residues and no change is observed when the length is >20 residues (Figure 3). These structural and functional observations converge to the mechanistically important conclusion that Lnk2 does not function in a fully untethered and extended conformation, contrary to the assumptions of recent models (29-31). In a fully extended Lnk2, the rate of prothrombin activation would decrease both below and above a critical length required for optimal alignment between the sites of cleavage and the active site of factor Xa (Figure 6), producing a bell shaped dependence contradicted by the data in Figure 3. On the other hand, a conformation where Lnk2 is allowed to bulge beyond a critical length is entirely consistent with the results in Figure 3.

The conformational plasticity of prothrombin should invite reconsideration of recent musings on the architecture of the zymogen bound to prothrombinase (29-31), or of the long list of alternative mechanisms of prothrombin activation (12,14-18,20). The approach presented here shows the benefits, if not the necessity, of combining multiple X-ray structures, site-directed mutagenesis and single molecule FRET to achieve a quantitative understanding of prothrombin and its key interactions in the blood coagulation cascade.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest.
AUTHOR CONTRIBUTIONS
NP and EDC designed research; NP and ZC carried out the work; NP, ZC and EDC analyzed results; NP and EDC wrote the manuscript.

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Figure 1. Conservation of Lnk2 and prothrombin constructs. In human prothrombin, Lnk2 is composed of 26 residues, from 144 to 169, and connects kringle-1 (65-143) to kringle-2 (170-248). The site of cleavage for thrombin and factor Xa, R155, defines the midpoint of the linker and the partitioning between proximal and distal portions. The result of the sequence alignment is shown as a sequence logo (Weblogo). Residues with frequencies $\geq 2$ are considered significant and marked with a star. The table lists the sequence and length for the prothrombin constructs of Lnk2 characterized in this study. Deletions of residues 145-168 in ProTA145-168 and residues 146-167 in ProTA146-167 remove 24 and 22 of the total 26 residues of Lnk2. ProTA154-167 lacks the distal portion of Lnk2 and retains most of the proximal one (red). ProTA146-156 lacks the proximal portion and retains most of the distal one (green). ProTA154-159 eliminates region of Lnk2 around R155. Scrambling the entire sequence 146-167 generates ProTsc146-167. The N-terminal half of the scrambled sequence of ProTsc146-167 (purple) was inserted in ProTins11 downstream of L167. The entire scrambled sequence of ProTsc146-167 (purple and yellow) was inserted in ProTins22 downstream of L167.

Figure 2A-E. Effect of perturbation of Lnk2 on the rate of prothrombin activation. The various constructs of Lnk2 presented in the Figure are listed in the scheme (bottom left). Other constructs listed in Table 2 are not included in the figure for clarity of presentation, but the best-fit results are shown below for the continuous assay and in Table 2 for the discontinuous assay. All experiments were carried out in triplicate. (A-C) Continuous assay of prothrombin activation started by addition of: (A) 20 nM factor Xa to 500 µM prothrombin; (B) 0.1 nM factor Xa and 20 µM phospholipids to 1 µM prothrombin; or (C) 1 pM factor Xa, 20 µM phospholipids, 10 nM cofactor Va to 1 µM prothrombin. The release of $p$-nitroaniline upon hydrolysis of FPF was monitored at 405 nm. Analysis of the progress curves yields the value of $k_{cat}/K_m$ for the activation of prothrombin when [ProT]<$K_m$ (A) and the value of $k_{cat}$ when [ProT]$>K_m$ (B-C). The best-fit values for $k_{cat}/K_m$ in panel A are: 440±10 M$^{-1}$s$^{-1}$ (ProTA145-168), 440±10 M$^{-1}$s$^{-1}$ (ProTA146-167), 500±20 M$^{-1}$s$^{-1}$ (ProTA154-167), 500±10 M$^{-1}$s$^{-1}$ (ProTA146-156), 430±10 M$^{-1}$s$^{-1}$ (ProTA154-159), 330±10 M$^{-1}$s$^{-1}$ (wild-type), 320±10 M$^{-1}$s$^{-1}$ (ProTsc146-167), 300±10 M$^{-1}$s$^{-1}$ (ProTins11) and 300±10 M$^{-1}$s$^{-1}$ (ProTins22). The best-fit values for $k_{cat}$ in panel B are: 0.17±0.02 s$^{-1}$ (ProTA145-168), 0.19±0.01 s$^{-1}$ (ProTA146-167), 0.16±0.01 s$^{-1}$ (ProTA154-167), 0.090±0.005 s$^{-1}$ (ProTA146-156), 0.042±0.001 s$^{-1}$ (ProTA154-159), 0.027±0.002 s$^{-1}$ (wild-type), 0.031±0.002 s$^{-1}$ (ProTsc146-167), 0.025±0.001 s$^{-1}$ (ProTins11) and 0.019±0.002 s$^{-1}$ (ProTins22). The best-fit values for $k_{cat}$ in panel C are: 8.6±0.2 s$^{-1}$ (ProTA145-168), 8.6±0.3 s$^{-1}$ (ProTA146-167), 11±1 s$^{-1}$ (ProTA154-167), 21±1 s$^{-1}$ (ProTA146-156), 32±1 s$^{-1}$ (ProTA154-159), 33±1 s$^{-1}$ (wild-type), 32±1 s$^{-1}$ (ProTsc146-167), 36±1 s$^{-1}$ (ProTins11) and 35±1 s$^{-1}$ (ProTins22). (D-E) Discontinuous assay of prothrombin activation in the absence (D) or presence (E) of cofactor Va. Experimental data obey Michaelis–Menten kinetics with best-fit parameter values reported in Table 2. Activity is expressed in s$^{-1}$ to facilitate direct comparison with the results from the continuous assay in panels B and C. Experimental conditions are: 150 mM NaCl, 5 mM CaCl$_2$, 0.1% PEG 8000, 20 mM Tris, pH 7.4 at 25°C.

