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Fluorescence approaches for the characterisation of the peripheral membrane binding of proteins applied for the blood coagulation protein prothrombin

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1 Introduction

The blood coagulation protein prothrombin, the zymogene of thrombin, is a single polypeptide chain glycoprotein which requires a high calcium stoichometry for membrane binding. It serves as the substrate of the prothrombinase complex consisting of the serine protease factor Xa that associates with cofactor Va on membrane surfaces containing negatively charged phospholipids in the presence of Ca^{2+} [1]. The catalytic activity of the prothrombinase complex strongly depends on the chemical structure of the phospholipid headgroup. Although several anionic phospholipids accelerate the prothrombin activation, the L-serine headgroup has been shown to be by far the most efficient one, promoting phosphatidyl-L-serine (PS) to the outstanding prothrombin-activating lipid [2-5]. The reason for this high lipid specificity is not known yet. However, the elucidation of the molecular mechanism of the interaction between the three proteins and the membrane surface will be a crucial step towards a more thorough understanding.

Fluorescence spectroscopy has been an important tool in the study of protein-membrane interactions due to its versatility and high sensitivity. Principally, the problem of protein-membrane interactions can be approached from two different sides. The first possibility is to take advantage of the intrinsic tryptophan (Trp) fluorescence or of fluorescent labels attached to the protein. In tryptophan studies the fluorescence properties of the protein in free and membrane bound state are compared [6]. Fluorescence changes in the intrinsic fluorescence may be interpreted in terms of a specific conformational change occurring during membrane binding. The use of bright covalently attached fluorescent labels allows for the application of techniques like fluorescence resonance energy transfer (FRET) [7,8] or fluorescence recovery after photobleaching (FRAP) [9-11]. The second possi-

bility is to focus on the phospholipid organisation of the membrane which might change due to protein binding. The characterisation of pyrene excimer formation [12,13] as well as the determination of fluorescence anisotropy of embedded membrane probes like 1,6-diphenylhexatriene (DPH) [14] have been widely used in studies of biological and model membranes. While both methods are well suited to monitor the dynamics of the interior part of a lipid bilayer [14] they turned out much less useful for the study of the organisation of the lipid headgroup region [14-16]. A fluorescence method which overcomes this limitation is the solvent relaxation method. Originally designed for the study of very fast solvation processes in non-viscous solvents [17] the method has been successfully adapted to the study of both the hydrophilic headgroup region and the hydrophobic interior of phospholipid bilayers [18-23]. This review summarises several contributions describing the use of fluorescence techniques in the characterisation of the calcium-mediated membrane binding of prothrombin and its N-terminal peptide fragment 1 (F1).

2 Prothrombin Binding to Negatively Charged Membrane Surface Characterised by Protein Fluorescence

The main advantages of exploiting the intrinsic (Trp) fluorescence in protein studies are the high sensitivity of the Trp fluorescence to changes in its microenvironment and the fact that the protein has not to be modified and, thus, the spectroscopic measurements can be performed using the native protein. Since the photophysics of even a single Trp residue in proteins appears complex in most cases, the applicability of tryptophan studies is limited to proteins with a low number of Trp residues. Moreover, the low quantum yield and the need for ultraviolet excitation (ideal excitation wavelength for Trp λ_{ex} = 295 nm) of Trp prevent the combination of Trp fluorescence with techniques employing fluorescence microscopy at the present time. Application of multiphoton excitation might be one way to overcome this limitation in the future [24]. Since prothrombin contains a rather high number of Trp residues (12 and 14 for human and bovine prothrombin, respectively [25]), tryptophan studies have been limited to its fragment 1 (F1) which contains only three Trp residues [6,26].

The application of bright covalently attached fluorescent labels in proteins studies, on the other hand, is not limited by the size of the protein or protein fragment. Moreover, this approach allows for the application of various fluorescence techniques and the use of microscopes. The main disadvantages, however, are that the covalent labelling might be rather unspecific regarding the site of attachment and that the label might change structure and function of the protein.

2.1 Intrinsic Protein Fluorescence: Picosecond Tryptophan Fluorescence of Membrane Bound Prothrombin Fragment 1 (F1)

The 1-156 N-terminal polypeptide F1 is believed to be the region predominantly responsible for the metal ion and membrane binding properties of prothrombin. Besides small but significant differences in the desorption rate, it displays basically very similar membrane binding characteristics as the entire protein [10]. The structure of F1 is commonly divided into the N-terminal "Gla domain", characterized by 10 γ-carboxyglutamic acid residues (Gla) and a region of disulfide linkages known as the "kringle region". Calcium ions bind (almost) exclusively to the Gla domain [27] and form the native conformation required for membrane binding [28]. Figure 1 depicts a sketch of the X-ray structure of Ca-bovine (B)F1 and shows the location of its three tryptophan residues and the seven Calcium ions bound to the Gla domain. Investigating possible molecular differences in the binding of the calcium-prothrombin complex to differently composed, negatively charged membrane surfaces, one might speculate whether such differences can be found in the conformation of membrane-bound Gla domains.

