# Comparison of Naturally Occurring Vitamin K-Dependent Proteins: Correlation of Amino Acid Sequences and Membrane Binding Properties Suggests a Membrane Contact Site†

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ABSTRACT: Membrane-binding properties of human and bovine forms of vitamin K-dependent proteins Z, S, and C were characterized. Each of these proteins showed unique properties and interspecies differences that correlated with specific amino acid sequence variations in the amino-terminal 45 residues. Protein Z showed 100-fold slower membrane binding and dissociation kinetics relative to other vitamin K-dependent proteins that have been tested. This property seemed to correlate with an extra $\gamma$-carboxyglutamic acid (Gla) residue at position 11 of protein Z. The interspecies difference for protein Z consisted of a higher packing density for the bovine protein on the membrane and a 9-fold slower dissociation rate. Higher affinity correlated with Asp at position 34 of bovine protein Z, where the human protein contains Asn. While both protein S species showed high affinity for the membrane, it was significantly greater for the human protein versus bovine protein S. Again, higher affinity correlated with an Asp (vs Asn) at position 34. Protein C was characterized by binding affinities that were 100−1000-fold lower than the other proteins. Low affinity appeared to be related to loss of Gla-32 (homologous to Gla-33 of protein Z). Interspecies differences of protein C appeared to be related to proline at position 10 (homologous to position 11 of protein Z) of bovine protein C, which produced at least 10-fold lower affinity than the human protein. Comparable substitutions at positions homologous to 11, 33, and 34 of protein Z may also underlie membrane binding behaviors of other vitamin K-dependent proteins. The three-dimensional structure of strontium−prothrombin fragment 1 [Seshadri et al. (1994) *Biochemistry 33*, 1087] shows that these positions are clustered on the protein surface near strontium-8, another possible candidate for membrane contact. A membrane contact mechanism consisting of an isolated protein−lipid ion pair is proposed. Comparison of naturally occurring vitamin K-dependent proteins has provided possible bases for divergent membrane binding and suggested future approaches to determine biological function.

Vitamin K-dependent proteins interact with membranes in a calcium-dependent process that has not been fully described. The X-ray structure of prothrombin fragment 1 shows seven calcium ions, many of which appear to serve entirely as protein structural elements (Soriano-Garcia et al., 1992). The X-ray structure of factor VIIa (Banner et al., 1996) and the NMR structure of factor IX (Freedman et al., 1995) are similar to fragment 1. This indicates very homologous tertiary structures, although biochemical evidence suggests the need to modify the structure of bovine prothrombin to a cis configuration for Pro-22 (Evans & Nelsestuen, 1996a).

A distinguishing structural feature is a cluster of three hydrophobic amino acid side chains of residues 5, 6, and 9 which projects from the protein, perpendicular to the plane in which the calcium ions are bound (Soriano-Garcia, 1992). Insertion of this hydrophobic cluster into the hydrocarbon region of the membrane, to a depth that allows some of the calcium ions to interact with the phospholipid head groups, is a central feature of a proposed membrane contact site (Sunnerhagen et al., 1995; Christiansen et al., 1995). Chemical modifications and site-directed mutations of amino acids in this region often influence the calcium affinity of the free protein (Welsch & Nelsestuen, 1988; Weber et al., 1992) or the calcium concentration required for membrane binding (Christiansen et al., 1995). At saturating calcium levels, which should isolate membrane contact for analysis, a surprising feature is the small impact of many changes. For example, replacement of Phe-4 by Gln in human protein C had very little impact on calcium or membrane binding (Christiansen et al., 1995). Substitutions of Gln for Leu-8 (Christiansen et al., 1995) and Ala for Leu-5 (Jalbert et al., 1996) of human protein C reduced membrane binding by 4-fold or less. Removal of the amino-terminal three residues of bovine prothrombin, which should substantially alter the hydrophobic cluster, reduced membrane affinity by only about 5-fold (Weber et al., 1992). Modification of lysine

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residues by acetylation and trinitrophenylation had small impact on membrane binding (Welsch et al., 1988; Welsch & Nelsestuen, 1988; Weber et al., 1992), even though Lys-3 of prothrombin should be found within the proposed membrane contact site. For comparison, an affinity change of 5-fold would represent only 10−15% of the free energy of protein−membrane binding (−7 to −10 kcal/mol, $K_d$ = $10^{-5}$ to $10^{-8}$ M). Finally, protein S represents a case where high affinity for membranes (Nelsestuen et al., 1978; Walker, 1980) is combined with low hydrophobicity of the homologous amino acids (Leu-4, Leu-5, and Thr-8; Lundwall et al., 1986) proposed to be important in membrane contact.

Biochemical properties are mixed with respect to a possible role of hydrophobic vs ionic forces in protein−membrane contact. Prothrombin displays almost no selectivity for binding to SUVs[1] vs LUVs (Lu & Nelsestuen, 1996a), despite differences in hydrophobic exposure that result in large differences for other proteins that exhibit membrane insertion. Large changes in surface pressure of phospholipid monolayers, which should directly affect the free energy change of hydrophobic interaction, produced only a 3-fold change in membrane affinity (Mayer et al., 1983). Direct calcium binding experiments indicate the membrane-bound prothrombin fragment 1 binds a greater number of calcium ions (Evans & Nelsestuen, 1994; Nelsestuen & Lim, 1977; Sommerville et al., 1986) than the free protein (Deerfield et al., 1987), suggesting that calcium bridging to the membrane may occur. Ionic interaction is also suggested by a high sensitivity of prothrombin−membrane binding affinity to acidic phospholipid content of the membrane (Lu & Nelsestuen, 1996a). However, protein−membrane interaction was quite insensitive to ionic strength as long as calcium was saturating (Resnick & Nelsestuen, 1980). Thus, the energetic forces driving membrane binding have not been clearly established as predominantly ionic or hydrophobic.

