Calcium-Induced Conformational Change in Fragment 1-86 of Factor X

A. HÄFNER,[1] F. MEROLA,[2] G. DUPORTAIL,[3] R. HUTTERER,[1] F. W. SCHNEIDER,[1] M. HOF[4]

[1] Institute of Physical Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

[2] Laboratoire pour L'Utilisation du Rayonnement Electromagnetique, Batiment 209D, Centre Universitaire Paris-Sud, B.P. 34, 91898 Orsay Cedex, France

[3] UMR CNRS 7034, Department of Pharmacy, Louis Pasteur University of Strasbourg, B.P. 24, 67401 Illkirch, France

[4] J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Dolejškova 3, 18223 Prague 8, Czech Republic

Received 19 October 1999; revised 28 December 1999; accepted 14 January 2000

ABSTRACT: Time-resolved fluorescence of the single tryptophan residue Trp41 in fragment 1-86 of factor X (FX F1-86) is studied using a time-correlated single photon counting technique with synchrotron radiation as the excitation source. Calcium ions are believed to induce a conformational change in the N-termini of the activated factor X and other vitamin K dependent proteins, which is accompanied by a decrease in fluorescence intensity. The titration with calcium yields a sigmoidal fluorescence titration curve with a transition midpoint concentration of 0.44 m*M*. The wavelength-dependent tryptophan fluorescence decays of the apo-FX F1-86 (in the absence of calcium) and Ca-FX F1-86 are characterized by conventional multiexponential analysis and fluorescence lifetime distribution analysis. In the *absence of calcium* there are *three* significant classes of fluorescence lifetimes (ns) that are nearly wavelength independent: 0.55 ± 0.08 (component A), 2.6 ± 0.1 (component B), and 5.3 ± 0.3 (component C). However, their preexponential amplitudes vary with wavelength. The decay associated emission spectra of the individual components show that components B and C contribute over 85% to the total fluorescence for all examined wavelengths. However, in the *presence of calcium*, the analysis of the time-resolved fluorescence data of Ca-FX F1-86 yields *four* wavelength-independent lifetimes (ns) of 0.30 ± 0.09 (component D), 0.65 ± 0.10 (component A), 2.7 ± 0.2 (component B), and 5.4 ± 0.3 (component C). Calcium addition to the apo-FX F1-86 leads to a decrease in the fluorescence intensities of components B and C while their decay times remain unaffected. In Ca-FX F1-86 an additional component D arises that has a decay time of 0.30 ns and that contributes up to 35% to the total fluorescence intensity. A comparison with a previous investigation of prothrombin fragment 1 demonstrates the extensive structural and functional homology between the N termini of prothrombin and factor X_a. © 2000 John Wiley & Sons, Inc. Biopolymers (Biospectroscopy) 57: 226–234, 2000

Keywords: calcium; fragment 1-86 of factor X; time-resolved fluorescence; time-correlated single photon counting; synchrotron radiation

Correspondence to: M. Hof (martin.hof@jh-inst.cas.cz).

Contract grant sponsor: Deutsche Forschungs Gemeinschaft (to M.H.).

Contract grant sponsor: Czech Academy of Sciences (to M.H); contract grant number: A4040905.

Contract grant sponsor: European Community; contract grant number: BF 005-97.

Biopolymers (Biospectroscopy), Vol. 57, 226–234 (2000)

INTRODUCTION

Blood coagulation consists of a cascade of proteolytic enzyme reactions. The last step of the cascade is the factor X_a catalyzed proteolysis of prothrombin by the prothrombinase complex. This membrane bound complex consists of factor X_a, cofactor V_a, and bound calcium ions. The membrane contains negatively charged phospholipids.[1] The acidic phospholipids exert a strong influence on the catalytic activity of the prothrombinase. Certain acidic phospholipids increase the turnover number of prothrombin considerably,[2–4] and phosphatidyl-L-serine is the most efficient.[2,3] The molecular background of this extreme lipid specificity is still unknown, which gives the motivation for studying the molecular mechanism of the interactions among the proteins, the calcium ions,[5] and the negatively charged membrane surface.[6]

The two proteins factor X and prothrombin, which are the enzyme and substrate of the prothrombinase reaction, respectively, belong to the class of vitamin K dependent proteins and are homologous in their N termini. It has been assumed that these proteins are bound to negatively charged phospholipid head groups by calcium ions. The region that is predominantly responsible for calcium and membrane binding is the γ-carboxyglutamic acid residues domain (Gla domain) where the calcium ions are chelated by Gla residues. In the case of factor X, 12 Gla residues chelate the calcium ions.[7] The calcium ions induce a conformational change in the Gla domain, yielding the native conformation of the protein that, in turn, is necessary for membrane binding.[8]

Recent picosecond time-resolved fluorescence studies[5] provided new insights that make a significant contribution to the understanding of the calcium-induced conformational change in prothrombin fragment 1 (BF1). BF1 consists of 156 amino acids that build the Gla domain and the "kringle" domain, and it contains three tryptophan residues: one tryptophan residue is located in the Gla domain (Trp42) while the other two are buried in the "kringle" domain.[9] In the present study we investigate the calcium-induced conformational change of the 1-86 amino acid N terminus of factor X_a (FX F1-86), which contains the N-terminal Gla domain followed by an epidermal growth factor like domain (Fig. 1). The Gla domain of factor X_a is highly homologous to that of BF1.[9] FX F1-86 contains only a single tryptophan residue (Trp41) that is located in the membrane- and calcium-binding part of the protein, which is the Gla domain (Fig. 1). The calcium-binding properties of the Gla domain are very similar to those of the entire protein.[10] Thus, the factor X fragment is an excellent model compound for investigating the interaction between the N-terminal end of vitamin K dependent proteins, calcium ions, and certain phospholipids.