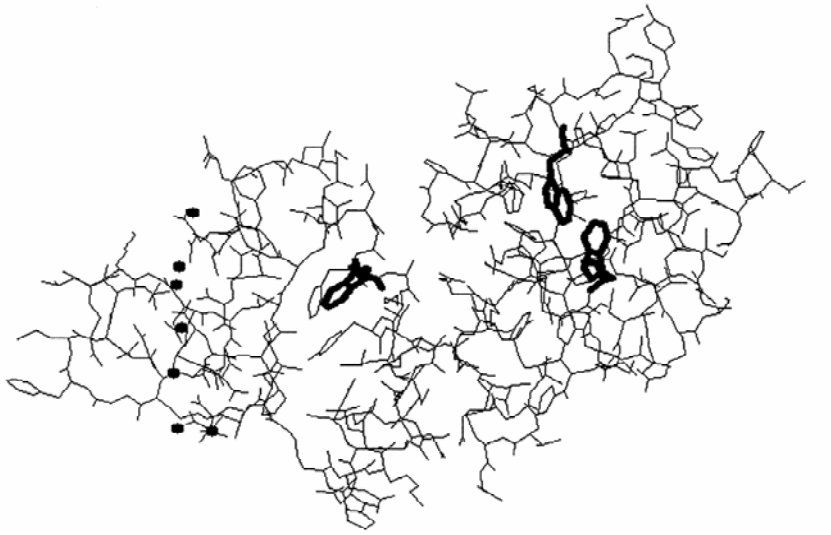

Fig. 1. A depiction of the X-ray structure of Ca-BF1. The right part of the protein is the kringle-domain, where Trp90 and Trp126 are located. The Gla-domain is the left part of the protein, containing Trp42 and seven calcium ions (dots). The coordinates where taken from the Brookhaven Protein Data Bank entries [29] and displayed via Rasmol V2.6 software (Glaxo Welcome Research Development, Sterenage, United Kingdom).

Using picosecond fluorescence time-correlated single photon counting, changes in the microenvironment of the three individual tryptophans can be separated from each other without either cleaving BF1 into the isolated Gla (containing Trp42) and kringle domains (containing Trp90 and Trp126) or modifying the protein by site-

directed mutagenesis. The sensitivity of this approach to conformational changes has been demonstrated by a time-resolved study of the calcium induced conformational change in BF1 [26]. The cited work [26] comprises a detailed analysis of the wavelength dependent fluorescence decays of apo-BF1 as well as of Ca-BF1. Fluorescence lifetime distribution (see figure 2) and conventional multiexponential analysis, as well as acrylamide quenching studies led to the identification of six distinguishable tryptophan excited states for the apo- as well as for the Ca-form of BF1. Accessibility to the quencher and the known structure have been used to associate the fluorescence decay of the tryptophan present in the Gla domain (Trp42) with two red shifted components (2.3 and 4.9 ns for apo-BF1). The two kringle domain tryptophans (Trp90 and Trp126) exhibit three decay times (0.24, 0.68 and 2.3 ns for apo-BF1) which are blue shifted. The 0.06 ns component remained unassigned due to the limited time-resolution of the experiment. It should be noted that the mentioned lifetime values result from a global analysis of the fluorescence decays detected at 305 to 425 nm.

