Prothrombin structure: unanticipated features and opportunities

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

Enrico Di Cera
Author for correspondence: Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA
Tel.: +1 314 977 9201
Fax: +1 314 977 9206
Email: enrico@slu.edu

The structure of prothrombin has eluded investigators for decades but recent efforts have succeeded in revealing the architecture of this important clotting factor. Unanticipated features have emerged outlining the significant flexibility of the zymogen due to linker regions connecting the γ carboxyglutamic domain, kringle-2 and protease domain. A new, structure-based framework helps in defining a molecular mechanism of prothrombin activation, rationalizes the severe bleeding phenotypes of several naturally occurring mutations and identifies targets for drug design.

Prothrombin, or coagulation factor II, is abundantly present in the blood where it circulates at a concentration of 0.1 mg/ml and a half-life of about 60 h [1]. In the penultimate step of the coagulation cascade, prothrombin is proteolytically converted to the active protease thrombin by the prothrombinase complex comprising the protease factor Xa and cofactor Va assembled on a phospholipid membrane in the presence of Ca²⁺. Thrombin catalyzes the conversion of fibrinogen to an insoluble fibrin clot, the activation of platelets via the protease-activated receptor 1 and the feedback control of the coagulation response via the thrombomodulin-dependent protein C pathway. Because of the crucial roles of thrombin, the body has an absolute requirement for its zymogen precursor and no living patient has ever been reported with undetectable levels of prothrombin in the blood [2]. However, the importance of prothrombin extends to other physiological processes. Prothrombin is involved in embryonic development [3,4], is elevated in the cerebrospinal fluid of patients suffering from progressive neurodegenerative diseases [5] and becomes the target of antibodies in the antiphospholipids syndrome [6]. Studies on the structure and function of prothrombin are therefore crucial to the understanding of processes that are essential for life and constitute a focus of therapeutic intervention.

Prothrombin is a vitamin K-dependent zymogen composed of a gamma carboxyglutamic (Gla) domain (residues 1–46), kringle-1 (residues 65–143), kringle-2 (residues 170–248) and the protease domain with the A chain (residues 285–320) and the catalytic B chain (residues 321–579). The prothrombinase complex converts prothrombin to thrombin by cleaving at R271 and R320, generating the intermediates prethrombin-2 and meizothrombin, respectively. Under conditions most relevant to physiology, that is, on the surface of platelets, prothrombinase activates prothrombin along the prethrombin-2 pathway [7]. On non-platelet surfaces or synthetic phospholipids, activation proceeds along the meizothrombin pathway [8]. The mechanism of how prothrombinase directs cleavage at R271 or R320 remains poorly understood at the molecular level and highly controversial [9–11]. Cofactor Va and phospholipids control both the rate and pathway of prothrombin activation, although the cofactor has the greatest effect in enhancing $k_{cat}$ by almost 2000-fold [12], but the molecular origin of this large effect remains unknown. Notwithstanding decades of intense investigation, our information on the factors that control the rate and pathway of prothrombin activation is largely phenomenological. For example, perturbations of the Gla domain anchoring

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prothrombin to the membrane switch the pathway of activation from meizothrombin to prethrombin-2 [13,14], but how this domain communicates with the sites of cleavage >80 Å away to inform the pathway of activation is not known. Active site occupancy of prothrombin also switches the pathway of activation from meizothrombin to prethrombin-2 [15], but again no compelling structural explanation has been offered. Kringle-1 and kringle-2 interact with cofactor Va [16,17] and so do residues in the autolysis loop [18] and exosite I [19] of the B chain. Factor Xa interacts with kringle-2 [20] and residues near exosite II of the B chain [21] as well as with the Gla domain [22]. Knowledge from these epitopes is most useful when complemented by structural information on the prothrombin–prothrombinase complex. The structure of a surrogate prothrombinase has been solved recently [23]. More relevant to human biology is the structure of prothrombin itself [24,25].

Three linkers connect kringle-1 to the Gla domain (Lnk1, residues 47–64), the two kringles (Lnk2, residues 144–169) and kringle-2 to the mature A chain (Lnk3, residues 249–284), respectively. The linkers are encoded by separate exons (IV, VII and VIII), but Lnk2 is unique insofar as it shares exon VII with kringle-2 in the longest coding region of the gene [26]. The architecture of the prothrombin gene supports an important role for the linkers, yet these structural elements have not attracted attention until recently [24,25]. Prothrombin has a highly plastic arrangement with the Gla domain, kringles and protease domain not vertically stacked and Lnk2 and Lnk3 highly disordered. Luminescence resonance energy transfer measurements in solution document the flexibility of Lnk2 that functions like a spring connecting the kringle-2/protease domain pair on the C-terminal side to the Gla domain/kringle-1 pair on the N-terminal side [24].