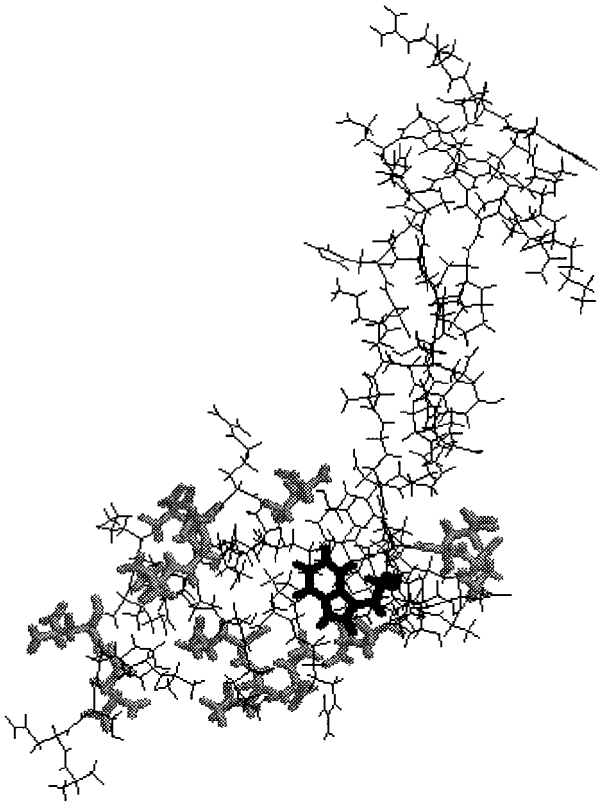

Figure 1. The 2-dimensional NMR structure of factor X fragment 1-86 without calcium (apo-FX 1-86). The coordinates were taken from the Brookhaven Protein Data Bank entry[7] and were displayed by RasMol software (R. Sayle, Glaxo Wellcome Research and Development, Stevenage, Hertfordshire, U.K.). The Trp41 is located in the Gla domain, which is shown on the right site; the EGF domain is on the left site. The black structures are the Trp41, and the hatched grey structures are the Gla residues.

MATERIALS AND METHODS

Protein

Bovine factor X was isolated according to the procedure of Esnouf et al.[11] Factor X fragment 1-86

was cleaved by tryptic digestion and purified by chromatography on a mono Q column by the method of Persson et al.[12] The homogeneity was checked by SDS-PAGE. The amino acid sequence of the Gla domain was determined by sequence analysis and it was in line with the literature sequence.[7] The concentrations of bovine factor X fragment 1-86 were determined by UV spectrophotometry at 280 nm ($E^{1\%}_{1cm}$ = 9.9, molecular mass = 10.3 kDa). The protein solutions were stored in Tris-buffered saline (0.05*M* Tris, 0.1*M* NaCl, pH 8.0) at −30°C before use. All experiments were carried out in Tris-buffered saline (0.05*M* Tris, 0.1*M* NaCl, pH 7.5).

Fluorescence Measurements

Steady-State Measurements

Stationary fluorescence spectra were obtained with a SLM Aminco–Bowman series 2 luminescence spectrometer. Fluorescence emission spectra for calculating the decay associated emission spectra (DAS) in the absence and presence of calcium ions were determined at an excitation wavelength of 295 nm. Bandwidths of 2 nm were set for excitation and for emission. The spectra were corrected for instrument response and Raman scattering.

Calcium Titration

Fluorescence titrations were performed in 1.0-mL volumes containing 2 μM FX F1-86 by adding aliquots of a 2 and 20 m*M* $CaCl_2$ stock solution. The fluorescence intensities were obtained by determining the mean value of the fluorescence emission intensity over 10 s at 350 nm (8-nm bandwidth) 1 min after adding the titrant. In addition, each fluorescence emission spectrum was integrated from 310 to 390 nm. The excitation wavelengths were set at 280 and 295 nm (2-nm bandwidth). All intensities were corrected for dilution.

The titration curve recorded at an excitation wavelength of 295 nm was fitted according to the Hill equation[13]:

$$R = \frac{f \times [Ca^{2+}]^n}{K_d + [Ca^{2+}]^n}$$

where R is defined as I/I_0; I is the observed fluorescence; I_0 is the intensity in the absence of Ca^{2+}; K_d is the dissociation constant; n is the Hill coefficient; and f is a constant value of R, when no further quenching occurs after calcium addition.

