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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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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.

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.

Keywords:

Factor VIIa, fluorescence quenching, fluorescence resonance energy transfer, tissue factor, fluorescein, rhodamine, conformational dynamics

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].

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 *E. coli* and purified using Q Sepharose and FVIIa affinity chromatography as previously described [9]. The protein concentration was calculated from absorption measurements using $\varepsilon_{280nm} = 37440\ M^{-1}cm^{-1}$.

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 minutes, 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 ºC. 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 ºC. 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.

3. Results and discussion

3.1. Preparation of fluorophore-labeled FVIIa

To obtain a specific handle for a rhodamine fluorophore (TMR) at one end of the FVIIa molecule, a Cys residue was introduced by site-directed mutagenesis in the N-terminal part of the Gla domain (F4C) (Fig.1). A fluorescein probe was attached to the PD at the other end of FVIIa by covalent linkage of the fluorescently labeled tripeptide inhibitor to His-193 in the active site. The level of modification was assessed by absorbance measurements and for TMR and fluorescein the degree of labeling was 1.14 and 0.97, respectively. In addition, the remaining enzyme activity of Fl-FVIIa after Fl labeling was determined to be below 1 %. Thus, these analyses together demonstrate that the probe positions have been completely utilized and because stoichiometric labeling is crucial for FRET measurements it was important to show that this requirement was fulfilled in our system. The Förster radius (55 Å; Molecular Probes: The Handbook, Invitrogen) of this FRET pair also appears suited for FRET measurements, since the distance between the active site and the N-terminal end in VIIa, as judged from the crystal structure of the sTF:FVIIa complex, is estimated to 94 Å**.**

3.2. Observation of FRET from Fl-FVIIa-TMR

Fluorescence spectra of two samples of 0.2 μM singly (Fl-FVIIa) and doubly (Fl-FVIIa-TMR) labeled FVIIa alone were recorded under identical conditions. Spectra of these labeled FVIIa samples in complex with sTF (0.3 μM) were also acquired (Fig. 2). The buffer solutions were examined and showed no trace of background fluorescence. As expected, the spectra of Fl-FVIIa alone and in complex with sTF were identical (within 0.5% of total intensity).

The emission intensity for doubly labeled FVIIa was lower, compared to the singly labeled protein (0.66 and 1.0, respectively; Fig. 2), as a consequence of the FRET effect, confirming that the fluorophore pair is suitable for FRET measurements in this particular protein. The relative emission intensity was increased to 0.74 upon binding to sTF. This suggests a more compact overall conformation of free FVIIa, since shorter distances between the probes would lead to a larger FRET effect and a lower emission intensity from the Fl donor.

3.3. Analysis of FRET in flexible protein structures

The usual procedure of FRET analysis is based on the fractional change of fluorescence intensity,

$$E = 1 - \frac{F_{DA}}{F_D} \quad . \qquad \text{eq.1}$$

Here F_{DA} and F_D are the observed fluorescence intensity with and without the presence of the acceptor, respectively. The change in intensity can then be related to the distance r between the fluorescent labels using the expression,

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad , \qquad \text{eq.2}$$

where R_0 is the Förster distance characteristic for a particular donor – acceptor pair. The Förster distance for fluorescein-TMR has been determined to 55 Å (Molecular Probes: The Handbook, Invitrogen). By using eqs. 1 and 2, the distance between the two fluorescent probes in free FVIIa and in the complex with sTF could be calculated to 61.4 and 65.5 Å, respectively. This is in sharp contrast to the distance between the attachment sites of 94 Å as judged from the crystal structure. However, in an earlier study we have investigated possible modes of interactions between inserted label(s) and protein(s) [9]. In all cases the lowest energy conformation for each label was detected in or close to the protein surface. Thus, the lengths of the probes including their linkers (12.7 Å for TMR and 26.7 Å for Fl measured

from alpha carbon to fluorophore center of gravity) and possible opposite orientations along the protein surface, could account for the significantly shorter distances estimated from the FRET measurements.

Moreover, because a protein in solution is considered to be much more flexible than what is expected from a crystal structure, some care was taken in the analysis of the apparent FRET distance. As judged from the FVIIa structure (Fig. 1), particularly the TMR probe attached to the Gla domain can be expected to wobble around quite freely. But it is also conceivable that the two halves of FVIIa (the Gla-EGF1 and EGF2-PD domain pairs) move relative to each other when not bound to TF. As the photo-physical FRET and fluorescence processes occur at a time scale much shorter than larger motions of domains one can anticipate that the protein with the donor and acceptor labels exists in a distribution of conformations with different distances between the two.