Figure 3. The length of Lnk2 influences the rate of prothrombin activation. Values of the specificity constant $k_{cat}/K_m$ for activation of prothrombin by factor Xa and phospholipids in the absence (orange) or presence (blue) of cofactor Va, plotted vs the length of Lnk2. The data are from Table 2 and portray an opposite dependence of the rate of activation that increases (-cofactor Va) or decreases (+cofactor Va) with the length of Lnk2. In the presence of phospholipids, prothrombin activation in the absence of cofactor Va is significantly enhanced when Lnk2 <15 residue long. On the other hand, a Lnk2 <15 residue long slightly compromises activation in the presence of cofactor. The overall effect of cofactor Va is highly dependent on the length of Lnk2 and is optimized when Lnk2 has a length of at least 20 residues, as documented in 29 different species (Figure 1).

Figure 4. Effect of perturbation of Lnk2 on the pathway of prothrombin activation. SDS-PAGE analysis of the conversion of prothrombin (1.4 µM) to thrombin by the prothrombinase complex (0.2 nM factor Xa, 20 µM...
Figure 5. Effect of perturbation of Lnk2 on the aPTT. aPTT values are shown as the ratio of the test to normal, prothrombin wild-type (30±2 s). Additional reference values are 32±3 s for plasma collected from a healthy donor (positive control, +) and 185±8 s for prothrombin deficient plasma (negative control, -). The solid orange line identifies the baseline and the dashed red line highlights the critical value of 1.5. Prothrombin constructs ProTA145-168, ProTA146-167 and ProTA154-167 shows a significant prolongation of the aPTT (1.50±0.03, 1.62±0.06 and 1.72±0.08 fold, respectively). Each measurement is the average of 6 individual determinations.

Figure 6A-F. Conformational flexibility of Lnk2 revealed by X-ray crystallography. Crystal structure of ProTA146-167 (A) reported previously (19), along with the new structures presented in this study for ProTA154-167 (B) and ProTA146-167 in a new conformation (C). The three structures are aligned over the rigid kringle-2/serine protease pair and then visualized separately in the same orientation. Individual domains are labeled and colored as follows: Gla domain (Gla, blue), kringle-1 (K1, red), kringle-2 (K2, green), A chain (Ac, orange), B chain (Bc, yellow). Linkers are colored in wheat. The flexibility of Lnk2, containing as little as 4 (ProTA146-167) or 12 (ProTA154-167) residues, causes the Gla domain/kringle-1 pair to move up to 40 Å (B vs A) or to rotate 180° (C vs A) relative to the rigid kringle-2/serine protease domain pair. Differences in the conformation of the Gla domain among the three structures are due to crystallization conditions (Ca²⁺ or Mg²⁺) and details are given in Figure 8D-F. The role of Lnk2 in orienting the two sites of cleavage at R271 (not visible and approximated by T274 in blue) and R320 (red) is readily appreciated when the three structures are docked on a phospholipid surface (D-F), with the Gla domain binding almost perpendicularly to the plane and inserting the hydrophobic ω-loop as proposed by Furie (57) and Tajkhorshid (58). The distance of each site of cleavage from the plane of the membrane is indicated, along with the position of the active site of factor Xa predicted by previous FRET measurements (59-61).