Time-Resolved Measurements

Fluorescence lifetime measurements were performed at the Laboratoire pour L'Utilisation du Rayonnement Electromagnetique (LURE, Orsay) using the single photon counting technique as summarized in the literature.[14,15] The optical and electronic parts of the instrumental setup are described elsewhere.[16] The synchrotron radiation of the SUPER-ACO at LURE was used for pulsed excitation of approximately 650 ps full width at half-maximum at a frequency of 8.3 MHz. The excitation wavelength of 295 nm was polarized and the emission was detected after passing a "magic angle" polarizer with a microchannel plate photomultiplier. Up to 50,000 counts of fluorescent photons were collected in the peak and the time scale was 24.93 ps per channel with 2048 channels collected. The fluorescence decays of apo-FX F1-86 and Ca-FX F1-86 were collected at emission wavelengths of 315–405 nm in 7.5-nm steps. Experiments under apo conditions (i.e., in the absence of calcium ions) were carried out in 5 μM FX F1-86 in Tris-buffered saline while the Ca experiments were performed in 10 μM FX F1-86 in Tris-buffered saline at 20°C. The $CaCl_2$ concentration in the Ca-FX F1-86 samples was 7.0 m*M*. The fluorescence was measured 30 min after $CaCl_2$ addition.

Data Analysis

Different analytical methods were applied for data analysis. A nonlinear least-squares iterative reconvolution procedure based on the Marquardt–Levenberg algorithm was used for the basic multiexponential analysis.[17,18] According to the following equations, the results of these analyses were used to calculate the fractional intensity f_i and fractional amplitude a_i of each component. In addition, the Edinburgh Analytical Instruments (EAI) distribution program[19] was employed to characterize the number of fluorescence lifetimes. In these programs the time shift calculation was included in the iteration. In addition to the EAI distribution program, a program employing the maximum entropy method (MEM)[20,21] was used for determining the number of lifetimes. In contrast to the conventional multiexponential analysis, there is no *a priori* assumption about the shape of the distribution. In-

stead, the two programs (EAI distribution and MEM) fit the fluorescence decay using an arbitrary sum of 100 (EAI) and 150 (MEM) lifetimes. In the MEM analysis a systematic correction for variable time shifts was applied as discussed by Garcia et al.[22] (and references therein). This correction is critical for a stable recovery of fluorescence lifetime distributions, especially in studies using variable settings of the emission monochromator. Avoiding possible compensations or crosstalks due to the simultaneous variation with wavelength of protein fluorescence and the optical path, the time offset over the detected emission range was determined independently using a solution of *n*-acetyltryptophanamide under identical optical settings. The variations of this time offset where found to be perfectly correlated with the monochromator position.

DAS

The DAS $F_i(\lambda)$ for each component i were obtained by combining time-resolved data with the total steady-state emission spectrum $F(\lambda)$ according to the following equation[23]:

$$F_i(\lambda) = \frac{A_i(\lambda) \times \tau_i(\lambda)}{A_i(\lambda) \times \tau_i(\lambda)} \times F_i(\lambda)$$

where A_i and τ_i are the preexponential contribution and lifetime of component i, respectively.

RESULTS

Steady-State Experiments

Calcium titration experiments were carried out using two different excitation wavelengths (λ_{exc}) of 280 and 295 nm. Excitation at 280 nm is known to create both tryptophan (Trp41) and tyrosine (Tyr44, Tyr68) excited states.[23] In analogy to Persson et al.[24] we used an excitation wavelength of 280 nm and an emission wavelength of 350 nm. We found titration curves similar to Persson et al.[24] who observed an initial fluorescence increase with a half-maximum at around 0.1 m*M* $CaCl_2$ followed by a fluorescence quenching of about 60%. (For details see figs. 10 and 11 in Persson et al.[24]) This observation is in agreement with the performed isolation procedure,[12] which yielded identical proteins as investigated in Persson et al.[24] We used excitation at 295 nm to exclusively excite the tryptophan residue Trp41. Again, the emission wavelength was set to 350 nm with a bandwidth of 8 nm. Here the titration curve differed markedly from the titration curve using 280-nm excitation. The addition of calcium ions led to a quenching of the intrinsic fluorescence emission of FX F1-86 with a decrease of the total fluorescence intensity of about 70%. As shown in Figure 2, a sigmoidal curve with a transition midpoint concentration (T_m) at 0.44 m*M* was observed. We did not find any evidence for the initial increase observed in Persson et al.[24] using 280-nm excitation. The sigmoidal shape indicated that binding of calcium ions was cooperative. The cooperative nature was also reflected in the parameters resulting from the fit according to the Hill equation (2.86 and 0.10 m*M* for the Hill coefficient and K_d, respectively). The observed fluorescence transition appeared to be saturated at 2 m*M* Ca^{2+}. Thus, the $CaCl_2$ concentration of 7 m*M* used in the characterization of the time-resolved fluorescence of Ca-FX F1-86 guaranteed that almost the entire amount of protein exhibited a Gla domain conformation that was responsible for the decrease of tryptophan fluorescence.