Since major elements of the membrane contact site of vitamin K-dependent proteins seem uncertain, alternative, testable models for membrane contact are needed. The naturally occurring vitamin K-dependent proteins display a 1000-fold range in membrane affinity (Nelsestuen et al., 1978). This study was initiated to provide a more detailed comparison of these proteins with the goal of identifying specific amino acids that may contribute to the divergence in membrane-binding properties. The results suggested that amino acids substitutions at positions 11, 33, and 34 may be responsible for most of the range of membrane contact properties. These residues are clustered on the surface of the protein, and an alternative membrane contact mechanism is suggested.

## MATERIALS AND METHODS

*Materials*. Bovine brain phosphatidylserine (PS) and dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine (dansyl-PE) were purchased from Avanti Polar Lipids, Inc. Egg phosphatidylcholine (PC) was purchased from Sigma Chemical Co. All phospholipids were of high purity (>98%, supplier's estimate). Other chemicals and reagents were from Sigma Chemical Co. and were of the highest grade available. Polycarbonate filters (0.1 μm diameter) were purchased from Nucleopore Corp. (Costar Co). Bovine protein Z was isolated from barium citrate eluates derived from plasma and purified according to Hashimoto et al. (1985). Bovine protein Z was highly pure as evidenced by Coomassie staining of SDS−polyacrylamide gels. Human protein Z was isolated from human plasma according to Sueyoshi et al. (1991). Bovine prothrombin was isolated according to Nelsestuen (1984). Bovine protein S (Dahlback et al., 1990) and bovine protein C (Stenflo, 1976) were purified and quantitated as described. Human protein S (DiScipio et al., 1977) and human protein C (Kisiel, 1978) were purified and quantitated as described.

Protein Z appeared to be monodisperse with no evidence of self-association. When chromatographed on a column of S-300 (48 cm × 0.7 cm in Tris buffer), both bovine and human protein Z (50−100 μg) eluted as a single peak at a position almost identical to that of bovine prothrombin. The elution profile was not affected by the presence of 5 mM calcium in the column buffer.

*Vesicle Preparations.* Chloroform solutions of phospholipids were mixed in appropriate ratios, and the organic solvent was evaporated by a stream of nitrogen. The sample was then placed under vacuum for 1 h. The dried phospholipids were dispersed in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.5) to a final concentration of 2−5 mg/mL. Small unilamellar vesicles (SUVs) were prepared as previously described (Huang, 1969) by sonication followed by gel filtration chromatography on Sepharose 4B. Large unilamellar vesicles (LUVs) were prepared by multiple freeze−thaw cycles and extrusion through polycarbonate membranes with a pore size of 0.1 μm (Hope et al., 1985). The vesicles were then dialyzed against Tris buffer (50 mM Tris, 0.1 M NaCl) to produce spherical shape. Phospholipid vesicle sizes were determined by dynamic light scattering as described previously (Bloomfield & Lim, 1978). SUVs gave average diameters of 29−30 nm and LUVs an average diameter of 110 nm. The molecular weights of the vesicles were estimated from the vesicle diameters, membrane thickness (3.7 nm for SUVs and 5 nm for LUVs), and the surface areas per phospholipid head group reported for SUVs (0.74 and 0.61 $nm^2$ for the outer and inner membrane leaflets, respectively; Huang & Mason, 1978) and LUVs (0.55 $nm^2$; Deamer & Bangham, 1976). Molecular weights were 5 × $10^6$ for SUVs and 100 × $10^6$ for LUVs.

*Equilibrium Binding of Proteins to Vesicles*. Protein binding to SUVs was monitored by light scattering and fluorescence energy transfer using a FluoroMax (JY/Spex Instruments SA, Inc.) fluorescence spectrophotometer. Light scattering at 90° was measured using light of 320 nm wavelength. The procedure and method for estimation of protein−membrane association from light scattering intensities were essentially as described by Nelsestuen and Lim (1977). Results were expressed as molecular weight ratios, $M_2/M_1$, where $M_1$ is the molecular weight of the vesicles alone and $M_2$ is the molecular weight of the protein−vesicle complex. Protein−membrane association was also monitored by fluorescence energy transfer from protein amino acids (excitation at 287 nm) to dansyl groups in the membrane (emission at 505 nm). The intensities are expressed relative to the fluorescence intensity of the dansyl

[1] Abbreviations: Gla, γ-carboxyglutamic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; Z, protein Z; S, protein S; PT, prothrombin; X, factor X; IX, factor IX; C, protein C; VII, factor VII. Protein sources are indicated by h (human) or b (bovine).

group due to direct excitation by the incident light. Fluorescence changes upon protein binding can also arise from changes in the environment of the dansyl groups. These effects were small as evidenced by almost no change in the dansyl fluorescence upon protein Z−membrane binding when excitation was at 350 nm. Thus, most of the fluorescence signal change was due to fluorescence energy transfer.

*Stopped-Flow Measurements.* Fast kinetics of protein binding to and dissociation from vesicles were monitored by fluorescence energy transfer and/or light scattering using a 4800C spectrofluorometer (SLM, Aminco) as described in detail elsewhere (Lu et al., 1995). Briefly, equal volume syringes were filled with appropriate solutions, and about 40 uL from each syringe was used per injection. The dead time of the instrument was about 6 ms as measured by the fluorescence reaction of pyramine with bovine carbonic anhydrase as described by the manufacturer. A typical experiment for estimation of protein−membrane association used a solution of protein in one syringe and vesicles in another. For dissociation experiments, one syringe contained protein and labeled vesicles and the other contained a concentration of unlabeled SUVs that was greater than 10 times the concentration of the labeled vesicles in the first syringe. This was sufficient to capture virtually all of the free protein and create irreversible dissociation of prothrombin from the labeled vesicles. For both light scattering and fluorescence energy transfer reactions, data points were collected at intervals of 0.025−0.1 s. Each point was derived from an average of measurements made every millisecond. The final intensity was measured after it had ceased to change with time. Data were fit to this as the final intensity. Control experiments involving protein alone, phospholipid alone, and protein and phospholipid without calcium were performed to ensure that signal changes were due to protein−membrane binding or dissociation events.

