Laser-Induced Europium(III) Luminescence as a Probe of the Metal Ion Mediated Association of Human Prothrombin with Phospholipid†

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ABSTRACT: $^7F_0 \rightarrow {}^5D_0$ excitation spectroscopy of Eu(III) has been used to investigate the Eu(III) and phospholipid binding properties of human prothrombin. The results indicate that human prothrombin contains four high-affinity Eu(III) binding sites which are distributed into two classes of binding sites. When 4 equiv of Eu(III) is bound to prothrombin, the prothrombin is capable of binding to phospholipid vesicles. The deuterium isotope effect on the lifetime of the Eu(III)–prothrombin complex and the Eu(III)–prothrombin–phospholipid complex was used to determine the number of water molecules coordinated to the Eu(III). In both complexes, each of the Eu(III)'s coordinated to 2.5 ± 0.5 water molecules. These results indicate that the binding of the Eu(III)–prothrombin complex to the phospholipid does not require the formation of a prothrombin–Eu(III)–phospholipid bridge.

The conversion of prothrombin to thrombin by factor Xa is greatly accelerated by Ca(II) and phospholipid. Prothrombin and factor Xa are vitamin K dependent proteins and contain γ-carboxyglutamic acid residues which are believed to be necessary for both the metal ion and phospholipid binding properties of these proteins [for a review, see Suttie & Jackson (1977)]. Although the actual mechanism for the interaction between prothrombin and phospholipid has not been fully clarified, the importance of a net negative charge on the phospholipid and the presence of γ-carboxyglutamic acid residues on prothrombin are well recognized (Bull et al., 1972; Esmon et al., 1975; Nelsestuen & Suttie, 1972; Papahadjopoulos & Hanahan, 1962; Stenflo & Ganrot, 1973). Lim et al. (1977) and Dombrose et al. (1979) have independently proposed a molecular model in which Ca(II) bridges link the γ-carboxyglutamic acid residues of prothrombin to the phosphate groups of the lipid. However, there is no direct evidence to support this model.

A major difficulty in studying the structure of the protein–Ca–phospholipid ternary complex is that Ca(II) is spectroscopically silent due to the closed electronic shell structure which does not permit a spectroscopic study of ligand interactions. Spectroscopically active trivalent lanthanide ions have been used to probe the interaction of Ca(II) with biologically important molecules (Ellis, 1977; Martin & Richardson, 1979; Nieboer, 1975; Reuben, 1979). Furie et al. (1976) have reported that several trivalent lanthanide ions can substitute for Ca(II) in the conversion of bovine prothrombin to thrombin by factor Xa. Recently, one of us (W.D.H.) has developed laser spectroscopic techniques which utilize lanthanide ion luminescence (Horrocks & Sudnick, 1979a,b, 1981; Horrocks et al., 1977) for probing the molecular mechanism of Ca(II)–protein complex formation in biological systems (Horrocks et al., 1980; Rhee et al., 1981; Snyder et al., 1981).

In this study, we report the use of two novel techniques to determine the nature of the metal ion mediated interaction of prothrombin with phospholipid. One is $^7F_0 \rightarrow {}^5D_0$ excitation spectroscopy of Eu(III) to determine the number of Eu(III) binding sites in prothrombin, and the other is the deuterium isotope effect on the reciprocal lifetime of excited Eu(III) to monitor the number of water molecules on Eu(III) before and after the reaction between prothrombin and Eu(III), and the prothrombin–Eu(III) complex and phospholipid.

Materials and Methods

D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238) was purchased from Kabi Diagnostica, Folch fraction III from Sigma Chemical Co., egg phosphatidylethanolamine from P-L Biochemicals, $EuCl_3$ from Alfa-Ventron, and *Echis carinatus* venom from Miami Serpentarium.

Human prothrombin was purified by the method of DiScipio et al. (1977). Human factor Xa was prepared as previously described (Kosow & Orthner, 1979). *E. carinatus* venom coagulant (ECV-P)[1] was purified by the method of Rhee et al. (1982). Phospholipid vesicles were prepared by a modification of the method of Kosow & Orthner (1979). Twenty-two milligrams of Folch fraction III was mixed with 50 mg of phosphatidylethanolamine in 2 mL of chloroform. The chloroform was removed by a stream of N_2, and the lipid was dispersed by direct probe sonication in 4 mL of 0.01 M Tris buffer, pH 7.5, containing 0.15 M NaCl and filtered through a Millipore membrane (type HA, 0.45 μm). The solution was diluted 5-fold with Tris buffer before use.