In this context it is relevant to characterize the dynamics of the proteins both isolated in solution and in complex. Recent MD simulations of the dynamic properties of FVIIa and sTF docked to a membrane have shown that there is a considerable difference in the dynamics between FVIIa in the free form and bound to sTF, the latter being much more rigid [6]. Domain flexibility of free FVIIa in solution has also been shown by small-angle X-ray scattering studies [15]. We extend the FRET analysis to investigate how protein dynamics can influence the apparent fluorescence signal. A flexible structure should be anticipated to give rise to a broader signal distribution than a more rigid structure. As the average of a time evolution of a stationary process is identical to the ensemble average (the ergodic principle) the results of such dynamic simulations can guide in the interpretation of our spectroscopic FRET data. We assume that the average distance between the probes is 65Å (close to our apparent experimental finding for the sTF:FVIIa complex). The dynamics can be interpreted

in terms of the uncertainty in positions and distances between residues as in the MD simulations [6]. The most straightforward approach is then to model this uncertainty in terms of a random distribution of distances using a Gaussian distribution. In Figure 3A the distance distributions centred at 65 Å are shown for different Gaussian variances of 2, 5, 10, and 15 Å, respectively. By setting the fluorescence intensity of the donor to unity we may use the Förster distance (55 Å) and eqs. 1 - 2 to calculate the apparent fluorescence signal from the FRET phenomenon weighted for each donor-acceptor distance. Such plots are shown as the dashed curves in Figure 3A. Integrating over all distances we obtain the total apparent fluorescence intensity for each distribution as shown in Figure 3B. Obviously, shorter distances give a larger reduction of the associated fluorescence signal due to the strongly nonlinear expression of eq. 2 and taken together over the whole distribution of distances, it results in a lower total fluorescence signal for a more spread or uncertain distribution of distances, even though they are centred around the very same average distance. For the narrowest distribution (2Å) we obtain the same value as expected directly from eqs.1 and 2 for a fixed distance: a reduction of the intensity from 1 to 0.75. However, when increasing the variance of the spread to 5, 10 and 15 Å the apparent "FRET-signal" will be reduced to 0.74, 0.71 and 0.67, respectively. We note that the first value (0.75) is close to the apparent FRET-signal for the sTF:FVIIa complex (experimental = 0.74), whereas the last value 0.67 is close to the FRET response for free FVIIa (experimental = 0.66). Therefore, the measured data might be interpreted as a random distribution centred around a mean value of approximately 65 Å (s.d.= 2 Å) for the sTF:FVIIa complex giving a FRET response around 0.74 and a more broad distribution (s.d. = 15 Å) centred around the very same average distance for free FVIIa giving a reduction to 0.66 from the FRET effect. In this context it is striking that similar average C^{α} mean square deviations were obtained from the simulations by Ohkubo and coworkers [6] as those discussed above (12.34 ± 3.91 Å for FVIIa and 2.94 ± 0.41 Å for the

sTF:FVIIa complex). Thus, when taking the possible dynamic properties of free or TF-bound FVIIa into account, the apparent difference in probe distance is close to zero which would, if true, indicate that the average length of the global conformation of FVIIa is almost unchanged upon binding to sTF. This is in line with the MD simulation data [6] concluding that FVIIa in free form seems to undergo large structural fluctuations that are significantly restricted in complex with sTF. Rapidly equilibrating ensembles of states containing FVIIa conformations resembling that in complex with sTF will facilitate association to sTF. Thus, the fact that FVIIa does not need to undergo significant global conformational changes upon ligation will of course make its docking to the rigid sTF cofactor easier leading to increased affinity and stability. However, given the extended conformation of FVIIa bound to TF [4] and the existence of a hinge around residue 88 located between the two EGF-like domains [2], one would intuitively expect the average distance between the two probes attached to FVIIa in this study to be shorter in the absence of TF.

3.4. TMR used as a quencher to reveal local conformational changes

The FRET effect between fluorescein and TMR in the doubly labeled FVIIa was employed in dynamic quenching studies of singly labeled Fl-FVIIa. The fluorescein fluorophore should be rather exposed, since it is positioned at the outer end of the tripeptide protruding from the active site of the PD. The more accessible the fluorescein label is to TMR, the more efficiently the FRET will be leading to quenching of the donor (fluorescein) emission. For relatively exposed fluorescent probes, like fluorescein in this case, a larger quenching molecule, like TMR, than those usually used (e.g. iodide, acrylamide) would be more sensitive to subtle conformational changes because the access would be more efficiently hindered. The use of a relatively large FRET acceptor as a quencher is a methodological approach that would be of general interest in studies of minor surface conformational changes.