*Data Analysis.* Association of several calcium-dependent proteins with phospholipid vesicles has been analyzed by pseudo-first-order conditions (Lu et al., 1995; Wei et al., 1982; Pusey et al., 1982). At low protein ratios (w/w of 0.4 or less) and high calcium concentrations, the number of binding sites on the vesicles is in excess over protein, and the reaction conforms to a pseudo-first-order reaction. The pseudo-first-order rate constant ($k_{app}$) was obtained from the time required to reach 40% of maximum reaction ($t_{0.4}$) using the relationship $k_{app} = (\ln 0.4)/t_{0.4}$. Forty percent reaction was within the linear portion of a first-order rate plot of the data. Second-order rate constants for protein binding to sites on the vesicle ($k_{on}$) were obtained from $k_{app}$ values determined at two or more vesicle (protein binding site) concentrations ([PL]) and the relationship in eq 1:

$$k_{app} = k_{on}[PL] + k_{off} \quad (1)$$

The number of protein binding sites per vesicle was determined from the $M_2/M_1$ ratio at apparent saturation and the molecular weights of the protein and vesicles.

Dissociation rate constants were measured directly, by mixing a large excess of unlabeled vesicles with a mixture of protein Z and fluorescent-labeled vesicles. Protein Z dissociation was monitored as a decrease in fluorescence intensity due to energy transfer. The unlabeled vesicles were used in a 7.5−20-fold molar excess over labeled vesicles.

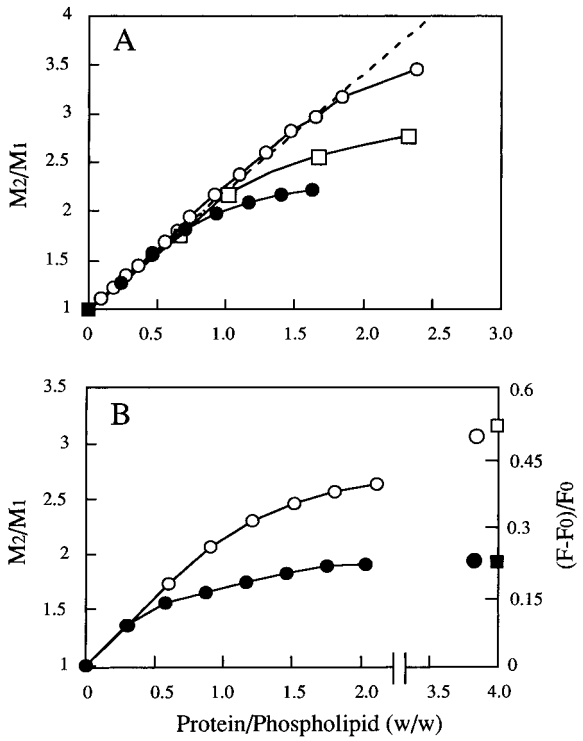


FIGURE 1: Association of protein Z with phospholipid vesicles. Panel A: Titrations of protein binding to SUVs (20 $\mu$g/mL; PS/PC, 50/50). Results for bovine Z (○), bovine prothrombin (□), and human Z (●) are shown. The calcium concentration was 5 mM. $M_2$ and $M_1$ are the molecular weights of the vesicle−protein complex and vesicles alone, respectively, and were calculated from light scattering intensities as described in Materials and Methods. In all cases, the addition of EGTA caused complete protein dissociation. The dotted line shows the values of $M_2/M_1$ expected if all of the added protein bound to the membrane. Panel B: Protein binding to SUVs of 25% PS measured by light scattering (○, ●) or fluorescence energy transfer (□, ■). Association of bovine Z (○) and human Z (●) with unlabeled SUVs (20 $\mu$g/mL; PS/PC, 25/75) as measured by light scattering ($M_2/M_1$). Binding to vesicles of PS/PC/dansyl-PE (25/65/10) also measured by fluorescence energy transfer for bovine Z (□) and human Z (■). $F$ is the fluorescence intensity of the sample, and $F_0$ is the intensity of the vesicles alone. The calcium concentration was 5 mM.

In this range, rates of dissociation were independent of unlabeled phospholipid concentration. Linear first-order rate plots indicated a single process without cooperativity. Curve fitting of the fluorescence data to the first order equation $F = F_0 - (F_0 - F_{fin}) \exp(-k_{off}t)$ was performed using the computer program Kaleidagraph to measure $k_{off}$. Correlation coefficients of the first-order fits were $R > 0.98$.

*Other Methods.* Protein concentrations were determined according to Bradford (1979) and phospholipid concentration was determined according to Chen (1956) assuming a 25:1 phospholipid to phosphorus weight ratio. The buffer system used throughout was 50 mM Tris and 100 mM NaCl, pH 7.5. The temperature was 25 ± 1 °C.

