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Effects on the conformation of FVIIa by sTF and Ca^{2+} binding: Studies of fluorescence resonance energy transfer and quenching

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ABSTRACT

The apparent length of FVIIa in solution was estimated by a FRET analysis. Two fluorescent probes, fluorescein (Fl-FPR) and a rhodamine derivative (TMR), were covalently attached to FVIIa. The binding site of Fl-FPR was in the protease domain whereas TMR was positioned in the Gla domain, thus allowing a length measure over virtually the whole extension of the protein. From the FRET measurements, the distances between the two probes were determined to be 61.4 for free FVIIa and 65.5 Å for FVIIa bound to soluble tissue factor (sTF). These seemingly short distances, compared to those anticipated based on the complex crystal structure, require that the probes stretch towards each other. Thus, the apparent distance from the FRET analysis was shown to increase with 4 Å upon formation of a complex with sTF in solution. However, considering how protein dynamics, based on recent molecular dynamics simulations of FVIIa and sTF:FVIIa (Y.Z. Ohkubo, J.H. Morrissey, E. Tajkhorshid, J. Thromb. Haemost. 8 (2010) 1044–1053), can influence the apparent fluorescence signal our calculations indicated that the global average conformation of active-site inhibited FVIIa is nearly unaltered upon ligation to sTF.

It is known from amidolytic activity measurements that Ca^{2+} binding leads to activation of FVIIa, but we have for the first time directly demonstrated conformational changes in the environment of the active site upon Ca^{2+} binding. Interestingly, this Ca^{2+}-induced conformational change can be noted even in the presence of an inhibitor. Forming a complex with sTF further stabilized this conformational change, leading to a more inaccessible active-site located probe.

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1. Introduction

The blood coagulation cascade is initiated by Ca^{2+}-dependent complex formation between factor VIIa (FVIIa) and its membrane bound cofactor tissue factor (TF). TF:FVIIa activates factor X (FX) and factor IX (FIX), subsequently leading to thrombin formation and finally a fibrin clot [1]. FVIIa is composed of a protease domain (PD), two EGF-like domains (EGF1 and EGF2), and a γ-carboxyglutamic acid (Gla) domain (Fig. 1). Crystal structures of FVIIa lacking its Gla domain show an extended conformation [2,3] similar to that observed for the full-length protein in complex with sTF [4]. Despite available structural data for FVIIa, the solution conformation of free full-length FVIIa is not clear, neither are the details of the structural changes in FVIIa upon Ca^{2+} or sTF binding. The Gla domain of FVIIa is assumed to be flexible in orientation, therefore the lack of structural data. Ca^{2+}-binding to this domain induces conformational changes that facilitate membrane and TF binding [5]. The complete FVIIa is a flexible molecule, based on MD simulation data [6], that becomes motionally restricted when bound to sTF [4].

In structural studies of proteins, where X-ray crystallography or NMR for some reason cannot be applied, labeling techniques offer an attractive approach. The site-directed labeling technique is based on the sensitivity of spectroscopic probes for changes in their surroundings. These fluorescent or spin probes are attached to cysteines introduced at specific positions of the protein of interest by site-directed mutagenesis [7,8]. We have previously exploited site-directed labeling to studies of the protein–protein interaction between sTF and FVIIa [9–11] and how their interacting regions are affected by Ca^{2+} binding [5] and by various inhibitors [12,13]. In addition, we have mapped the area involved in the binding between sTF and FXa in the sTF:FVIIa:FXa:$TFPI_{(1-161)}$ complex by the labeling technique [5].

Abbreviations: EGF, epidermal growth factor-like domain; FVIIa, activated coagulation factor VII; Fl, fluorescein FPR-chloromethyl ketone; Gla, γ-carboxyglutamic acid; PD, protease domain; sTF, soluble tissue factor (residues 1-219); TF, tissue factor; TMR, tetramethylrhodamine-5-maleimide.

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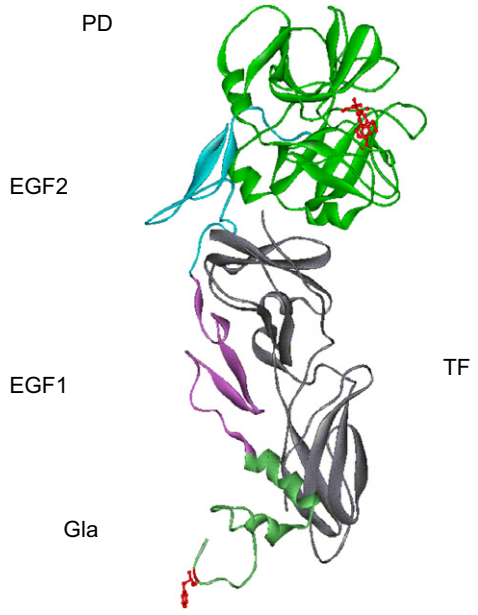

Fig. 1. Picture of sTF:FVIIa, with sTF in grey and the four domains of FVIIa shown. The location of fluorescein is represented by FFR (displayed in red) in the active site of FVIIa and the location of rhodamine is represented by Phe in position 4 (shown in red) in the Gla domain.