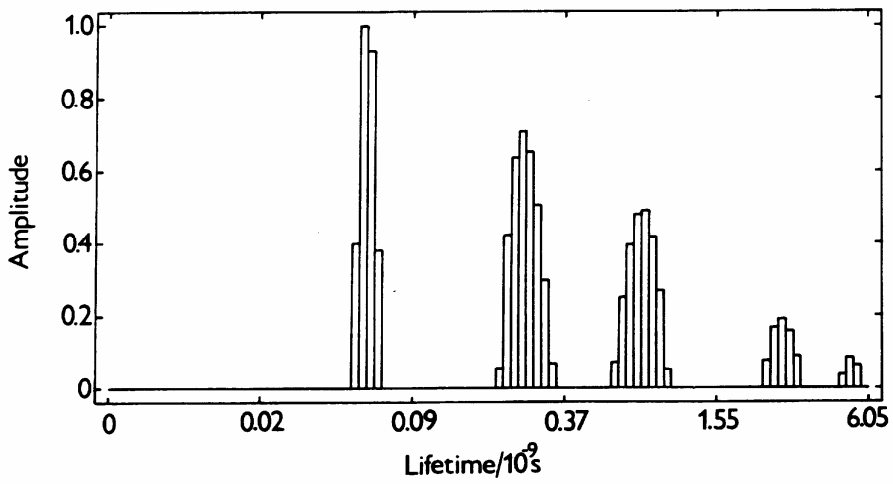

Fig. 2. Amplitude profile of the fluorescence lifetimes distribution of apo-BF1 fluorescence decay (λ_{ex} = 295 nm; λ_{em} = 330 nm; 4 μM in Tris-buffer) resulting from Edinburgh Analytical Instruments distribution analysis illustrating the existence of five separate classes of fluorescence lifetimes [26]. Note that the 2.3 ns component is due to the fluorescence of Gla and kringle tryptophans.

Addition of calcium ions did not change fluorescence lifetimes and intensities of those components which have been assigned exclusively to the two kringle tryptophans Trp90 and Trp126 (0.24 ns and 0.68 ns) – a result that argues against a calcium binding site in the kringle domain [27]. On the other hand, we found that the overall fluorescence quenching is due to a "static-like" quenching of the Gla-Trp (Trp42) components D and E (2.3 ns and 5.1 ns for Ca-BF1, respectively) as a consequence of a ground state interaction between Trp42 and the Cys18-Cys23

disulfide bridge. For illustration see figure 3, comparing the decay associated spectra (DAS) of the two Gla-components in absence and presence of Calcium. It is important to note that an observed decrease in the fluorescence intensity in the 5.1 ns component of 85% is by far larger than the calcium-induced changes in the parameters determined by circular dichroism [30], by antibody binding experiments [31], differential scanning calometric studies [32,33] and fourier transform infrared spectroscopy [34].

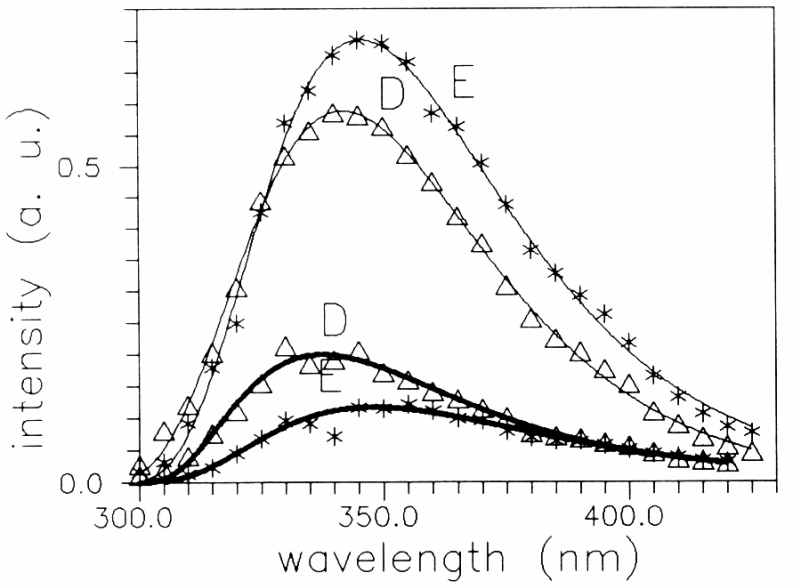

Fig. 3. Decay-associated spectra of components D (Δ) and E (*) for apo-BF1 (2.3±0.2 ns (D) and 4.9±0.3 ns (E)) and for Ca-BF1 (2.3±0.3 ns (D) and 5.1±0.4 ns (E)). Shown are the experimental points and the log-normal fits to the data. Thick lines represent the decay-associated spectra of Ca-BF1. The emission maxima are 341.9 nm (D) and 345.5 nm (E) for apo-BF1 and 337.4 nm (D) and 347.6 nm (E) for Ca-BF1.

The high sensitivity of the time-resolved Trp42 fluorescence to conformational changes in the Gla domain was one motivation to reexamine the hypothesis of possible lipid-induced conformational changes in the Gla-domain of prothrombin by picosecond tryptophan fluorescence spectroscopy of BF1 [6]. Therefore, the wavelength-dependent tryptophan fluorescence decays of Ca-BF1 in presence of pure phosphatidylcholine (PC) small unilamellar vesicles (SUV) and PC-SUV containing either 25% phosphatidylserine (PS) or 40% phosphatidylglycerol (PG) were characterized. Based on the determined apparent membrane dissociation constant K_d (The K_d -values are 0.9±0.1 μM and 0.8±0.1 μM for 25 mol % PS, and 40 mol % PG, respectively), Ca-BF1 (4 μM) should have been > 90% bound to

