Importance of *cis*-Proline 22 in the Membrane-Binding Conformation of Bovine Prothrombin†

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ABSTRACT: Upon addition of calcium to the metal-free protein, bovine prothrombin displays a conformational change with behavior of a classic *trans*- to *cis*-proline isomerization. The change is accompanied by a decrease of the intrinsic protein fluorescence and is essential to creating the membrane-binding conformation of prothrombin. This study showed that an identical conformational change was displayed by a peptide corresponding to residues 1−45 of prothrombin. This peptide contains a single tryptophan that underwent extensive quenching upon calcium addition. The kinetics were slow ($t_{1/2}$ = 2.7 min at 24 °C) and displayed an activation energy of 24 kcal/mol. These properties overlapped precisely with the behavior of bovine prothrombin fragment 1 (residues 1−156). Consistent with studies on prothrombin and other vitamin K-dependent proteins that have been modified or truncated, the 1−45 peptide required about 10-fold higher calcium to elicit these behaviors than did fragment 1. The conformational change was necessary for membrane binding by the 1−45 peptide. The only proline in this sequence is at position 22. This proline is of the trans configuration in a crystallized form of calcium−bovine prothrombin fragment 1 [Soriano-Garcia, M., et al. (1992) *Biochemistry 31*, 2554]. Unless the protein conformational change is based on another behavior, this study showed that biochemical properties of the protein are inconsistent with structure solutions. Further studies are needed to reconcile structure/function in membrane association. Proline 22 in bovine prothrombin may constitute a useful biochemical marker for the membrane-binding conformation of a vitamin K-dependent protein.

Prothrombin is one of several vitamin K-dependent proteins that rely on γ-carboxyglutamic acid residues for calcium and membrane binding [for a review, see Nelsestuen (1984)]. Calcium serves at least two roles: one in forming the correct protein conformation and the other in supporting membrane interaction (Nelsestuen & Lim, 1977). All of the vitamin K-dependent proteins show protein conformational change upon addition of calcium. The γ-carboxyglutamate region of the protein (approximately residues 1−40) appears to be largely disordered in the absence of calcium but refolds in its presence (Freedman et al., 1995a,b; Park & Tulinsky, 1986; Seshadri et al., 1991; Soriano-Garcia et al., 1992; Sunnerhagen et al., 1995; Tulinsky et al., 1988).

The vitamin K-dependent proteins display a number of unique biochemical properties. For example, bovine prothrombin undergoes a calcium-dependent protein folding event with classic behavior of a *trans*- to *cis*-proline isomerization (Marsh et al., 1979). This behavior includes an initial ratio of 25% cis:75% trans configuration, slow kinetics with a half-time of several minutes at room temperature and an activation energy of about 21 kcal/mol (Nelsestuen, 1976). This protein folding event was essential to formation of the membrane-binding conformation. Bovine prothrombin is also unique among the vitamin K-dependent proteins in that it contains a proline at position 22, within a highly conserved 6-residue disulfide loop. Originally, it was postulated that this proline was responsible for the protein folding kinetics and behavior (Marsh et al., 1979). However, structural studies of bovine prothrombin fragment 1 showed this proline in the trans configuration (Soriano-Garcia et al., 1992). The proline responsible for the conformation change was suggested to be proline 54, in a second disulfide loop of prothrombin (residues 48−61) which is also implicated in forming tight calcium binding, although it is not essential to the membrane interaction (Schwalbe et al., 1989).

The crystal structure did not reveal a simple membrane-binding structure. It contained seven calcium ions, many of which were buried in the protein. It also showed a cluster of three hydrophobic amino acid side chains projecting from the protein surface. A binding interaction for vitamin K-dependent proteins has been suggested to be comprised of the hydrophobic amino acid side chains that penetrated into the hydrocarbon region of the membrane to the depth of the calcium ions which could then interact by sharing ligands with sites on the membrane (Christiansen et al., 1995; Zhang & Castellino, 1994).

