Received: 3 August 2022 | Revised: 8 September 2022 | Accepted: 22 September 2022

DOI: 10.1002/rth2.12830

rpth research & practice in thrombosis & haemostasis

STATE OF THE ART ISTH 2022

# Cryo-EM structures of coagulation factors

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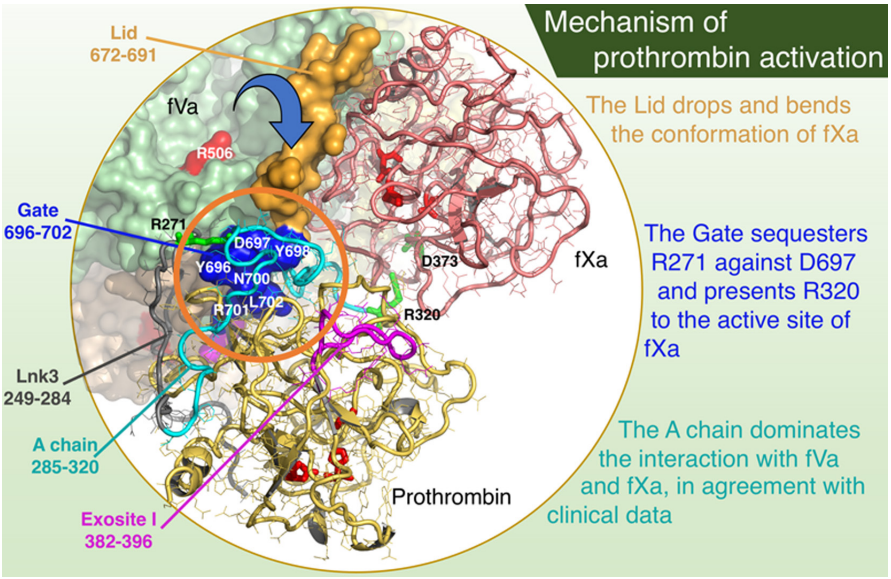
**Funding information**
National Heart, Lung, and Blood Institute, Grant/Award Number: HL049413, HL139554 and HL147821

**Handling Editor:** Prof. Yotis Senis

## Abstract

A State of the Art lecture titled "Cryo-EM structures of coagulation factors" was presented at the ISTH Congress in 2022. Cryogenic electron microscopy (cryo-EM) is a revolutionary technique capable of solving the structure of high molecular weight proteins and their complexes, unlike nuclear magnetic resonance (NMR), and under conditions not biased by crystal contacts, unlike X-ray crystallography. These features are particularly relevant to the analysis of coagulation factors that are too big for NMR and often recalcitrant to X-ray investigation. Using cryo-EM, we have solved the structures of coagulation factors V and Va, prothrombinase on nanodiscs, and the prothrombin-prothrombinase complex. These structures have advanced basic knowledge in the field of thrombosis and hemostasis, especially on the function of factor V and the molecular mechanism for prothrombin activation, and set the stage for exciting new lines of investigation. Finally, we summarize relevant new data on this topic presented during the 2022 ISTH Congress.

**KEYWORDS**
blood coagulation, factor V, factor Va, prothrombin, vitamin K-dependent clotting factors



**Essentials**

- Cryo-EM is revolutionizing the structural analysis of coagulation factors and their complexes.
- We review cryo-EM structures of factors V and Va, prothrombinase free and bound to prothrombin.
- A mechanism for prothrombin activation along the meizothrombin pathway is presented.
- Future directions in the field are discussed.

## 1 | INTRODUCTION

Vascular injury triggers a cascade of proteolytic events in which inactive zymogens of the coagulation system are converted to their active proteases to carry out biological function.[1,2] The cascade starts with exposure of tissue factor from the damaged endothelium that recruits factor VIIa (fVIIa) to the extrinsic tenase complex that in turn activates fX to fXa. During this initiation phase of the coagulation response, the small quantities of fXa convert prothrombin to thrombin, which activates the cofactors fV and fVIII[3] and the zymogen fXI.[4] At this point, fVa and fXa assemble with $Ca^{2+}$ and phospholipids in the prothrombinase complex[5] on the surface of platelets, red blood cells, or the endothelium and lead to an explosive acceleration of the conversion of prothrombin to thrombin[6] in a cofactor-dependent activation that is paradigmatic of analogous reactions of the blood coagulation and complement cascades.[1,7] Further acceleration in the production of fXa and assembly of prothrombinase is achieved along the intrinsic pathway where fXIa converts fIX to fIXa and the fIXa-fVIIIa complex efficiently activates fX to fXa.[8] Once thrombin is generated from prothrombin by the prothrombinase complex, it goes on to target fibrinogen and PAR1 to promote clot formation and platelet activation to restore hemostasis.[9] Hence, elucidation of the steps that lead to assembly of the prothrombinase complex and of the mechanism of prothrombin activation by prothrombinase bears broad significance to blood physiology and warrants utmost attention by structural biology.

Cryogenic electron microscopy (cryo-EM) is a revolutionary technique capable of solving the structure of high molecular weight proteins and their complexes, unlike nuclear magnetic resonance (NMR), and under conditions that do not require crystal growth, unlike X-ray crystallography.[10,11] The technique has become a cost-effective alternative or complement to X-ray crystallography for high-resolution structural studies and offers investigation of a broad spectrum of macromolecular systems, their interactions, and dynamic conformational states under conditions highly relevant to physiological function. Rapid progress in cryo-EM technology has ushered in a new era of structural biology,[10,11] best illustrated by the elucidation of the architecture of the ribosome,[12] several GPCRs,[13] and ATPases.[14] The technique has recently achieved resolutions <2 Å for macromolecular complexes in the 100–150 kDa molecular weight range.[15] The cryo-EM structure of proteins as "small" as hemoglobin, with a molecular weight (64 kDa) in the same range as that of prothrombin (72 kDa) or protein C (PC) (68 kDa), have been solved recently.[16] Our laboratory has been among the pioneers in the application of cryo-EM to the study of coagulation factors,[17–20] which are either too big for NMR or recalcitrant to X-ray investigation. Specifically, we have solved the structures of fV and fVa,[19] as well as of prothrombinase on nanodiscs and of the prothrombin-prothrombinase complex.[20] These structures, reviewed in the following section, show that macromolecular interactions relevant to thrombosis and hemostasis can now be approached directly to complement information derived from biochemical studies.

## 2 | Cryo-EM STRUCTURE OF fV

Coagulation fV is a large (2224 residues) precursor of fVa that, together with fXa, $Ca^{2+}$, and phospholipids, defines the prothrombinase complex.[21,22] Human fV circulates in the plasma at a concentration of 20 nM and approximately 20% of the total is contained in platelet α-granules, where it is partially fragmented and secreted during platelet activation.[23] After removal of a signal peptide of 28 residues, fV secreted to plasma features the domain structure A1-A2-B-A3-C1-C2 (Figure 1A) analogous to that of coagulation fVIII, with the three A domains homologous to ceruloplasmin. Sequence comparison shows that the A and C domains are highly conserved among human, bovine, and murine fV sequences, but the B domain, whose main function is to keep fV in an inactive state,[22] is poorly conserved.[24] The A1 domain is connected to the A2 domain by a short segment composed mainly of basic amino acids (residues 304–316). An acidic segment (residues 657–709) transitions the A2 domain to the large B domain (836 residues) that continues into the A3, C1, and C2 domains. A longstanding interest in unraveling the architecture of fV has focused on the sites of cleavage by thrombin at R709, R1018, and R1545,[21,22] as well as on the sites of inactivation by activated protein C (APC), R306 and R506, with the latter associated with the clinically important variant $fV^{Leiden}$ (R506Q) causing resistance to APC and a thrombotic phenotype.[25] More recently, the role of the B domain in keeping fV in its inactive state has acquired centerstage[3,22,26,27] with the discovery of splice variants like fV short.[28,29] These variants are constitutively active in the prothrombinase complex and interact with tissue factor pathway inhibitor α (TFPIα) and protein S (PS) to hijack fXa[3,22,26,27] in a resulting fV short/TFPIα/PS/fXa complex responsible for a bleeding phenotype.[28,29]

The cryo-EM structure of fV was solved at atomic (3.3 Å) resolution[19] and reveals the overall organization of the six domains of the protein (Figure 1B). The C1 and C2 domains align edge to edge to define a membrane binding platform on which the A domains

