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Sequential coagulation factor VIIa domain binding to tissue factor ☆

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Abstract

Vessel wall tissue factor (TF) is exposed to blood upon vascular damage which enables association with factor VIIa (FVIIa). This leads to initiation of the blood coagulation cascade through localization and allosteric induction of FVIIa procoagulant activity. To examine the docking pathway of the FVIIa–TF complex, various residues in the extracellular part of TF (sTF) that are known to interact with FVIIa were replaced with cysteines labelled with a fluorescent probe. By using stopped-flow fluorescence kinetic measurements in combination with surface plasmon resonance analysis, we studied the association of the resulting sTF variants with FVIIa. We found the docking trajectory to be a sequence of events in which the protease domain of FVIIa initiates contact with sTF. Thereafter, the two proteins are tethered via the first epidermal growth factor-like and finally the γ-carboxyglutamic acid (Gla) domain. The two labelled sTF residues interacting with the protease domain of FVIIa bind or become eventually ordered at different rates, revealing kinetic details pertinent to the allosteric activation of FVIIa by sTF. Moreover, when the Gla domain of FVIIa is removed the difference in the rate of association for the remaining domains is much more pronounced.

Keywords: Tissue factor; Factor VIIa; Protein–protein interaction; Probe; Kinetics

Protein–protein interactions are fundamental for biological processes. To gain insight into physiologically relevant protein complexes, it is of paramount importance to understand the specificity and forces that govern protein recognition [1]. To shed more light on the initiation of blood coagulation, we have studied the complex formed by the extracellular part of tissue factor (sTF) and coagulation factor VIIa (FVIIa) [2–5]. Tissue factor (TF) is the cellular receptor for FVIIa, and the binary complex activates factors IX and X and triggers the coagulation cascade. TF is present on fibroblasts and smooth muscle cells surrounding the vessels and is exposed to FVIIa in the circulation upon vessel injury [6].

The binding pathways and kinetics underlying the formation of complexes between proteins are particularly elusive and therefore poorly understood, primarily due to technical difficulties in monitoring the establishment of intermolecular contacts and the ensuing structural changes. To try to elucidate the docking mechanism of FVIIa and sTF, we recently performed a Φ-value analysis of the complex formation [7]. Using this approach it is possible to describe the extent of interaction at specific positions in the transition state of FVIIa–sTF complex formation. The determined Φ-values showed that the transition state comprises a more established interaction at the interface between the protease domain (PD) of FVIIa and sTF,

☆ *Abbreviations:* FVIIa, coagulation factor VIIa; TF, tissue factor; sTF, extracellular part of tissue factor; SPR, surface plasmon resonance.

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and less established interactions between the first epidermal growth factor-like (EGF1) domain and, in particular, the γ-carboxyglutamic acid (Gla) domain of FVIIa and sTF. Based on these findings a sequential docking mechanism was proposed in which the initial event in the association of FVIIa and sTF is the binding of the PD to sTF followed by binding of the EGF1 and finally the Gla domain. Real-time kinetic evidence is a prerequisite to prove this domain-by-domain docking pathway. Therefore, we have made an attempt to monitor the association of the various domains of FVIIa to sTF by using stopped-flow fluorescence spectroscopy experiments. sTF was subjected to site-directed fluorophore labeling to report on the complex formation at various sites along the interface between these two proteins.

Materials and methods

Protein preparation. The different sTF variants were produced in *Escherichia coli* and purified as previously described [8]. Human recombinant FVIIa and des(1–44)-FVIIa were prepared according to published methods [9–11] and optionally modified with the active site inhibitor FFR-chloromethyl ketone. Protein concentrations were calculated by measuring the absorption at 280 nm, using extinction coefficients of 37,440 M^{-1} cm^{-1} (sTF(F140C, I22C, V207C)), 36,160 M^{-1} cm^{-1} (sTF(Y94C)), 31,750 M^{-1} cm^{-1} (sTF(W45C, W158C)), 63,380 M^{-1} cm^{-1}(FVIIa), and 56,265 M^{-1} cm^{-1} (des(1–44)-FVIIa) [12]. The Cys residue inserted in sTF was labeled with IAEDANS [5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid] according to a published method [8], and the degree of labeling was determined by measuring the absorbance at 337 nm using $\varepsilon = 5700$ M^{-1} cm^{-1}.

SPR measurements. To characterize the global binding of AEDANS-labeled sTF variants to FVIIa and des(1–44)-FVIIa inhibited by biotin-FPR chloromethyl ketone, we used a Biacore instrument as described elsewhere [2]. FVIIa was immobilized and the concentrations of sTF variants were 3–15 times the dissociation constant derived from experiments in which the amidolytic activity of FVIIa was measured as a function of the concentrations of the different sTF variants.