Protein–Phospholipid Binding Determination. Human prothrombin (1.3 nmol) in 0.01 M imidazole buffer, pH 6.5, containing 0.15 M NaCl (referred to as "imidazole buffer") and 0–8 equiv of $EuCl_3$ was incubated at room temperature with 60 nmol of phospholipid for at least 10 min in a final volume of 0.14 mL. The amount of prothrombin bound was determined by centrifuging 0.12 mL of the reaction mixture in a Beckman airfuge. The supernate was removed, and the phospholipid pellet was resuspended. The prothrombin concentrations of the resuspended pellet and the supernate were determined by converting the prothrombin to thrombin with 0.5 μg of ECV-P and assaying the thrombin by the method of Orthner & Kosow (1980). Alternatively, the prothrombin concentration of the supernate was determined from the ab-

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[1] Abbreviations: ECV-P, *Echis carinatus* venom coagulant; Tris, tris(hydroxymethyl)aminomethane.

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sorbance at 280 nm by employing an $E^{1\%}_{280nm}$ of 14. Controls were incubated in the absence of phospholipid to ensure that the binding to phospholipid, and not nonspecific protein precipitation, was being measured.

Eu(III) Excitation Spectra of the $^7F_0 \rightarrow {}^5D_0$ Transition. Human prothrombin (2×10^{-5} M) in 0.2 mL of imidazole buffer was irradiated with a pulsed dye laser and continuously scanned from 578 to 580 nm (rhodamine 6G dye) after each addition of Eu(III). The emission intensity was then recorded as a function of wavelength with the aid of a cooled photon-counting system and multichannel analyzer.

Determination of the Number of Water Molecules on Eu(III) in Prothrombin. To human prothrombin (2×10^{-5} M) in 0.1 mL of imidazole buffer was added 1 μL of Eu(III) solution (8.64×10^{-3} M), and the reciprocal lifetime of excited Eu(III) in prothrombin was measured in the presence of 0%, 50%, and 75% D_2O. The value of the reciprocal lifetime in pure D_2O was obtained by linear extrapolation to 100% D_2O (Horrocks et al., 1977). The effect of phospholipid on the reciprocal lifetime of the Eu(III)–prothrombin complex was determined by titrating 100 μL of 2×10^{-5} M prothrombin containing 4 equiv of Eu(III) with phospholipid and observing the change of the reciprocal lifetime of Eu(III) in the prothrombin–Eu(III) complex. Measurement of the reciprocal lifetime of Eu(III) in the Eu(III)–prothrombin complex was achieved by irradiating the sample with the pulsed dye laser at both 578.8 and 579.5 nm and monitoring the decay of luminescence at 612 nm with the photon-counting system and multichannel analyzer. A more detailed description of the instrumentation may be found elsewhere (Horrocks & Sudnick, 1981; Sudnick, 1979).

Activation of Prothrombin by Factor Xa and Phospholipid in the Presence of either Eu(III) or Ca(II). To 0.2 mL of human prothrombin (2×10^{-5} M) in imidazole buffer containing various amounts of either Eu(III) or Ca(II) was added 20 μL of phospholipid (1.8×10^{-4} M) followed by 1 μL of human factor Xa (8.8×10^{-6} M). At various time intervals, an aliquot of the reaction mixture was removed and assayed for thrombin by the method of Orthner & Kosow (1980).

Results

Eu(III) Titration of Prothrombin Monitored by $^7F_0 \rightarrow {}^5D_0$ Excitation Spectra. The excitation spectra of prothrombin having 1–5 equiv of Eu(III) are shown in Figure 1. At 1 equiv of Eu(III), the spectrum shows a peak centered at 579.3 nm which gradually increases as the Eu(III)/protein ratio is increased up to a value of 4. It is notable that the maximum peak position is shifted to a higher energy after 2 equiv of Eu(III) is added. This suggests that another peak is appearing as more Eu(III) is added. Curve analysis shows that the second peak is positioned at 579.0 nm. After the addition of 4 equiv of Eu(III), there was no more increase of intensity, indicating that the protein is saturated with Eu(III). When more than 5 equiv of Eu(III) was added, the protein precipitated, making it difficult to carry out the experiments due to light scattering.