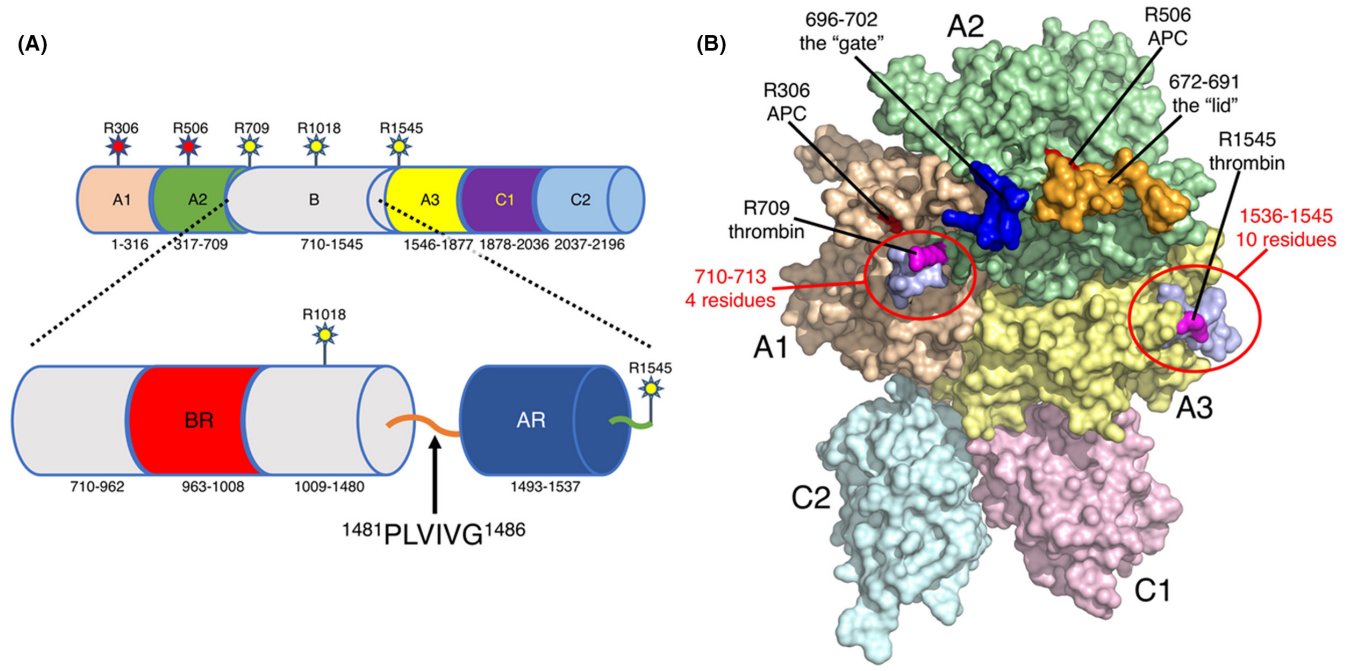



**FIGURE 1** Cryo-EM structure of fV. (A) Schematic representation of the A1-A2-B-A3-C1-C2 domain organization of human fV (2196 residues total) and its B domain containing the basic (BR) and acidic (AR) regions that interact to keep fV inactive. The short hydrophobic patch is unmasked in the splice variant fV short and contributes to TFPIα binding, along with the acidic region. (B) The protein is rendered in surface representation with the constitutive domains colored in wheat (A1), pale green (A2), light blue (B), pale yellow (A3), light pink (C1), and pale cyan (C2). The structure of fV (7KVE[19]) was solved at 3.3 Å resolution and features the C domains aligned "edge-to-edge" into a platform involved in membrane binding and upon which the A domains rest side by side. The A1-A2-A3-C1-C2 domain assembly is resolved in its entirety. The sites of thrombin activation at R709 and R1545 (magenta) are clearly visible in the A2 and B domains and exposed to solvent for proteolytic attack. The sites of APC cleavage at R306 and R506 (red) are 75% buried. Also shown are the gate (blue) and the lid (orange) that play an important role in prothrombin activation. The B domain is very dynamic and only a total of 14 residues are resolved (red circles) in the connection to the A2 and A3 domains.

rest side by side. This arrangement was first observed in a pioneering X-ray structure of bovine fVai.[30] New for the cryo-EM structure is assignment of the important A2 domain and very limited portions of the B domain corresponding to residues $^{710}$SFRN$^{713}$ following the site of thrombin activation at R709 and residues $^{1536}$PDNIAAWYLR$^{1545}$ at the C-terminal end. The additional information from the cryo-EM structure is sufficient to visualize, for the first time, two of the three sites of activation by thrombin at R709 and R1545 exposed to solvent for proteolytic attack and the sites of cleavage by APC at R306 and R506 that are 75% buried. In addition, two regions of the A2 domain to be referred to as the "gate" (residues 696–702) and the "lid" (residues 672–691) make an important contribution to prothrombin activation. Extensive disorder in the B domain precludes assignment of the site of activation by thrombin at R1018 and regions that are assumed to keep fV in its inactive state[3,22,26,27] (i.e., a basic region near the N-terminal [residues 963–1008] interacting intramolecularly with an acidic region at the C-terminal [residues 1493–1537]) (Figure 1A). Removal of the basic region by proteolysis[3,22,26,27] or alternative splicing as in fV short[28,29] yields derivatives constitutively active in the prothrombinase complex and unmasks a binding site in the acidic region for TFPIα that leads to formation of the anticoagulant fV short/TFPIα/PS/fXa complex. The hydrophobic patch $^{1481}$PLVIVG$^{1486}$ (Figure 1A) identified recently[31] works with the acidic region in the recognition of TFPIα and PS to promote formation of this complex, which contributes to the role of TFPIα as a regulator of the assembly of prothrombinase[31,32] and blood coagulation.[33] Much remains to be learned about the B domain of fV and the architecture of its splice variants from future cryo-EM analysis.

## 3 | CRYO-EM STRUCTURES OF fVa FREE AND BOUND TO fXa IN THE PROTHROMBINASE COMPLEX

Conversion of fV to fVa is catalyzed by thrombin upon cleavage at the highly conserved residues R709, R1018, and R1545[34] and release of the entire B domain that splits the protein into the A1-A2 heavy chain and A3-C1-C2 light chain that assemble as a noncovalent $Ca^{2+}$-linked heterodimer. This form of fVa is extremely stable and its partial inactivation is produced proteolytically by cleavage at R506 in the A2 domain by APC with the assistance of PS. Complete inactivation requires two additional cleavages at R306 in the A1 domain and R679 in the C-terminal of the A2 domain. Interestingly, cleavage of fV at R506 by APC produces an alternative, anticoagulant form of fV that promotes APC inactivation of

fVIIIa with the assistance of PS.[22,35,36] The regulation of thrombin generation is critically dependent on the levels of fVa and failure to control fVa activity may result in either bleeding or thrombotic complications. Complete deficiency of fV in mice results in midembryogenic lethality or fatal perinatal hemorrhage.[37] In contrast, the bleeding manifestation of severe fV deficiency in human patients varies significantly.[22] The physiological importance of the down-regulation of fVa activity by APC is demonstrated by the variant fV$^{Leiden}$ (R506Q)[25] that is a common genetic risk factor for venous thrombosis in humans.[38,39]

The cryo-EM structure of fVa solved at nearly atomic (4.4 Å) resolution reveals the organization of the A1-A2-A3-C1-C2 domains[19] (Figure 2A). Gone is the B domain and its substantial disorder, with a resulting clear visualization of the sites of APC cleavage at R306 and R506 that are 77% exposed to solvent and ready for proteolytic attack. However, the structure of fVa contains a substantial degree of disorder, with numerous residues missing from the density map in the A2 (99 of 393 total) and A3 (37 of 332 total) domains. Disorder in the A2 domain removes all information about the gate and the lid and shows that release of the B domain upon activation of fV has a direct influence on the dynamics of the A2 and A3 domains of fVa. Therefore, the B domain not only keeps fV in its inactive state but also stabilizes the ordered conformations of the A2 and A3 domains.

