

Binding of prothrombin and its fragment 1 to phospholipid membranes studied by the solvent relaxation technique

R. Hutterer ^a, F.W. Schneider ^a, W.Th. Hermens ^b, R. Wagenvoort ^b, M. Hof ^{a, c, *}

^a *Institute for Physical Chemistry, University of Würzburg, D-97074 Würzburg, Germany*

^b *Cardiovascular Research Institute, Maastricht University, NL-6200 Maastricht, Netherlands*

^c *J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Dolejškova 3, CZ-18223 Prague 8, Czech Republic*

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Abstract

The phospholipid headgroup mobility of small unilamellar vesicles composed of different mixtures of phosphatidyl-L-serine (PS) and phosphatidylcholine is characterized by the solvent relaxation behavior of the polarity sensitive dyes 6-propionyl-2-(dimethylamino)naphthalene (Prodan) and 6-palmitoyl-2-[trimethylammoniummethyl]-methylaminonaphthalene chloride (Patman). If the PS content exceeds 10%, the addition of calcium leads to a substantial deceleration of the solvent relaxation of both dyes, indicating the formation of Ca(PS)_2 complexes. Addition of prothrombin and its fragment 1 leads to a further decrease of the headgroup mobility, as explained by the binding of more than two PS-molecules by a single protein molecule. Prodan monitors the outermost region of the bilayer and it clearly distinguishes between the binding of prothrombin and its fragment 1. The deeper incalated Patman does not distinguish between both proteins. The validity of the solvent relaxation technique for the investigation of the membrane binding of peripheral proteins is demonstrated by the studies of prothrombin induced changes in the steady-state fluorescence anisotropies of 1,6-diphenyl-1,3,5-hexatriene. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The prothrombinase complex catalyzes the activation of prothrombin to thrombin. It consists of the serine protease factor Xa which associates with co-factor 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 yet known. However, the understanding of the molecular mechanism of the interaction between the three proteins and the membrane surface seems to be a key step.

In this work, we focus on the interaction of the substrate prothrombin with membrane surfaces con-

* Corresponding author: J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Dolejškova 3, CZ-18223 Prague 8, Czech Republic.
Fax: +42 (2) 858-2307; E-mail: hof@jh-inst.cas.cz

taining PS. The N-terminal region of prothrombin contains 10 γ -carboxyglutamic acid residues (Gla) and is thus negatively charged. Similar to other vitamin K-dependent coagulation factors (factor VII, IX, X and protein C+S), this region is believed to be predominantly responsible for the membrane binding properties of prothrombin. The X-ray structure of the isolated N-terminus of prothrombin (fragment 1) in the presence of Ca^{2+} reveals three Gla residues reasonably located to be involved in a calcium-mediated interaction with negatively charged phospholipids [6]. The binding isotherms obtained by dynamic light scattering suggest the existence of four equivalent lipid binding sites per prothrombin molecule [7]. It has to be stressed that the number of Gla molecules found by Soriano-Garcia et al. (three) [6] cannot be directly correlated with the number of binding sites concluded by Cutsforth et al. (four) [7], but it is obvious, on the basis of a calcium mediated binding model, that both studies find roughly similar numbers of binding sites in prothrombin and/or its fragment 1. Since two or three PS molecules were found to be associated with each surface bound prothrombin molecule, it appears likely that each bound PS molecule is coordinated with one individual lipid binding site ('1:1 complex'). This conclusion is in agreement with the chelate model for the interaction of vitamin K-dependent proteins with PS molecules, which assumes that calcium ions form a coordination complex with Gla residues of the protein and the negatively charged headgroups of PS [3–5]. Since all three fixed charges in the polar headgroup of PS are believed to be involved in the formation of a stable chelate complex [5] and considering the maximum coordination number of six for the calcium central atom, a '1:1 complex' seems to be likely.

Studies comparing the binding behavior of different fragments of prothrombin led to the postulate of a calcium independent binding site of prothrombin in its non-fragment 1 portion, when bound to PS-containing membranes [8,9]. Although the differences in the binding constants of prothrombin and its fragment 1 are small [10] and the calcium-independent binding constant (K_d) of prothrombin to PS containing membranes is only slightly smaller than the K_d of unspecific binding to neutral membranes, it has been speculated that this interaction might be one key for

the understanding of the described lipid specificity [8,9].

Since the binding of vitamin K-dependent proteins should predominantly influence the organization of the interfacial region, we have characterized the headgroup organization in lipid bilayers in the absence and presence of protein binding. The applied 'solvent relaxation technique' has been originally used for picosecond relaxation dynamics in polar solvents [11] and it has been most recently applied for the investigation of different aspects of headgroup organization in lipid bilayers [12–15]. Using this approach, we try to shed some light on the understanding of the molecular mechanism of the membrane binding of prothrombin and its fragment 1.