These results indicate that human prothrombin has four high-affinity Eu(III) binding sites which are distributed into two classes of binding environments. Each of these binding environments consists of two binding sites. The binding curve (Figure 1, inset) is suggestive of positive cooperativity. When the Eu(III) is added to phospholipid vesicles, the excitation spectrum (Figure 1B) shows a single peak centered at 579.1 nm while free aquoeuropium(III) shows an excitation peak at 578.7 nm indicating all the Eu(III) ions are bound to phospholipid under these experimental conditions. However,

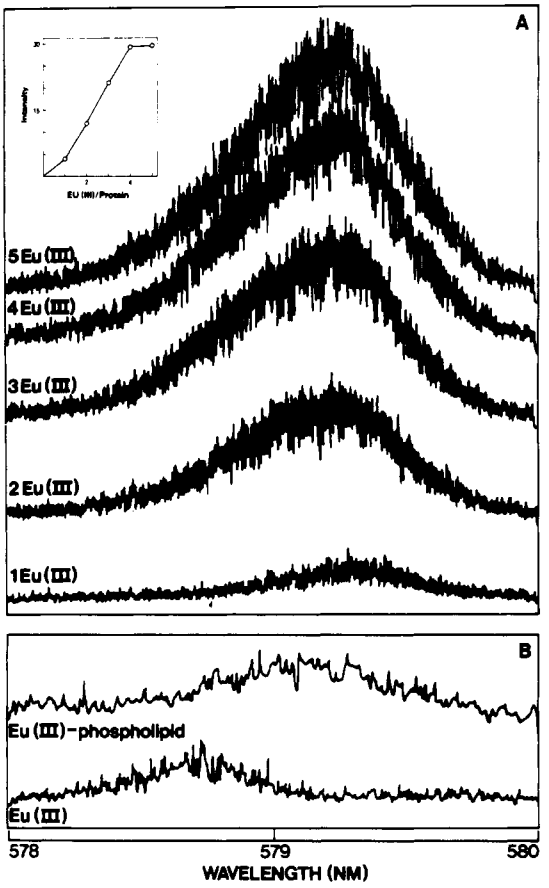

FIGURE 1: (A) $^7F_0 \rightarrow {}^5D_0$ excitation spectra of the Eu(III)–prothrombin complexes containing (from bottom to top) 1.0, 2.0, 3.0, 4.0, and 5.0 equiv of Eu(III). The prothrombin concentration was constant at 2×10^{-5} M. The inset is a plot of the intensity at 579.5 nm vs. the Eu(III)/prothrombin ratio. (B) $^7F_0 \rightarrow {}^5D_0$ excitation spectra of the Eu(III)–phospholipid complex (top) and free Eu(III) (bottom) at a concentration of 2×10^{-5} M.

the affinity of Eu(III) for phospholipid is about 10 times lower than the affinity of Eu(III) for prothrombin.[2]

Association of Prothrombin with Phospholipid. It is well established that the association of prothrombin with phospholipid requires Ca(II) (Suttie & Jackson, 1977). However, the ability of Eu(III) to substitute for Ca(II) in this reaction has not been reported, although Nelsestuen et al. (1976) have shown that both La(II) and Tb(III) support prothrombin–phospholipid complex formation. For demonstration that Eu(III) can mediate prothrombin binding to phospholipid, mixtures of prothrombin and phospholipid were titrated with Eu(III), and complex formation was measured as described under Materials and Methods.

When phospholipid–prothrombin complex formation was determined by centrifugation (Figure 2), only about 1.3% of the prothrombin was detected in the phospholipid pellet in the absence of metal ions. The amount of prothrombin bound reached a plateau value of about 60% when 4 mol of Eu(III) per mol of prothrombin was bound. When 1 mM Ca(II) was the metal ion, 77.7% of the prothrombin was bound to phospholipid. It can therefore be concluded that Eu(III) supports prothrombin binding to phospholipid vesicles.

Activation of Eu(III) Prothrombin by Factor Xa. When a native protein molecule is modified by a structural probe, it is essential to demonstrate that the original structure and function of the protein are not drastically altered. In this

[2] M.-J. Rhee, W. DeW. Horrocks, Jr., and D. P. Kosow, unpublished experiments.