Disorder in the A2 and A3 domains of fVa is almost entirely removed upon binding of fXa and assembly of the prothrombinase complex (Figure 2B). A cryo-EM structure of the complex was solved on nanodiscs to reproduce conditions closest to physiological. The resolution is only 5.3 Å but sufficient to reveal for the first time the relative arrangements of fVa and fXa in a complex of 1752 residues missing only 10 residues ($^{1546}$SNNGNRRNYY$^{1555}$) in the N-terminus of the A3 domain. The entire C-terminal segment 654–709 of the A2 domain containing the lid (residues $^{672}$ESTVMATRKMHDRLEPEDEE$^{691}$) and the gate (residues $^{696}$YDYQNRL$^{702}$) missing in the free form of fVa because of an intrinsic disorder[19] become structured upon fXa binding and change their conformation relative to that seen in fV. Specifically, the lid drops >7 Å to engage the protease domain of fXa with numerous contacts and the gate rearranges by shifting Y696 and L702. Notably, assembly of prothrombinase reduces the solvent exposure of R306 and R506 to 31% and 48%, respectively, in agreement with biochemical evidence that fXa binding protects fVa from inactivation by APC.[40,41] Exposure of R306 is reduced even further (to 23%) upon binding of prothrombin (see the following section). The architecture of fXa is fully resolved and includes the Gla domain missing from all current X-ray structures[42,43] to facilitate crystallization. The Gla, EGF1, EGF2, and protease domains of fXa align along the C1, A3, and A2 domains of fVa in a curved arrangement that is accentuated by a kink between the Gla and EGF1 domains. This feature of fXa in the prothrombinase complex positions the active site region about 60 Å above the plane

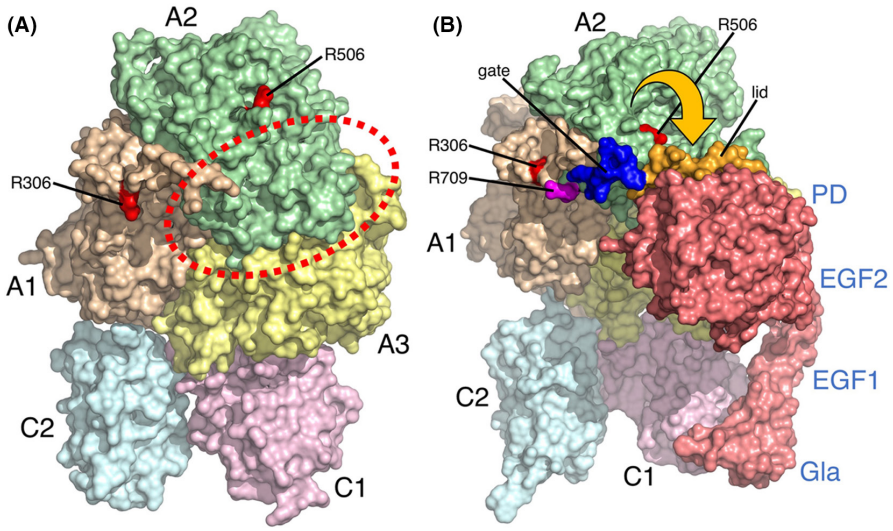



**FIGURE 2** Cryo-EM structure of fVa free and bound to fXa. (A) The protein is rendered in surface representation with the constitutive domains colored as in Figure 1B. Overall, the arrangement of the A1-A2-A3-C1-C2 domains is like that of fV. The structure of fVa (7KXY[19]) was solved at 4.4 Å resolution and is more disordered than that of fV, with fewer (1181 of 1360 total) residues resolved in the A1 (294 of 316 total), A2 (294 of 393 total), A3 (295 of 332 total), and C2 (139 of 160 total) domains. The disorder in fVa removes all information about the gate and lid (dotted circle). (B) The structure of prothrombinase was solved on nanodiscs (7TPQ[20]) at 5.3 Å resolution and shows fVa and fXa in surface representation, with fVa colored as in Figure 1 and fXa in red. The architecture of the complex is solved in almost its entirety (1742 residues of 1752), except for the N-terminal $^{1546}$SNNGNRRNYY$^{1555}$ sequence of the A3 domain immediately downstream of the site of thrombin activation at R1545. The overall arrangement of fVa is like that of the free form (A) but the gate and lid are fully resolved and change their conformation relative to fV (Figure 1B). The architecture of fXa is also fully resolved for the first time and shows a curved conformation, enhanced by a 90° kink at the EGF1-Gla domain junction, that positions the active site about 60 Å over the plane of the nanodiscs. The enzyme aligns along the A2, A3, and C1 domains of fVa, with most of the contacts between the A2 domain of fVa and the protease domain (PD) of fXa (Table 1).

of the membrane. Extensive interactions with the A2 domain of fVa fix the orientation of the active site region of fXa for optimal engagement of substrate. It is unclear if the curved conformation of fXa preexists in solution and is selected by fVa for assembly in the prothrombinase complex or is the result of an induced fit caused by interaction with fVa. Additional structural work and single molecule measurements as done recently for prothrombin[44,45] will address this issue.

## 4 | THE PROTHROMBIN-PROTHROMBINASE INTERACTION

Success in solving the structure of prothrombinase made it possible to tackle the more challenging complex with prothrombin. Prothrombin is one of the most abundant proteins circulating in the blood and features a modular assembly composed of the N-terminal Gla domain, kringle-1, kringle-2, and a C-terminal protease domain containing the A and B chains (Figure 3), with three linkers connecting the Gla domain to kringle-1 (Lnk1), the two kringles (Lnk2), and kringle-2 to the protease domain (Lnk3). The interaction of prothrombin with prothrombinase has been studied by several groups,[46–51] and its biochemistry is well understood. Conversion of prothrombin to thrombin requires cleavage at R271 in Lnk3 and R320 in the A chain (Figure 3). Cleavage at R271 sheds the auxiliary Gla domain and kringles and generates the inactive intermediate prethrombin-2. The alternative cleavage at R320 separates the A and B chains, which remain connected through the C293-C439 disulfide bond, and generates the active intermediate meizothrombin. Thrombin is generated as a fully active protease by a final autoproteolytic cleavage at R284.[9] The pathway of prothrombin activation by prothrombinase is context dependent. On the surface of platelets activation proceeds along the prethrombin-2 pathway.[52] On nonplatelet surfaces such as red blood cells[53] and the endothelium,[6] activation proceeds along the meizothrombin pathway. In vitro, the presence of fVa drives activation along the meizothrombin pathway,[48,49] with and without phospholipids.[48,54,55] In the absence of fVa, activation proceeds along the prethrombin-2 pathway[46] or with cleavage at R155 to generate prethrombin-1.[47] Importantly, the site at R320 is cleaved preferentially in the presence of fVa, regardless of its sequence, whereas the sequence at the R271 site is preferred in the absence of fVa, regardless of position.[56] Hence, based on the biochemistry of activation in vitro, a structure of the prothrombin-prothrombinase complex is expected to capture the predominant, meizothrombin pathway where fVa promotes cleavage at R320.

Important insight into the mechanism of prothrombin activation by prothrombinase comes from consideration of general properties of the hydrolysis of peptide bonds by a trypsin-like protease such as fXa. Three independent rate constants are involved: the second-order rate of productive diffusion of substrate into the active site, $k_{on}$; the first-order rate of dissociation of the enzyme-substrate complex into the parent species, $k_{off}$; and the rate-limiting step for catalysis, $k_2$, measuring substrate acylation.[57,58] These rates define the independent Michaelis–Menten parameters as $k_{cat} = k_2$ and $k_{cat}/K_m = k_{on}k_2/(k_{off}+k_2)$. The drastic (>10,000-fold) increase of $k_{cat}/K_m$ because of $k_{cat}$, as observed for fVa enhancement of prothrombin activation by fXa,[2,6,47] requires an increase of both $k_2$ and $k_{on}$ to a similar extent. The increase in $k_2$ depends on properties of the enzyme–substrate complex, and the increase in $k_{on}$ depends on properties of the enzyme and substrate before the complex is formed. Therefore, the molecular origin of the low specificity of prothrombin for fXa in the absence of fVa must reside in factors that hinder productive formation of the complex (effect on $k_{on}$) and efficient turnover of substrate by the enzyme-substrate complex (effect on $k_{cat}$). fVa corrects these defects by acting on the free enzyme and/or substrate to increase

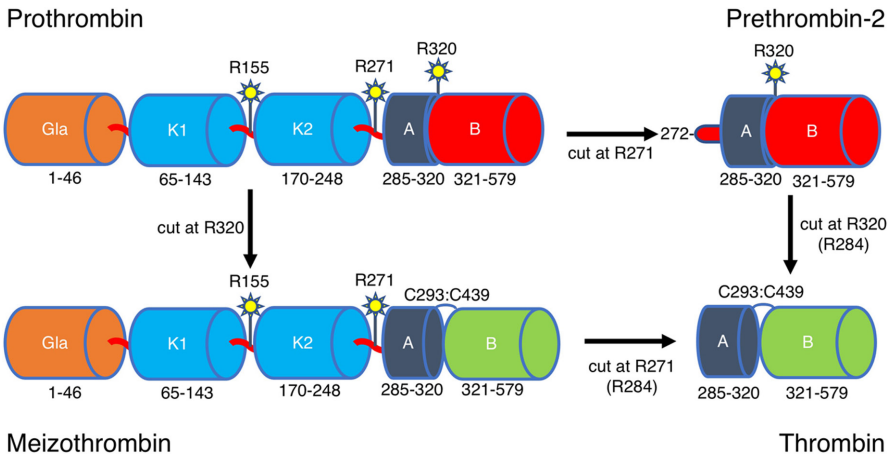



**FIGURE 3** Prothrombin activation pathways. Schematic representation of prothrombin activation by the prothrombinase complex. The conversion to thrombin entails cleavage at R271 in Lnk3 and R320 in the A chain, along two alternative pathways generating the intermediates prethrombin-2 (cleavage at R271 first) or meizothrombin (cleavage at R320 first). An additional autoproteolytic cleavage at R284 produces thrombin. The presence of fVa, in vitro, promotes activation along the meizothrombin pathway. In the absence of fVa, activation proceeds along the prethrombin-2 pathway or through cleavage at R155 to produce prethrombin-1.

