Maturation of coagulation factor IX during Xase formation as deduced using factor VIII-derived peptides

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Blood coagulation involves extrinsic and intrinsic pathways, which merge at the activation step of blood coagulation factor X to factor Xa. This step is catalysed by the extrinsic or intrinsic Xase, which consists of a complex of factor VIIa and its cofactor tissue factor or factor IXa (FIXa) and its cofactor coagulation factor VIIIa (FVIIIa). Upon complex formation with FVIIIa, FIXa is conformationally activated to the Xase complex. However, the mechanistic understanding of this molecular recognition is limited. Here, we examined FVIIIa-FIXa binding in the context of FIXa’s activation status. Given the complexity and the labile nature of FVIIIa, we decided to employ two FVIII-derived peptides (558-loop, a2 peptide) to model the cofactor binding of FIX(a) using biosensor chip technology. These two FVIII peptides are known to mediate the key interactions between FVIIIa and FIXa. We found both of these cofactor mimetics as well as full-length FVIIIa bind more tightly to zymogenic FIX than to proteolytically activated FIXa. Consequently and surprisingly, we observed that the catalytically inactive FIX zymogen can outcompete the activated FIXa from the complex with FVIIIa, resulting in an inactive, zymogenic Xase complex. By contrast, the thrombophilic Padua mutant FIXa-R170 in complex with the protein–substrate analogue BPTI bound tighter to FVIIIa than to the zymogen form FIX-R170L, suggesting that the active Xase complex preferentially forms in the Padua variant. Together, these results provide a mechanistic basis for the thrombophilic nature of the FIX-R170L mutant and suggest the existence of a newly discovered safety measure within the coagulation cascade.

Blood coagulation is a cascade-like molecular system to stop blood loss upon vessel injury. The reaction cascades can be subdivided into an extrinsic and intrinsic pathway, which serve to initiate, maintain and eventually stop blood coagulation. Both pathways merge at the activation step of blood coagulation factor X to factor Xa which is catalysed by the extrinsic or intrinsic Xase, consisting of the complex of factor VIIa and its cofactor tissue factor or factor IXa and its cofactor VIIIa, respectively [1–3].

The homologous coagulation factors IX and X are trypsin-like serine proteases, which obligatorily require the protein cofactors factor VIII and factor V for their clotting activity. Also the cofactors V and VIII share an homologous domain architecture, consisting of three paralogous A domains (A1, A2, A3) and two C domains (C1, C2) (Fig. 1). While the C domains confer binding to the membrane of activated platelets, the A domains mediate binding to the substrate (A1) and the protease (A2, A3). A so-called B domain of

Abbreviations
BPTI, basic pancreatic trypsin inhibitor; Factor IXa, activated factor IX; IB, inclusion body; PMSF, phenylmethylsulfonyl fluoride.
variable length and post-translational modification is interspersed between the A2 and A3 domains. The A2 domain of factor VIIIa and factor Va is particularly important as it conformationally activates the corresponding coagulation protease, that is factor IXa in complex with factor VIIIa and factor Xa in complex with factor Va. These two protein complexes activate the downstream substrates factor X and prothrombin and are accordingly termed Xase and prothrombinase [2–5].

All protein factors participating in the Xase and prothrombinase complexes require proteolytic activation to achieve enzymatic activity. Activation of factor VIII to VIIIla, and correspondingly V to Va, is carried out by thrombin. Thrombin cleavages of factor VIII (and analogously in factor V) release the B domain by cleaving after Arg1689 and Arg740 (FV: Arg709, Arg1018 and Arg1545; sequence numbering of the mature proteins after cleavage of the signal peptides), and followed by the cleavage of the A1-A2 linker after Arg372 in FVIII [6].

In the protease factors IX and X, proteolytic activation at Arg180 (FX: Arg194) and Arg145 releases an activation peptide, triggering a conformational disorder-to-order transition in the serine protease domain (Figs 2 and S1). The activation peptide is positioned between the light chain (consisting of the N-terminal Gla, EGF1 and EGF2 domains) and the trypsin-like serine protease domain. Light and heavy chains remain covalently linked via a disulfide bond. In both factor IX and factor X, the activation peptide harbours an intriguing high-affinity binding site for benzamidine/arginine, exceeding the affinity towards the active site by an order of magnitude [7]. By contrast, macromolecular factor IX/X inhibitors such as serpins or Kunitz inhibitors such as BPTI do not bind to the activation peptide but to the active site exclusively.