the membrane surface at a lipid concentration of 1.3 mM in both investigated lipid systems. In both cases the lifetime analysis identified the existence of five wavelength independent lifetimes. Specific binding to PS-containing membranes did neither change the fluorescence lifetimes nor the corresponding wavelength dependent amplitudes. Apparently, the membrane binding part of calcium-prothrombin remains in its native conformation when bound to the highly procoagulant PS-containing membrane surface. In contrary to the PS-results, the tryptophan studies of the PG-bound BF1 yielded an interesting new result, i.e. a lipid induced conformational change in the Gla-domain, observed by a significant prolongation of lifetime E. At protein/lipid concentration ratios ensuring that the majority of the protein is bound to the 40% PG / 60% PC surface, the lifetime of component E shifts from 5.1 to 7.5 ns, when compared with Ca-BF1 in solution. The prolongation of the Trp42 fluorescence lifetime can be observed as well by an apparent shift of the component D from 2.2 ns to 2.8 ns. On the other hand, as in the case of the binding to PS containing surfaces, the kringle components B and C remain unchanged. Since component D is due to the fluorescence of Gla and kringle tryptophans, the constant kringle tryptophan fluorescence portion in D might mask the entire magnitude of the lifetime shift in the Gla-portion of component D.

For the N-terminal membrane binding part the comparison of the PS- with the PG-results leads to the conclusion that Ca-BF1 exhibits already the "perfect" conformation for binding and proteolysis and, thus, retains its conformation when bound to PS surfaces. The PG-induced conformational disruption of the Gla domain possibly might affect the protein conformation in the non-fragment 1 part of the protein and/or the lateral diffusion on the membrane surface. Both scenarios could yield a possible explanation for the lower procoagulant activity of PG when compared to PS.

2.2 Overview on Investigations Applying Fluorescently Labelled Prothrombin

In the majority of the studies on fluorescently labelled prothrombin a combination of substrate-supported planar model membranes and the use of evanescent illumination with laser-based, quantitative fluorescence microscopy has been employed [9-11,35,36]. Measurement of the steady-state, surface-associated fluorescence can be used to examine the thermodynamic properties of prothrombin at membranes [35]. Combined with fluorescence recovery after photobleaching (FRAP), this technique provides information about membrane-binding kinetics. On the other hand, combined with fluorescence pattern photobleaching recovery (FPPR) [9,10], measurement of the translational diffusion coefficients of prothrombin bound to membranes is possible [11,36].

In the first paper of this series, fluorescein-labelled bovine prothrombin and its amino- and carboxy-terminal peptides, prothrombin fragment 1 and prethrombin 1, were added at various concentrations in the presence or absence of Ca^{2+} to substrate-supported planar membranes composed of 1-palmitoyl-2-oleoyl-3-sn-phosphatidylcholine (POPC), POPC / PS (70:30 mol/mol), or POPC / 1,2-dioleoyl-3-sn-phosphatidylglycerol (DOPG) (70:30 mol/mol). Measurement of the steady-

state surface-associated fluorescence was employed for the determination of equilibrium dissociation constants (k_D). Although the calcium independent binding constant (k_D) of prothrombin has been shown to be only slightly higher than the k_D of unspecific binding to neutral membranes, the authors postulated a calcium independent binding site of prothrombin in its non-fragment 1 portion, when bound to PS containing membranes [35]. In the subsequent two studies of this group evanescent field excitation has been combined with FRAP (see figure 4) and used to compare the membrane binding characteristics of fluorescein-labelled bovine prothrombin and fluorescein-labelled bovine prothrombin fragment 1 to supported planar membranes composed of mixtures of PS (2-10 mol %) and DOPC in presence of Ca^{2+}. Equilibrium binding measurements showed that the k_D-values increased with decreasing molar fractions of PS and that the dissociation constants were somewhat lower for intact prothrombin. Kinetic measurements, using FRAP, showed that the measured dissociation rates were approximately equivalent for prothrombin and fragment 1 and did not change with the protein solution concentration or the molar fraction of phosphatidylserine. The kinetic data also implied that the surface binding mechanism for both proteins is more complex than a simple reversible reaction between monovalent proteins and monovalent surface sites.