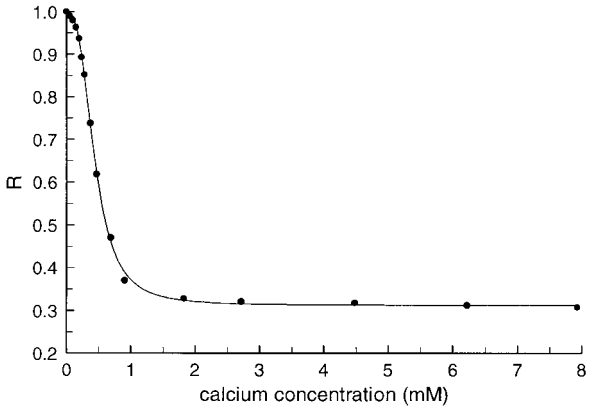

Figure 2. The calcium titration curve of FX F1-86 for the fractional fluorescence ($I/I_0 = R$) versus Ca^{2+} concentration; λ_{exc} = 295 nm.

Characterization of Fluorescence Decay of apo-FX F1-86 and χ^2 values

In the absence of calcium a multiexponential analysis of the fluorescence decay of FX F1-86 (detected from 315 to 405 nm) yielded four components at all wavelengths. The lifetimes of all components obtained from 337.5 to 405 nm did not change with increasing emission wavelength (Fig. 3). The lifetimes of components B and C at

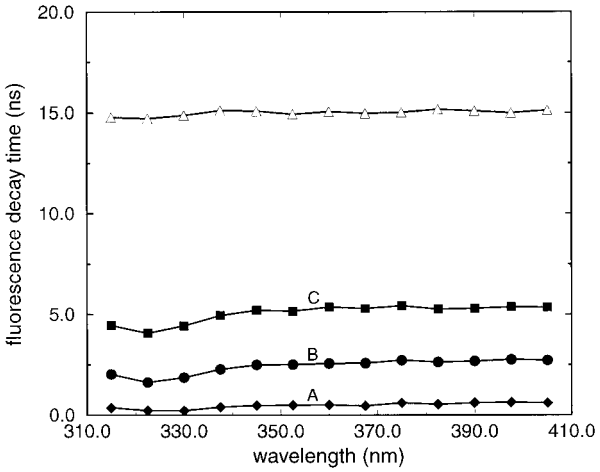

Figure 3. The fluorescence decay times of apo-FX F1-86 (5 μM) that are dependent on the emission wavelengths in Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). The lifetime values (ns) of the significant components are (♦) 0.55 ± 0.08 (component A), (●) 2.60 ± 0.1 (component B), and (■) 5.3 ± 0.2 (component C). (△) The 15-ns component with a contribution of <2%.

315, 322.5, and 330 nm differed slightly from these wavelength-independent values. Strictly speaking, these deviations, which were most pronounced at 322.5 nm for components B and C, might argue against a model with three wavelength-independent major components. However, the obtained high χ^2 values (>1.4) at these wavelengths indicated that the quality of those data was somewhat lower than the quality of the data collected at higher wavelengths ($\chi^2 < 1.2$). The lifetime-linked global analysis of the entire wavelength data set yielded a global χ^2 of 1.32, which certainly does not argue against wavelength-independent lifetime values. We favor a model with three wavelength-independent lifetimes for components A, B, and C where the respective lifetime values are 0.54 ± 0.08, 2.6 ± 0.1, and 5.3 ± 0.2 ns. For example, at 367.5 nm, the fractional intensities are 0.05, 0.50, and 0.44 and the corresponding fractional amplitudes are 0.25, 0.52, and 0.23 for the three major components A–C, respectively, at 0.54 (component A), 2.6 (component B), and 5.3 (component C) ns. Moreover, a long-lifetime component (≈15 ns) was detected at all wavelengths in the absence of calcium. It is neglected, because the fractional intensities and corresponding fractional amplitudes of this component were less than 0.02 and 0.005, respectively, at all examined emission wavelengths, although we are aware of discussions concerning "long lifetime components" in tryptophan fluorescence.[25] It should be stressed that the existence of four different lifetimes was supported by two different methods (EAI and MEM) of lifetime data analysis. Combining steady-state intensities with the various percentage contributions of the decays led to the DAS. As shown in Figure 4, components B (2.6 ns) and C (5.3 ns) contributed over 85% to the total fluorescence at all examined wavelengths. The emission maxima (nm) of the polynomial fits of the DAS were 333 (component A), 346 (component B), and 350 (component C).

Characterization of Fluorescence Decay of Ca-FX F1-86

The fluorescence decays in the presence of calcium were detected from 315 to 405 nm in 7.5-nm steps. To characterize the decay behavior of Ca-FX F1-86, the apo-FX F1-86 analysis procedure was applied. Multiexponential analysis of the time-resolved data of Ca-FX F1-86 yielded four decay times (ns): 0.30 ± 0.09 (component D), 0.65 ± 0.10 (component A), 2.7 ± 0.2 (component B), and 5.4 ± 0.3 (component C). The individual contributions of the components were again illustrated by the fractional intensities of 0.17 (component D), 0.17 (component A), 0.41 (component B), and 0.25 (component C) or corresponding frac-