$k_{on}$, and on the prothrombin-fXa complex to increase $k_{cat}$. The effect on $k_{cat}$ must ultimately involve residues of the catalytic triad of fXa as they attack the scissile bonds of prothrombin at R271 and R320 but it may be difficult to capture by structural biology. Subtle changes in mobility of the catalytic Ser translate in large effects on the rate of substrate hydrolysis.[59] In fact, replacement of Ser with the bulkier Thr drastically reduces the fVa-dependent enhancement of prothrombin activation by fXa, as well as other allosteric effects like the thrombomodulin dependent enhancement of PC activation by thrombin and $Na^+$ activation of thrombin, fXa, and APC.[59] The effect of fVa on $k_{on}$ requires optimization of the enzyme-substrate encounter through pre-organization of prothrombin and fXa for a "rigid-body" association.[60] This role of fVa is revealed by the cryo-EM structure of the prothrombin-prothrombinase complex[20] as a culmination of the progress made in the elucidation of the structural architecture of its separate components. Numerous X-ray crystal structures of fXa have been solved,[42,43] all lacking the Gla domain and featuring an almost vertical alignment of the protease and EGF domains. This alignment is not confirmed by the cryo-EM structure of the prothrombinase complex (Figure 2B) that shows fXa full length for the first time and in a curved rather than elongated conformation. Whether this conformation preexists in solution or is the result of changes induced by fVa remains to be established by future studies. Structures of prothrombin have revealed two conformations in equilibrium, open and closed, also confirmed by single molecule studies in solution.[44,45,61–64] Relevant to the mechanism of activation is that the open form is preferentially cleaved at R271 and initiates the prethrombin-2 pathway, whereas the closed form dominates in solution and is preferentially cleaved at R320 to initiate the meizothrombin pathway.[44,45,63] Hence, a structure of the prothrombin–prothrombinase complex is expected to show fVa promoting cleavage at R320 by optimizing the encounter between fXa and prothrombin in the closed conformation.

## 5 | CRYO-EM STRUCTURE OF THE PROTHROMBIN–PROTHROMBINASE COMPLEX

The cryo-EM structure of the prothrombin–prothrombinase complex was solved without nanodiscs at a resolution of 4.1 Å, with 2300 of the 2323 total residues assigned in the density maps and prothrombin missing only residues 157–170 in the flexible Lnk2 region[62] (Figure 4A). Under the conditions used for cryogenic freezing, >3 million particles were collected that visualized fVa free and bound to prothrombin and fXa (Figure 4B). More than 60,000 particles of the prothrombin-fVa-fXa complex were selected for final refinement and the relative arrangement of the three proteins was revealed for the first time. The conformation of prothrombinase bound to prothrombin is practically identical to that detected free on nanodiscs at lower resolution (Figure 2B), with numerous interactions involving mainly the A2 and A3 domains of fVa with the protease and EGF2 domains of fXa (Table 1). The similarity of the two structures, obtained with and without nanodiscs, suggests that the architecture of prothrombinase is driven predominantly by protein–protein interactions and that the membrane accelerates the kinetics mainly through a template mechanism that reduces the dimensionality of the system.[60] The prothrombin–prothrombinase complex is shaped like a dome, with the top housing the A2 domain of fVa and the bottom defining a membrane binding module with the Gla domain of prothrombin widely (>100 Å) separated from the Gla domain of fXa and the C1 and C2 domains of fVa. Prothrombin aligns along the C2, A1, and A2 domains of fVa and contacts prothrombinase only through the protease domain that binds fVa at the gate, with minor contacts with T305 and K309 in the A1 domain, and fXa at the entrance of the active site region (Table 1). Formation of the ternary complex buries a surface area of 6318 $Å^2$, which is only 5.5% of the total accessible surface area of fVa. The limited surface of interaction coupled with a large $k_{cat}/K_m$ suggests that prothrombin and prothrombinase are preorganized for binding through what could be defined as a rigid body association.[60] Importantly, the cryo-EM structure traps prothrombin in a curved conformation that resembles the closed form identified by single molecule measurements in solution[44,61,63] and X-ray structures carrying removal of the Gla domain,[65] full or partial deletion of Lnk2,[66] or artificial cross-linking of kringle-1 to the protease domain.[44] The cryo-EM structure also supports the conclusion that the closed form promotes activation along the meizothrombin pathway.[44,45,63] In fact, R320 penetrates the active site region of fXa fully exposed to solvent and ready to engage D373 in the primary specificity pocket. On the other hand, the alternative sites of cleavage at R155 and R271 sit 47 Å and 37 Å away from D373, both inaccessible to fXa in the observed conformation of prothrombinase. Overall, the structure reveals fVa directing R320 into the active site of fXa and preventing alternative cleavages at R271 and R155, consistent with biochemical evidence[47–49,54–56,62,67] and especially with the role of fVa in directing cleavage R320 in a position-dependent and sequence-independent manner.[56]

Knowledge of the precise arrangement of the three components of the prothrombin–fVa–fXa complex points to new targets for future mutagenesis studies (Table 1) and offers rigorous validation of existing paradigms, biochemical, and modeling data.[6,21,67–76] Not all the epitopes assigned in previous studies are confirmed by the cryo-EM structure. The A2 domain fixes the orientation of fXa with numerous contacts involving the $Na^+$ site, the 170 segment and the C-terminal helix (Figure 5). Notable interactions include R347 of fXa with D628 and D577 of fVa, along with a possible cation-π interaction with F576. Residue R347 was identified previously as being critical for fVa binding.[77,78] However, the long segment $^{680}$KMHDRLEPEDEESDADYDYQNRLAAALGIR$^{709}$ in the A2 domain implicated in prothrombin binding and prothrombinase function[74,79–84] contacts prothrombin only through the gate (residues $^{696}$YDYQNRL$^{702}$). Highly relevant in this context is a previous study on the peptide sequence $^{695}$DYDYQ$^{699}$ functioning as a competitive inhibitor of prothrombin activation by prothrombinase and capable of switching the pathway of activation from meizothrombin to prethrombin-2.[80,81,83] The lid (residues