## RESULTS

*Equilibrium Binding of Protein Z to Membranes.* The binding of protein Z to SUVs of 50% PS was evaluated by light scattering measurements (Figure 1A). At low protein/phospholipid ratios (P/PL), both proteins displayed quantitative binding to the membrane, suggesting a high-affinity interaction. However, there was a substantial difference in the final amount of protein bound to the vesicle. Bovine

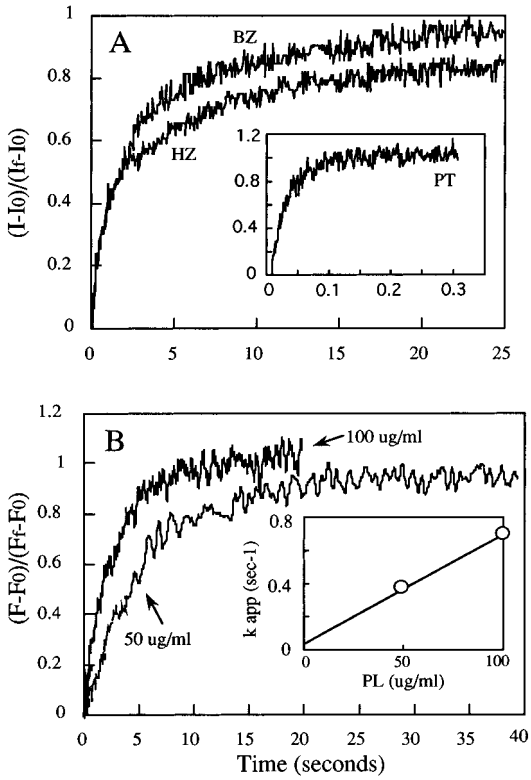


FIGURE 2: Kinetics of protein Z association with phospholipid vesicles. Panel A: Comparison of human and bovine protein Z. The reaction conditions were the same for all proteins (40 $\mu$g/mL after mixing). Binding to SUVs (100 $\mu$g/mL after mixing; PS/PC, 25/75) was measured by light scattering intensity at 320 nm. $I$ is the light scattering intensity of the sample, $I_0$ is the intensity of the vesicles alone, and $I_f$ is the final intensity measured after more than 2 min. The calcium concentration was 5 mM. Inset: Prothrombin−membrane binding. The prothrombin concentration (40 $\mu$g/mL), SUVs (25% PS, 120 $\mu$g/mL), and calcium (2 mM) were described by Lu and Nelsestuen (1996). Panel B: Rate of bovine Z association with vesicles, measured by fluorescence energy transfer. Protein (50 $\mu$g/mL after mixing) was mixed with labeled SUVs (100 or 50 $\mu$g/mL after mixing; PS/dansyl-PE/PC, 25/10/65), and binding was followed by the increase in fluorescence intensity (excitation at 287 nm, emission at 500 nm). $F_0$, $F$, and $F_{max}$ represent fluorescent intensity at the beginning, at time $t$, and at the end of the reaction, respectively. Inset: Dependence of pseudo-first-order rate constant ($k_{app}$) on phospholipid concentration. The $k_{app}$ was determined from $t_{0.4}$ as described in Materials and Methods.

protein Z reached $M_2/M_1$ ratios of more than 3.0, while human protein Z plateaued at $M_2/M_1$ of just over 2. Bovine prothrombin was intermediate between the protein Z species.

Binding to SUVs of 25% PS was measured by both light scattering and fluorescence energy transfer (Figure 1B). Protein Z is unique among the vitamin K-dependent proteins in that fluorescence energy of the protein can be transferred to dansyl groups in the membrane. Emission intensity at 520 nm due to direct excitation of the dansyl groups at 350 nm showed almost no change upon protein Z binding, suggesting that the increased signal was due to energy transfer and not to changes in the environment of the dansyl group. These vesicles also showed differences in the amount of human and bovine protein Z that were bound at saturation. The intensity of fluorescence energy transfer was proportional to the amount of protein Z that was bound, suggesting that similar groups in both proteins were responsible for fluorescence energy transfer.

*Protein Z−Membrane Association Kinetics*. The velocity of protein Z association with SUVs of 25% PS was measured by light scattering (Figure 2A). The kinetic parameters for the human and bovine proteins were very similar, with only a small difference at longer times (Figure 2A). Association rate constants, $k_{app}$ and $k_{assn}$, were obtained by methods described in Materials and Methods. Human and bovine protein Z gave similar rates of association (Table 1).

Table 1: Kinetic Properties of the Protein Z−Membrane Interaction

| protein | vesicle size (nm) | $N$ | $k_{assn}$[c] ($\times 10^{-5}$ $s^{-1}$ $M^{-1}$) | $k_{dssn}$ ($s^{-1}$) | $K_d$ (nM) | $[P_f]_{25/vesicle}$ (nM)[d] |
|---|---|---|---|---|---|---|
| bovine Z | 29.5 | 182[a] | 1.95 | 0.0063 | 32 | 5.1 |
| | 110 | 2716[b] | 1.33 | 0.0030 | 23 | |
| human Z | 29.5 | 91[a] | 3.36 | 0.057 | 170 | 64 |
| bovine | 27.5 | 58 | 176 | 1.9 | 110 | 84 |
| prothrombin[e] | 119 | 1420 | 43 | 1.0 | 230 | |

[a] Protein binding sites per vesicle were estimated from maximum binding capacity ($M_2/M_1$ of 2.0 for human protein Z and 3.0 for bovine protein Z) and molecular weights of small vesicles ($5 \times 10^6$) and protein Z (55 000). [b] Protein binding sites per vesicle were estimated on the basis of the same binding capacity of SUVs, the molecular weight of large vesicles ($100 \times 10^6$) and protein Z, and the relative amount of phospholipid on the outer leaflet of vesicles of different sizes (67% for SUVs and 50% for LUVs). [c] Values expressed per binding site. [d] Free protein concentration at 25 protein molecules bound per vesicle of $M_r$ $5 \times 10^6$. [e] Data from Lu and Nelsestuen (1996).

The most striking feature of both human and bovine protein Z was that their rates for binding were approximately 100-fold slower than those reported for other vitamin K-dependent proteins. This slow association appeared to be a property of interaction of monomeric protein Z with the membrane. The reaction displayed the anticipated dependence on phospholipid concentration, whether measured by light scattering or fluorescence energy transfer (Figure 2B). In addition, gel filtration analysis showed no evidence of protein Z self-association (Materials and Methods). Structural features that are responsible for these slow association kinetics should be common to human and bovine protein Z.