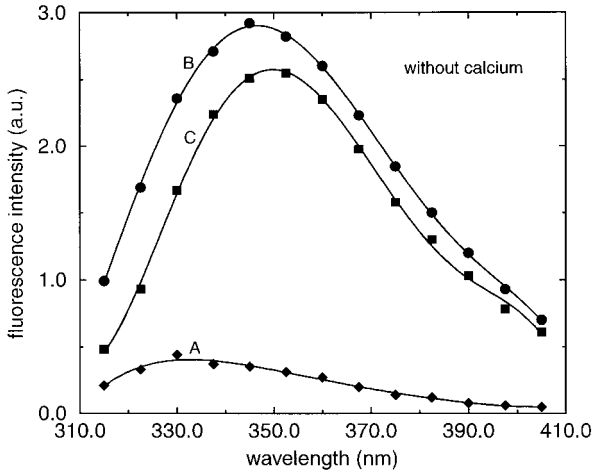

Figure 4. Decay-associated emission spectra of apo-FX F1-86 in Tris-buffered saline (λ_{exc} = 295 nm). The emission maxima (nm) of the polynomial fits are (♦) 332.0 (component A), (●) 345.9 (component B), and (■) 349.9 (component C). The experimental points are the symbols, and the polynomal fit functions are the solid lines.

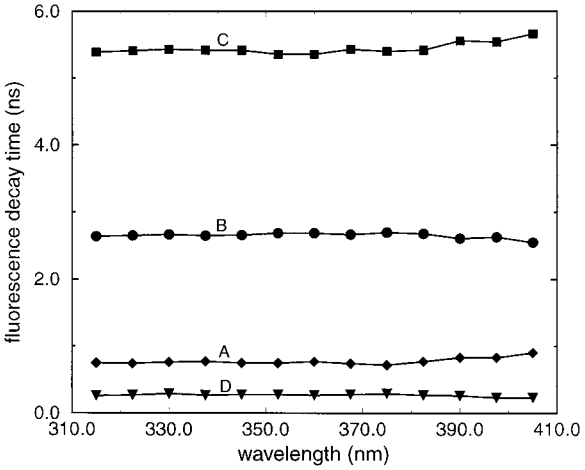

Figure 5. Fluorescence decay times of Ca-FX F1-86 (10 μM) in Tris-buffered saline that are dependent on the emission wavelength (λ_{exc} = 295 nm). The lifetime values (ns) are (▼) 0.30 ± 0.09 (component D), (♦) 0.65 ± 0.10 (component A), (●) 2.70 ± 0.2 (component B), and (■) 5.40 ± 0.3 (component C).

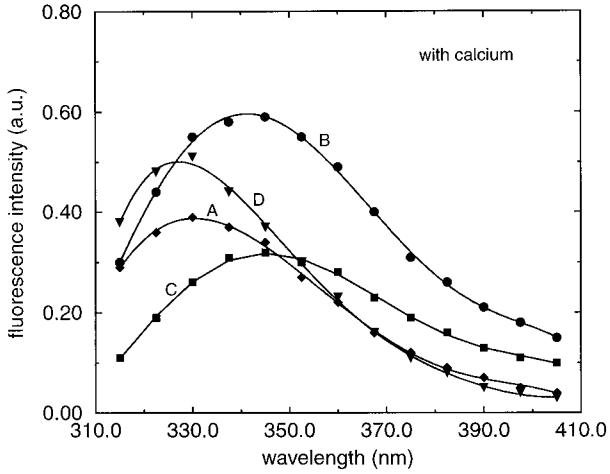

Figure 7. Decay-associated emission spectra of Ca-FX F1-86 in Tris-buffered saline (λ_{exc} = 295 nm). The experimental points are the symbols, and the polynomial fit functions are the solid lines. The emission maxima (nm) of the polynomial fits are (▼) 326.5 (component D), (♦) 330.3 (component A), (●) 341.0 (component B), and (■) 345.2 (component C).

tional amplitudes of 0.55 (component D), 0.25 (component A), 0.15 (component B), and 0.05 (component C) for the four components (0.30 ns for component D, 0.65 ns for component A, 2.7 ns for component B, and 5.4 ns for component C) obtained by the analysis of the data collected at 367.5 nm. Thus, an additional subnanosecond component with a decay time of ≤300 ps appeared. For all detected emission wavelengths, the decay times remained nearly constant (Fig. 5) while the amplitudes varied with wavelength.

These results were confirmed by a MEM distribution analysis as shown for the decay at 360 nm (Fig. 6). Polynomial fits of the DAS (Fig. 7) yielded the following fluorescence emission maxima: component A at 330 nm, component B at 341 nm, component C at 345 nm, and component D at 327 nm. The addition of calcium ions led to a strong decrease of the fluorescence intensities for the B and C components (Fig. 8). By contrast, the inten-