The proteolytic activation of factor IX (or X) to IXa is insufficient to achieve significant Xase activity. Instead, the cofactor VIIIa is critically needed to achieve physiologically relevant Xase activity. However, the mechanism of cofactor stimulation is incompletely understood. Functional and structural studies of this central haemostatic complex are hampered, among others, by the difficulty to obtain factor VIII(a) in sufficient amount and quality. To shed light on the cofactor triggered activation, we set out to study the interaction of factor IX with the cofactor VIIIa and VIIIa-derived peptides mimicking important interactions.

**Materials and methods**

**Expression of FIX constructs in Escherichia coli cells and purification**

Factor IX protein variants were expressed in *Escherichia coli*, refolded, purified and activated as described elsewhere [8–10].

**Factor VIII-derived peptides**

The factor VIII-derived peptides Ser558-Gln565 (SVDQGRGNQ) and Lys713-Arg740 (KHTGDSY-sYEDSsYEDISAYLLSKNNAIEPR; sY: sulfotyrosine) were purchased from JPT Peptide Technologies. Quality was controlled by HPLC and mass spectrometry and confirmed to be of better than 90% homogeneity.

**Activation of FVIII with thrombin**

Pro-FVIII was generously provided by Baxter Bio-sciences (Vienna, Austria). Human thrombin was purchased from Sigma. For activation, 100 μg FVIII lyophilisate was dissolved in ddH2O and spin-dialysed by repeated concentration in 30 kDa cut-off Amicon concentrators to a final concentration of ~0.2 mg·mL⁻¹. FVIII was activated by thrombin similar to protocols described elsewhere [11,12]. Briefly, thrombin was dissolved in 20 mM Hepes, pH 7.5, 100 mM NaCl and 5 mM CaCl₂. 1 unit of thrombin was added to

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**Fig. 1.** Factor VIII assists in orienting the FIXa-FX interaction. Physiologically, FIXa acts on the activated platelets’ membrane surface, which expose negatively charged lipids during plug formation. FIXa has a very low amidolytic activity; only in the presence of its cofactor, factor VIIIa, the activity of FIXa is significantly increased. FVIIIa enhances both $k_{cat}$ and $k_{m}$ of the FIXa-catalysed FX activation. In this instance, FVIIIa displays considerable affinity to both enzyme (FIXa) and substrate (FX) and pre-orients both in a productive interaction.
1000 units of FVIII (100 µg) and incubated for 15 min at 37 °C without shaking. The protein mixture was then dialysed by in total four centrifugation steps against a 4 °C cold wash buffer consisting of 50 mM Hepes, pH 7.4, 300 mM NaCl and 2.5 mM CaCl₂. The reaction was stopped after the second concentration step by adding 20 µL 50 mM PMSF to inhibit thrombin. The activation of FVIIIa was controlled by SDS/PAGE to observe the A1, A2 and A3-C1-C2 at ~55 kDa, 40 kDa and 70 kDa, respectively.

Surface acoustic wave measurements via SAM5 device (SAW Instruments)

Chip calibration

Prior to the chip calibration at 22 °C, the instrument was flushed with a flow rate of 40 µL·min⁻¹ for 5 to 10 min. Chip calibration was performed using the SAM5 program with gain of 180 and a phase difference of 0.363.

SAM5 ligand online immobilization

The SAM5 instrument with a dextran-COOH-coated gold chip (NanoTemper Technologies) was equilibrated using filtered and degassed PBS running buffer, and the chip was calibrated as described above. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were dissolved in ddH₂O to a final concentration of 400 mM and 100 mM, respectively. To activate the chip, EDC and NHS were mixed at equal volume, resulting in the final concentrations of 200 mM EDC and 50 mM NHS. The mixture was rapidly placed into position A1 of the tray holder. If carried out properly, the baseline will increase. Subsequently, the ligand (protein ligands at 250–500 nM concentration, peptidic ligands at 100 µM to 1 mM concentration) was placed into the tray holder at position A1. The binding of the ligand to the dextran matrix is based on electrostatic interactions. Therefore, positively charged ligands are required, that is the pH has to be adjusted smaller than the ligand’s pI, but larger than pH 3.5 (pI > pH > pH 3.5). The increase in the baseline resulting from successful ligand coating is larger than that resulting from EDC-NHS activation. To cap the unspecific protein binding sites, 1 M ethanolamine, pH 8.5, was applied into the tray holder at position A3. The system was equilibrated against the running buffer.