Unfortunately, several biochemical properties have appeared inconsistent with details of this proposed interaction. The proposed structure does not show three or four additional calcium ions that have been reported to bind to the protein−membrane complex (Evans & Nelsestuen, 1994; Nelsestuen & Lim, 1977; Sommerville et al., 1986). In addition, vitamin K-dependent proteins are almost unique among peripheral membrane-binding proteins in that they display almost no selectivity for interaction with small unilamellar vesicles (SUV)[1] versus large unilamellar vesicles (LUV) (Lu & Nelsestuen, 1996). The head group spacings in these structures differ substantially [0.55 nm^2 for LUV (Deamer & Bangham, 1976) and 0.74 nm^2 for SUV (Huang & Mason,

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1978)], making the hydrocarbon region much more accessible in SUV than in LUV. Many proteins show high selectivity for interaction with SUV. Even blood clotting factor V, which may have limited penetration into the hydrocarbon region of the membrane, favored interaction with SUV by 20−100-fold over LUV (Abbott & Nelsestuen, 1987). While proteins that interact with the hydrocarbon region would be anticipated to favor SUV, prothrombin showed only a 2-fold preference. Although the kinetics of binding and dissociation may differ substantially, equilibrium binding of prothrombin to SUV, LUV, phospholipid monolayers, and supported phospholipid bilayers was surprisingly constant (Andree et al., 1994; Mayer et al., 1983a; Nelsestuen & Broderius, 1977; Pearce et al., 1992, 1993).

Ultimate solution of structure−function relationships requires agreement of biochemical and structural studies. This investigation examined biochemical properties of short peptide segments of bovine prothrombin in order to clarify the sites responsible for various behaviors. Although earlier studies had suggested that the peptide corresponding to residues 1−45 of bovine prothrombin showed considerable nonideal behavior [aggregation upon a several hour incubation (Pollock et al., 1988)], this study found that use of short times eliminated this problem. The 1−45 peptide, which contains a single proline at residue 22, showed classic *trans*- to *cis*-proline isomerization behavior upon calcium addition. This was accompanied by fluorescence quenching and was necessary to form the membrane-binding conformation of the peptide. From these studies, we suggest that proline 22 is of the cis configuration in the membrane-binding conformation of bovine prothrombin. Structure determinations may have to be reassessed. More detailed analysis may offer an opportunity to observe a protein conformational change in depth.

MATERIALS AND METHODS

Materials. Highly purified phospholipids, phosphatidylserine (PS) from bovine brain, phosphatidylcholine (PC) from egg, and dansylphosphatidylethanolamine (dansyl-PE), were purchased from Sigma Chemical Co. TNBS and TLCK-treated α-chymotrypsin were from Sigma Chemical Co. Bovine prothrombin and fragment I were prepared as described previously (Heldebrant & Mann, 1973; Nelsestuen & Suttie, 1973). Other reagents were purchased from Sigma Chemical Co. and were of the highest purity available. A mixture of the 1−42 and 1−44 peptides from human prothrombin was as described (Schwalbe et al., 1989). Amino acid composition analysis was conducted by the University of Minnesota Microchemical Facility. Unless otherwise stated, standard buffer was 50 mM Tris, 100 mM NaCl, pH 7.5, and reactions were carried out at 22 ± 2 °C.