2. Materials and methods

Egg yolk phosphatidylcholine (PC) and bovine brain phosphatidyl-L-serine (PS) were supplied by Fluka. Prodan (6-propionyl-2-(dimethylamino)naphthalene), Patman (6-palmitoyl-2-[trimethylammoniumethyl]methylamino]naphthalene chloride) and DPH (1,6-diphenyl-1,3,5-hexatriene) were purchased from Molecular Probes. All other chemicals were of the highest purity commercially obtainable. Small unilamellar vesicles (SUV) were prepared essentially as described by Bashford et al. [16]. In brief, the respective dye (in EtOH) was added to the lipid in CHCl_3 to yield a lipid/dye ratio of 100:1. The solvent was removed under a stream of N_2 and the lipid-dye mixture was further dried under vacuum overnight. After addition of Tris buffer (pH 7.5, 100 mM NaCl) the lipid suspension (1 mM) was sonicated at room temperature for 10 min, allowed to anneal for 30 min [17] and centrifuged for 15 min to remove any titanium particles. For the time-resolved measurements, lipid concentrations of 300 μM were used. Isolation and purification of prothrombin [18] and prothrombin fragment 1 [19] were carried out as described elsewhere.

Steady-state spectra and anisotropies were recorded using an Aminco Bowman II spectrometer. All steady-state data were corrected for instrument response. Temperature was controlled within 0.2°C. Fluorescence decays ($\lambda_{\text{ex}} = 337 \text{ nm}$) were recorded

with commercial single photon counting equipment (Edinburgh Instruments 199S) and analyzed using an iterative reconvolution technique, as described before [20]. Mean decay times were calculated according to

$$\langle \tau \rangle = (A_1\tau_1 + A_2\tau_2)/100 \quad (1)$$

where the $\tau_{1,2}$ are the individual decay times and the $A_{1,2}$ are the corresponding relative amplitudes. Time-resolved emission spectra (TRES) were calculated from the fit parameters of the multi-exponential decays detected from 390 to 530 nm and the corresponding steady-state intensities [11]. The TRES were fitted by log-normal functions [21]. Correlation functions $C(t)$ are calculated from the emission maxima $v(t)$ of the TRES at defined time t after excitation:

$$C(t) = \frac{v(t) - v(\infty)}{v(0) - v(\infty)} \quad (2)$$

where $v(0)$ and $v(\infty)$ are the emission maxima (in cm^{-1}) at times zero and ∞ , respectively. The latter two values have been assessed by inspection of the reconstructed TRES [11]. In all cases, the solvent response cannot be satisfactorily described by a monoexponential relaxation model. In order to characterize the overall time scale of the solvation response, we use an (integral) average relaxation time:

$$\langle \tau \rangle \equiv \int_0^\infty C(t) dt \quad (3)$$

3. Results

3.1. Decay behavior

The wavelength-dependent fluorescence decays of Patman and Prodan in SUV composed of different ratios of PS and PC were determined at 25°C which is far above the phase transition temperature for the given phospholipid systems. The fluorescence decays can be well described using a bi-exponential model for both dyes at all emission wavelengths ($\chi^2 < 1.2$). For $\lambda_{\text{em}} \geq 470$ nm one decay component is always obtained with a negative pre-exponential factor. An increase in the mean decay times is observed with increasing λ_{em} , as expected for increasing contributions of relaxed states.

3.2. Solvent relaxation of Prodan and Patman in PC/PS SUV in the absence and presence of Ca^{2+}

Similar to other liquid phase phospholipid systems [12–15], the reconstructed TRES of Prodan and Patman in PC/PS SUV show large time-dependent Stokes shifts of ≈ 50 nm. The TRES of Patman are significantly blue shifted compared to those of Prodan (15 nm). As for pure 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) SUV in the fluid phase [12], the solvent response of Patman is about two-fold slower than that of Prodan for all investigated PS/PC mixtures. A recent publication deals exclusively with the comparison of both dyes [12]. Results obtained by fluorescence and ^1H -NMR spectroscopy led to the conclusion that Patman is incalated deeper between the lipid acyl chains than Prodan, sensing a less polar and/or more restricted environment. Prodan, on the other hand, is located very close to the membrane/water interface.