SAM5 ligand and protein interaction measurements

Firstly, pumps were washed using filtered and degassed running buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM CaCl₂). The flow rate was set to 40 µL·min⁻¹. After a stable baseline was reached, protein was injected by using the SAM5 sequence master program in an increasing concentration series (10 nM to 250 nM). The sensogram SAM5 data were exported to Origin and TraceDrawer, allowing to analyse and quantify the protein binding affinities, as described in the SAM5 user manual.

Results

Recombinant factor IX was activated to FIXa using factor Xla (Figs S2, S3) and factor VIII to FVIIIa using thrombin (Fig. S4).
Interaction of factor VIII with zymogenic and activated factor IX and IXa variants

The sequential order of factor IX and VIII activation and complex formation is not clearly established. Therefore, we asked whether not only the activated factor IXa but also the zymogenic factor IX would be able to bind coagulation factor VIIIa as well as its proform, factor VIII. We carried out the corresponding binding measurements with the cofactor being immobilized on a chip (Fig. 3). We found the zymogenic factor IX (FIX wt) to bind to the cofactor VIII with more than 10-fold higher affinity than the activated factor IXa (FIXa wt). This was confirmed also when activated factor VIIIa was used as a binding partner, in the presence of both benzamidine and BPTI (Table 1).

This finding was surprising to us and indicates that the tightest complex is the ‘pro-Xase’ formed by the zymogenic FIX with either FVIII or FVIIIa. The preference of FVIII(a) binding to the inactive FIX may serve as another regulation level. Zymogenic factor IX and activated factor IXa not only differ by the presence of the activation peptide, but also conformationally, whereby the zymogen FIX is considerably more flexible and disordered as compared to activated FIXa [13–15]. To further test the relevance of the factor IX(a) conformational rigidity for cofactor VIII(a) binding, we employed the triple factor IX mutant (Y94F-K98T-Y177T), which is known to be more active and conformationally more rigid than wt FIX [8]. The binding of the pro-cofactor VIII or activated cofactor FVIIIa to the triple mutant factor IX (FIX 3M) in the presence of benzamidine or BPTI was qualitatively similar to the wt FIX(a), that is the zymogenic FIX-3M bound with more than fourfold higher affinity than the activated FIXa-3M, with FIX(a)-3M having a slightly higher affinity than wt FIX(a) (Table 1).

| Table 1. Dissociation constants of the interaction of FVIII or FVIIIa with various zymogenic FIX and activated FIXa constructs |
|---|---|---|
| FVIII analyte | Benzamidine | Aprotinin (BPTI) |
| | $K_d$ (nM) | $K_d$ (nM) |
| **A** | | |
| FIX wt | 81.48 ± 86.77 | FIX wt | 65.77 ± 21.16 |
| FIXa wt | 948.00 ± 749.65 | FIXa wt | 107.62 ± 60.78 |
| FIX-R170L | 40.43 ± 57.65 | FIX-R170L | 46.66 ± 34.99 |
| FIXa R170L | 587.29 ± 937.70 | FIXa R170L | 73.10 ± 28.36 |
| FIX 3M | 50.64 ± 69.09 | FIX 3M | 56.00 ± 9.15 |
| FIXa 3M | 421.75 ± 392.66 | FIXa 3M | 192.25 ± 107.32 |
| **B** | | |
| FIX wt | 83.62 ± 140.31 | FIX wt | 72.25 ± 60.21 |
| FIXa wt | 1304.13 ± 1381.53 | FIXa wt | 204.05 ± 169.08 |
| FIX-R170L | 202.29 ± 404.48 | FIX-R170L | 173.75 ± 281.43 |
| FIXa R170L | 249.89 ± 484.14 | FIXa R170L | 44.94 ± 32.26 |
| FIX 3M | n.d. | FIX 3M | 88.57 ± 76.30 |
| FIXa 3M | 817.75 ± 752.89 | FIXa 3M | 438.25 ± 250.87 |

The analytes were incubated with 1 mM benzamidine (left) or with 1 mM aprotinin/BPTI (right).

Section A describes the interaction of nonactivated factor VIII; section B describes that of activated factor VIIIa. Dissociation constants were determined using the SAM5 Blue device.