Purification of the 1−45 Peptide of Fragment I. The first 45 amino acids of bovine prothrombin fragment I were isolated as described previously (Pollock et al., 1988), with slight modification. Bovine prothrombin fragment I was incubated with α-chymotrypsin (1:3000, mol/mol) for 2 h at room temperature in buffer. The reaction was stopped by addition to a Mono Q column (Pharmacia, HR 5/5) which was washed extensively with standard buffer. Elution was with a 0.1−0.75 M NaCl gradient at a 1 mL/min flow rate. The 1−45 peptide eluted as the second retained peak, and its identity was verified by amino acid composition. An $E^{1\%}$ at 280 nm of 8.3 and a molecular weight of 5600 were used to calculate peptide concentration (Pollock et al., 1988). The sequence of the 1−45 peptide of prothrombin fragment 1 is ANKGFLXXVR KGNLXRXCLX XPCSRXXAFX ALX-SLSATDAFWAKY, where X represents Gla residues. The determined and predicted (in parentheses) amino acid composition was $D_{2.81(3)}$ $T_{1.14(1)}$ $S_{3.06(3)}$ $E_{8.67(10)}$ $P_{1.28(1)}$ $G_{2.19(2)}$ $A_{5.67(6)}$ $C_{0.31(2)}$ $V_{1.16(1)}$ $M_{0(0)}$ $I_{0(0)}$ $L_{5.37(5)}$ $Y_{0.80(1)}$ $F_{3.01(3)}$ $H_{0(0)}$ $K_{3.20(3)}$ $R_{3.21(3)}$.

Preparation of Phospholipid Vesicles. Phospholipids in organic solvent were mixed at the desired ratio, and the organic solvent was evaporated by a stream of N_2. The resulting film of phospholipids was resuspended in buffer and used to prepare small unilamellar vesicles (Bazzi & Nelsestuen, 1987; Huang, 1969). The solution was subjected to intermittent sonication (Heat Systems Model W185 sonicator) for a total of 5 min. The sonicated vesicles were gel-filtered on a Sepharose 4B column. Column fractions containing vesicles with a diameter of ≤39 nm (determined using quasi-elastic light scattering with a Langley-Ford LSA 2 photon correlation spectrometer and a Model 1096 correlator) were pooled and used for subsequent study. Phospholipid concentration was determined by the phosphorus assay (Chen et al., 1956) assuming a phosphorus to phospholipid weight ratio of 1:25.

Protein Fluorescence Change. Changes in the intrinsic protein fluorescence were monitored using a SPEX FluoroMax spectrophotometer with an excitation wavelength of 284 nm and an emission wavelength of 344 nm. A water-jacketed cuvette maintained constant temperature. Kinetic data were acquired by following fluorescence intensity after calcium addition. Equilibrium measurements utilized a separate sample for each experimental determination. For equilibrium titrations, fluorescence intensity was monitored until a stable value was reached. The addition of EDTA caused the fluorescence intensity to return to ≥90% of its pre-calcium level.

Intrinsic fluorescence (excitation 284 nm, emission 344 nm) is expressed in two ways. For general purposes, $I100/I_0$ is used where I is the fluorescence intensity of the sample in the presence of calcium and I_0 is the pre-calcium value. For equilibrium and rate analysis, $\Delta I100/\Delta I_{max}$ was used where ΔI represents the fluorescence intensity in the absence of calcium minus the value in its presence and ΔI_{max} is the fluorescence intensity of the calcium-free sample minus the intensity at maximum time and/or at maximum calcium concentration.

Protein−Membrane Binding. Association of the 1−45 peptide with vesicles was followed by fluorescence energy transfer from aromatic groups of the protein (excitation at 284 nm) to dansyl (dansyl-PE, emission at 520 nm) in the membrane. The temperature was controlled with a water-jacketed cuvette. Membrane binding was assessed by the relationship: $(I - I_0)100/(I_f - I_0)$, where I is the total intensity at time t and I_0 is the intensity due to direct excitation of dansyl by incident light. The latter is the

[1] Abbreviations: 1−45 peptide, amino acid residues 1−45 of bovine prothrombin; Gla, γ-carboxyglutamic acid; SUV, small unilamellar vesicles (about 30 nm in diameter); LUV, large unilamellar vesicles (about 120 nm in diameter); PS, L-α-phosphatidyl-L-serine; PC, L-α-phosphatidylcholine; dansyl-PE, *N*-dansyl-L-α-dipalmitoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; TNBS, trinitrobenzenesulfonic acid.

intensity before protein−membrane interaction. The difference $I - I_0$ is the intensity arising from energy transfer from tryptophan to the dansyl group. I_f is the maximum fluorescence intensity attained at long time and/or high calcium concentration.