Addition of 3 mM CaCl_2 does not affect the solvent relaxation behavior of pure PC vesicles, as monitored by both dyes. Thus, the zwitterionic PC headgroup seems not to interact with the Ca^{2+} ions at the given experimental conditions. In the absence of Ca^{2+} , a small increase in the relaxation times of Patman with increasing PS content is observed, while Prodan shows no effect. The presence of Ca^{2+} yields a much stronger dependence of the solvent relaxation on the PS content. For Prodan, the average relaxation times increase from 1.1 ns for 100% PC vesicles to 1.5 ns for vesicles composed of an equimolar PC/PS mixture. For Patman a continuous increase from 2.1 to 3.4 ns is observed. These results are summarized in Fig. 1A. Information about the relative number of excited states contributing to the emission at given times after excitation can be obtained from the halfwidths of the reconstructed TRES. In all cases, the halfwidths increase with time after excitation and reach a maximum at a time near to the average relaxation time. At longer times after the excitation, we observe a decrease in the halfwidths corresponding to a reduced number of excited states. The observed halfwidth/time after excitation profile indicates that during the lifetime of the excited state, solvent relaxation completes and that the major part of the solvent relaxation process is detectable with the given subnanosecond time resolution of the experiment

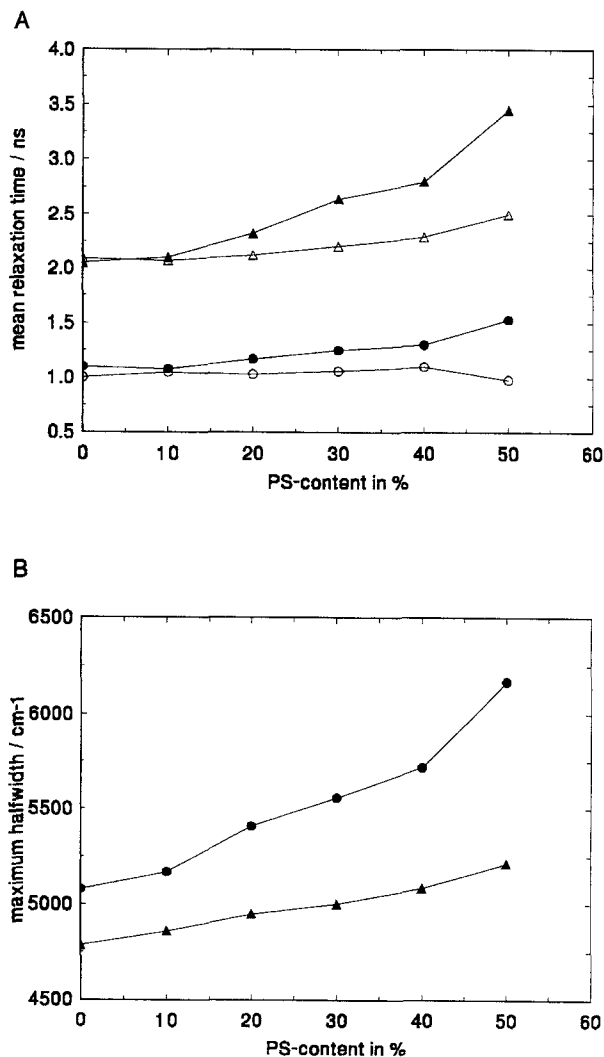


Fig. 1. (A) Mean solvent relaxation times calculated according to Eq. 3 for Prodan (circles) and Patman (triangles) as a function of the PS content in the absence (open symbols) and presence of 3 mM Ca^{2+} . The temperature was 25°C. (B) Maximum halfwidths of the time-resolved emission spectra for Prodan (circles) and Patman (triangles) as a function of the PS content in the presence of 3 mM Ca^{2+} . The temperature was 25°C.

[15]. Thus, observed changes in the solvent relaxation kinetics can be interpreted without discussing intrinsic experimental limitations [15]. Increasing PS content broadens the TRES at all times after excitation showing a larger heterogeneity of the dye environment (Fig. 1B; presence of 3 mM CaCl_2). Moreover, a comparison of the halfwidths of Patman with those of Prodan indicates a less heterogeneous localization of Patman, probably due to its long acyl chain.

3.3. Patman and Prodan do not bind significantly to proteins in the presence of SUV

For the application of the solvent relaxation method described above to the protein- Ca^{2+} -PS interaction, it had to be verified that the used fluorophores do not bind significantly to the proteins in the presence of lipid. This was done comparing steady-state spectra of Prodan and Patman in Tris buffer, in the 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 not change the emission spectra, addition of vesicles in the presence of the protein led to a new blue shifted emission band caused by the binding of Prodan and Patman, respectively, to the membrane (Fig. 2).