The incomplete structural data for full-length FVIIa including its Gla domain and the known flexibility of FVIIa lead to the aim of this study, namely investigation of the conformation of FVIIa in solution and the structural changes upon Ca^{2+} and sTF binding. We have employed a fluorescent labeling approach using FRET between derivatives of two well known fluorescent donor–acceptor pairs, rhodamine and fluorescein. Specifically, tetramethylrhodamine-5-maleimide (TMR) was attached to the Gla domain of FVIIa by coupling to a Cys residue introduced by site-directed mutagenesis. A fluorescein derivative was attached to the active site in the PD via a covalently-linked tripeptide inhibitor, enabling FRET measurements on full-length FVIIa in solution, both free and in complex with sTF. By this method global conformational changes in FVIIa can be detected and distances between the two positions estimated. We also studied quenching of fluorescein-labeled FVIIa by free TMR to detect local conformational changes in the PD of FVIIa caused by Ca^{2+} and sTF binding.

2. Material and methods

2.1. Reagents

TMR was from Molecular Probes, (Eugene, OR, USA) and fluorescein FPR-chloromethylketone (Fl) from Hematologic Technologies, Inc. (Essex Junction, VT, USA). sTF was expressed in *Escherichia coli* and purified using Q Sepharose and FVIIa affinity chromatography as previously described [9]. The protein concentration was calculated from absorption measurements using $\varepsilon_{280nm} = 37{,}440\ M^{-1}\ cm^{-1}$.

2.2. FVIIa mutagenesis and purification

Residue Phe-4 in FVII was replaced by a Cys residue by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA, USA). F4C-FVII was stably expressed in baby hamster kidney cells and purified by a combination of anion-exchange (Q Sepharose, GE Healthcare) and affinity chromatography (sTF-Sepharose). Activation to FVIIa was done by incubation at room temperature for 3 days. The concentration of FVIIa was determined by absorbance measurements ($\varepsilon_{280\ nm} = 63\ 380\ M^{-1}\ cm^{-1}$).

2.3. Fluorescent probe labeling

Fl, in 5-fold molar excess, was reacted with F4C-FVIIa or FVIIa over night at room temperature. Excess inhibitor was removed by Q Sepharose chromatography. For labeling of Fl-F4C-FVIIa with TMR, 50 μl of a slurry of TMR (3.7 mM) was added to a 250-μl mixture of Fl-F4C-FVIIa (0.6 mg/ml), reduced glutathione (0.5 mM), oxidized glutathione (15 μM) and glutaredoxine 2 (10 μM), in 50 mM Hepes, pH 7.0, containing 0.1 M NaCl and 10 mM $CaCl_2$. After 30 min, the redox reaction was terminated by adding cysteine to a final concentration of 0.21 mM, followed by application to a column of MonoQ (GE Healthcare) and elution by Ca^{2+}.

2.4. Labeling degree

The labeling degree was assessed by measuring the absorbance maxima for the fluorescein probe ($\varepsilon_{492\ nm=}\ 79{,}000\ M^{-1}\ cm^{-1}$; Molecular Probes) and for the tetramethylrhodamine probe ($\varepsilon_{541\ nm} = 95{,}000\ M^{-1}\ cm^{-1}$; Molecular Probes). The calculated probe concentration was then related to the protein concentration of FVIIa. The labeling degree of the inhibitor-linked fluorescein was also estimated by measuring the residual amidolytic FVIIa activity using the chromogenic substrate S-2288 (Chromogenix). The residual activity was ~0.5%, i.e. the labeling degree was ~99.5%.

2.5. FRET measurements

Fluorescence emission spectra were recorded on a Hitachi F-4500 spectrophotometer with a thermostated cell compartment at a constant temperature of 20 °C. All measurements were carried out using a 0.5-cm quartz cell and the slits were set to 5 nm for both excitation and emission. Excitation was performed at 469 nm and the emission maximum of the fluorescein probe at 521 nm was used for the FRET calculations. The concentration of the doubly labeled FVIIa (Fl-FVIIa-TMR) in these measurements was 0.2 μM and the concentration of sTF for the complex formation was 0.3 μM. The same concentrations were used as above for the singly labeled FVIIa (Fl-FVIIa) and sTF. The proteins were buffered with 50 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4.

2.6. Fluorescence quenching measurements

Fluorescence emission spectra were recorded on FluoroMax-4 (Horiba Jobin Yvon) thermostated at 20 °C. A 1-cm quartz cell was used and the excitation and emission slits were 2 and 1 nm, respectively. Concentrations of 0.2 μM Fl-FVIIa and 2 mM EDTA were used for the Ca^{2+} free FVIIa sample. A Ca^{2+} containing FVIIa was prepared from 0.2 μM Fl-FVIIa and 5 mM $CaCl_2$. The sTF: FVIIa complex was formed from 0.2 μM Fl-FVIIa, 0.3 μM sTF and 5 mM $CaCl_2$. The proteins were buffered with 50 mM Hepes, 0.15 M NaCl, pH 7.4. All samples were quenched by titration with TMR (0.06 M stock solution). The quenching data were fitted to Stern–Volmer equation [14]:

$$F_o/F = 1 + K_{SV}[Q]$$

where F_o and F are the fluorescence intensities in the absence and presence of the quencher, respectively, [Q] is the concentration of quencher, and K_{sv} is the dynamic quenching constant.

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