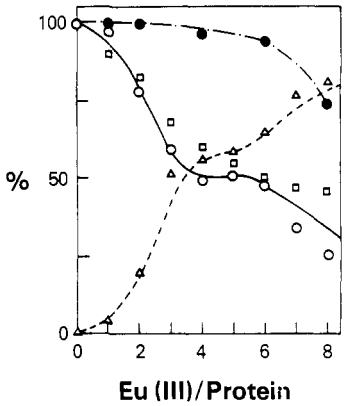

FIGURE 2: Binding of prothrombin to phospholipid vesicles as a function of the ratio of Eu(III) to prothrombin. Prothrombin (1.3 nmol) was incubated in the presence (△, □, ○) or absence (●) of 60 nmol of phospholipid. The amount of prothrombin in the phospholipid pellet (△) or remaining in solution after centrifugation (□, ○, ●) was determined by activity measurements (△, ○) or absorbance at 280 nm (□, ●) as described in the text.

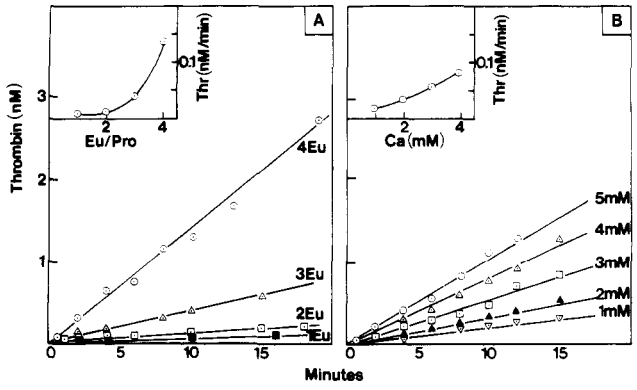

FIGURE 3: Conversion of prothrombin to thrombin by factor Xa in the presence of phospholipid and Eu(III) or Ca(II). Prothrombin (17 μM) was incubated at room temperature with 16 μM phospholipid and 38 nM factor Xa. (A) Eu(III)/prothrombin ratio was 1 (■), 2 (□), 3 (△), or 4 (○). (B) Ca(II) concentration was 1 (▽), 2 (▲), 3 (□), 4 (△), or 5 mM (○).

regard, we have shown that Eu(III) supports the binding of prothrombin to phospholipid (Figure 2). However, it is also necessary to demonstrate that the Eu(III)-mediated binding of prothrombin to phospholipid leads to a functionally active complex. Therefore, prothrombin was activated by factor Xa and phospholipid in the presence of either Eu(III) or Ca(II). The kinetic data (Figure 3) show that Eu(III) ions support prothrombin activation by factor Xa as effectively as Ca(II) ions.

Determination of the Number of Water Molecules Coordinated to Eu(III) in Prothrombin, Phospholipid, and the Prothrombin–Phospholipid Complex. Since the excitation spectra (Figure 1A) show peaks at 579.0 and 579.3 nm, the reciprocal lifetimes of prothrombin-bound excited Eu(III) were measured at 578.8 and 579.5 nm in order to obtain values for each class of binding site with minimal interference from Eu(III) bound to the other site. The data shown in Table I and Figure 4 indicate that in the presence of prothrombin, the reciprocal lifetime (τ^{-1}) of bound Eu(III) is significantly less than that of free Eu(III). From the linear extrapolation of the deuterium isotope effect (Figure 4) and eq 1 (Horrocks & Sudnick, 1979a, 1981) where q is the number of water

$$q = 1.05(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O}) \quad (1)$$

molecules coordinated to Eu(III), we can calculate that when Eu(III) is bound to prothrombin there is a concomitant loss

Table I: Number of Water Molecules Coordinated to Eu(III) and to Eu(III) Bound to Prothrombin and/or Phospholipid

	$\tau^{-1}_{H_2O}$ (ms^{-1})[a]	$\tau^{-1}_{D_2O}$ (ms^{-1})	q[b]
Eu(III)[c]	9.6	0.38	9.6
Eu(III)-prothrombin[c]	3.12	0.82	2.4
Eu(III)-prothrombin[d]	3.32	0.82	2.6
Eu(III)-phospholipid[c]	8.2	1.6	6.9
Eu(III)-prothrombin-phospholipid[c]	3.4	1.0	2.5

[a] $\tau^{-1}_{H_2O}$ and $\tau^{-1}_{D_2O}$ are the luminescence decay constants of excited Eu(III) in H_2O and D_2O, respectively. The constants were measured in the presence of 0, 50%, and 75% D_2O. The $\tau^{-1}_{D_2O}$ was determined by linear extrapolation to 100% D_2O (Horrocks et al., 1977). [b] q is the number of H_2O molecules coordinated to Eu(III) and was calculated by using the equation of Horrocks & Sudnick (1979a, 1981): $q = 1.05(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O})$. All values are ±0.5 due to uncertainty of the correlation between the luminescence decay values and the actual number of water molecules (Horrocks & Sudnick, 1979a). [c] Excitation wavelength was 579.5 nm. [d] Excitation wavelength was 578.8 nm.