This behavior is also reflected by time-resolved measurements which are summarized as an illustration for Prodan and BF1 in Table 1. The decay times and amplitudes of Prodan in buffer remain constant after addition of the protein, which indicates that no protein-binding occurs. It has to be stressed, that the aim of this studies is to demonstrate the protein and membrane binding behavior of Prodan, and not to reopen the discussion upon the origin of the non-monoexponential decay behavior of Prodan in fluid solvents [22,23]. Gradually adding increasing lipid

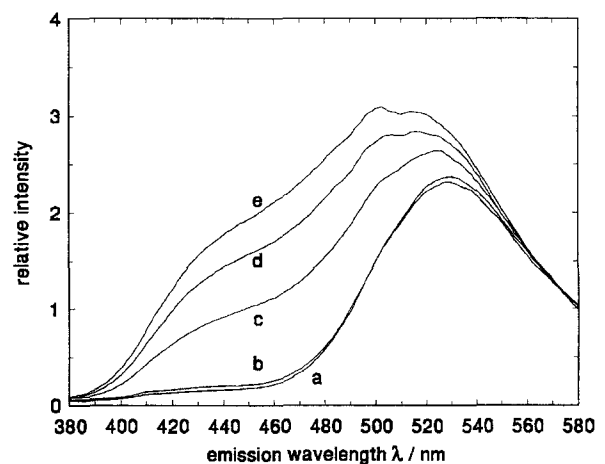


Fig. 2. Emission spectra of Prodan in buffer (10^{-5} M) (a) in the presence of 40 μM BF1 (b); and in the presence of increasing amounts of vesicles (PC/PS 60:40): c, 75 μM lipid; d, 150 μM lipid; e, 225 μM lipid; excitation wavelength was 360 nm; the temperature was 25°C.

Table 1

Fluorescence decay behaviour of Prodan in buffer (10^{-5} M), in the presence of 40 μ M BF1 and in the presence of different lipid concentrations ($\lambda_{\text{ex}} = 337$ nm, $\lambda_{\text{em}} = 430$ nm)

System	τ_1 (ns)	τ_2 (ns)	A_1 (%)	A_2 (%)	$\langle\tau\rangle$ (ns)
Prodan (buffer A)	0.76	2.06	67.7	32.3	1.18
Prodan+40 μ M BF1 (buffer B)	0.76	2.10	66.6	33.4	1.21
B+75 μ M PC/PS 60:40	0.99	2.52	43.9	56.1	2.33
B+150 μ M PC/PS 60:40	1.10	3.50	30.9	69.1	2.76
B+225 μ M PC/PS 60:40	1.23	3.61	22.8	77.2	3.13

The temperature was 25°C. The presence of 30 μ M prothrombin yielded very similar results.

concentration changes both, lifetimes and their amplitudes. Obviously, an increasing number of membrane bound dye prolongs the apparent average fluorescence lifetime recorded at 430 nm. Since the solvent relaxation kinetics of Prodan bound to lipid systems occurs on the nanosecond time scale [12–15], the amplitudes and lifetimes of membrane bound Prodan have to be interpreted in terms of an excited state process rather than by a fluorescence of excited state Prodan in two defined environments or conformations. All the shown data suggest that no significant binding of the dyes to the proteins does occur.

3.4. Influence of prothrombin and its fragment 1 on the relaxation behavior

Prothrombin and its fragment 1 have been added to vesicles composed of 60:40 and 80:20 PC/PS mixtures. As shown in Tables 2 and 3 the binding of both proteins lead to substantial increases of the relaxation times $\langle\tau_r\rangle$. This general trend has been observed for both Patman and Prodan.

Since the solvent relaxation method has not been used for the characterization of protein binding up to

now, the effect of increasing amounts of protein on the solvent relaxation times was investigated exemplary for the Patman/Prothrombin/20% PS system. Table 2 shows that the SR kinetics becomes slower with increasing protein concentration up to a saturation level, which is apparently reached at a prothrombin concentration of 12 μ M. Averaging over the $\langle\tau_r\rangle$ values for protein concentrations at the apparent saturation concentrations (i.e. 12, 16, and 20 μ M) yields a total deceleration of 0.7 ns, corresponding to 30% relative to the corresponding system without protein. Table 2 indicates a direct connection between amount of surface coverage and solvent relaxation kinetics and the first step (i.e. from 0 to 2 μ M prothrombin), which can be considered as the physiological relevant one, shows the largest change in $\langle\tau_r\rangle$. The observed binding behavior appears to be qualitatively in line with binding isotherms determined by other methods [7]. It has to be stressed, however, that the experimental errors are too large, preventing use of this method for the determination of binding constants. The deceleration at saturation concentrations, however, is significant and will be used for the comparison of prothrombin versus fragment 1 binding.