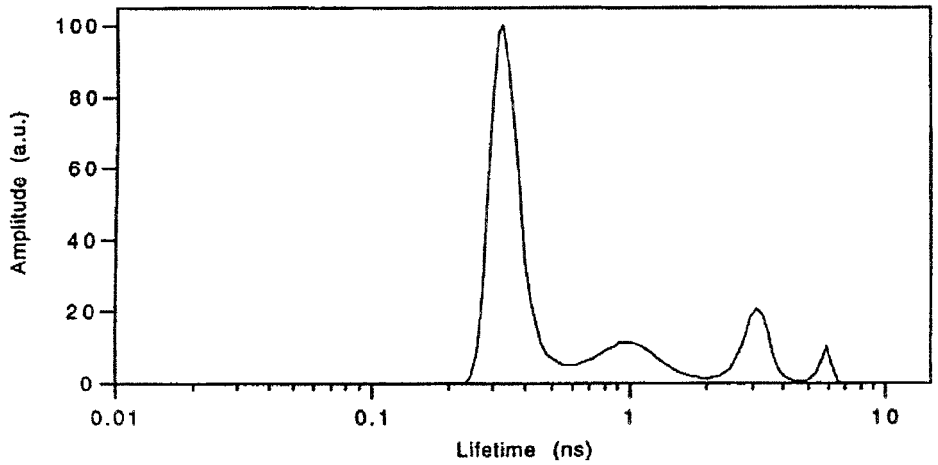

Figure 6. The MEM distribution of lifetimes analyses of Ca-FX F1-86 shown for the emission wavelength of 360 nm. The lifetime values (ns) are 0.35 ± 0.06 (component D), 1.01 ± 0.16 (component A), 3.05 ± 0.13 (component B), and 5.73 ± 0.03 (component C).

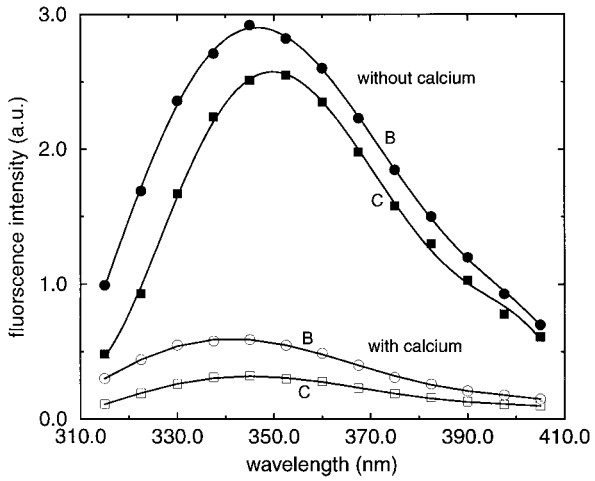

Figure 8. A comparison of the DAS of components (■, □) B and (●, ○) C (■, ●) before and (□, ○) after calcium addition.

sity of component A (0.65 ns) remained nearly unchanged upon calcium addition.

DISCUSSION

The calcium titration resulted in a sigmoidal shaped curve with a T_m value of 0.44 m*M*, which is in line with analogous investigations on prothrombin fragment 1 (0.18 m*M* in Bajaj et al.,[26] 0.19 m*M* in Bloom and Mann,[27] 0.35 m*M* in Prendergast and Mann,[28] and 0.4 m*M* in Nelsestuen[29]). Considering the extensive homology between the Gla domains of prothrombin and factor X/X_a, this was certainly not a surprising result. At first glance, however, our calcium titration results were in disagreement with the experiments performed in another group.[24] However, the experimental conditions (excitation at 280 nm instead of 295 nm as in this study) in Persson et al.[24] showed that, in addition to Trp41, the two tyrosines (Tyr44, Tyr68) present in FX F1-86 contribute to the fluorescence. Thus, the measured signal in Persson et al.[24] is a superposition of both intrinsic tryptophan and tyrosine fluorescence[23] and it is therefore not a valid indicator for any conformational changes in the microenvironment of Trp41 alone.

The fluorescence decay behavior of apo-FX F1-86 was characterized by the three components at 0.54 ± 0.08 (component A), 2.6 ± 0.1 (component B), and 5.3 ± 0.2 ns (component C), which might be assigned to three different conformational states of the microenvironment of the single Trp41 residue. The conformational substates B and C with respective lifetimes of 2.6 and 5.3 ns contribute over 85% to the total fluorescence (see DAS in Fig. 4) whereas the fractional intensities of component A are less than 10% for all examined wavelengths (Fig. 4). As a matter of fact, the lifetime values of components B and C are in good agreement with the decay times determined for the comparable Trp42 in the apo-prothrombin fragment 1 (BF 1, 2.5 and 5.3 ns),[5] which gives further evidence for an extensive structural homology within the Gla domains of prothrombin and factor X/X_a.