Fig. 3. Sensogram of FIX binding to immobilized FVIII-derived 558-loop. FVIIIa-derived peptide S558-Q565 was immobilized on a carboxymethyl-dextran chip, and zymogenic FIX wild-type was applied in the mobile phase with six concentrations: 10 nM (light blue), 50 nM (yellow), 100 nM (green), 150 nM (dark blue), 200 nM (red) and 250 nM (black). The measurement was conducted at a flow rate of 40 µL·min$^{-1}$ with 4-min association with zymogenic FIX wild-type, followed by 4-min dissociation. For curve-fitting, we used the one-to-one two-state model, which is shown by the thin black lines.
Additionally, we investigated the thrombophilic Padua variant (R170L) which was suggested in the literature to have higher affinity to cofactor VIIIa [16]. However, the mechanism of thrombophilia and increased Xase activity is unknown. Overall, the Padua variant showed a similar binding pattern as the wt factor IX; however, we found one notable exception: in the wt FIX, the zymogen binds tighter to FVIII(a) than the activated FIXa independent of the ligand. By contrast, in the presence of BPTI we found the highest affinity for the Padua variant in the activated form FIXa(Padua) to the activated FVIIIa, that is the productive complex is preferred in the presence of a substrate analogue (Table 1), in stark contrast to other factor IX variants. The Padua variant thus prefers the complex formation with the proteolytically activated, that is enzymatically productive, components. This property may relate to the thrombophilic symptoms found in the Padua mutation.

To interpret these surprising findings, we further tried to reduce the interaction to known factor VIII interaction hotspots, that is the 558-loop (Ser558-Gln565) and the C-terminal a2 peptide.

**Interaction of factor VIII-derived 558-loop with zymogenic and activated factor IX(a) variants**

Albeit less pronounced, zymogenic and activated factor IX(a) variants exhibited analogous binding affinities towards the FVIII-derived 558-loop peptide as to protein cofactor VIII. The affinity of zymogenic wt FIX towards the 558-loop was approximately threefold higher ($K_d = 172 \text{ nM}$) than that of activated wt FIXa ($K_d = 457 \text{ nM}$) (Table 2). A similar relation was observed for the triple mutant factor IX-3M and the Padua variant (R170L): the zymogenic factor IX variants had an approximately twofold higher affinity towards the 558-loop than the activated factor IXa variants (Table 2). These relations were also observed in the presence of benzamidine. Importantly, in the presence of the substrate analogue BPTI, the FIXa Padua variant had a higher binding affinity than the zymogenic FIX (Padua), mirroring the already described result for protein FVIIIa. The binding affinities of factor IX(a) to the 558-loop thus parallel those observed for the binding towards the protein cofactor VIIIa.

**Interaction of factor VIII-derived a2 peptide with zymogenic and activated factor IX(a) variants**

We further investigated the binding to the a2 peptide, located at the C terminus of the A2 domain (Lys713-Arg740), which is also known to contribute binding affinity to the Xase complex by interacting with the heparin-binding site [17]. We found that the a2 peptide had an approximately twofold higher affinity towards the zymogen factor IX than the activated factor IXa, independent from the presence of (substrate-analogous) ligands or point mutations, that is FIX-3M or FIX-R170L (Padua) (Table 3). Different from the 558-loop for the a2 peptide, we did not find a switch in the binding affinity of the zymogenic and activated Padua variants in the presence of BPTI (Tables 2 and 3). This suggests that the 558-loop plays the dominant role in tuning the activation status of the Xase complex. This conclusion is consistent with the proposed binding sites of the 558-loop and the a2 peptide.

**Discussion**

The here described binding affinities of zymogenic and activated factor IX to factor VIII(a) and factor VIII-derived peptides are partly surprising, but can be rationalized when considering the conformational transitions in factor IX.
Like all chymotrypsinogen-like proteases, factor IX undergoes a conformational disorder–order transition upon proteolytic activation. Almost one third of the serine protease domain is conformationally flexible in the zymogenic state, including the activation pocket and the surrounding of the active site [18]. The proteolytic activation of factor IX to factor IXa thus involves surface patches that are proposed to participate in cofactor binding (Fig. 4) [17]. Therefore, it was to be expected that activation of factor IX to factor IXa should affect cofactor-binding affinity, which was indeed the case, case (Tables 1 and 2). Additional conformational transitions are induced in activated factor IXa by binding to peptidic substrates, which are known to further mature the active site region [19]. Consequently, substrate binding should also influence the binding affinity towards factor VIIIa(a). We mimicked the substrate-bound factor IXa by using the canonical (i.e. substrate-like) protein inhibitor BPTI and observed a change in binding affinity towards the activated cofactor VIIIa or the VIIa-derived peptide Ser558–Gln565. In all factor IXa variants, the presence of BPTI significantly improved the affinity towards FVIIIa.