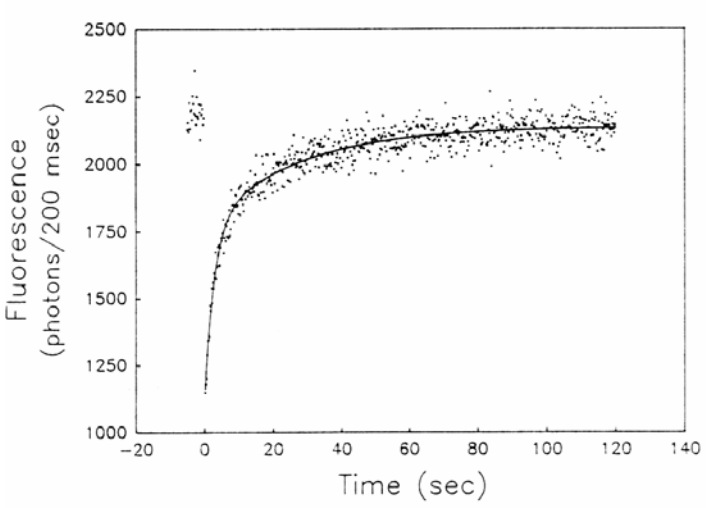

Fig. 4. Representative FRAP intensity versus time profile for the binding of prothrombin (1 μM) to planar PC-bilayers containing 6 mol% PS in presence of 10 mM Ca^{2+} using evanescent field excitation. Experimental data points are given together with the best fit using a biexponential model [10].

In the last work of this series [11], fluorescence pattern photobleaching recovery (FPPR) with evanescent interference patterns has been used to measure the translational diffusion coefficients of membrane bound prothrombin fragment 1. In this method, two internally reflected laser beams are collided to create a periodic eva-

nescent intensity pattern that illuminates a region of a solid/liquid interface [11]. The results show that the translational diffusion coefficient on fluid-like PS/PC planar membranes is about 5×10^{-9} cm^2/s and is reduced when the fragment 1 surface density is increased. In addition, no translational diffusion was detected for fragment 1 on solid-like (gel phase) membranes. FPPR combined with evanescent field excitation was also used to measure the diffusion coefficient of a fluorescent lipid in PS/PC planar membranes. These measurements yielded a diffusion coefficient of approximate 10^{-8} cm^2/s which is consistent with that measured by conventional FRAP [37] or fluorescence correlation spectroscopy (FCS) [38]. The authors conclude that the translational diffusion of fragment 1 requires and mimics membrane fluidity suggesting that fragment 1 does not skim over the membrane surface [11].

An important issue in the study of membrane binding of prothrombin and other coagulation proteines are possible conformational changes of the protein during Ca^{2+}-mediated association with negatively charged lipids, especially PS. Fluorescence resonance energy transfer (FRET) from a donor-labelled protein to an acceptor-labelled bilayer can be used to study protein-membrane interactions and changes in protein conformation. Chen and Lentz [8] applied this method to test the hypothesis of a membrane-induced conformational change in prothrombin compared to meizothrombin, an active intermediate in prothrombin activation formed by initial cleavage at Arg^{323}-Ile [39]. Based on a R_0-value for the given donor-acceptor pair of 52 Å the authors calculated the distance from the selectively fluorescein-labelled C-terminus of prothrombin to the rhodamine-labelled phosphatidylethanolamin in the PC/PS bilayer to be 94±3 Å versus 114±2 Å for meizothrombin. As the overall length of prothrombin in solution was estimated to be about 113 Å these data suggest that binding with PS-containing membranes induces the prothrombin molecule to "fold up" internally to achieve the shorter fluorescein-to-rhodamine distance. It was concluded that these conformational changes might help to align the bond Arg^{323}-Ile in prothrombin with the active site of membrane bound factor Xa which is known to be 61 Å above the membrane surface in absence of another cofactor, factor Va [7]. It should be stated, however, that the employed method requires several assumptions, e.g. concerning the value of κ^2 used in the calculation of R_0, and data corrections; thus, the accuracy of the donor-acceptor distances given by Chen and Lentz appears quite optimistic.

3 Prothrombin Induced Changes in the Organisation of Phospholipid Bilayers

3.1 Solvent Relaxation (SR)

3.1.1 Solvent Relaxation Probed by the Headgroup Labels Prodan and Patman

During the last years solvent relaxation monitored by time-resolved fluorescence measurements has become an extremely useful method in membrane research [18-

22,40,41]. It has been shown that suitable fluorescent dyes allow for direct observation of viscosity and polarity changes in the vicinity of the probe molecule which can be intentionally located in the hydrophobic backbone or in the hydrophilic headgroup region of the phospholipid bilayer. Such dyes described in recent publications were based on 2-amino-substituted naphthalene [19,20] and anthracene-9-carboxylic acid [21]. Representatives for 2-amino-substituted naphthalenes are the dyes Prodan (6-propionyl-2-(dimethylamino)-naphthalene) and its long alkyl chain derivative Patman (6-palmitoyl-2-[[trimethyl-ammoniumethyl]methylamino]-naphthalene chloride) whose chromophores are known to be located within the hydrophilic headgroup region of the membrane. It might seem obvious considering that Ca^{2+}-assisted membrane binding of peripheral proteins should first of all influence the organisation and dynamics of the lipid headgroup region. Thus, Prodan and Patman were considered as ideal probes for the study of the Ca^{2+}-induced prothrombin-membrane interaction.