Peptide Modification. Derivatization with TNBS was carried out as described previously (Haynes et al., 1967; Schwalbe et al., 1989). TNBS (10 mM) was added to peptide (8 μM) in 3% $NaHCO_3$, pH 8.5, and the solution was allowed to react at 37 °C for 2 h. Unreacted TNBS was removed by dialysis. The reaction should derivatize all free amino groups.

Reductive methylation was as described (Jentoft & Dearborn, 1979; Schwalbe et al., 1989). Peptide (0.19 mg/mL) in 100 mM HEPES, pH 7.5, was made 20 mM in $NaCNBH_4$ followed by the addition of formaldehyde to 20 mM. The reaction proceeded at room temperature for 16 h. Unreacted materials were removed by dialysis. As above, the reaction should derivatize all free amino groups.

RESULTS

Earlier reports of the amino-terminal 45 residue peptide of bovine prothrombin revealed considerable aggregation and nonideal behavior (Pollock et al., 1988) when exposed to calcium for several hours. To avoid this problem, calcium concentrations were restricted to less than 2 mM. Subsequent studies with the homologous peptide from human prothrombin suggested that higher calcium concentrations were needed to support peptide−membrane binding but that aggregation and irreversibility were serious problems (Schwalbe et al., 1989). This investigation found that use of bovine protein and shorter incubation times allowed studies to be conducted with nearly complete reversibility. Prolonged incubation with calcium ($\geq$1 h) caused further changes and irreversible behavior (data not shown) and was avoided. All of the studies shown below were conducted under conditions that gave $\geq$90% reversibility upon EDTA addition.

Intrinsic Fluorescence Quenching of the 1−45 Peptide. The addition of calcium to the 1−45 peptide of bovine prothrombin caused a time-dependent decrease of the intrinsic protein fluorescence (Figure 1A). The kinetics showed two phases: a rapid initial component (10% decrease) and a second, slower component (40% further decrease with a $t_{1/2}$ of 2.7 min). Fluorescence intensity was fully restored by addition of excess EDTA. A first-order rate plot of the slow phase yielded a single component (Figure 1A, inset) with a rate constant of 0.0043 s^{-1}. Similar behavior was observed for bovine prothrombin fragment 1 (Nelsestuen, 1976). Further correlations were seen in the overlapping Arrhenius plots for bovine fragment 1 and the 1−45 peptide (Figure 1B). An activation energy (E_a) of 24 kcal/mol agreed well with the E_a for proline isomerization and with the previously reported fragment 1 conformational change (Brandts et al., 1975; Cheng & Bovey, 1977; Marsh et al., 1979).

Association of the 1−45 Peptide and Phospholipid Vesicles. Membrane binding by the 1−45 peptide was monitored by fluorescence energy transfer from aromatic amino acids to dansyl groups in the membrane. Binding closely followed the kinetics of the calcium-induced protein fluorescence quenching process (Figure 2). Thus, in the presence of calcium, the 1−45 peptide underwent a conformational change that produced a decrease of intrinsic fluorescence and was essential to form a competent membrane-binding conformation. As was reported for the homologous peptides from human prothrombin (Schwalbe et al., 1989), the 1−45 bovine prothrombin peptide caused calcium-dependent ag-