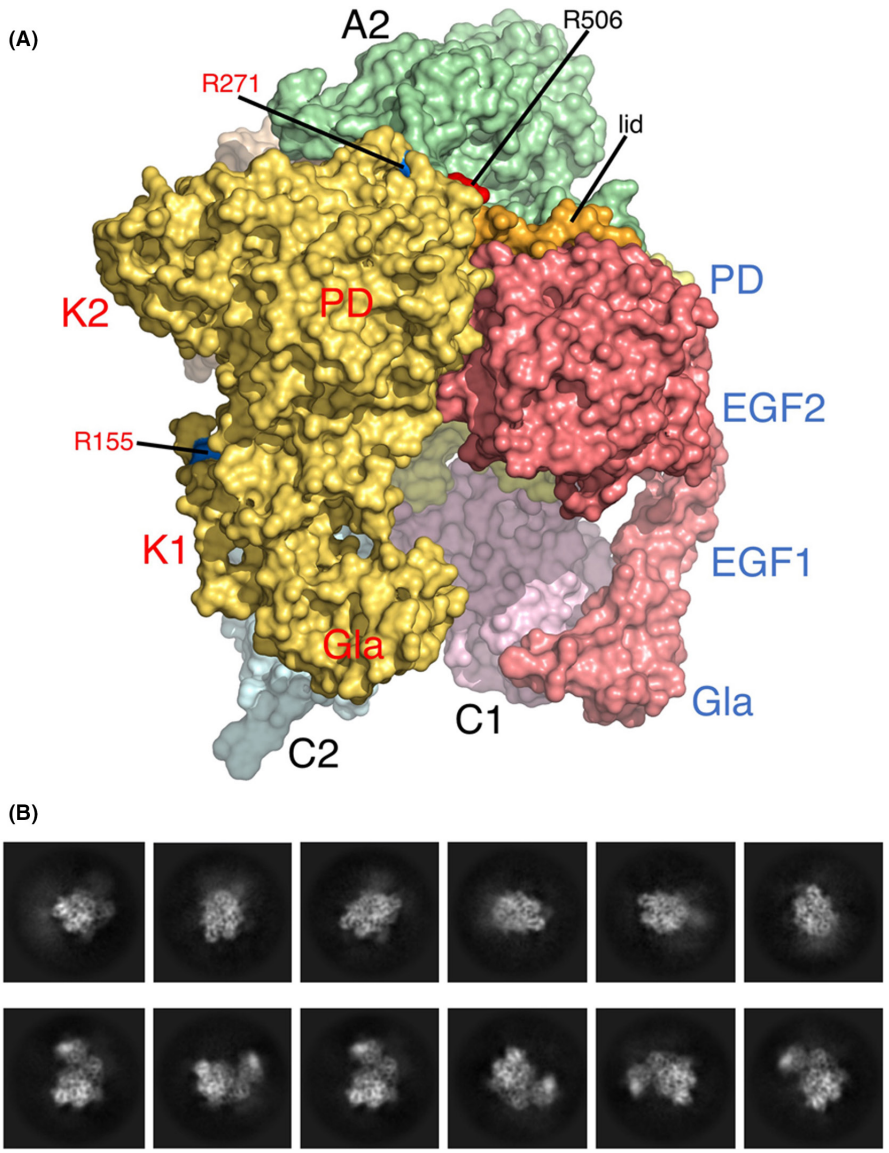



**FIGURE 4** Cryo-EM structure of the prothrombin-prothrombinase complex. (A) The structure (7TPP[20]) was solved at 4.1 Å resolution and shows prothrombinase in the same arrangement found on nanodiscs (Figure 2B) and prothrombin (yellow) aligning along the C2, A1, and A2 domains of fVa in a curved conformation similar to the closed form identified crystallographically.[44] The architecture of the complex is solved in almost its entirety (2307 residues of 2331). Remarkably, prothrombinase engages prothrombin only through the protease domain (PD) that binds to the gate of fVa (covered) and the active site region of fXa. The Gla domain of prothrombin aligns with the Gla domain of fXa and the C1 and C2 domains of fVa to define a membrane binding module but remains 100 Å separated from prothrombinase. The site of cleavage at R320 penetrates the active site of fXa (see Figure 5). The structure depicts prothrombinase in the process of cleaving prothrombin at R320 to initiate activation along the meizothrombin pathway. (B) Representative 2D class averages of the prothrombin-prothrombinase complex (bottom) and images of fVa (top) document the distribution of free and bound particles obtained under the conditions used for cryo-EM structure determination.

$^{672}$ESTVMATRKMHDRLEPEDEE$^{691}$) is flanked by the short segment $^{659}$DDDED$^{663}$ influencing the rate of cleavage of prothrombin by prothrombinase,[85] but only E662 is in electrostatic interaction with R306 of fVa. Likewise, E467 and R652[74,86] and the entire segment $^{323}$EYFIAAEEV$^{331}$[86,87] deemed important for fXa binding make no contacts with the enzyme. Additional minor contacts are observed between the A3 domain of fVa and EGF2 of fXa and a weak interaction between the C1 domain and the Gla domain. Overall, the fVa–fXa interaction involves a total of 15 residues of fVa (Table 1), far fewer than the numerous entries tabulated recently.[74] Likewise, the fVa–prothrombin interaction involves only eight residues of fVa (Table 1) and does not involve the A3 and C1 domains, as suggested recently.[74] The segment $^{473}$GKGQPSVLQVVNLPI$^{487}$ of prothrombin proposed as an fVa binding epitope[88] is mostly buried and makes no contacts with fVa. There are no contacts between exosite I (residues 382–396) or the autolysis loop (residues 466–477) of prothrombin with either fXa or fVa, contrary to the suggestions of molecular models[71–73] and biochemical studies.[70,88–90] Deletion of kringles of prothrombin has implicated direct involvement of these domains in the interaction with fVa,[91,92] but there is no evidence of such contacts in the cryo-EM structure. Likewise, interactions of fXa with prothrombin residues 205–220 of kringle-2,[93] the C-terminus of the protease domain,[94] and the Gla domain[95] assigned from competition experiments are not confirmed by the cryo-EM structure.

## 6 | MOLECULAR MECHANISM OF PROTHROMBIN ACTIVATION ALONG THE MEIZOTHROMBIN PATHWAY

Several residues of prothrombin contribute to the docking of R320 onto the protease domain of fXa to initiate activation along the meizothrombin pathway (Figure 5). The segment $^{307}$KTERELLE$^{314}$ has been shown to influence thrombin function and its generation from prothrombin.[96] Residue K307 at P14 is in electrostatic interaction with Q240 in the loop above the entrance to the active site of

**TABLE 1** Molecular contacts <5 Å among components of the prothrombin-fVa-fXa complex

| fVa-fXa | Prothrombin-fXa | Prothrombin-fVa |
|---|---|---|
| A511-L352 | K307-Q240 | L260-T305 |
| A511-I357 | D318-R405 | D261-K309 |
| F576-R347 | D318-K408 | D265-K309 |
| D577-R347 | R320-E372 | R266-A694 |
| T579-K351 | E323-K330 | E269-R505 |
| T626-N348 | D326-K242 | R271-D697 |
| D628-R347 | | R310-Y698 |
| E662-R306 | | L312-Y698 |
| E662-K420 | | L313-L702 |
| E669-R306 | | P534-Y696 |
| E672-R306 | | F535-Y696 |
| E672-Q360 | | |
| E672-K414 | | |
| V675-R424 | | |
| E686-K276 | | |
| S1598-E82 | | |
| T1679-F84 | | |
| H1683-L91 | | |
| Y2021-E39 | | |

*Note*: The list should be compared with a recent summary[74] of biochemical and computational data on the contacts made by fVa with fXa and prothrombin. The summary lists a total of 91 residues (56 in the A2 domain, 35 in the A3 domain) interacting with fXa and 51 residues (23 in the A2 domain, 18 in the A3 domain, 10 in the C1 domain) interacting with prothrombin. The cryo-EM structure documents only 15 residues (11 in the A2 domain, three in the A3 domain, one in the C1 domain) interacting with fXa and eight residues (two in the A1 domain, six in the A2 domain, none in the A3 and C1 domains) interacting with prothrombin.

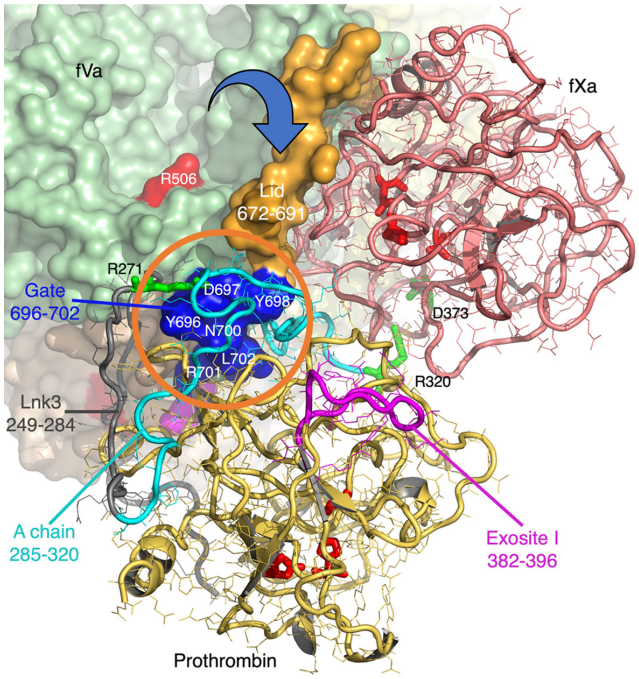



**FIGURE 5** Mechanism of prothrombin activation along the meizothrombin pathway. Top view of the prothrombin-prothrombinase complex showing fVa in surface representation (green) and the protease domains of fXa (red) and prothrombin (yellow) as cartoons. The lid drops >7 Å on the protease domain of fXa (blue arrow) and fixes the orientation of the active site region to optimize interaction with prothrombin. The protease domain of fXa makes extensive interactions (Table 1) with the A2 domain of fVa, especially through the $Na^+$ binding site, the 350 segment and the C-terminal helix. The $Ca^+$ binding site is on the opposite side of the fVa-fXa interface. Residues of the catalytic triad are in red and D373 in the primary specificity pocket is shown in green. The sites of activation of prothrombin, R320 and R271, are shown in green and are widely separated. Important regions of the zymogen are Lnk3 (gray) that supports R271 and continues on to the A chain (cyan) hosting R320 ready to engage D373 of fXa. The B chain (yellow) contains exosite I (magenta) that makes no contacts with prothrombinase. The only segment of prothrombin contacting prothrombinase is the A chain (cyan), in agreement with clinical data on naturally occurring mutations associated with severe bleeding.[97] The mechanism of prothrombin activation involves a drop of the lid on fXa to curve its conformation and fix orientation of the active site and a conformational change of the gate that sequesters R271 against D697 on one side and provides a surface on the other side on which the A chain slides down to present R320 to the active site of fXa.