*Kinetics of Protein Z−Membrane Dissociation*. Fluorescence energy transfer was employed to follow the dissociation of bovine and human protein Z from labeled SUVs. In both cases, dissociation was fit to a first-order process (see Materials and Methods) to obtain the dissociation rate constant ($k_{dssn}$). Single-phase behavior was observed throughout the release process, indicating noninteractive sites and the absence of protein−protein interactions on the membrane.

Two striking reaction features were noted. First of all, both proteins showed very slow dissociation rates in comparison with other vitamin K-dependent proteins (compare to prothrombin, Table 1). Second, the two protein Z species were substantially different. Bovine Z gave a $k_{dssn}$ of 0.0063 $s^{-1}$ (Figure 3A, Table 1), 9-fold slower than the $k_{dssn}$ of 0.057 $s^{-1}$ for human Z (Figure 3B, Table 1).

The ratio of $k_{dssn}/k_{assn}$ ($=K_d$) provided values for protein Z that were not greatly different from those of bovine prothrombin (Table 1). The slow association and dissociation events were compensating at equilibrium. Since both maximum binding capacity and equilibrium binding constants varied for these proteins, another term, $[P_f]_{25/vesicle}$, the concentration of free protein at 25 protein molecules bound per vesicle, is shown in Table 1. This value showed a 13-fold difference between human and bovine protein Z. Bovine protein Z bound with substantially better affinity than bovine prothrombin, while human protein Z had slightly less membrane interaction ability than prothrombin (Table 1).

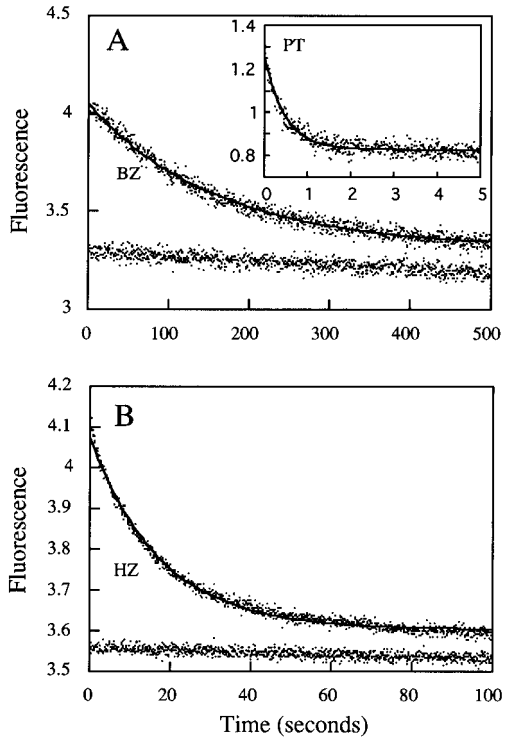


FIGURE 3: Kinetics of protein Z dissociation from small unilamellar vesicles. Panel A: A solution of bovine Z (BZ, 100 $\mu$g/mL after mixing) and labeled SUVs (50 $\mu$g/mL after mixing; PS/dansyl-PE/PC, 25/10/65) was mixed at time 0 with a solution of unlabeled SUVs (800 $\mu$g/mL after mixing; PS/PC, 25/75). All solutions contained 5 mM calcium. The fluorescence signal was monitored (excitation at 287 nm, emission at 500 nm). After the signal had ceased to change with time, a second measurement (lower, horizontal data) was made to determine the final intensity. The solid line corresponds to a first-order dissociation reaction with $k_{dssn} = 0.0063$ s$^{-1}$. Inset: Prothrombin (PT) dissociation kinetics under experimental conditions similar to those found in panel A. The solid line shows a first-order dissociation reaction with $k_{dssn} = 1.9$ s$^{-1}$. Panel B: Dissociation of human Z (HZ) from labeled SUVs. The experimental conditions and protein amd phospholipid concentrations were the same as described for bovine Z in panel A. The solid line shows a first-order dissociation reaction with a $k_{dssn}$ of 0.057 s$^{-1}$. The lower horizontal line shows the final intensity after 2 min.

*Kinetics of Protein Z Interactions with Large Unilamellar Vesicles.* Since SUVs have greater surface area per phospholipid on their outer layer than LUVs, proteins which penetrate into the hydrocarbon region of the membrane should display selectivity for vesicle size. The low selectivity of prothrombin for large versus small vesicles suggested little hydrophobic contact (Lu & Nelsestuen, 1996a). Proteins Z are unusual in that the hydrophobic cluster (Leu 5, Leu 6, and Val 9) is enhanced by tyrosine at positions 4 and 10 (Table 2). Thus, if insertion of hydrophobic residues into the hydrocarbon region of the membrane is the predominant interaction, protein Z should display accentuated behavior.

The kinetic behavior of protein Z binding to LUVs was examined by fluorescence energy transfer. The pseudo-first-order association rate constant showed a dependence on phospholipid concentration, consistent with pseudo-first-order conditions (Figure 4A, inset). In agreement with studies with prothrombin (Lu & Nelsestuen, 1996a), association rate constants for protein Z differed only slightly for LUVs versus SUVs. Dissociation rate constants for bovine Z release from labeled LUVs (Figure 4B) also gave values very similar to those for SUVs (Table 1). The first-order process suggested noninteracting proteins and binding sites. Overall, bovine Z showed little selectivity for vesicle size or surface curvature.

*Membrane Binding of Proteins S and C.* Species differences between human and bovine protein S and C are apparent in both mechanisms of biological function and membrane binding properties (Nelsestuen et al., 1978; Walker, 1981; Bakker et al., 1992). As shown below, human protein C has substantially higher affinity for membranes than bovine protein C, a property that that may be linked to different cofactor/enzyme behavior by human and bovine protein S and activated protein C.