In summary, we propose a four-state model to explain the observed results (Fig. 5): zymogenic FIX (I) is enzymatically inactive, conformationally flexible and plastic. Upon proteolytic activation, FIX is converted to FIXalatent [immature FIXa (II)], which has its active site preformed, and is conformationally rigidified, thereby losing its plasticity. Importantly, however, the conformation of FIXalatent is partly incompatible with cofactor binding, and it takes energy to adapt to the cofactor-binding conformation, thus reducing the affinity of binding. By contrast, zymogenic FIX (I) can more easily adapt to the conformation consistent with cofactor binding. The conformation of FIXa(II) is modulated in the complex with the protein–substrate analogue BPTI (III). Different from benzamidine, BPTI has an extensive interaction interface with FIXa and is thereby able to convert FIXalatent (II) into FIXaintermEDIATE (III), resulting in...
approximately equal affinities of FXα$_{\text{intermediate}}$ FVIIIa (III) and FIX-FVIIIa (I). This assimilation in affinity is seen in the complex of FIX(a) with the 558-loop (Ser558-Gln565) and most pronounced in the complex with FVIII and FVIIIa, but not in the a2 peptide (Lys713 – Arg740). The latter observation is consistent with the proposed binding site of a2 to FIXa near the heparin-binding site, away from the active site [17].

Remarkably, the clinically relevant thrombophilic mutant R170L is able to further improve the binding affinity of the activated FIXa towards FVIIIa in the ternary complex with the protein–substrate analogue BPTI, not benzamidine or the apo form. An analogous effect was observed for the FVIIia-derived 558-loop, but not for the C-terminal a2 peptide. This latter effect is consistent with the R170L mutation being positioned directly at the binding interface to the 558-loop (Ser558-Gln565), see Fig. 4, whereas the binding epitope of the a2 peptide (Lys713-Arg740) is more than 30 Å away from the mutation site. This analysis rationalizes the molecular basis of the thrombophilic effect of the R170L mutation which is not caused by an increase in the binary FIXa-FVIIIa binding affinity but by an increase in the binding affinity of the ternary FVIIIa-FIXa substrate complex. Finally, we propose that the correct physiological substrate FX would be able to induce an even more compatible conformation in FIXa (IV).

**Conclusions**

The conformational changes accompanying the proteolytic activation of factor IX to IXa affect not only its enzymatic activity but also its binding properties towards the cofactor VIIIa. Unexpectedly, the affinity of the complex of zymogen FIX towards the cofactor VIII(a) is higher than that of the activated FIXa to FVIIia Xase complex. Consequently, the zymogenic ‘pro-Xase’ outcompetes the catalytically active Xase, keeping blood coagulation under tight control. In the ternary complex with the additional presence of a substrate analogue, the Xase complex gets tightened, enabling an efficient substrate turnover. Intriguingly, the Padua variant of active factor IXa had a particularly strong affinity in the ternary complex, explaining its thrombophilic property.

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Conflict of interest
The authors declare no conflict of interest.

Author contributions
HF conducted most experiments and wrote the manuscript. TZ supervised the work. HB supervised the work and wrote the manuscript.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.
**Fig. S1.** Domain organization of the homologous factor IX and X. (A) Benzamidine binding sites in zymogenic factor IX / X. (B) Benzamidine binding sites in the activated factor IX a/ Xa.

**Fig. S2.** SDS/PAGE of the SEC fractions of zymogenic FIX wt. The sample containing FIX wt after Q sepharose purification is referred to as load. The most pure fractions were pooled and stored at -20 °C for further experiments.

**Fig. S3.** Zymogenic FIX wt activation by hFXIa. The *E. coli* expressed recombinant FIX wt was activated by hFXIa. The 4 lanes on the left side show samples loaded with dithiothreitol. The remaining 4 samples at the right side were without DTT. The samples without DTT showed an extra thin band on the top, which corresponds to dimeric hFXIa. Wild-type and mutant zymogenic FIX were activated by human blood coagulation FXIa (hFXIa), cleaving at Arg145-Ala146 and Arg180-Val181. After the first cleavage (at the Arg145-Ala146; after 1 h) an activation intermediate FIXx was observed, which migrated at an increased apparent size. Activation was completed after 16 hours, corresponding to resembling cleavages at position Arg145-Ala146 and Arg180-Val181, with the activation peptide being released.

**Fig. S4** FVIII activation via human thrombin. FVIII was incubated with human thrombin for 30 min at 37 °C without shaking to generate the activated form. The samples were loaded without (marked in blue) and with (marked in red) DTT. The B domain deleted pro-FVIII with an approximate molecular mass of 280 kDa was activated by human thrombin and converted into three major bands: A1 domain approx. 50 kDa, A2 domain approx. 40 kDa, and A3-C1-C2 domain approx. 80 kDa.