Since the solvent relaxation method cannot be considered as a standard fluorescence technique up to know, its principles will be shortly outlined. The electronic excitation of a chromophore causes an ultrafast change of the probe´s charge distribution, but does not affect the position or orientation of the surrounding solvent molecules. The solvent molecules are, thus, forced to adapt to the new situation and start to reorient themselves in order to find an energetically favored position with respect to the excited dye. The dynamic process starting from the originally created non-equilibrium Franck-Condon state and gradually establishing a new equilibrium in the excited state is called solvent relaxation (SR). This relaxation red shifts the probe's emission spectrum continuously from the emission maximum frequency of the Franck-Condon state ($\nu(0)$ for $t = 0$) to the emission maximum of the fully relaxed state ($\nu(\infty)$ for $t = \infty$). The time-resolved emission spectra (TRES) are usually determined by "spectral reconstruction" [17-21]. The TRES are fitted by an empirical "log-normal function" [42]. From the fitted spectra the emission maximum frequencies $\nu(t)$ and the full width at half maximum (FHWM) of the TRES are usually derived. The maximum of the time-zero ($t=0$) spectrum $\nu(0)$ can be estimated quite accurately when both the absorption and fluorescence spectra in a non-polar reference solvent and the absorption spectrum in the system of interest are known [17,41,43]. Since $\nu(t)$ contains both information about polarity ($\Delta\nu$) and viscosity of the reported environment, the spectral shift $\nu(t)$ is normalised to the total shift $\Delta\nu$. The resulting "correlation functions" $C(t)$ (equation 1) describe the time course of the solvent response.

$$C(t) = \frac{\nu(0) - \nu(\infty)}{\Delta\nu} \qquad (1)$$

In order to characterise the overall time scale of the solvation response, we use an (integral) average relaxation time:

$$\langle \tau_r \rangle \equiv \int_0^\infty C(t)\, dt \qquad (2)$$

The time-dependent Stokes-shift $\Delta\nu$ ($\Delta\nu = \nu(0) - \nu(\infty)$) depends both on the solvent polarity and on the change in the solute's dipole moment. Since Prodan and Patman contain practically identical chromophores, detected differences in $\Delta\nu$ (e.g. 3750 cm^{-1} and 3000 cm^{-1} for Prodan and Patman in PC-SUV at room temperature, respectively [41]) directly reflect microenvironments of different polarity of the chromophores. It has been shown that the chromophore of Prodan is located in the headgroup region of phospholipid bilayers close to the lipid/water interface [18-20]. Smaller $\Delta\nu$ values for Patman indicate that its chromophore is embedded deeper in the bilayer [18-20,41]. A comparison of $\nu(0)$ values obtained by the time-zero spectrum estimation with those obtained by TRES reconstruction shows that about 90% of the SR in PC-SUV in the liquid crystalline state probed by both dyes occurs on the subnanosecond to nanosecond timescale [41]. This conclusion is confirmed by the time evolution of the FWHM. In SUV composed of PC as well as of PC/PS mixtures the FWHM increase at early times and reach their maxima followed by a decrease of the spectral width. The observed profiles prove that during the lifetime of the excited state SR completes and almost the whole relaxation process is captured by an equipment providing subnanosecond time-resolution. The resulting characteristic SR time for Prodan (e.g. 1.1 ns in PC-SUV [20] are significantly smaller than obtained for Patman (e.g. 2.1 ns in PC-SUV [20]) in all investigated phospholipid bilayer systems so far [18-20,22,23,40].

3.1.2 Influence of Prothrombin and its Fragment 1 on the Phospolipid Headgroup Organisation

For the application of the solvent relaxation method described above to the protein-Ca^{2+}-PS interaction we had to assure that the used fluorophores do not bind significantly to the proteins in presence of lipid. This was done comparing steady-state spectra of Prodan and Patman in Tris-buffer, in presence of 40 μM BF1 or 30 μM prothrombin, respectively, and after addition of different amounts of vesicle suspension. While the addition of the proteins alone did neither change the emission spectra nor the decay times, addition of vesicles in presence of the protein led to both a new blue shifted emission band caused by the binding of Prodan and Patman, respectively, to the membrane and considerably increased decay times [20].

In order to establish the correlation between the solvent relaxation kinetics of Prodan and Patman with protein coverage of the membrane surface, small unilamellar vesicles (SUV) composed of PC/PS = 80/20 (mol:mol) in presence of 5 mM Ca^{2+} were titrated with prothrombin. An increase in $\langle\tau_r\rangle$ for Patman from 2.3 ns in absence of prothrombin to about 3.0 ns in presence of saturating concentrations of prothrombin (> 12 μM) was observed with the largest increase for the first addition of 2 μM prothrombin. This behaviour is in qualitative agreement with binding isotherms established by other methods [44].