Figure 5A shows that human protein S bound to membranes with higher affinity than bovine protein S. This result corroborated a previous comparison (Schwalbe et al., 1990). Saturation of the membrane surface with human protein S was accompanied by some apparent aggregation, and these titrations were not carried to that level. Qualitatively, however, human protein S appeared to bind to a higher density (higher $M_2/M_1$ ratios) on the membrane than bovine protein S. The free protein concentration at a $M_2/M_1$ ratio of 1.29 (25 proteins bound per vesicle) was 34 nM for human protein S and 103 nM for bovine protein S.

Figure 5B shows that human protein C−membrane interaction was readily detectable under the conditions used in this study. If a maximum capacity of 1 g of human protein C/g of phospholipid was assumed, the binding data in Figure 5B suggested a $K_d$ of 1500 nM. Bovine protein C has been reported to have a $K_d$ for similar membranes of 17 000 nM (Nelsestuen et al., 1978). The results shown in Figure 5B were consistent with a $K_d$ of $\geq$17 000 nM.

## DISCUSSION

The naturally occurring vitamin K-dependent proteins show striking variety in membrane binding properties (Table 2), despite homology in sequence and the structures that have been reported. The biological roles for variant properties may be related to simultaneous association of proteins with membranes and other proteins. High affinity, created by coordinate binding to two components, can limit exchange of protein from the membrane and can become the rate-limiting process in an enzymatic reaction such as prothrombinase (Lu & Nelsestuen, 1996b). Proteins with low affinity for the membrane (protein C and factor VII), or slow binding kinetics (protein Z), may have special features related to regulating affinity in protein−protein complexes on the membrane surface. Elucidating the membrane contact site and the mechanism by which diversity of affinity is created will contribute to an understanding of the structural role of $\gamma$-carboxyglutamic acid. It should also allow production of specific protein mutants that alter membrane affinity and study of the role of membrane binding and exchange rates in biological systems.

This study was also stimulated by a need to reconcile physical properties of protein−membrane interaction, which seem in conflict with either ionic or hydrophobic binding mechanisms. Recent evidence suggests that the membrane contact sites of prothrombin and factor X are fully expressed in a peptide of residues 1−37 from the amino termini of these proteins (Evans & Nelsestuen, 1996b). This study also suggested effective contribution by residue 34. Two extremes of affinity, in bovine protein C and bovine factor X,

Table 2: Comparison of Amino Acid Sequences of Vitamin K-Dependent Proteins in the GLA Domain

| | | | | | | | | | | | | | | | protein [j] | $K_d$ (nM) | $[P_f]_{25/ves}$ [i] (nM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | 10 | | | 20 | | | 30 | | | | 40 | | | | |
| AG[a]S | **Y**[a]LL | XXL | F**X**[a]G | HLX | KXC | WXX | ICV | YXX | ARX | VFX | **D**[c]**D**[c]X | TTD | XFW | RTY | bZ | 32 | 5.1 |
| AG[a]S | **Y**[a]LL | XXL | F**X**[a]G | NLX | KXC | YXX | ICV | YXX | ARX | VFX | NXV | VTD | XFW | RRY | hZ | 170 | 64 |
| ANS | −LL | XXT | KQG | NLX | RXC | IXX | LCN | KXX | ARX | VFX | N**D**[c]P | XTD | YFY | PKY | hS | 51[h] | 34 |
| ANT | −LL | XXT | KKG | NLX | RXC | IXX | LCN | KXX | ARX | IFX | NNP | XTE | YFY | PKY | bS | 154[h] | 103 |
| ANK | GFL | XXV | RKG | NLX | RXC | LXX | PCS | RXX | AFX | ALX | SLS | ATD | AFW | AKY | bPT | 110[d] | 84 |
| ANT | −FL | XXV | RKG | NLX | RXC | VXX | TCS | YXX | AFX | ALX | SST | ATD | VFW | AKY | hPT | ND | |
| ANS | −FL | XXV | KQG | NLX | RXC | LXX | ACS | LXX | ARX | VFX | **D**[c]AX | QTD | XFW | SKY | bX | 40[e] | 10 |
| ANS | −FL | XXM | KKG | HLX | RXC | MXX | TCS | YXX | ARX | VFX | DSX | KTN | XFW | NKY | hX | ND | |
| YNS | GKL | XXF | VQG | NLX | RXC | MXX | KCS | FXX | ARX | VFX | NTX | KTT | XFW | KQY | bIX | 1000 [f] | |
| YNS | GKL | XXF | VQG | NLX | RXC | MXX | KCS | FXX | ARX | VFX | NTX | RTT | XFW | KQY | hIX | ND | |
| ANS | −FL | XXL | RH**S**[b] | SLX | RXC | IXX | IC**D**[b] | FXX | AKX | IF**Q**[b] | NVD | DTL | AFW | SKH | hC | 1500 | >1000 |
| ANS | −FL | XXL | R**P**[b]G | NVX | RXC | SXX | VC**X**[b] | FXX | ARX | IF**Q**[b] | NTX | DTM | AFW | SFY | bC | >15000 [f] | >10000 |
| ANA | −FL | XXL | R**P**[b]G | SLX | RXC | KXX | QCS | FXX | ARX | IF**K**[b] | DAX | RTK | LFW | ISY | hVII | 17000 [g] | >10000 |
| ANG | −FL | XXL | L**P**[b]G | SLX | RXC | RXX | LCS | FXX | AHX | IF**R**[b] | NXX | RTR | QFW | VSY | bVII | 17000 [f] | >10000 |
| | | | * | | | | | | | * | * | | | | | | |