fXa. Residue Y698 of fVa is in hydrophobic contact with L312 and in cation-π interaction with R310, whereas L313 contacts L702 that flips 180° from its position in fV. The docking is also stabilized by strong electrostatic interactions between D318 with both R405 and K408 in the $Na^+$ binding loop, E323 with K330, and D326 with K242 in the loops that define access to the active site of fXa. The A chain of prothrombin plays no significant role in thrombin function[96] but is the single most important structural component that interacts with prothrombinase (Figure 5). The critical role of the A chain supported by the cryo-EM structure explains the wealth of clinical data on naturally occurring mutations associated with severe bleeding that affect predominantly this structural segment of prothrombin.[97] Particularly important is the interaction of R296 with E300 and E309 on top of the gate that turns the A chain toward the active site of fXa. Prothrombin Denver (E300K/E309K)[98] is associated with severe bleeding from defective prothrombin activation and the mutant R296A is stabilized in an open-like form that promotes activation along the prethrombin-2 pathway and significantly slows down clotting.[63]

The cryo-EM structure of the prothrombin-prothrombinase complex reveals a simple mechanism for selection of the meizothrombin pathway of activation through the combined action of the lid and the gate (Figure 5). The lid drops >7 Å and curves the conformation of fXa at the EGF1-Gla domain junction to position the active site region 60 Å above the plane of the membrane to optimally engage the closed form of prothrombin selected from the preexisting open-closed equilibrium in solution. The gate changes shape and separates the two sites of cleavage by sequestering R271 in Lnk3 on one side through interaction with D697 and causing the A chain to slide down on the other side to direct R320 toward the active site of fXa. This mechanism underscores the importance of the A2 domain of fVa as the main determinant of prothrombin activation. The same molecular mechanism is unlikely to apply to

cleavage at the alternative site R271, which initiates the prethrombin-2 pathway. A rotation of the entire protease domain of prothrombin is necessary to position R271 toward the active site of fXa. This would require prothrombin to switch to the more elongated open form[44,45,62,64] and fXa to extend upward and push the lid to the resting position observed in fV (Figure 1B). A switch of prothrombin activation from the meizothrombin to the prethrombin-2 pathway would require conformational changes of all components of the prothrombin–prothrombinase complex, contrary to several published proposals.[67–69,72,75,76]

## 7 | ISTH MEETING REPORT

Interesting new data on the structural enzymology of coagulation factors were presented at the meeting, especially on the rapidly evolving investigation of fV short and its interaction with TFPIα, PS, and fXa. Magdalena Gierula reported on the kinetics of the TFPIα-PS and TFPIα-fV short interactions and elucidated the basis of the synergistic enhancement of fXa inhibition. The presentation provided a valuable segue to the state-of-the-art talk from her mentor Josefine Ahnstrōm on a closely related topic. Bjorn Dahlback reported the identification of a hydrophobic patch (residues 1481–1486) preceding the acidic region of the B domain of fV (Figure 1A) as a key epitope for the recognition of TFPIα by fV short and assembly of the fV short/TFPIα/PS complex hijacking fXa. Unrelated to this dominant topic, Jonas Emsley reported new X-ray structural data on the heavy chain of fXII, a key component of the contact activation pathway. The structure reveals a torc shape with a head to tail interaction between the fibronectin type II and kringle domains that promotes an unanticipated dimerization.

## 8 | FUTURE DIRECTIONS

The structure of the prothrombin-prothrombinase complex is an important first step toward future cryo-EM studies of this and related interactions relevant to blood coagulation. The role of the gate and lid in the A2 domain of fV should be addressed further with mutagenesis and the structures of fVa variants with these domains deleted. A complex of prothrombinase with prothrombin mutants stabilized in the open form or meizothrombin will be needed to reveal the conformational changes linked to cleavage at R271. A better understanding of the architecture of the B domain as a follow up of the structures of fV and fVa will clarify the role of the acidic and basic regions in keeping fV inactive. Ordering of the B domain may be obtained by removal of large portions, as in fV short, to enable visualization of the epitopes that recognize TFPIα and PS. In turn, this may facilitate solution of a cryo-EM structure of the challenging fV short/TFPIα/PS/fXa complex. Complexes of fV bound to physiological activators such as thrombin, fXa, and meizothrombin may also produce a more ordered B domain and reveal the mechanism of recognition of the sites of activation at R709, R1018, and R1545. Likewise, a complex of fVa with APC and PS will shed light on a key interaction down-regulating the progression of the coagulation response. Success with the prothrombin–prothrombinase complex will motivate cryo-EM investigation of PC free and bound to the thrombin–thrombomodulin complex, as well as of similar macromolecular interactions in the intrinsic pathway like the fVIIIa/fIXa/fX and fXIa/fIX complexes. These are only few of many new possible lines of investigation whose results will revolutionize our understanding of macromolecular interactions in the blood coagulation cascade.

### AUTHOR CONTRIBUTIONS

All authors cowrote the manuscript.

### ACKNOWLEDGMENTS

We gratefully acknowledge Tracey Baird for her assistance with illustrations and Dr. Eliza Ruben for her past contributions. This study was supported in part by the National Institutes of Health Research Grants HL049413, HL139554, and HL147821.

### RELATIONSHIP DISCLOSURE

The authors declare no financial interests.

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### REFERENCES

1. Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry*. 1991;30(43):10363-10370.
2. Mann KG. Thrombin formation. *Chest*. 2003;124(3 suppl):4S-10S.
3. Camire RM, Bos MH. The molecular basis of factor V and VIII procofactor activation. *J Thromb Haemost*. 2009;7(12):1951-1961.
4. Gailani D, Broze GJ Jr. Factor XI activation in a revised model of blood coagulation. *Science*. 1991;253(5022):909-912.
5. Wood JP, Silveira JR, Maille NM, Haynes LM, Tracy PB. Prothrombin activation on the activated platelet surface optimizes expression of procoagulant activity. *Blood*. 2010;117:1710-1718.
6. Krishnaswamy S. The transition of prothrombin to thrombin. *J Thromb Haemost*. 2013;11(suppl 1):265-276.
7. Krem MM, Di Cera E. Evolution of enzyme cascades from embryonic development to blood coagulation. *Trends Biochem Sci*. 2002;27(2):67-74.
8. Shamanaev A, Emsley J, Gailani D. Proteolytic activity of contact factor zymogens. *J Thromb Haemost*. 2021;19(2):330-341.
9. Di Cera E. Thrombin. *Mol Aspects Med*. 2008;29(4):203-254.
10. Bonomi M, Vendruscolo M. Determination of protein structural ensembles using cryo-electron microscopy. *Curr Opin Struct Biol*. 2018;56:37-45.
11. Cheng Y. Single-particle cryo-EM-how did it get here and where will it go. *Science*. 2018;361(6405):876-880.
12. Bassler J, Hurt E. Eurokaryotic ribosome assembly. *Annu Rev Biochem*. 2018;88:281-306.