Table 2

Mean relaxation times $\langle\tau_r\rangle$ in ns according to Eq. 3 of Patman in PC/PS 80:20 vesicles in presence of different amounts of prothrombin

PC/PS Fluorophore	Prothrombin			
	0 μ M	2 μ M	5 μ M	8 μ M
80:20 Patman	2.3 \pm 0.2	2.7 \pm 0.2	2.6 \pm 0.3	2.8 \pm 0.4
	12 μ M	16 μ M	20 μ M	25 μ M
80:20 Patman	3.0 \pm 0.3	3.1 \pm 0.3	2.9 \pm 0.3	3.0 \pm 0.2

The temperature was 25°C.

Table 3

Increases of $\langle\tau_r\rangle$ in the presence of protein relative to the relaxation times in the absence of proteins

PC/PS	Fluorophore	PT	BF1
80:20	Patman	30%	30%
60:40	Patman	35%	30%
80:20	Prodan	100%	50%
60:40	Prodan	92%	58%

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 μ M for PC/PS 60:40 and 12, 16, 20 μ M for PC/PS 80:20). The temperature was 25°C.

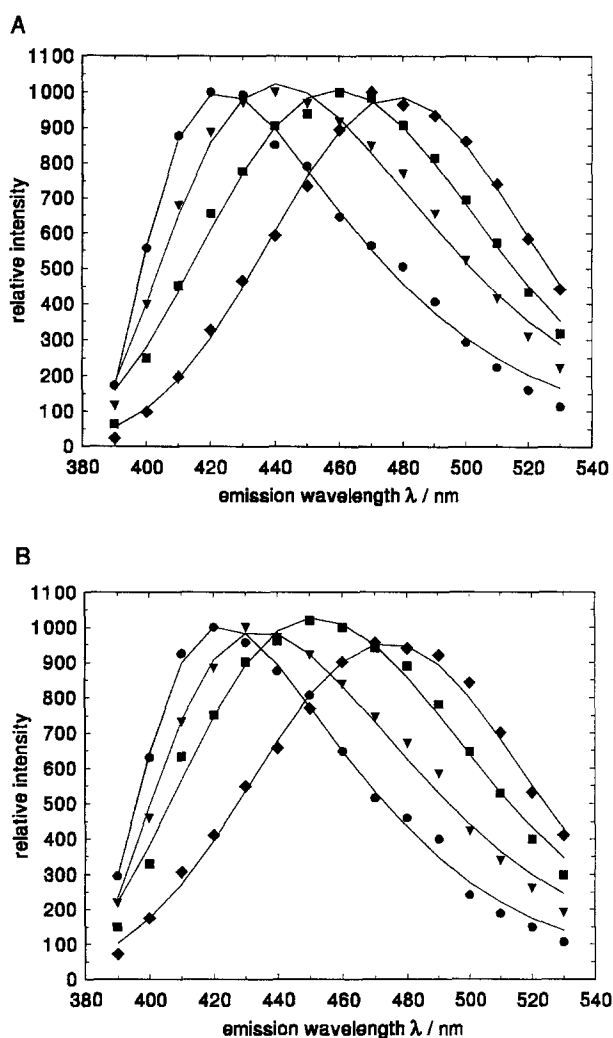


Fig. 3. Time-resolved emission spectra for Patman in PC/PS 60:40 vesicles in the presence of 5 mM Ca^{2+} without protein (A) and in the presence of 16 μM prothrombin (B). The temperature was 25°C. Spectra are shown at 0.2 ns after excitation (circles), 1.0 ns (triangles), 2.0 ns (boxes) and 5.0 ns (diamonds).

The effects of binding of prothrombin on the solvent relaxation kinetics of Prodan and Patman are compared with those of its fragment 1 using SUV composed of PC/PS 60:40 and 80:20 at saturating protein concentrations (8, 12, 16 and 12, 16, 20 μM for PC/PS 60:40 and 80:20, respectively). In Fig. 3 the reconstructed TRES are shown for Patman in the absence (Fig. 3A) and presence (Fig. 3B) of 16 μM prothrombin. Fig. 4 gives the corresponding correlation functions for Patman (Fig. 4A) and Prodan (Fig. 4B) in the absence and presence of proteins. For Patman (Fig. 4A) both proteins yield nearly

identical effects on the mean relaxation times. For both lipid systems and both proteins the averaged increase in $\langle\tau_r\rangle$ at saturating concentrations was $\approx 30\%$ (Table 3).