Figure 7A-B. Conformational flexibility of Lnk2 revealed by single molecule FRET. (A) Structures of ProTA146-167 (4003 wheat, 5EDK marine), with the Gla domain removed for clarity. The two structures overlap significantly (rmsd = 0.56 Å) at the level of the kringle-2/protease domain pair, but differ sharply in the orientation of kringle-1 (red). The change is due to the conformation of Lnk2 that switches from α-helix (4003, position 1) to β-strand (5EDK, position 2). Residues S101 and S210 are represented as green and magenta spheres, with arrows indicating Cα-Cα distances. (B) FRET histograms for the mutant ProTA146-167/S101C/S210C. The lower panel shows a two-dimensional histogram of stoichiometry, S, vs FRET efficiency, E, for each diffusing molecule that contains both AF555 and AF647 fluorophores (i.e., molecules with 0.25＜S＜0.75). The upper panel shows a one-dimensional E histogram of the molecules in the lower panel. The E distribution was fit to a double Gaussian distribution (red). The center and percentage of the population in each Gaussian is indicated. FRET efficiencies were converted to distances using a theoretical R0=51 Å and a random orientation factor k=2/3.

Figure 8A-F. Structural features of prothrombin mutants proTA146-167 and proTA154-167. Artificial connections between kringle-1 and kringle-2 of proTA146-167 (A), proTA154-167 (B) and the new conformation of proTA146-167 (C) showing the plasticity of Lnk2. The electron density 2Fo-Fc map is...
countered at 1σ. Polar interactions are shown as dash lines, with kringle-1 (red), kringle-2 (green) and Lnk2 (wheat). Lnk2 acts as a molecular switch between α-helix (C) or β-strand (A and B) configurations. Notably, most of the 12 residues of Lnk2 in proTΔ154-167 form an uninterrupted β-sheet with the adjacent 139-143 strand of kringle-1 stabilized by additional contacts between T153, R81 and C170. Structure of the Gla domain of ProTΔ146-167 (19) (D), bovine prothrombin fragment 1 (57) (E) and ProTΔ154-167 (F) bound to Ca2+ (D and E) or Mg2+ (F). Overall, the architecture of the Ca2+ and Mg2+ bound forms are superimposable in the N12-A46 region (rmsd=0.48 Å) and show a similar globular shape with three α-helices spanning and two connecting loops. The difference between Ca2+ and Mg2+ bound forms is restricted to the first 10 residues from the N-termini, in agreement with previous NMR studies on the peptide 1-47 of factor IX (62). Formation of a functional ω-loop is impeded in the presence of Mg2+ (F). Valence calculations (63) identify a total of five binding sites for Mg2+. Mg1, Mg3, Mg4 and Mg5 in ProTΔ154-167 (F) occupy the same sites as Ca1, Ca2, Ca5 and Ca6 in the structure of bovine prothrombin fragment-1, suggesting direct competition in these positions.
Table 1. Crystallographic data for prothrombin mutants ProTΔ146-167 and ProTΔ154-167.

|                  | ProTΔ146-167 | ProTΔ154-167 |
|------------------|--------------|--------------|
| Buffer           | 200 mM Na₂HPO₄ | 100 mM MES, 1.6 M MgSO₄, pH 6.5 |
| PEG              | 3350 (20%)   | -            |
| PDB ID           | 5EDK         | 5EDM         |

**Data collection:**

|                  | ProTΔ146-167 | ProTΔ154-167 |
|------------------|--------------|--------------|
| Wavelength (Å)   | 1.54         | 1.54         |
| Space group      | P4₁,2,2      | C222₁        |
| Unit cell dimensions (Å) | a=84.2, b=84.2, c=346.4 | a=109.9, b=168.7, c=144.3 |
| Molecules/asymmetric unit | 1 | 1 |
| Resolution range (Å) | 40-3.2 | 40-2.2 |
| Observations     | 89160        | 486501       |
| Unique observations | 20053  | 67055       |
| Completeness (%) | 94.5 (94.0)  | 98.0 (97.8)  |
| Rsym (%)         | 15.5 (44.1)  | 8.7 (48.7)   |
| I/σ(I)           | 7.0 (2.0)    | 17.0 (3.0)   |

**Refinement:**

|                  | ProTΔ146-167 | ProTΔ154-167 |
|------------------|--------------|--------------|
| Resolution (Å)   | 40-3.2       | 40-2.2       |
| R<sub>crys</sub>, R<sub>free</sub> | 0.29, 0.32 | 0.20, 0.24 |
| Reflections (working/test) | 18675/1009 | 63362/3409 |
| Protein atoms    | 4242         | 4414         |
| Ca<sup>2+</sup> ions | 4 | - |
| Mg<sup>2+</sup> ions | - | 6 |
| Solvent molecules | - | 464 |
| Rmsd bond lengths<sup>a</sup> (Å) | 0.006 | 0.012 |
| Rmsd angles<sup>a</sup> (°) | 1.2 | 1.7 |
| Rmsd ΔB (Å<sup>2</sup>) (mm/ms/ss)<sup>b</sup> | 3.07/2.39/2.31 | 2.66/2.98/3.29 |
| <B> protein (Å<sup>2</sup>) | 87.2 | 58.4 |
| <B> Ca<sup>2+</sup> ions (Å<sup>2</sup>) | 59.6 | - |
| <B> Mg<sup>2+</sup> ions (Å<sup>2</sup>) | - | 57.7 |
| <B> solvent (Å<sup>2</sup>) | - | 57.6 |