Stopped-flow fluorescence measurements. Complex formation between sTF and FFR-FVIIa was monitored as the change in extrinsic fluorescence, using an Applied Photophysics SX-17MV sequential stopped-flow spectrofluorometer at 25 °C. To ensure pseudo-first-order kinetics, we used 0.75 μM sTF together with 15 μM FFR-FVIIa and 0.3 μM sTF together with 6 μM of des(1–44)-FFR-FVIIa. All analyses were performed in 50 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, at pH 7.5. The AEDANS moiety was excited at 350 nm, whereafter the fluorescence emission was recorded in the time range 1 ms–0.5 s using a 408 nm cut-off filter. Complex formation between FFR-FVIIa and each AEDANS-labeled sTF variant was measured 5–8 times in duplicate series. Rate constants were calculated by fitting the fluorescence signal to a first-order rate equation using Table curve 2D v.4 (AISN Software). To simplify comparison with SPR data, pseudo-first-order rate constants were transformed into second-order rate constants by dividing the obtained data by the FVIIa concentration used for the stopped-flow measurements. Standard deviations were then determined. Control experiments were performed using different excesses of FVIIa giving similar second-order rate constants.

Results and discussion

Residues involved in the sTF–FVIIa binding interface

The sTF–FVIIa complex is elongated in shape, 115 Å long and 40–50 Å in diameter, and possesses a contact area of 1800 $Å^2$. The interface between FVIIa and sTF can be divided into three major regions [13]: the PD–sTF interface, the EGF1 domain–sTF interface, and the Gla domain–sTF interface (Fig. 1). These binding regions, comprising discrete domains of FVIIa, can be assumed to bind individually to sTF [2]. However, due to their direct linkage it is reasonable to believe that, although these regions within the interface can form productive interactions with different rates of association, they are influenced by the neighboring domains.

To investigate the appearance of local interface structures upon FVIIa–sTF association, we have studied the energies involved in the transition state [7] and found evidence of a sequential domain binding pathway.

In this work, we have performed a kinetic investigation to further substantiate this sequential binding mechanism. For this purpose we used the six single-cysteine sTF mutants constructed for the transition state analysis [7]. The free cysteines were specifically labeled with the fluorescent probe IAEDANS and used in stopped-flow and accompa-

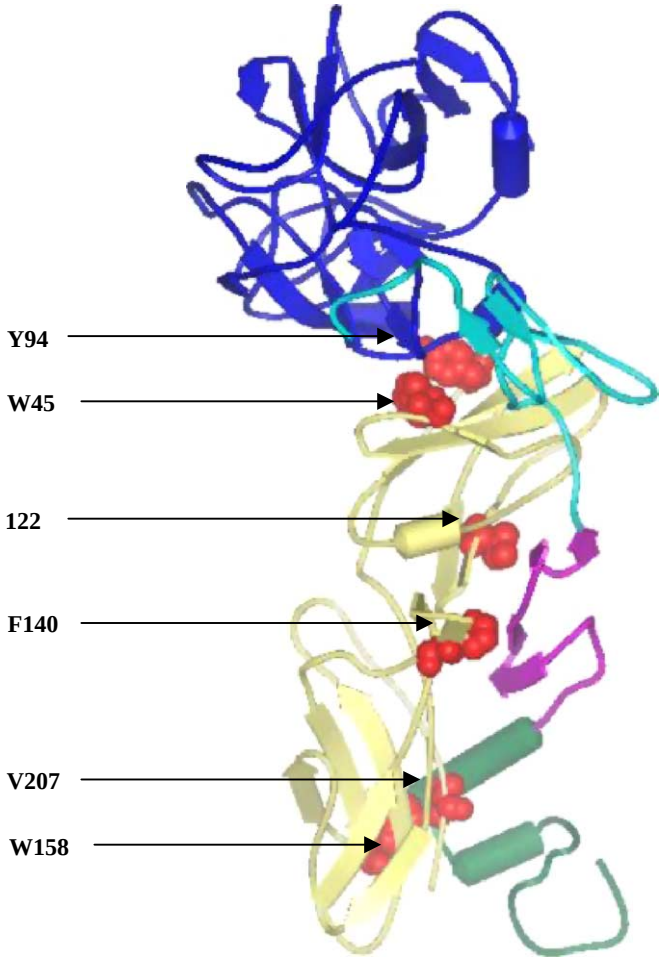

Fig. 1. The crystal structure of sTF (yellow) in complex with FVIIa [13]. The three major binding regions of FVIIa include: the protease domain (PD) (dark blue), the first epidermal growth factor (EGF1) domain (purple), and the γ-carboxyglutamic acid (Gla) domain (green). The mutated positions in sTF are highlighted in red.