In contrast to Patman which obviously could not differentiate between both proteins, a clear difference was detected using Prodan as the fluorophore (Fig. 4B). Addition of BF1 yielded an increase of $\langle\tau_r\rangle$ of 50 and 58% relative to the system without protein for 20 and 40% PS vesicles, respectively, while the corresponding increases for prothrombin were as high as 100 and 92%, respectively (Table 3). Thus, Prodan

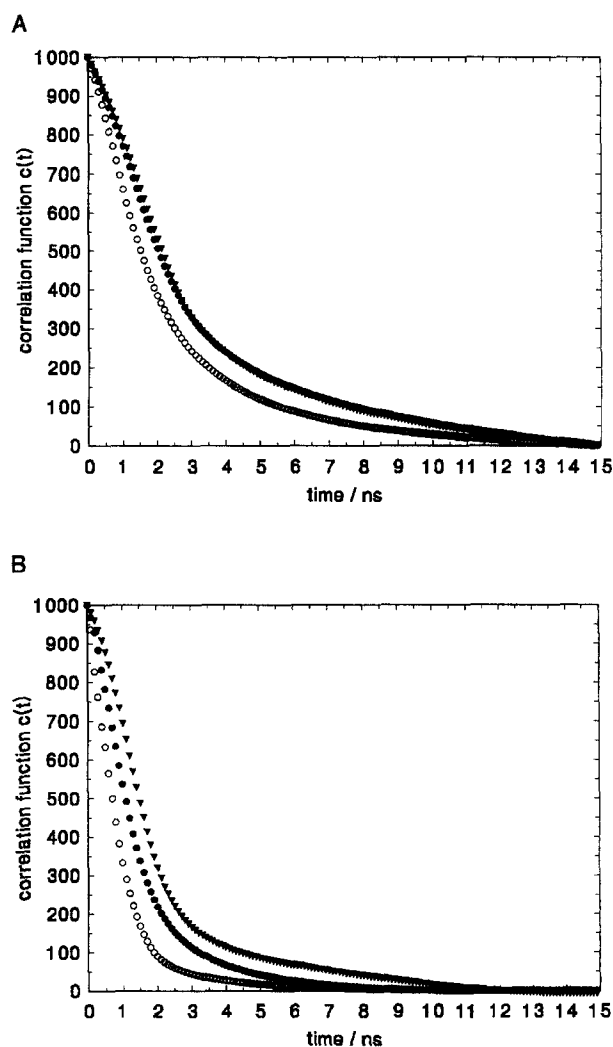


Fig. 4. Correlation functions $C(t)$ (according to Eq. 2) for Patman (A) and Prodan (B) in PC/PS 60:40 vesicles in the presence of 5 mM Ca^{2+} without protein (open circles), in the presence of 16 μM BF1 and prothrombin (triangles). The temperature was 25°C.

which has been shown to be localized closer to the lipid/water interface [12] reacts considerably more sensitive to the binding of different proteins than does Patman.

3.5. Effect of prothrombin and its fragment 1 on PC/PS membrane order

The determination of the so-called ‘membrane order’ by steady-state fluorescence anisotropy measurements of DPH [24] is certainly the most frequently used tool for the characterization of the physical state of a membrane system [25]. The method has been used for the investigation of the prothrombin binding to SUV composed of different ratios of DMPC and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylserine (DMPS) [8]. Nevertheless, we have determined the dependence of the DPH anisotropy in SUV composed of PC/PS 60:40 mixtures on the binding of prothrombin and its fragment 1. The temperature scan (10–35°C) of the DPH anisotropy is shown in Fig. 5. Adding both proteins to a concentration leading to nearly surface coverage slightly increases the membrane order. The effect of prothrombin appears to be larger than that of BF1. It should be noted that the increases in the anisotropies expressed as percentages, averaged over all temperatures are only 5 and 3% for prothrombin and its fragment 1, respectively.

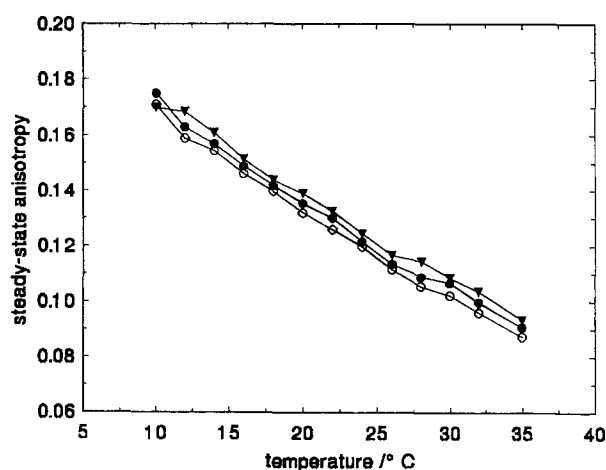


Fig. 5. Steady-state anisotropies of DPH (0.5 μ M) in PC/PS 60:40 vesicles (50 μ M) in the presence of 5 mM Ca^{2+} without protein (open circles), 10 μ M BF1 (closed circles) and 10 μ M prothrombin (triangles). The excitation wavelength was 360 nm, emission was detected at 430 nm.