After calcium addition we observed four discrete lifetimes: 0.30 ± 0.09 (component D), 0.65 ± 0.10 (component A), 2.7 ± 0.2 (component B), and 5.4 ± 0.3 ns (component C). Apparently, the lifetime values of components A, B, and C are very close to those found for apo-FX F1-86. While the DAS of component A appears to not be affected by calcium addition, the intensities of components B and C strongly decrease (Figs. 7 and 8). Thus, a decrease of the population of the fluorescent substates B and C appeared to be responsible for the observed total fluorescence decrease of about 70%. We must stress that we found essentially the same quenching patterns for the two main fluorescence components B and C of the Gla-tryptophan in a previous study on prothrombin fragment 1. While the lifetime values (2.3 and 5 ns, respectively) did not change within experimental error after the addition of calcium, the DAS of both components showed large intensity decreases. The X-ray structure of Ca-BF1 showed that calcium addition stabilized the interaction between the disulfide bridge Cys18-Cys23 and the aromatic cluster Phe41, Trp42, and Tyr45. Fluorescence and phosphorescence studies of other proteins gave strong evidence for the existence of specific disulfide–tryptophan or disulfide–tyrosine interactions.[30–34] Fluorescence studies on tyrosine and tryptophan residues in model compounds and proteins indicated that the interaction between the aromatic π system and the disulfide bridge leads to a quenching pattern that is more static (formation of a ground-state complex) than dynamic.[33,34] The Gla domain of factor X_a displays extensive homology to residues 1–48 of prothrombin: the Gla-tryptophan Trp41 is part of an aromatic cluster with residues Phe40 and Tyr44, and a disulfide bridge (Cys17-Cys22) is located in the vicinity of Trp41. Furthermore, the extensive homology among the N termini of both

proteins is demonstrated by the nearly identical lifetime values of the 2.5- and 5-ns components of the Gla-tryptophans in the presence and absence of calcium. Although no structural data about Ca-FX F1-86 are available at the present time, in our opinion the striking similarities in the fluorescence lifetimes and the calcium-induced quenching pattern, together with the homology derived from the amino acid sequence, justify the speculation that a ground-state adduct between Trp41 and Cys17-Cys22 might be formed.

The second significant calcium-induced change in the fluorescence of FX F1-86 was the appearance of the 300-ps component. As seen from the calcium-induced increase of the 0.23-ns component of prothrombin fragment 1 (fig. 7 in Hof et al.[5]), the calcium-induced appearance of such a highly quenched component is another common feature of Ca binding to BF1 and FX F1-86. We interprete this component in terms of a conformation that is characterized by the close proximity (<0.5 nm) of Cys17-Cys22 to Trp41, which is not present in apo-FX F1-86. Apparently, the tryptophan-disulfide ground-state complex formed fluoresces and cannot be considered as a purely dark, statically quenched complex between the fluorophore and the quenching agent (i.e., Cys17-Cys22). The ratios between the average lifetimes of Ca-FX F1-86 and apo-FX F1-86, however, were substantially larger than the corresponding ratios of the steady-state fluorescence intensities at all examined wavelengths. A 69% decrease in the steady-state intensity and a 48% decrease in the first-order average fluorescence lifetime, both detected at 337.5 nm, might serve as an illustration. A possible explanation for this obvious difference might be that the highly efficient quenching leading to the 300-ps component was accompanied by static quenching in terms of the existence of a dark complex. Analogous to phosphorescence studies on proteins exhibiting such disulfide–π interactions,[32] one might speculate that an electron transfer from the tryptophan excited state to the disulfide bridge might have resulted in the observed fast (300 ps) quenching process. Further arguments for the suggested electron transfer model for the quenching of tryptophan fluorescence by disulfides are given by Mérola et al.[30] Although our original motivation for investigating FX F1-86 was to furnish more precise information about a possible lipid-specific conformational change in the Gla domain, it should be mentioned that FX F1-86 represents a good model compound for more detailed photophysical studies of such disulfide–π interactions.

The simplest explanation for the remaining fluorescence of components B and C after calcium addition (Fig. 8) would be a substantial residual portion of apo-FX F1-86. Considering the calcium titration curve (Fig. 2) and a calcium concentration of 7 m*M*, however, the possibility of a significant portion of apo-FX F1-86 in the time-resolved experiment has to be neglected. As for BF1, we suggest that the remaining fluorescence of components B and C after calcium addition could be due to Gla conformations of Ca-FX F1-86, which are characterized by microenvironments of Trp41 that are similar to those in apo-FX F1-86. If this hypothesis is correct, an equilibrium between the Trp41 microenvironment in its unquenched form and the tryptophan–disulfide adduct exists, which was already suggested for prothrombin fragment 1.[5]

Because the two tryptophan residues (Trp90 and Trp126) that are present in the second protein domain of BF1 ("kringle") mainly fluoresce with a lifetime of 0.67 $\pm$ 0.02 ns, it is not be possible to resolve a possible 0.55 $\pm$ 0.08 ns component of Trp42 in BF1. Thus, the appearance of component A in apo-FX F1-86 has to be explained exclusively on the basis of the presented FX F1-86 data. It is certainly possible that component A again accounts for a third unquenched major conformation of the microenvironment of Trp41. One might speculate that the calcium-induced disulfide–tryptophan interaction might lead to an additional contribution in the 0.5-ns range that masks an expected population decrease of the third unquenched conformation (component A). However, on the basis of the presented data, the observation that the intensity of component A is not affected by calcium addition remains unexplained.