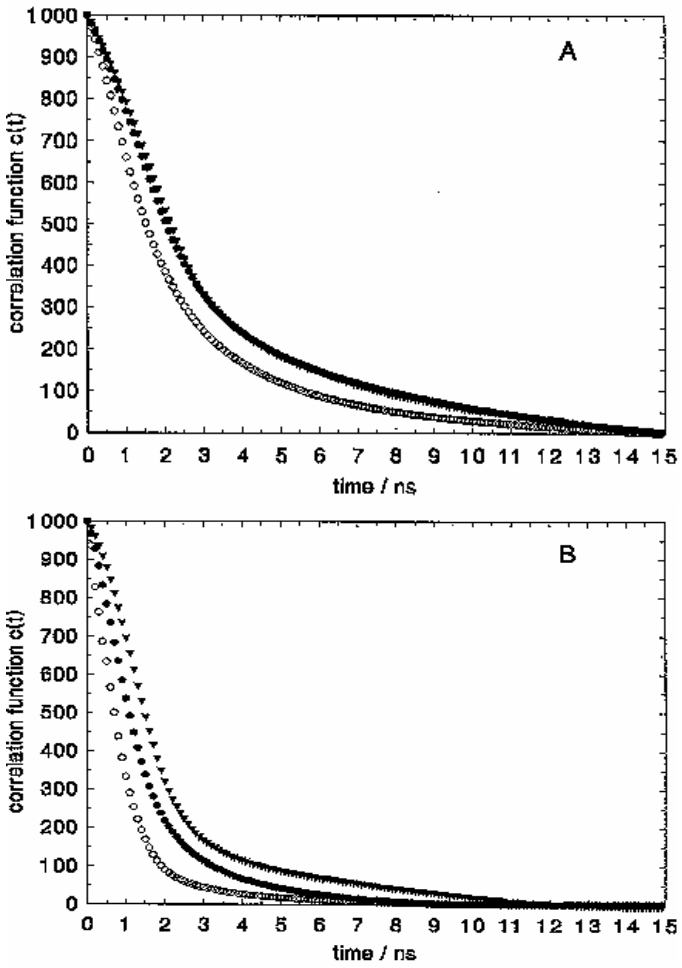

Fig. 5. Correlation functions C(t) for Patman (A) and Prodan (B) in PC/PS 60:40 vesicles in presence of 5 mM Ca^{2+} without protein (open circles), in presence of 16 μM BF 1 and prothrombin (triangles). The temperature was 25 °C.

To compare the effect of binding of prothrombin and its fragment 1 on the headgroup organisation of PC/PS-SUV the SR-kinetics of Prodan and Patman were determined at saturating protein concentrations (16 μM). In figure 5 some exemplary correlation functions in absence and presence of proteins for Prodan and Patman in SUV composed of PC/PS = 60/40 (mol:mol) are shown. As shown by the data in table 1, protein binding significantly reduces the mobility of the dye microenvironment in all investigated systems.

For Patman both proteins at saturating concentrations yield nearly identical effects on the mean relaxation times, i.e an increase of 30 % (table 1). In contrast to Patman which obviously could not differentiate between both proteins, a clear

difference was detected using Prodan as the fluorophore. The binding of the complete prothrombin induces a higher rigidity than binding of the N-terminal fragment alone. Thus, Prodan which has been shown to be localised closer to the lipid/water interface [19] reacts considerably more sensitive to the binding of different proteins than does Patman.

Table 1. Percentual increases of $\langle\tau_r\rangle$ in presence of protein relative to the relaxation times in absence of proteins. Final values of $\langle\tau_r\rangle$ are obtained by averaging the $\langle\tau_r\rangle$ values obtained at apparent saturation protein concentrations (8, 12, 16 μM for PC/PS 60:40 and 12, 16, 20 μM for PC/PS 80:20).

PC/PS	*fluorophore*	*prothrombin*	*BF 1*
80:20	Patman	30 %	30 %
60:40	Patman	35 %	30 %
80:20	Prodan	100 %	50 %
60:40	Prodan	92 %	58 %

The observation that the increase in solvent relaxation times monitored by Patman is identical for both prothrombin and its fragment 1 indicates similarities in the binding mechanism of both proteins. Located closer to the lipid/water interface, Prodan, however, is more affected by prothrombin binding. A possible explanation for the more rigid outermost region of the negatively charged phospholipid headgroup due to binding of the entire protein could be a tighter binding of the fragment 1 portion when associated with the non-fragment 1 part of prothrombin.