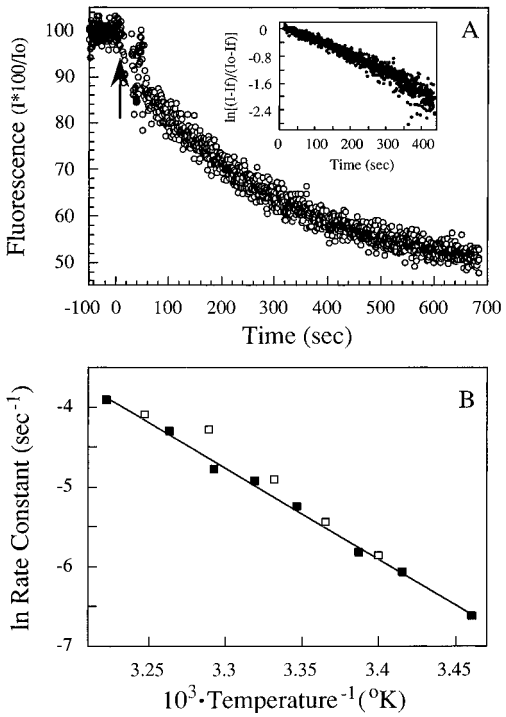

FIGURE 1: Kinetics of the 1−45 peptide fluorescence change. (Panel A) The intrinsic fluorescence (I) of residues 1−45 (17 μg/mL) of bovine prothrombin was measured at 24 °C as described under Materials and Methods. I_0 is the intensity of the calcium-free protein. The arrow indicates the point of calcium (30 mM) addition. (Panel A, inset) First-order rate plot. The fluorescence change in panel A was analyzed as a first-order reaction by assigning the fluorescence intensity at 700 s equal to I_f. A plot of ln $[(I - I_f)/(I_0 - I_f)]$ versus time is shown. The best linear fit to the data corresponded to a first-order rate constant of 0.0043 s^{-1}. (Panel B) Arrhenius plot. Data collected for the 1−45 peptide (□) and, separately, for bovine prothrombin fragment I (■) were obtained as in panel A. Rate constants were obtained by fitting 0−90% of the maximum fluorescence change. The line shown in panel B is the best fit for the fragment I data (solid line). A least-squares fit for the 1−45 peptide data yielded an E_a of 24 kcal/mol.

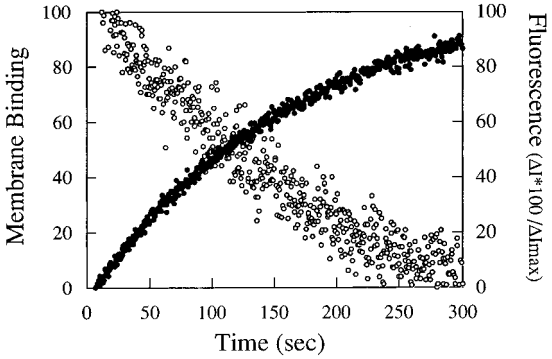

FIGURE 2: Kinetics of fluorescence change and membrane association. The rate of fluorescence quenching of the 1−45 peptide (17 μg/mL) was determined in the presence of phospholipid (PS/PC, 25:75, 180 μg/mL) and calcium (6.5 mM) (○). This was compared with the rate of membrane association of the 1−45 peptide (6 μg/mL) (●). Membrane (PS/dansyl-PE/PC, 30:5:65, 17.4 μg/mL) binding was monitored by fluorescence energy transfer. The intensity values (ΔI, ΔI_{max}) are defined under Materials and Methods. Both experiments were conducted at 24 °C.