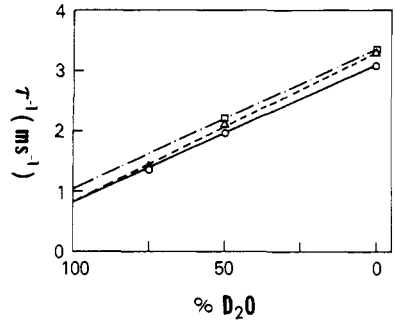

FIGURE 4: Reciprocal lifetime of excited Eu(III) in prothrombin as a function of the D_2O concentration. Excitation was at 578.8 (△) or 579.5 nm (○, □). When present (□), phospholipid was at a 37.5-fold molar excess. The experimental point at 75% D_2O in the presence of phospholipid could not be determined due to excessive light scattering.

of seven molecules of H_2O from the Eu(III) (Table I). The prothrombin–Eu(III) complex is capable of being bound to phospholipid vesicles. The effect of phospholipid on the number of water molecules coordinated to Eu(III) in the prothrombin–Eu(III) complex was investigated by titrating a solution of 2×10^{-5} M prothrombin containing 4 equiv of Eu(III) with phospholipid and measuring the effect on τ^{-1}. The results indicate that the τ^{-1} values are not affected by the presence of phospholipid within experimental error (Figures 4 and 5). Thus, no water molecules were displaced from the Eu(III)–prothrombin complex. If water molecules are displaced by phospholipid, τ^{-1} values would be expected to decrease as shown by the theoretical lines (Figure 5). In order to calculate the theoretical lines, we made the assumptions that, under our experimental conditions, 60% of Eu(III)–prothrombin is bound to phospholipid and that four Eu(III) ions are involved in the interaction with phospholipid (see Figure 2). These data strongly suggest that the coordination sphere of Eu(III) in prothrombin is not disturbed when the prothrombin–Eu(III) complex binds to phospholipid vesicles.

Discussion

Sarasua et al. (1980) have studied Eu(III) coordination with γ-carboxyglutamic acid containing synthetic di- and tripeptides. Their results indicate that two of the nine water molecules are lost from the Eu(III) inner coordination sphere per γ-carboxyglutamic acid residue bound. Work from the same laboratory (Marsh et al., 1981) demonstrated that at an equimolar ratio of Eu(III) and bovine prothrombin frag-

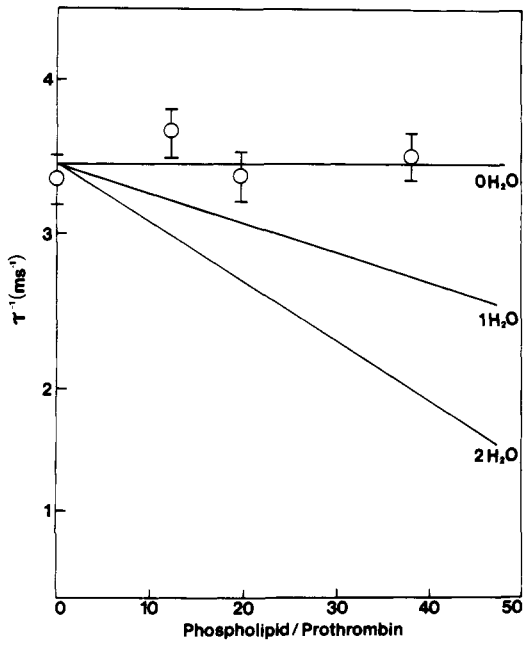

FIGURE 5: Effect of phospholipid on the reciprocal lifetime of Eu(III) bound to prothrombin. Excitation was at 579.5 nm. Prothrombin (2×10^{-5} M) in imidazole buffer containing 4 equiv of Eu(III) was titrated with phospholipid up to 37-fold excess over prothrombin. The two theoretical lines show the calculated τ^{-1} for the loss of the one and two water molecules from Eu(III) of the Eu(III)–prothrombin complex.