13. Draper-Joyce CJ, Khoshouei M, Thal DM, et al. Structure of the adenosine-bound human adenosine A1 receptor-Gi complex. *Nature*. 2018;558(7711):559-563.
14. Hiraizumi M, Yamashita K, Nishizawa T, Nureki O. Cryo-EM structures capture the transport cycle of the P4-ATPase flippase. *Science*. 2019;365(6458):1149-1155.
15. Merk A, Bartesaghi A, Banerjee S, et al. Breaking Cryo-EM resolution barriers to facilitate drug discovery. *Cell*. 2016;165(7):1698-1707.
16. Khoshouei M, Radjainia M, Baumeister W, Danev R. Cryo-EM structure of haemoglobin at 3.2 a determined with the volta phase plate. *Nat Commun*. 2017;8:16099.
17. Fuller JR, Knockenhauer KE, Leksa NC, Peters RT, Batchelor J. Molecular determinants of the factor VIII/von Willebrand factor complex revealed by BIVV001 cryo-electron microscopy. *Blood*. 2021;137(21):2970-2980.
18. Gish JS, Jarvis L, Childers KC, et al. Structure of blood coagulation factor VIII in complex with an anti-C1 domain pathogenic antibody inhibitor. *Blood*. 2021;137(21):2981-2986.
19. Ruben EA, Rau MJ, Fitzpatrick J, Di Cera E. Cryo-EM structures of human coagulation factors V and Va. *Blood*. 2021;137(22):3137-3144.
20. Ruben EA, Summers B, Rau MJ, Fitzpatrick J, Di Cera E. Cryo-EM structure of the prothrombin-prothrombinase complex. *Blood*. 2022;139(24):3463-3473.
21. Mann KG, Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood*. 2003;101(1):20-30.
22. Camire RM. A new look at blood coagulation factor V. *Curr Opin Hematol*. 2011;18(5):338-342.
23. Hayward CP, Furmaniak-Kazmierczak E, Cieutat AM, et al. Factor V is complexed with multimerin in resting platelet lysates and co-localizes with multimerin in platelet alpha-granules. *J Biol Chem*. 1995;270(33):19217-19224.
24. Pittman DD, Marquette KA, Kaufman RJ. Role of the B domain for factor VIII and factor V expression and function. *Blood*. 1994;84(12):4214-4225.
25. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. 1994;369(6475):64-67.
26. Petrillo T, Ayombil F, Van't Veer C, Camire RM. Regulation of factor V and factor V-short by TFPIalpha: relationship between B-domain proteolysis and binding. *J Biol Chem*. 2020;296:100234.
27. Toso R, Camire RM. Removal of B-domain sequences from factor V rather than specific proteolysis underlies the mechanism by which cofactor function is realized. *J Biol Chem*. 2004;279(20):21643-21650.
28. Vincent LM, Tran S, Livaja R, Bensend TA, Milewicz DM, Dahlback B. Coagulation factor V(A2440G) causes East Texas bleeding disorder via TFPIalpha. *J Clin Invest*. 2013;123(9):3777-3787.
29. Broze GJ Jr, Girard TJ. Factor V, tissue factor pathway inhibitor, and East Texas bleeding disorder. *J Clin Invest*. 2013;123(9):3710-3712.
30. Adams TE, Hockin MF, Mann KG, Everse SJ. The crystal structure of activated protein C-inactivated bovine factor Va: implications for cofactor function. *Proc Natl Acad Sci U S A*. 2004;101(24):8918-8923.
31. Dahlback B, Tran S. A hydrophobic patch (PLVIVG; 1481-1486) in the B-domain of factor V-short is crucial for its synergistic TFPIalpha-cofactor activity with protein S and for the formation of the FXa-inhibitory complex comprising FV-short, TFPIalpha, and protein S. *J Thromb Haemost*. 2022;20(5):1146-1157.
32. Santamaria S, Reglinska-Matveyev N, Gierula M, et al. Factor V has an anticoagulant cofactor activity that targets the early phase of coagulation. *J Biol Chem*. 2017;292(22):9335-9344.
33. Mast AE, Ruf W. Regulation of coagulation by tissue factor pathway inhibitor: implications for hemophilia therapy. *J Thromb Haemost*. 2022;20(6):1290-1300.
34. Yang TL, Cui J, Rehumtulla A, et al. The structure and function of murine factor V and its inactivation by protein C. *Blood*. 1998;91(12):4593-4599.
35. Dahlbäck B. Pro- and anticoagulant properties of factor V in pathogenesis of thrombosis and bleeding disorders. *Int J Lab Hematol*. 2016;38(Suppl 1):4-11.
36. Rosing J, Tans G. Coagulation factor V: an old star shines again. *Thromb Haemost*. 1997;78(1):427-433.
37. Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. *Nature*. 1996;384(6604):66-68.
38. Eitzman DT, Westrick RJ, Shen Y, et al. Homozygosity for factor V Leiden leads to enhanced thrombosis and atherosclerosis in mice. *Circulation*. 2005;111(14):1822-1825.
39. Rosing J, Tans G. Factor V. *Int J Biochem Cell Biol*. 1997;29(10):1123-1126.
40. Solymoss S, Tucker MM, Tracy PB. Kinetics of inactivation of membrane-bound factor Va by activated protein C. protein S modulates factor Xa protection. *J Biol Chem*. 1988;263(29):14884-14890.
41. Norstrom EA, Tran S, Steen M, Dahlback B. Effects of factor Xa and protein S on the individual activated protein C-mediated cleavages of coagulation factor Va. *J Biol Chem*. 2006;281(42):31486-31494.
42. Shi Y, Li C, O'Connor SP, et al. Aroylguanidine-based factor Xa inhibitors: the discovery of BMS-344577. *Bioorg Med Chem Lett*. 2009;19(24):6882-6889.
43. Kamata K, Kawamoto H, Honma T, Iwama T, Kim SH. Structural basis for chemical inhibition of human blood coagulation factor Xa. *Proc Natl Acad Sci U S A*. 1998;95(12):6630-6635.
44. Chinnaraj M, Chen Z, Pelc LA, et al. Structure of prothrombin in the closed form reveals new details on the mechanism of activation. *Sci Rep*. 2018;8(1):2945.
45. Pozzi N, Bystranowska D, Zuo X, Di Cera E. Structural architecture of prothrombin in solution revealed by single molecule spectroscopy. *J Biol Chem*. 2016;291(35):18107-18116.
46. Esmon CT, Owen WG, Jackson CM. The conversion of prothrombin to thrombin. V. The activation of prothrombin by factor Xa in the presence of phospholipid. *J Biol Chem*. 1974;249(24):7798-7807.
47. Rosing J, Tans G, Govers-Riemslag JW, Zwaal RF, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. *J Biol Chem*. 1980;255(1):274-283.
48. Rosing J, Zwaal RF, Tans G. Formation of meizothrombin as intermediate in factor Xa-catalyzed prothrombin activation. *J Biol Chem*. 1986;261(9):4224-4228.
49. Krishnaswamy S, Mann KG, Nesheim ME. The prothrombinase-catalyzed activation of prothrombin proceeds through the intermediate meizothrombin in an ordered, sequential reaction. *J Biol Chem*. 1986;261(19):8977-8984.
50. Malhotra OP, Nesheim ME, Mann KG. The kinetics of activation of normal and gamma-carboxyglutamic acid-deficient prothrombins. *J Biol Chem*. 1985;260(1):279-287.
51. Mann KG, Nesheim ME, Church WR, Haley P, Krishnaswamy S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood*. 1990;76(1):1-16.
52. Haynes LM, Bouchard BA, Tracy PB, Mann KG. Prothrombin activation by platelet-associated prothrombinase proceeds through the prethrombin-2 pathway via a concerted mechanism. *J Biol Chem*. 2012;287(46):38647-38655.
53. Whelihan MF, Zachary V, Orfeo T, Mann KG. Prothrombin activation in blood coagulation: the erythrocyte contribution to thrombin generation. *Blood*. 2012;120(18):3837-3845.
54. Boskovic DS, Giles AR, Nesheim ME. Studies of the role of factor Va in the factor Xa-catalyzed activation of prothrombin, fragment 1.2-prethrombin-2, and