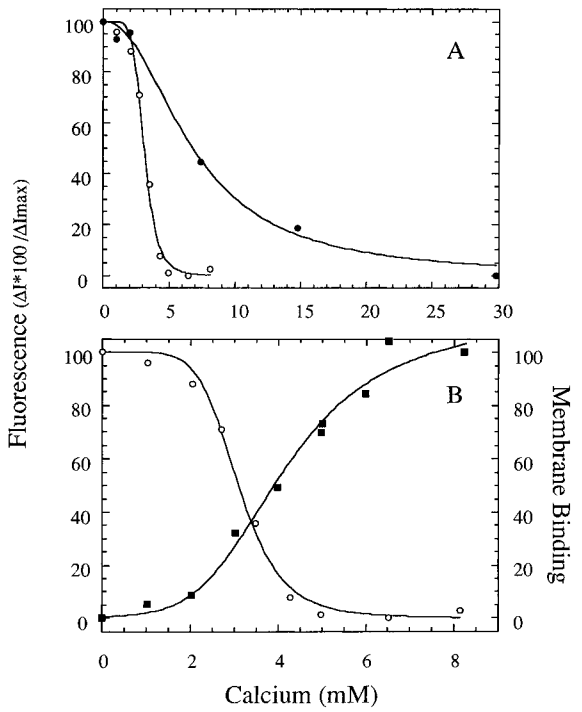

FIGURE 3: Calcium requirement of fluorescence change and membrane association. (Panel A) Fluorescence intensity of the 1−45 peptide (17 μg/mL) as a function of calcium in the presence (○) and absence (●) of phospholipid (PS/PC, 25:75, 180 μg/mL). The smooth curves drawn are theoretical for reactions that have midpoints of 3.1 and 6.8 mM calcium (left to right), respectively. The cooperativity coefficients were 2.1 and 6.0, respectively. Fluorescence values (ΔI, ΔI_0) are described under Materials and Methods. (Panel B) Calcium titration of 1−45 peptide (4.2 μg/mL) binding to phospholipid vesicles (PS/dansyl-PE/PC, 30:5:65, 17 μg/mL) (■). Membrane association was monitored by fluorescence energy transfer as described, and the intensity values (ΔI, ΔI_{max}) are defined under Materials and Methods. The calcium concentration at the midpoint was 3.8 mM. A calcium titration of the intrinsic fluorescence change is also shown (○). Conditions were identical to those described in panel A in the presence of phospholipid.

gregation of vesicles (not shown). Thus, light scattering could not be used to monitor peptide−membrane association. Due to this property, correlations between protein conformation change and membrane binding are viewed as qualitative rather than precise. Membrane association (and aggregation) was completely reversed by addition of excess EDTA.

At equilibrium, the extent of protein fluorescence was dependent on the calcium concentration. Titrations in the presence and absence of phospholipid showed similar saturation levels (Figure 3A) at 65% and 55% of initial intensities for titrations in the presence and absence of phospholipids, respectively. Phospholipids lowered the calcium required for half-maximal change from 6.8 to 3.1 mM (Figure 3A). Although these concentrations of calcium were higher than those required to cause similar changes in fragment 1, both curve shape and the impact of phospholipid were similar (Nelsestuen, 1976). Previous work has demonstrated that truncation of vitamin K-dependent proteins decreased their calcium affinity by up to 10-fold (Schwalbe et al., 1989). Both titration curves were sigmoidal and corresponded to cooperativity coefficients of 2.1 and 6 in the absence and presence of membranes, respectively. Again, this mimicked the property of fragment 1, where the cooperativity coefficient was 2.6 in the absence of phospholipids and 4.5 in its presence (Nelsestuen, 1976).

Calcium titration of peptide binding to vesicles displayed a midpoint of 3.8 mM (Figure 3B). Again, the close correlation of membrane binding, measured by fluorescence energy transfer, and the intrinsic protein fluorescence quenching process suggested that the conformation change was necessary for membrane association. This agreed with the results in Figure 2 and with earlier studies of bovine prothrombin and fragment 1 (Nelsestuen, 1976).

Peptide Modifications and the Human Gla Domain. Derivatization of the free amino groups in the 1−45 peptide with TNBS did not alter the qualitative features of membrane binding by the native peptide (data not shown). However, reductive methylation, which is also specific for amino groups, abolished membrane association (data not shown). This behavior was similar to that of bovine prothrombin fragment 1 (Welsch & Nelsestuen, 1988), blood clotting factors IX and X, and amino-terminal peptides from human prothrombin and factor X (Schwalbe et al., 1989).

The slow reaction outlined in this study appeared to be limited to the species that contained proline at position 22. A mixture of peptides corresponding to residues 1−42 and 1−44 of human prothrombin [described by Schwalbe et al. (1989)] was also studied. These peptides lack proline. Upon calcium addition, the peptides from human prothrombin displayed rapid intrinsic fluorescence quenching and membrane association. The reactions were complete within the time required for manual sample mixing (*ca*. 15 s).