[a] Residues unique to slow association. [b] Residues unique to lowest affinity interactions. [c] Residues unique to highest affinity interactions. [d] Lu & Nelsestuen, 1996a. [e] Data not shown. [f] Nelsestuen et al., 1978. [g] Unpublished. [h] Calculated assuming a maximum capacity of 1 g/(g of protein)$^{-1}$ (phospholipid)$^{-1}$. [i] $[P_f]_{25/vesicle}$ = concentration of free protein at 25 proteins bound per vesicle of $M_r$ $5 \times 10^6$. [j] References for protein sequences are as follows: bZ, Hojrup et al., 1985; hZ, Ichinose et al., 1990; hS, Lundwall et al., 1986; bS, Dahlback et al., 1986; hPT, Degen et al., 1983; bPT, Magnusson et al., 1975; bX, Enfield et al., 1980; hX, Fung et al., 1985; bIX, Katayama et al., 1979; hIX, Kurachi & Davie, 1982; hC, Plutzky et al., 1986; bC, Fernlund & Stenflo, 1982; bVII, Takeya et al., 1988; hVII, Hagen et al., 1986. Asterisks indicate positions which may be involved in membrane binding.

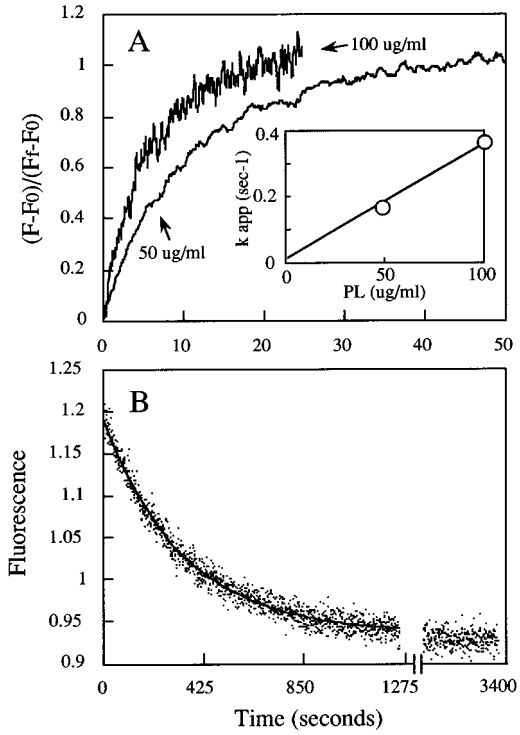


FIGURE 4: Bovine Z association and dissociation kinetics with large unilamellar vesicles. Panel A: Association of bovine Z (50 $\mu$g/mL after mixing) with labeled LUVs (100 and 50 $\mu$g/mL after mixing; PS/dansyl-PE/PC, 25/10/65). Pseudo-first-order rate constants were determined from $t_{0.4}$. The calcium concentration was 5 mM. Inset: Dependence of the pseudo-first-order association rate constant on phospholipid composition. Panel B: Dissociation of bovine Z (100 $\mu$g/mL after mixing) from labeled LUVs (50 $\mu$g/mL after mixing; PS/dansyl-PE/PC, 25/10/65). The complex was mixed with a solution of unlabeled SUVs (800 $\mu$g/mL after mixing; PS/PC, 25/75) at time 0. The fluorescence signal was followed until it had ceased to change (excitation at 287 nm, emission at 500 nm). The solid line shows a first-order dissociation process with a $k_{dssn}$ of 0.0030 s$^{-1}$.

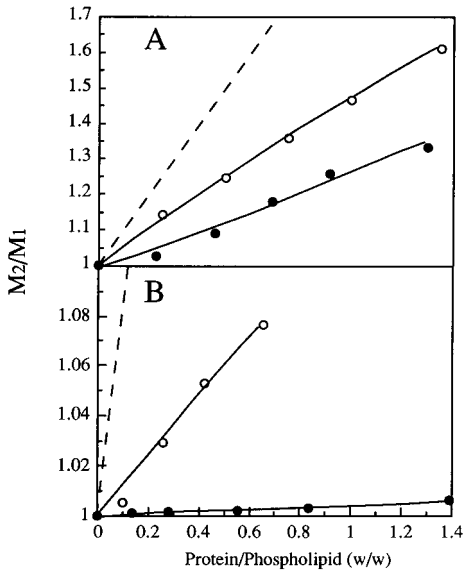


FIGURE 5: Protein S and protein C association with membranes. Relative light scattering was used to measure protein S− and protein C−membrane binding as described in Materials and Methods. Panel A: Association of human protein S (○) and bovine protein S (●) with SUVs (14.4 $\mu$g; PS/PC, 25/75). Protein S was added sequentially to the levels shown. The reaction was conducted in 0.75 mL of 50 mM Tris, 0.1 M NaCl, pH 7.5, buffer containing 2 mM $CaCl_2$. Panel B: Association of human protein C (○) and bovine protein C (●) with SUVs (14.4 $\mu$g; PS/PC, 25/75). Protein C was added sequentially to the levels shown. The reaction was conducted in 0.75 mL of 50 mM Tris, 0.1 M NaCl, pH 7.5, buffer containing 2 mM $CaCl_2$. Protein−membrane binding is reported as $M_2/M_1$. In both panels, the dashed line represents the theoretical values if all of the added protein associated with the phospholipid vesicles. The observed values represent the average of three experiments.

show only five significant differences in the amino-terminal 34 residues (Table 2). The locations of these residues and their relationships to the sequence and properties of other proteins may offer suggestions for a membrane contact site.