**Ramachandran plot:**

|                  | ProTΔ146-167 | ProTΔ154-167 |
|------------------|--------------|--------------|
| Most favored (%) | 96.3         | 99.4         |
| Generously allowed (%) | 2.4 | 0.2 |
| Disallowed (%) | 1.3          | 0.4          |

<sup>a</sup>Root-mean-squared deviation (Rmsd) from ideal bond lengths and angles and Rmsd in B-factors of bonded atoms. <sup>b</sup>mm, main chain-main chain; ms, main chain-side chain; ss, side chain-side chain.
### Table 2. Kinetic parameters for activation of prothrombin wild type and mutants of Lnk2

|          | - Cofactor Va | + Cofactor Va |
|----------|---------------|---------------|
|          | $k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_m$ (µM) | $k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_m$ (µM) | $L^a$ |
| wt       | 0.12±0.01     | 0.016±0.003   | 0.13±0.02 | 270±20 | 32±3 | 0.12±0.01 | 26 |
| ProTΔ145-168 | 0.92±0.08     | 0.11±0.01     | 0.12±0.01 | 100±10 | 11±1 | 0.11±0.01 | 2  |
| ProTΔ146-167 | 1.2±0.1       | 0.13±0.02     | 0.11±0.01 | 110±10 | 11±1 | 0.10±0.01 | 4  |
| ProTΔ154-167 | 0.63±0.06     | 0.095±0.005   | 0.15±0.02 | 120±10 | 10±1 | 0.085±0.008 | 12 |
| ProTΔ146-156 | 0.29±0.02     | 0.041±0.005   | 0.14±0.02 | 190±10 | 21±2 | 0.11±0.01 | 16 |
| ProTΔ154-159 | 0.13±0.01     | 0.018±0.002   | 0.14±0.01 | 270±20 | 25±2 | 0.091±0.008 | 20 |
| ProTscr146-167 | 0.16±0.01     | 0.016±0.003   | 0.10±0.02 | 240±20 | 26±2 | 0.11±0.01 | 26 |
| ProTins11 | 0.12±0.01     | 0.015±0.003   | 0.13±0.02 | 320±20 | 32±2 | 0.10±0.01 | 37 |
| ProTins22 | 0.10±0.01     | 0.011±0.002   | 0.11±0.01 | 280±20 | 31±2 | 0.11±0.01 | 48 |

Experimental conditions are: 20 µM phospholipids, 150 mM NaCl, 5 mM CaCl$_2$, 0.1% PEG8000, 20 mM Tris, pH 7.4 at 25 °C. The concentration of factor Xa in the absence of cofactor Va is 0.1-7.5 nM, depending on the prothrombin construct used, and 10 pM in the presence of cofactor. $^a$Length of the linker (in amino acid residues) connecting the two kringles.
| Construct     | Sequence                                                   | Length |
|--------------|------------------------------------------------------------|--------|
| Ins22        | GQDQVTVMTPRSEGSSVNLSPPLEQ                                  | 48     |
| Ins11        | GQDQVTVMTPRSEGSSVNLSPPLEQ                                 | 37     |
| scr146-167   | GQTDPVMQSTGEVPSPLSVLREQ                                    | 26     |
| WT           | GQDQVTVMTPRSEGSSVNLSPPLEQ                                  | 26     |
| Δ154-159     | GQDQVTVMANT-------SVNLSPPLEQ                               | 20     |
| Δ146-156     | GQ----------------SEGSSVNLSPPLEQ                            | 16     |
| Δ154-167     | GQDQVTVMANT----------------EQ                              | 12     |
| Δ146-167     | GQ------------------E                                       | 4      |
| Δ145-168     | G-------------------                                       | 2      |
Figure 3

\[ \log \left( \frac{k_{\text{cat}}}{K_m} \right) \text{ (M}^{-1}\text{s}^{-1}) \]

Length of Lnk2 (aa residues)
Figure 4
How the Linker Connecting the Two Kringles Influences Activation and Conformational Plasticity of Prothrombin
Nicola Pozzi, Zhiwei Chen and Enrico Di Cera

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