4. Discussion

The purpose of this work is two-fold: first, we present the ‘solvent relaxation technique’ as a sensitive method for the investigation of structural changes in the phospholipid headgroup region induced by e.g. binding of peripheral membrane proteins; and second, we apply this method to get further insight into the nature of the prothrombin–PS interaction in the presence of calcium ions.

The TRES and the solvent relaxation kinetics of Prodan and Patman in all investigated systems support our previous conclusion [12,15] that this pair of fluorophores is capable to distinguish between structural changes at the membrane/water interface (Prodan) and in the region between the lipid headgroups and the first C-atoms of the hydrophobic acyl chains (Patman).

The slight deceleration of the solvent relaxation monitored by Patman due to increasing PS content in the absence of Ca^{2+} suggests a lower flexibility of the PS headgroup compared to the PC headgroup. This conclusion is in agreement with NMR studies, having shown that the rotational mobility of the PS headgroup is lower than for phosphatidylglycerol, which is on the other hand less mobile than the PC headgroup [26,27]. In contrast to pure PC vesicles, the addition of 3 mM Ca^{2+} leads to a considerable deceleration of the solvent relaxation in PS-containing vesicles as observed by both dyes. This effect increases with increasing PS content (Fig. 1A). Thus, the deceleration of the headgroup mobility with increasing PS content is much more pronounced when Ca^{2+} is present. These results indicate a tighter phospholipid headgroup packing with increasing PS content and suggest a bridging of PS molecules by Ca^{2+} within the plane of the membrane leading to a decrease in lipid mobility. A Ca^{2+} -induced increase of the lateral packing density in bilayers has already been suggested by studies investigating the phase transition of 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol/1,2-dimyristoyl-3-*sn*-phosphatidylcholine SUV [28] and more recently, by the fractal analysis of the monomer fluorescence decay of pyrene-labeled phosphatidylglycerol in PC vesicles [29].

On the molecular level, the observed tighter headgroup packing can be explained by a coordinative Ca–PS binding. The first possibility of 1:1 calcium/

PS complexes would only lead to an increase of the molecular mass of the serine headgroup and cannot explain the observed large increase in the mean relaxation times with increasing PS content. A much more likely explanation is the formation of Ca(PS)_x complexes ($x > 1$). Considering the calcium coordination number of six and the presence of two negative charges in the PS headgroup, x should be 2.

In order to apply the solvent relaxation method described above to the protein– Ca^{2+} –PS interactions, it had to be verified that the used fluorophores do not bind significantly to BF1 or prothrombin in the presence of lipid. This was shown comparing steady-state spectra of Prodan and Patman in buffer, in the presence of high protein concentrations, and after addition of different amounts of vesicle suspension (Fig. 2). While neither the addition of prothrombin nor BF1 changed the emission spectra or the decay behavior, addition of vesicles in the presence of the proteins led to a new blue shifted emission band and a pronounced increase of the lifetimes (Table 1) caused by the binding of Prodan and Patman, respectively, to the membrane. Thus, it could be assumed that significant binding of the dyes to the proteins does not occur.

The validity of the solvent relaxation approach for the investigation of the prothrombin binding is demonstrated by a comparison of both the TRES (Fig. 3) and the changes in the mean relaxation times expressed as percentages (Fig. 4 and Table 3) with effects observed in the steady-state anisotropies (Fig. 5). While the latter shows only small increases in the membrane order due to saturation binding of prothrombin and its fragment 1 (5 and 3%, respectively), the determined changes in the solvent relaxation kinetics nearly reach 100%. It has to be mentioned that the influence of prothrombin and fragment 1 binding on the membrane order has been intensively studied before [8]. These authors did not detect any effect of BF1 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 show similar trends, with the major difference, however, that we observe a detectable, though very small effect of BF1 and a smaller prothrombin induced increase of membrane order than detected by Tendian and Lentz [8]. One reason for

this discrepancy might be the different lipids used, with very different phase transition temperatures. The other major difference is the choice of the temperatures used for the comparison of the anisotropies in the presence and absence of prothrombin and fragment 1. In this work, we simply compare the anisotropies at absolute temperatures far above the phase transition, while Tendian and Lentz defined a relative temperature (5°C above the respective phase transition) for each experiment. 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. On the other hand, the phospholipid headgroup region becomes considerably more rigid, as demonstrated by our solvent relaxation data.