The low affinity of protein C for membranes may be influenced by proline at position 11, which is shared with factor VII, also of low affinity. A contribution of that residue to reduced membrane binding is supported by the higher membrane affinity of human protein C, which contains histidine at this position (Table 2). In addition, the impor-

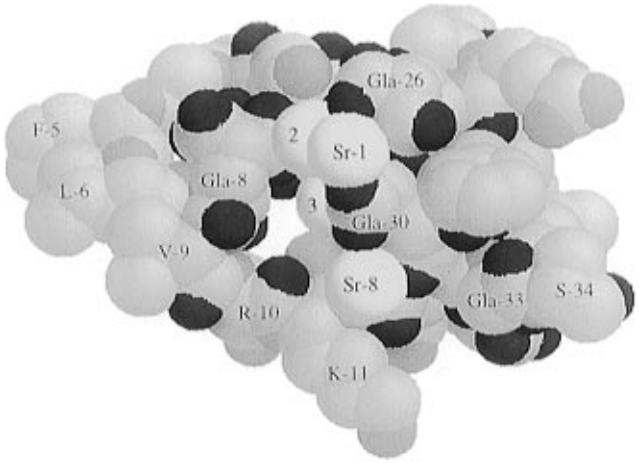


FIGURE 6: Proximity of important amino acids. Residues 1−34 of strontium-complexed prothrombin fragment 1 (Protein Data Base reference 2spt.pdb) are depicted by the Rasmol program in space-filling, CPK form. The side chain of Gla-33 is not shown. Metal ions are in yellow. Sr-1 and Sr-8 are on the protein surface, with Sr-2 through Sr-7 proceeding inward as a column along a pore in the protein. Amino acids 5, 6, and 9 form a hydrophobic cluster on the left. Residues 11, 33, and 34 are located on this surface.

tance of position 11 may be supported by proteins Z, which display unusual membrane binding kinetics and have the unique placement of Gla at that residue.

The absence of Gla-33 also correlated with low membrane affinity. Site-directed mutation of this amino acid in human prothrombin suggested that it is moderately important to membrane binding (Ratcliffe et al., 1993). The difference between human protein C and prothrombin (Table 2) may reflect a similar influence of the Gla residue. Factor VII, which contains substitutions at both positions (11 and 33), displays the lowest membrane affinity.

Interspecies comparisons called attention to amino acid 34. Bovine protein Z, with a higher affinity for membranes than human protein Z, contains Asp at position 34 versus Asn (Table 2). A related correlation existed for human vs bovine protein S, where the presence of Asp at position 35 corresponded with higher affinity (Table 2). A similar correlation can be made for factor X, a high-affinity protein which contains Asp at position 34. Proteins containing Asp-34 also showed increased binding capacity, as illustrated by bovine versus human protein Z (Figure 1) and factor X vs prothrombin (Nelsestuen & Broderius, 1977).

The three-dimensional structure of strontium−prothrombin fragment 1 (Seshadri et al., 1994; Figure 6) shows that residues 11, 33, and 34 are located on the surface of the protein. These are in close proximity to strontium-8, a metal ion that is not present in the calcium structure (Soriano-Garcia et al., 1992) and which may be a candidate for one of the additional ions found in the protein−phospholipid complex.

Spatial clustering of these components suggests an intriguing possibility for membrane contact. That is, an unusual feature of this region is a pore in the protein (Figure 6A), containing Sr-1, -2, -3, and -8. Insertion of a single phospholipid head group into the pore, to the depth of Sr-3, might allow formation of an ion pair in an isolated environment. In a medium of dielectric constant 4, an ion pair provides a free energy change of about −20 kcal/mol. Metal ions -1 and -8 form a nearly equilateral triangle with Sr-3, which may be appropriate for three-site attachment with the oxygen atoms of a phosphate from the head group.

This arrangement may have support from previous structural and biochemical studies. Association of phospholipase $A_2$ (Scott et al., 1991) with phospholipid substrate includes the formation of an isolated calcium−phosphate ion pair that may serve as a precedent for the proposed membrane interaction. The proposal might also explain the low impact of ionic strength on prothrombin−membrane binding (Resnick et al., 1980) but high dependence on acidic phospholipid content (Lu & Nelsestuen, 1996a); univalency provides relatively small affinity change with ionic strength, while the number of binding sites and overall affinity could increase with anionic lipid content. Uniform dissociation rate behavior at widely differing protein densities on the membrane (Figures 3 and 4B) and low clustering of acidic phospholipids by prothrombin (Tendian & Lentz, 1990) suggest little competition for anionic phospholipids, which would correlate with a major role of univalent interaction. Reliance on an isolated ion pair may explain the pH dependence of prothrombin binding to phosphatidic acid. PA was a poor substrate for prothrombin binding at neutral pH but was nearly equal to PS at alkaline pH (Resnick & Nelsestuen, 1980), where integer charge on one oxygen atom (Nelsestuen, 1988) would be consistent with univalent binding. Factor Xa has been reported to bind soluble PS with low affinity ($K_d$ = 73 $\mu$M), producing the same impact on enzyme activity as factor Xa association with phospholipid bilayers. A specific binding site for soluble PS (Koppaka et al., 1996) might correlate with the membrane contact site proposed here.

Special importance of the metal ions near the pore is also suggested by site-directed mutations of human prothrombin. Three "critical" Gla residues, 17, 27, and 30 (Ratliffe et al., 1993), were identified; these provide most of the ligands to the metals, Sr-1, -2, -3, and -8 (Seshadri et al., 1994), which line the pore. In addition, the peptide backbone of residues 8−12, which border the pore, may be disrupted by proline at position 11, correlating with reduced affinity.

Membrane interaction may also be mediated by other metal ions and residues. For example, Gla-33 and Asp-34 may bridge to the membrane and/or participate in binding of metal ion 8. Hydrophobic amino acids may enhance calcium affinity of the free protein and aid in membrane contact, in a role analogous to hydrophobic residues that line the protein surface near the substrate binding pore in phospholipase $A_2$ (Scott et al., 1991).

Although speculative, the proposed contact mechanism may reconcile apparent conflictions in biophysical properties of membrane binding. The potentially important amino acids and proposed sites on the protein provide targets for future experimentation.

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