The unanticipated plasticity of prothrombin due to its flexible linkers influences both the rate and pathway of activation. Deletion of Lnk1 perturbs Ca$^{2+}$ binding to the Gla domain and the level of γ-carboxylation and results in a switch of the pathway of prothrombin activation from meizothrombin to prethrombin-2. Lnk1 directs prothrombinase to cleave at R320 by channeling information from the Gla domain to the activation domain. The crystal structure of prothrombin suggests how this communication is established at the molecular level [24,25]. Lnk1 comprises two helices connected by the C47–C60 disulfide bond [25]. Through this hinge region, the Gla domain anchored to the membrane affects the position of kringle-1 and propagates conformational changes to the rest of the molecule using the flexible Lnk2, thereby influencing long-range the directionality of cleavage. The importance of this region of Lnk1 is further demonstrated by the naturally occurring mutant prothrombin Mumbai (C47S) that is associated with profoundly altered coagulation parameters and intracranial bleeding [27]. Lnk2 also contributes to direct prothrombinase at R320. Deletion of Lnk2 reduces the preference for the meizothrombin pathway and drastically increases the rate of activation in the absence but not the presence of cofactor Va. Most of Lnk2 can be deleted with a small perturbation of the rate of prothrombin activation by prothrombinase [25], which implies that the recent structure of prothrombin devoid of Lnk2 is an intriguing representation of the conformation of prothrombin optimized for catalysis by binding of cofactor Va in the prothrombinase complex. A molecular picture of prothrombin activation begins to emerge from structural biology [24,25]. Factor Xa and prothrombin anchored to the membrane by their Gla domain need to align properly for efficient thrombin generation. The flexibility of Lnk2 enables prothrombin to sample multiple conformations, and this may constitute an entropic barrier to optimal interaction with factor Xa that itself undergoes conformational transitions on the plane of the membrane. Binding of cofactor Va, acting in concert with the membrane, relieves this entropy cost by providing a scaffold that aligns both factor Xa and prothrombin for optimal catalysis. A key component of this action is to ‘compress’ prothrombin in a way that is mimicked by the structure devoid of Lnk2 [25]. This model offers a testable framework for studies of the structure and dynamics of the zymogen free and bound to components of the prothrombinase complex using single molecule detection. The discovery of prothrombin plasticity due to its linker regions has also pointed to effective strategies for crystallization of the zymogen under conditions most relevant to physiology.

The crystal structure of prothrombin enables a rational approach to the identification of epitopes for factor Xa and cofactor Va binding and reveals docking sites for the design of small molecules aimed at interfering with the interaction of prothrombin with prothrombinase. Charged and aromatic residues >70% exposed to solvent become prime candidates for mutagenesis and offer a critical assessment of targets identified in previous studies [16–22]. Additional targets are provided by naturally occurring mutations of prothrombin interspersed with residues singled out by the solvent exposure criterion. Some of these mutations are buried but point to important determinants of recognition. For example, the severe bleeding reported in prothrombin Puerto Rico (T122M/R457Q) is associated with normal antigen levels [28] and may be due to mutation of R457, a residue in the B chain making a strong ionic interaction with D306 in the A chain [28]. The A chain contains four residues arranged in an ion quartet where R296 is caged by the side chains of E300, D306 and E309. D306 also ion-pairs with R457. Each of the four Ala mutants of the quartet has been reported to activate poorly due to the lack of cleavage by prothrombinase at R271 [29]. Remarkably, prothrombin Denver (E300K and E309K) causes severe bleeding because of direct perturbation of prothrombin activation [30], and so does prothrombin Puerto Rico (T122M/R457Q) [28]. Through the structure of prothrombin, a molecular understanding of the bleeding phenotype associated with naturally occurring mutations becomes possible.

A new, structure-based framework has emerged for the study of prothrombin. The unanticipated important role of the linkers and the flexibility of the zymogen have a direct bearing on
the mechanism of activation and warrant proper attention in future studies. The structure of prothrombin also offers a molecular interpretation of the phenotypes associated with naturally occurring mutations and points to new targets for drug design.

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References

25. Pozzi N, Chen Z, Pel CA, et al. The linker connecting the two kringles plays a key role in prothrombin activation. Proc Natl Acad Sci USA 2014;111:7630-5