Considering the results of Cutsforth et al. [7], showing that up to three PS molecules are bound to one prothrombin molecule, it seems understandable that this extensive bridging of the serine headgroups by a single calcium/protein complex lowers the headgroup mobility. The deceleration of the solvent relaxation process due to BF1 binding is larger for Prodan which is bound near the surface of the membrane, than for Patman which is located closer to the hydrocarbon region (Fig. 4 and Table 3). Apparently, the binding of the proteins predominantly affects the outermost region of the membrane, where the amino and the carboxyl group are supposed to be located. Our observations argue against a hydrophobic protein membrane interaction [30] and favor the formation of a coordination complex by the PS headgroup, calcium ions and the Gla residues of the proteins [5].

The influence of the prothrombin binding on the physical structure of the membrane shares many common features with that of BF1. The only substantial difference observed is the solvent relaxation kinetics of Prodan. Obviously, the interaction of prothrombin and its fragment 1 with the anionic lipid surface is different for the outermost region of the bilayer. Patman, on the other hand, located slightly deeper in the membrane, cannot distinguish between binding of the entire protein and its fragment 1. As for BF1, this observation argues against a proposed hydrophobic interaction of the non-fragment 1 por-

tion of prothrombin with the phospholipid bilayer [30]. There are several reports in the literature about differences in the binding behavior of prothrombin and its fragment 1 [8–10,30]. Yet, the origin of the observed differences is not clear and several hypotheses have been discussed. One possible explanation is that prothrombin, but not fragment 1, experiences a Ca^{2+} -independent interaction with PS-containing membranes [8–10]. Such an interaction would increase the number of protein bound lipid molecules by one, leading to a somewhat more dense headgroup packing. Following the trend observed for the calcium and BF1 binding, one would expect a decrease in the solvent relaxation dynamics for both Prodan and Patman. The fact that Patman does not experience any differences does not support this argumentation.

An alternative explanation is that in the entire prothrombin molecule, the PS-specific sites in the fragment 1 region are more readily occupied by membrane associated PS than in the case of the isolated fragment 1 [10]. In other words, the presence of the non-fragment 1 part of prothrombin strengthens the coordinative binding between the PS headgroups and the Ca fragment 1 complex. In order to judge this hypothesis on the basis of our results, we reconsider the basic principles of the solvent relaxation approach. The process observed is the reorientation dynamics of the dye microenvironment after the instantaneous change of the dipole moment of the chromophore. Supposing that Prodan is located in the close vicinity of the serine group forming the chelate complex, the reorientation of the Prodan microenvironment will depend on the binding strength of the calcium coordination of the PS ligands. Assuming that prothrombin strengthens this coordination compared to fragment 1, an increase in the solvent relaxation time monitored by Prodan should be the consequence. On the other hand, Patman, which is located closer to the acyl chain region, will not sense this enforced binding; thus its relaxation behavior is essentially the same for either prothrombin or BF1 binding. Of course, this interpretation is speculative. In summary, however, our results rather favor a tighter fragment 1 binding in the case of prothrombin compared with the isolated BF1, than support the possibility of a further

PS binding site in the non-fragment 1 portion of prothrombin.

5. Conclusions

The herein presented results certainly demonstrate, that the ‘solvent relaxation technique’ is a useful method for mechanistic investigations of the membrane binding of peripheral membrane proteins. Binding of more than one negatively charged phospholipid molecule to a single divalent cation or a single protein molecule rigidifies significantly the phospholipid headgroup region. When using membrane labels, which are located at different depths within the bilayer, information about possible hydrophobic interactions can be gained. Comparison of the changes in the solvent relaxation times, induced by either prothrombin or its fragment 1 monitored by either Patman or Prodan, together with the comparatively small changes in the membrane order detected by DPH, indicate that the negatively charged headgroup region of PS-containing membranes is predominantly responsible for the calcium-mediated prothrombin binding. The observation that the increase in the solvent relaxation times monitored by Patman is identical for prothrombin and its N-terminal fragment 1 indicates similarities in the binding mechanisms of both proteins. The solvent relaxation kinetics of Prodan, however, which is located closer to the lipid/water interface than Patman, is more affected by the binding of prothrombin than by its fragment 1. A possible explanation for the more rigid outermost region of the negatively charged phospholipid headgroup due to the binding of the entire protein could be a tighter binding of the fragment 1 portion in the case of the entire prothrombin molecule when compared to the isolated fragment 1.

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