By measuring the ability of TMR to quench Fl-FVIIa we attempted to detect local conformational changes in (FPR-inhibited) FVIIa. It is well-known that Ca^{2+} binding to FVIIa influences enzymatic activity. In the absence of Ca^{2+}, FVIIa exhibits only ~10% enzymatic activity [16-18]. Thus, it would be of particular interest to probe the conformation of the active site of free FVIIa in the absence and presence of Ca^{2+} as well as with sTF in the presence of Ca^{2+}. The results of the measurements in terms of the resulting Stern-Volmer plots are shown in Figure 4. In the absence of Ca^{2+} (EDTA-treated protein), the fluorescein probe was efficiently quenched with a quenching constant of $0.012 \pm 0.0013\ \mu M^{-1}$. The addition of Ca^{2+} reduced the quenching efficiency to approximately half ($0.006 \pm 0.0005\ \mu M^{-1}$). Thus, Ca^{2+} binding to FVIIa appeared to alter the active-site environment in a way that reduced the accessibility of the fluorescein probe. Furthermore, complex formation of Fl-FVIIa with sTF led to a quite inaccessible fluorescein probe. Since the interaction area between FVIIa and sTF is located on the opposite side of the active-site bound probe no direct reduction in probe accessibility can come from the complex formation *per se*. Evidently, sTF binding to FVIIa instead allosterically leads to a conformational change in the vicinity of the probe, and the active-site environment is most likely altered via stabilization of the so-called 170 loop in a position that protects the fluorophore located in the S3/S4 subsite region.

4. Concluding remarks

The distance between the probes located in the PD and the Gla domain of FVIIa, alone and in complex with sTF, were determined by FRET measurements. This could lead to the conclusion that inhibited FVIIa does not seem to undergo large structural changes upon binding to sTF when taking the dynamics of free FVIIa into account. As previously mentioned, to allow for the relatively short distance as measured in the FVIIa:sTF complex it would be required that the probes stretch towards each other. It adds to the complexity of data

interpretation, if not only different dynamics, but also potentially different probe orientations in free and TF-bound FVII are considered. Because the probes are not located at the cofactor interface it is, however, most likely that they are oriented in a similar way in free FVIIa and in the complex.

Throughout this study FVIIa was inhibited by the fluorescein FPR-chloromethylketone fluorophore attached to the active site, and chloromethylketone-inhibited FVIIa is known to have a conformation resembling the most active form of the protein, that is with a stabilized PD and the N-terminus inserted into the activation pocket [2,3]. Conformational changes in the environment of the active-site upon Ca^{2+} binding have previously been inferred from Ca^{2+}-stimulated enzyme activity and other indirect measurements [18], but are now for the first time directly demonstrated. Interestingly, this Ca^{2+}-induced conformational change can be noted even in the presence of the inhibitor.

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Figure legends

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.

Fig. 2. Emission spectra of singly and doubly labeled FVIIa with and without sTF. The excitation wavelength was 469 nm. Curve colors: Fl-FVIIa (green); Fl-FVIIa + sTF (blue); Fl-FVIIa-TMR (black); Fl-FVIIa-TMR + sTF (red). The spectra of Fl-FVIIa (green) and Fl-FVIIa : sTF (blue) were normalized to have unit amplitude. The spectra of Fl-FVIIa-TMR (black) and Fl-FVIIa-TMR: sTF (red) are plotted using the same normalization constant as for the singly labeled protein.

Fig. 3. (A) Random Gaussian distribution of protein structures with average distance between donor-acceptors 65 Å (solid lines) and with standard deviation 2 (black), 5 (red), 10 (green) and 15 Å (blue). Note that each distribution is normalized to represent the same total number of conformations. The associated fluorescence 'FRET intensities' calculated using eqs. 1 and 2 as each weighted with the distributions of conformations are also shown (dashed curves). (B) The total integrated fluorescence signal due to FRET for a distribution of donor-acceptor distances with the same average distance but different spread in terms of standard deviation of a random (Gaussian) ensemble. Color code for standard deviations is as in figure 3A.

Fig. 4. TMR quenching of the fluorescein fluorescence in Fl-FVIIa. The graphs are fitted to the Stern-Volmer equation, where F and F_o represent the area of the fluorescence intensity

with and without the quencher, respectively. Curve colors: Fl-FVIIa in the absence of Ca^{2+} (black squares), Fl-FVIIa in the presence of Ca^{2+} (green diamonds), and sTF:Fl-FVIIa in the presence of Ca^{2+} (red triangles).