DISCUSSION

Structural and biochemical studies provide complementary information that can be used to assure correct structure/function assignments. This study has examined a vitamin K-dependent protein. Since annexins bind to membranes in a calcium-dependent manner, they provide an interesting comparison (see below).

A number of biochemical and structural properties of bovine prothrombin fragment 1 are consistent. For example, direct calcium binding measurements (Deerfield et al., 1987) and the X-ray crystal structure show seven calcium ions bound to the free protein (Soriano-Garcia et al., 1992). Both types of information show that calcium causes a conformational change involving the amino-terminal region of the protein. Biochemical measurements showed that loss or certain modification of peptide beyond about residue 45 decreased calcium-binding affinity; this region was found to interact with the amino-terminal residues of bovine prothrombin in the crystal structure.

Despite these similarities, other biochemical information has not been easily reconciled with structural information. The differences are of several types which may be solved by various means. For example, biochemical measurements suggest that fragment 1, in complex with phospholipid, binds about 10 calcium ions (Evans & Nelsestuen, 1994; Nelsestuen & Lim, 1977; Sommerville et al., 1986). As in the case of annexins, certain calcium ions may be involved in bridging to the membrane and may only bind in the presence of a membrane component. Recent proposals for the interaction of vitamin K-dependent proteins, which are based on only seven calcium ions, may need to be reconsidered (Christiansen et al., 1995; Zhang & Castellino, 1994). This may be accomplished by new proposals for the protein−membrane interaction. A problem may be that alternative

methods of membrane contact are not readily apparent in the structure.

A second problem concerns biochemical properties of the membrane interaction. The proposed prothrombin−membrane contact contains a core of hydrophobic amino acid side chains that penetrate into the hydrocarbon region of the membrane (Christiansen et al., 1995; Zhang & Castellino, 1994). However, biochemical studies show that vitamin K-dependent proteins are rather unusual in that they show very low selectivity for large versus small unilamellar vesicles (Lu & Nelsestuen, 1996). Although not definitive, this lack of selectivity is unexpected for a protein that penetrates into the hydrocarbon of the membrane. Another biochemical property is a low air−water surface pressure of bovine prothrombin fragment 1 in the presence or absence of calcium (Mayer et al., 1983b). This suggests relatively little of the amphipathic structure that would be expected for a protein which penetrates into the hydrocarbon region of the membrane. Again, this possible inconsistency of biochemical and structural properties might be solved by new proposals for the mode of interaction between prothrombin and the membrane.

In contrast to these earlier types of information, the current study presented a direct dissimilarity between biochemical and structural properties that may require reevaluation of structure and/or identification of quite unexpected biochemical properties. This study showed that a peptide corresponding to residues 1−45 of bovine prothrombin retained membrane-binding ability and underwent a conformational change that displayed characteristics identical to those of intact prothrombin and to a *trans*- to *cis*-proline isomerization. This conformational change is critical to forming the membrane-binding site on bovine prothrombin. The only proline in the 1−45 sequence is at position 22. No other vitamin K-dependent protein or peptide, including human prothrombin, displayed the unusual kinetics of this conformational change. The X-ray crystal structure of bovine prothrombin fragment 1 shows this proline in the trans configuration. Thus, structural information may require reanalysis.

One possible explanation for the biochemical behavior is that the conformational change represents an event other than trans to *cis*-proline isomerization. This possibility seems remote. The conformational change shows the unusually slow rate, the unusually large activation energy, and the initial distribution of conformers (about 3:1) that characterize proline isomerization. The slow process is not observed either in human prothrombin or in the amino-terminal peptide from human prothrombin. The level of coincidence required for an alternative conformational change seems extraordinary, and testing this explanation would seem a last resort.