3.2 Fluorescence Anisotropy: Influence of Prothrombin and its Fragment 1 on PC/PS Membrane Order

It is instructive to compare the effects of protein binding using the solvent relaxation approach with those obtained by steady-state fluorescence anisotropy measurements [15,20]. In the PC/PS-system used by us adding both proteins to a concentration leading to nearly surface coverage slightly increases the membrane order as monitored by the DPH anisotropy in the temperature range 10–35 °C. In the former investigation [15] the authors did not detect any effect of BF 1 binding on the membrane order of fluid phase DMPC/DMPS-SUV. On the other hand they found a substantial increase due to prothrombin binding. Our experiments using PC/PS-SUV showed similar trends, with the major difference, however, that we observed a detectable, though very small effect of BF 1 (≈ 3 %) and a smaller prothrombin induced increase of membrane order (≈ 5 %) than detected by Tendian and Lentz [15]. Summarizing both these investigations it can be stated that

binding of prothrombin and its BF1 leads, if at all, only to a very small increase in packing density of the hydrocarbon region of the bilayer.

3.3 Pyrene Fluorescence: Influence of Fragment 1 on Membrane Order Monitored by the Excimer/Monomer Ratio

The excited pyrene molecule can exist as either a monomer or an excited dimer (excimer), both forms exhibiting different fluorescence properties, i.e. different emission maxima. The ratio of excimer to monomer emission (E/M) increases with increasing pyrene concentration and with increasing rates of diffusion [45]. Thus, changes in the E/M-ratio have been taken as a qualitative reflection of changes in the probe´s microenvironment, i.e. changes in the bilayer microviscosity. For example, the presence of cholesterol in DMPC vesicles results in a lower E/M-ratio, reflecting slower pyrene diffusion [46]. Pyrene-labeled phospholipids have been used to detect temperature-induced lateral phase separations and changes in lipid phase transitions associated with the interaction of membranes with Ca^{2+} [47] and extrinsic membrane proteins [48].

Jones and Lentz [16] reported the use of pyrene-labeled phospholipids with either anionic or neutral headgroups to probe the effect of prothrombin fragment 1 binding on the lipid organisation within phosphatidylglycerol/phosphatidylcholine (PG/PC) bilayers. Similar to PS, PG supports the binding of prothrombin and its fragment 1 in presence of Ca^{2+}. Saturating amounts of fragment 1 and 5 mM Ca^{2+} have been added to pure dioleoylphosphatidylglycerol (DOPG) vesicles containing pyrene-PG. No change in the E/M-ratio could been observed, indicating that the diffusion of pyrene-PG was not significantly decreased by the presence of fragment 1. These data are in agreement with the results obtained by DPH anisotropy measurements, supporting the view that the membrane fluidity in the inner part of the bilayer is not substantially affected by the binding of prothrombin or its fragment 1.

On the other hand, the phospholipid headgroup region becomes considerably more rigid, as demonstrated by the solvent relaxation data. The comparison of all three membrane focussed methods to characterise protein-membrane interactions supports our view that the solvent relaxation method is superior to both steady-state anisotropy and pyrene excimer formation for the study of headgroup organization and dynamics.

4 Conclusion

The described investigations employing fluorescence spectroscopy clearly demonstrate that calcium ions as well as negatively charged lipid surfaces are essential for the membrane binding of prothrombin. Kinetic data obtained by fluorescence recovery after photobleaching imply that the surface binding mechanism is more complex than a simple reversible reaction between monovalent proteins and mono-

valent surface sites. The N-terminal peptide fragment 1 displays basically very similar membrane binding characteristics as the entire prothrombin. However, fluorescence recovery after photobleaching experiments as well as solvent relaxation experiments using the membrane labels Prodan and Patman indicate a slightly tighter binding of the fragment 1 portion in the case of the entire prothrombin molecule compared to the isolated fragment 1. Investigations based on the fluorescence of membrane labels show that the negatively charged headgroup region of phosphatidylserine-containing membranes is predominantly responsible for the calcium-mediated prothrombin binding; there are no hydrophobic membrane binding sites in the 'non-fragment 1' part of prothrombin. The lateral diffusion coefficient of fragment 1 bound to phosphatidylserine-containing membranes appears to be within the same range as that determined for lateral lipid diffusion within fluid bilayer systems.

The tryptophan fluorescence characteristics of fragment 1 changes when bound to differently composed membrane surfaces. Binding to a phospholipid surface with high prothrombin binding affinity but low procoagulant activity disrupts the Gla-domain conformation. The membrane binding part of the calcium-prothrombin, on the other hand, remains in its native conformation when bound to highly procoagulant (phosphatidylserine) containing surfaces.

Acknowledgement

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