dansyl-L-glutamyl-glycyl-L-arginine-meizothrombin in the absence of phospholipid. *J Biol Chem*. 1990;265(18):10497-10505.
55. Tans G, Janssen-Claessen T, Hemker HC, Zwaal RF, Rosing J. Meizothrombin formation during factor Xa-catalyzed prothrombin activation. Formation in a purified system and in plasma. *J Biol Chem*. 1991;266(32):21864-21873.
56. Stojanovski BM, Di Cera E. Role of sequence and position of the cleavage sites in prothrombin activation. *J Biol Chem*. 2021;297(2):100955.
57. Page MJ, Di Cera E. Serine peptidases: classification, structure and function. *Cell Mol Life Sci*. 2008;65(7–8):1220-1236.
58. Hedstrom L. Serine protease mechanism and specificity. *Chem Rev*. 2002;102(12):4501-4524.
59. Pelc LA, Chen Z, Gohara DW, Vogt AD, Pozzi N, Di Cera E. Why ser and not Thr brokers catalysis in the trypsin fold. *Biochemistry*. 2015;54:1457-1464.
60. Di Cera E. Mechanisms of ligand binding. *Biophys Rev*. 2020;1(1):011303.
61. Stojanovski BM, Pelc LA, Zuo X, Pozzi N, Di Cera E. Enhancing the anticoagulant profile of meizothrombin. *Biomol Concepts*. 2018;9(1):169-175.
62. Pozzi N, Chen Z, Di Cera E. How the linker connecting the two Kringles influences activation and conformational plasticity of prothrombin. *J Biol Chem*. 2016;291:6071-6082.
63. Acquasaliente L, Pelc LA, Di Cera E. Probing prothrombin structure by limited proteolysis. *Sci Rep*. 2019;9(1):6125.
64. Chakraborty P, Acquasaliente L, Pelc LA, Di Cera E. Interplay between conformational selection and zymogen activation. *Sci Rep*. 2018;8(1):4080.
65. Pozzi N, Chen Z, Gohara DW, Niu W, Heyduk T, Di Cera E. Crystal structure of prothrombin reveals conformational flexibility and mechanism of activation. *J Biol Chem*. 2013;288(31):22734-22744.
66. Pozzi N, Chen Z, Pelc LA, Shropshire DB, Di Cera E. The linker connecting the two kringles plays a key role in prothrombin activation. *Proc Natl Acad Sci U S A*. 2014;111(21):7630-7635.
67. Brufatto N, Nesheim ME. Analysis of the kinetics of prothrombin activation and evidence that two equilibrating forms of prothrombinase are involved in the process. *J Biol Chem*. 2003;278(9):6755-6764.
68. Orcutt SJ, Krishnaswamy S. Binding of substrate in two conformations to human prothrombinase drives consecutive cleavage at two sites in prothrombin. *J Biol Chem*. 2004;279:54927-54936.
69. Kim PY, Nesheim ME. Further evidence for two functional forms of prothrombinase each specific for either of the two prothrombin activation cleavages. *J Biol Chem*. 2007;282(45):32568-32581.
70. Bock PE, Panizzi P, Verhamme IM. Exosites in the substrate specificity of blood coagulation reactions. *J Thromb Haemost*. 2007;5(Suppl 1):81-94.
71. Lee CJ, Wu S, Pedersen LG. A proposed ternary complex model of prothrombinase with prothrombin: protein-protein docking and molecular dynamics simulations. *J Thromb Haemost*. 2011;9(10):2123-2126.
72. Lechtenberg BC, Murray-Rust TA, Johnson DJ, et al. Crystal structure of the prothrombinase complex from the venom of *Pseudonaja textilis*. *Blood*. 2013;122(16):2777-2783.
73. Shim JY, Lee CJ, Wu S, Pedersen LG. A model for the unique role of factor Va A2 domain extension in the human ternary thrombin-generating complex. *Biophys Chem*. 2015;199:46-50.
74. Schreuder M, Reitsma PH, Bos MHA. Blood coagulation factor Va's key interactive residues and regions for prothrombinase assembly and prothrombin binding. *J Thromb Haemost*. 2019;17(8):1229-1239.
75. Bianchini EP, Orcutt SJ, Panizzi P, Bock PE, Krishnaswamy S. Ratcheting of the substrate from the zymogen to proteinase conformations directs the sequential cleavage of prothrombin by prothrombinase. *Proc Natl Acad Sci U S A*. 2005;102(29):10099-10104.
76. Lee CJ, Wu S, Eun C, Pedersen LG. A revisit to the one form kinetic model of prothrombinase. *Biophys Chem*. 2010;149(1–2):28-33.
77. Rudolph AE, Porche-Sorbet R, Miletich JP. Substitution of asparagine for arginine 347 of recombinant factor Xa markedly reduces factor Va binding. *Biochemistry*. 2000;39(11):2861-2867.
78. Rudolph AE, Porche-Sorbet R, Miletich JP. Definition of a factor Va binding site in factor Xa. *J Biol Chem*. 2001;276(7):5123-5128.
79. Kalafatis M, Beck DO, Mann KG. Structural requirements for expression of factor Va activity. *J Biol Chem*. 2003;278(35):33550-33561.
80. Kalafatis M. Coagulation factor V: a plethora of anticoagulant molecules. *Curr Opin Hematol*. 2005;12(2):141-148.
81. Beck DO, Bukys MA, Singh LS, Szabo KA, Kalafatis M. The contribution of amino acid region ASP695-TYR698 of factor V to procofactor activation and factor Va function. *J Biol Chem*. 2004;279(4):3084-3095.
82. Toso R, Camire RM. Role of hirudin-like factor Va heavy chain sequences in prothrombinase function. *J Biol Chem*. 2006;281(13):8773-8779.
83. Bukys MA, Kim PY, Nesheim ME, Kalafatis M. A control switch for prothrombinase: characterization of a hirudin-like pentapeptide from the COOH terminus of factor Va heavy chain that regulates the rate and pathway for prothrombin activation. *J Biol Chem*. 2006;281(51):39194-39204.
84. Bakker HM, Tans G, Thomassen MC, et al. Functional properties of human factor Va lacking the Asp683-Arg709 domain of the heavy chain. *J Biol Chem*. 1994;269(32):20662-20667.
85. Hirbawi J, Vaughn JL, Bukys MA, Vos HL, Kalafatis M. Contribution of amino acid region 659-663 of factor Va heavy chain to the activity of factor Xa within prothrombinase. *Biochemistry*. 2010;49(39):8520-8534.
86. Steen M, Villoutreix BO, Norstrom EA, Yamazaki T, Dahlback B. Defining the factor Xa-binding site on factor Va by site-directed glycosylation. *J Biol Chem*. 2002;277(51):50022-50029.
87. Kalafatis M, Beck DO. Identification of a binding site for blood coagulation factor Xa on the heavy chain of factor Va. Amino acid residues 323-331 of factor V represent an interactive site for activated factor X. *Biochemistry*. 2002;41(42):12715-12728.
88. Yegneswaran S, Mesters RM, Fernandez JA, Griffin JH. Prothrombin residues 473-487 contribute to factor Va binding in the prothrombinase complex. *J Biol Chem*. 2004;279(47):49019-49025.
89. Chen L, Yang L, Rezaie AR. Proexosite-1 on prothrombin is a factor Va-dependent recognition site for the prothrombinase complex. *J Biol Chem*. 2003;278(30):27564-27569.
90. Akhavan S, De Cristofaro R, Peyvandi F, Lavoretano S, Landolfi R, Mannucci PM. Molecular and functional characterization of a natural homozygous Arg67His mutation in the prothrombin gene of a patient with a severe procoagulant defect contrasting with a mild hemorrhagic phenotype. *Blood*. 2002;100(4):1347-1353.
91. Deguchi H, Takeya H, Gabazza EC, Nishioka J, Suzuki K. Prothrombin kringle 1 domain interacts with factor Va during the assembly of prothrombinase complex. *Biochem J*. 1997;321(Pt 3):729-735.
92. Kotkow KJ, Deitcher SR, Furie B, Furie BC. The second kringle domain of prothrombin promotes factor Va-mediated prothrombin activation by prothrombinase. *J Biol Chem*. 1995;270(9):4551-4557.
93. Taneda H, Andoh K, Nishioka J, Takeya H, Suzuki K. Blood coagulation factor Xa interacts with a linear sequence of the kringle 2 domain of prothrombin. *J Biochem*. 1994;116(3):589-597.
94. Yegneswaran S, Mesters RM, Griffin JH. Identification of distinct sequences in human blood coagulation factor Xa and prothrombin essential for substrate and cofactor recognition in the prothrombinase complex. *J Biol Chem*. 2003;278(35):33312-33318.
95. Harlos K, Holland SK, Boys CW, Burgess AI, Esnouf MP, Blake CC. Vitamin K-dependent blood coagulation proteins form heterodimers. *Nature*. 1987;330(6143):82-84.

96. Papaconstantinou ME, Bah A, Di Cera E. Role of the a chain in thrombin function. *Cell Mol Life Sci*. 2008;65:1943-1947.
97. Lancellotti S, Basso M, De Cristofaro R. Congenital prothrombin deficiency: an update. *Semin Thromb Hemost*. 2013;39(6):596-606.
98. Lefkowitz JB, Haver T, Clarke S, et al. The prothrombin Denver patient has two different prothrombin point mutations resulting in Glu-300→Lys and Glu-309→Lys substitutions. *Br J Haematol*. 2000;108(1):182-187.

**How to cite this article:** Di Cera E, Mohammed BM, Pelc LA, Stojanovski BM. Cryo-EM structures of coagulation factors. *Res Pract Thromb Haemost*. 2022;6:e12830. doi:10.1002/rth2.12830