Another possibility is that the 1−45 peptide forms a different membrane-binding structure than does intact prothrombin. Again, the biochemical properties are so distinctive that this seems unlikely. In addition to the unusual conformational change displayed by both intact prothrombin and the peptide, sensitivity and insensitivity to chemical modifications were identical. For example, reductive methylation abolishes membrane binding by prothrombin and had the same impact on the peptide. In contrast, modification of amino groups by trinitrobenzylation did not abolish membrane binding by either the peptide or the native protein. It seems most unlikely that two significantly different modes of membrane binding would retain such striking similarities. In addition, peptides that contain γ-carboxyglutamate residues do not automatically bind to membranes, as illustrated by the lack of membrane association by the 12−44 peptide of prothrombin (Nelsestuen et al., 1975). Thus, it seems likely that the 1−45 peptide retains the main features of the membrane-binding site on the native protein, except for reduction of calcium affinity, a process that also occurs upon modification of a peptide region (cleavage at residues 52 and 70) in the second disulfide loop of protein S (Schwalbe et al., 1990). How the latter region contributes to higher calcium affinity is not known and will require further studies.

Unless major coincidence is responsible for biochemical observations, it may be necessary to reevaluate structural information. It may be possible that *cis*-proline 22 can fit the structural information. This might alter the protein structure in a critical manner that allows membrane interaction. However, a more radical possibility is that fragment 1 provides an unusual example where a rare form of the protein, corresponding to a non-membrane-binding conformation, has crystallized. Magnusson and co-workers described the difficulties in obtaining crystals of bovine prothrombin fragment 1 in the presence of calcium (Olsson et al., 1980). When formed, the protein had been modified by cleavage of the C-terminal 12 amino acid residues. These residues were also absent or disordered in the crystal structure (Soriano-Garcia et al., 1992). Thus, it is possible that new methods of crystallization must be sought.

Recently, the NMR structure of a homologous factor IX peptide has been reported (Freedman et al., 1995a). Comparison of corresponding residues from the X-ray crystal structure of fragment 1 revealed no large variation in residues 12−47. However, deviations in amino acids 1−11 were reported. This illustrates the lack of a consensus structure for vitamin K-dependent proteins. Unfortunately, the need to perform NMR analysis in urea/guanidine hydrochloride, in order to prevent aggregation, makes it difficult to establish whether the structure observed is the correct membrane-binding conformation. Low calcium affinity by these peptides and the potential for interactions of calcium with malonyl groups in a manner that is unrelated to the native protein structure make definitive conclusions difficult. In this respect, bovine prothrombin and the *cis*-proline at position 22 may provide an internal standard and an ideal molecule for future studies.

Annexins may provide a model for consistency of biochemical and structural studies. Initially, structure studies detected only five calcium sites (Huber et al., 1990) in the free protein, while biochemical studies suggested 8 (Bazzi & Nelsestuen, 1991b) or 10 (Evans & Nelsestuen, 1994) calcium ions bound per annexin V−membrane complex. More recent structural studies included phospholipid head groups and reported 10 calcium ions in the annexin complex (Swairjo, 1996; Swairjo et al., 1995). Agreement supports a correct stoichiometry in both types of study. Extensive biochemical studies for annexins also appear to support other features of the proposed membrane interaction. For example, free annexins bind calcium very weakly, but the annexin−membrane complex has high affinity. The low calcium affinity of the free protein is supported by structural studies which show relatively few protein ligands to each calcium ion but high ligand occupancy in complexes with phospholipid head groups. The high cooperativity for calcium

binding that is suggested by these properties was also found in biochemical studies which suggest an unusual model of "sequential annexin−membrane binding" (Bazzi & Nelsestuen, 1991a). Biochemical studies show an exceptionally high affinity of annexins for membranes, a property that is consistent with the large number of protein−calcium−membrane contact points suggested by the structure. Thus, extended investigation has produced a high level of consistency between biochemical and structural properties for the annexin−membrane complex.

Overall, this study has shown the value of biochemical information in corroboration of a structure or in suggesting the need for continued studies. The 1−45 peptide of bovine prothrombin is a useful tool in the study of membrane-binding structures, and its *trans*- to *cis*-proline isomerization may be a biochemical marker for the correct membrane-binding structure of bovine prothrombin. Peptides containing this proline may be useful in future structural studies.

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