CONCLUSION

Factor X fragment 1-86 was used as a model compound for the detection of calcium-induced conformational changes in the Gla domain of vitamin K dependent proteins. The investigations support the results of former experiments with prothrombin fragment 1. The addition of calcium ions led to a conformational change in the Gla domain resulting in "static-like" quenching of the fluorescence of the tryptophan residue located in the Gla domain.[5] In both experiments the intensities of

the components strongly decreased with decay times in the range of 2.5 and 5 ns of the two comparable tryptophan residues Trp41/Trp42 of FX F1-86 and BF1, respectively. Moreover, in both proteins calcium addition led to the appearance of a 200–300 ps component. These studies give strong evidence for the structural and functional homology between BF1 and FX F1-86. The catalytic activity of the prothrombinase complex strongly depends on the composition of the membrane. The influence of membrane binding on the conformation of BF1 was investigated in previous experiments[6] and it was shown that certain lipids alter the conformation of the Gla domain.[6] Further work on lipid-specific conformational changes in the Gla domain of factor X_a is in progress.

The authors acknowledge the help from the Technical Staff of Laboratoire pour L'Utilisation du Rayonnement Electromagnetique.

REFERENCES

1. Suttie, J. W.; Jackson, C. M. Physiol Rev 1977, 57, 1–70.
2. Jones, M. E.; Lentz, B. R.; Dombrose, F. A.; Sandberg, H. Thromb Res 1985, 39, 711–724.
3. Rosing, J.; Speijer, H.; Zwaal, R. F. A. Biochemistry 1988, 27, 8–11.
4. Comfurius, P.; Smeets, E. F.; Willems, G. M.; Bevers, E. M.; Zwaal, R. F. A. Biochemistry 1994, 33, 10311–10324.
5. Hof, M.; Fleming, G. R.; Fidler, V. Proteins Struct Funct Genet 1996, 24, 845–894.
6. Hof, M. Biochim Biophys Acta 1998, 1388, 143–153.
7. Sunnerhagen, M.; Olah, G. A.; Stenflo, J.; Forsen, S.; Drakenberg, T.; Trewhella, J. Biochemistry 1996, 35, 11547–11559.
8. Nelsestuen, G. L. J Mol Biol 1976, 251, 5648–5656.
9. Soriano-Garcia, M.; Padmanabhan, K.; de Vos, A. M.; Tulinsky, A. Biochemistry 1992, 31, 2554–2566.
10. Persson, E.; Björk, I.; Stenflo, J. J Biol Chem 1991, 266, 2444–2452.
11. Esnouf, M. P.; Lloyd, P. H.; Jesty, J. Biochem J 1973, 131, 781.
12. Persson, E.; Selander, M.; Linse, S.; Drakenberg, T.; Öhlin, A.-K.; Stenflo, J. J Biol Chem 1989, 284, 16897–16904.
13. Gutfreund, H. Enzymes: Physical Principles; Wiley: New York, 1972; pp 76–79.
14. Demas, J. N. Excited State Lifetime Measurements; Academic: New York, 1983.
15. O'Connor, D. V.; Phillips, D. Time-Correlated Single Photon Counting; Academic: London, 1984.
16. Brochon, J. C.; Tauc, P.; Merola, F.; Schoot, B. M. Anal Chem 1993, 65, 1028–1034.
17. Hof, M.; Schleicher, J.; Schneider, F. W. Ber Bunsen Phys Chem 1989, 93, 1377–1381.
18. Marquardt, D. W. J Soc Ind Appl Math 1963, 11, 431–441.
19. Hof, M.; Vajda, S.; Fidler, V.; Karpenko, V. Collect Czech Chem Commun 1996, 61, 808–818.
20. Livesey, A. K.; Brochon, J. C. Biophys J 1987, 52, 693–706.
21. Jaynes, E. T., Rosenkrantz, R. D., Eds. Collected Works. Papers on Probability, Statistics and Statistical Physics; Reidel: Dordrecht, Holland, 1983.
22. Garcia, P.; Mérola, F.; Receveur, V.; Blandin, P.; Minard, P.; Desmadril, M. Biochemistry 1998, 37, 7444–7455.
23. Permyakov, E. A. Luminescence Spectroscopy of Proteins; CRC Press: Boca Raton, FL, 1993; p. 86.
24. Persson, E.; Björk, I.; Stenflo, J. J Biol Chem 1991, 266, 2444–2452.
25. Döring, K.; Beck, W.; Konermann, L.; Jähnig, F. Biophys J 1997, 72, 326–334.
26. Bajaj, S. P.; Butkowski, R. J.; Mann, K. G. J Biol Chem 1975, 250, 2150–2156.
27. Bloom, J. W.; Mann, K. G. Biochemistry 1978, 17, 4430–4438.
28. Prendergast, F. G.; Mann, K. G. J Biol Chem 1977, 252, 840–850.
29. Nelsestuen, G. L. J Biol Chem 1976, 251, 5648–5656.
30. Mérola, F.; Rigler, R.; Holmgren, A.; Brochon, J.-C. Biochemistry 1989, 28, 3383–3398.
31. Li, Z.; Bruce, A.; Galley, W. C. Biophys J 1992, 61, 1364–1371.
32. Schlyer, B. D.; Lau, E.; Maki, A. H. Biochemistry 1992, 31, 4375–4383.
33. Swandesh, J. K.; Mui, P. W.; Scheraga, H. A. Biochemistry 1987, 26, 5761–5769.
34. Sanyal, G.; Kim, E.; Thompson, F. M.; Brady, K. E. Biochem Biophys Res Commun 1989, 165, 772–781.