ment 1 (the peptide which contains all the γ-carboxyglutamic acid residues of native prothrombin) approximately two water molecules remain in the inner coordination sphere of Eu(III). Our results demonstrate that a plot of the reciprocal of the Eu(III) luminescence lifetime (τ^{-1}) vs. the percent of D_2O in an H_2O–D_2O solvent mixture is linear in the presence of prothrombin, indicating that the observed changes in τ^{-1} are a function of the number of water molecules in the inner coordination sphere of Eu(III) (Horrocks et al., 1977). At a Eu(III) to prothrombin ratio of 4, we calculate that 2.4 ± 0.5 water molecules are coordinated to Eu(III) in one of the two classes of binding sites in prothrombin and that 2.6 ± 0.5 water molecules are coordinated to Eu(III) in the other. This indicates that all four binding sites in prothrombin as well as the binding site in bovine prothrombin fragment 1 studied by Marsh et al. (1981) provide the same number of ligands to Eu(III), although each class of binding sites has a slightly different geometry as inferred from the excitation spectra. Since aqueous Eu(III) contains 9.6 ± 0.5 water molecules in its inner coordination sphere (Horrocks & Sudnick, 1979a), if the only ligands for Eu(III) binding are the γ-carboxyglutamic acid residues of prothrombin (Suttie & Jackson, 1977) and if at least two water molecules are displaced per γ-carboxyglutamic acid residue coordinated to Eu(III) (Sarasua et al., 1980) then at least three γ-carboxyglutamic acid residues would be required to displace six to eight water molecules from the Eu(III). Interestingly, Osterberg et al. (1980) calculated that it would require three malonate residues per Ca(II) bound to prothrombin in order to account for the high ($>10^5$) stability constant of the prothrombin–Ca(II) complex.

In order to use Eu(III) luminescence as a probe of the role of metal ions in the binding of prothrombin to phospholipid, it was first necessary to demonstrate that Eu(III) can support the binding of prothrombin to phospholipid and its conversion to thrombin by factor Xa. Nelsestuen et al. (1976) reported that Tb(III) and La(III) could substitute for Ca(II) in the binding of bovine prothrombin to phospholipid vesicles, and Furie et al. (1976) demonstrated that Gd(III), Tb(III), La(III), Dy(III), Pr(III), and Sm(III) could substitute for Ca(II) in the phospholipid-enhanced activation of bovine prothrombin by factor Xa. The data presented in Figures 2 and 3 clearly demonstrate that Eu(III) ions are functioning as effectively as Ca(II) ions in terms of formation of a prothrombin–phospholipid complex and in the activation of prothrombin to thrombin by factor Xa. Therefore, it is reasonable to assume that the mechanism of prothrombin binding to phospholipid in the presence of Eu(III) is very similar, if not the same, to that in the presence of Ca(II). From this assumption, we postulate that if the function of the metal ion is to bridge between the negative carboxyl groups of the protein and the phosphate head groups or carboxyl group of the phospholipid, then fewer water molecules should occupy the inner coordination sphere of the Eu(III) in the Eu(III)–prothrombin–phospholipid ternary complex than in the Eu(III)–prothrombin binary complex. The data of Table I and Figure 4 demonstrate that the Eu(III)–phospholipid complex retains seven water molecules, indicating that phospholipid can provide at least two ligands to Eu(III). However, as shown in Figure 5, titration of the Eu(III)–prothrombin complex with phospholipid, while monitoring the reciprocal lifetime of Eu(III) in prothrombin, shows that within experimental error, the presence of phospholipid does not affect the reciprocal lifetime of Eu(III), even though up to 60% of the Eu(III)–prothrombin is bound to phospholipid under the conditions used.

From the lifetime measurements of Eu(III) in the prothrombin–phospholipid complex, we conclude that the mechanism for the metal ion mediated binding of prothrombin to phospholipid is not via metal ion bridges. It has been proposed that bovine prothrombin and fragment 1 undergo a Ca(II)-induced transition which is necessary for their interaction with phospholipid (Nelsestuen, 1976; Marsh et al., 1979). We therefore suggest that a possible alternative mechanism to the metal ion bridge hypothesis is that the metal ions induce a conformational change in the fragment 1 region of prothrombin which exposes a hydrophobic site and/or an electrically charged site which has a high affinity for phospholipid. In this regard, it is of interest to note that Marsh et al. (1981) have shown that Eu(III) and Ca(II) induce similar but not completely equivalent conformational changes in bovine prothrombin fragment 1 as judged by immunological techniques.

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