Fourier transform infrared spectroscopic study of Ca^{2+} and membrane-induced secondary structural changes in bovine prothrombin and prothrombin fragment 1

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ABSTRACT Fourier transform infrared (FTIR) spectroscopy was used to monitor secondary structural changes associated with binding of bovine prothrombin and prothrombin fragment 1 to acidic lipid membranes. Prothrombin and prothrombin fragment 1 were examined under four different conditions: in the presence of (*a*) Na_2EDTA, (*b*) 5 mM $CaCl_2$, and in the presence of $CaCl_2$ plus membranes containing 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) in combination with either (*c*) bovine brain phosphatidyl-serine (bovPS) or (*d*) 1,2-dioleoyl-phosphatidylglycerol (DOPG). The widely reported Ca^{2+}-induced conformational change in bovine prothrombin fragment 1 was properly detected by our procedures, although Ca^{2+}-induced changes in whole prothrombin spectra were too small to be reliably interpreted. Binding of prothrombin in the presence of Ca^{2+} to procoagulant POPC/bovPS small unilamellar vesicles produced an increase in ordered secondary structures (2% and 3% increases in α-helix and β-sheet, respectively) and a decrease of random structure (5%) as revealed by spectral analysis on both the original and Fourier-self-deconvolved data and by difference spectroscopy with the undeconvolved spectra. Binding to POPC/DOPG membranes, which are less active as procoagulant membranes, produced no detectable changes in secondary structure. In addition, no change in prothrombin fragment 1 secondary structure was detectable upon binding to either POPC/bovPS or POPC/DOPG membranes. This indicates that a membrane-induced conformational change occurs in prothrombin in the nonmembrane-binding portion of the molecule, part of which is activated to form thrombin, rather than in the membrane-binding fragment 1 region. The possible significance of this conformational change is discussed in terms of differences between the procoagulant activities of different acidic lipid membranes.

INTRODUCTION

The prothrombinase complex, which consists of a serine protease (factor Xa), a protein cofactor (factor Va), Ca^{2+}, and an appropriate acidic lipid membrane surface, plays a key role in blood coagulation by converting prothrombin to thrombin (Zwaal and Hemker, 1982; Mann, 1987). Because prothrombin is efficiently converted to thrombin only by the membrane-bound prothrombinase in the presence of Ca^{2+}, much effort has gone into characterizing the interactions of prothrombin with Ca^{2+} and acidic lipid membranes. Intrinsic fluorescence quenching experiments (Nelsestuen, 1976; Prendergast and Mann, 1977), circular dichroism experiments (Bloom and Mann, 1978; Marsh et al., 1979), antibody binding experiments (Borowski et al., 1986), and differential calorimetric studies (Ploplis et al., 1981) all have indicated that prothrombin undergoes one or two Ca^{2+}-induced conformational changes that are necessary for binding to acidic lipid membranes (Jackson et al., 1975; Nelsestuen, 1976).

Binding of prothrombin to membranes containing acidic phospholipids has been proposed to occur via calcium bridging of γ-carboxyglutamic acid residues (GLA[1]), located in the *N*-terminal fragment 1 portion of the molecule, to negatively charged phospholipids in the membrane (Lim et al., 1977; Dombrose et al., 1979; Wei et al., 1982). Analysis of the binding of these proteins to membranes has utilized a binding model that assumes the existence of protein "binding sites" on a membrane (Lim et al., 1977). The binding site has been proposed to be a local pool or "domain" of negatively charged phospholipid that condenses under the surface-bound protein (Lim et al., 1977; Dombrose et al., 1979; Mayer and Nelsestuen, 1981). More recently, both because of acidic-lipid specificity in the prothrombinase reaction (Jones et al., 1985) and because of physical studies that failed to detect domains (Lentz et al., 1985; Jones and Lentz, 1986), we have demonstrated that the binding of prothrombin to membranes can be explained in terms of interactions of acidic lipids with only three or four

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[1]*Abbreviations used in this paper:* DEAE-cellulose, diethylaminoethyl-cellulose; D_2O, deuterium oxide; DOPG, 1,2-dioleoyl-3-*sn*-phosphatidylglyceral; DPH, 1,6-dipheny-1,3,5-hexatriene; FSD, Fourier Transform self-deconvolution; FT-IR, Fourier Transform Infrared Spectroscopy; GLA, γ-carboxyglutamic acid; MOPS,3-[*N*-Morpholino] propane-sulfonic acid; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; PS, phosphatidylserine; S-2238, phenylalanyl-pipecolyl-arginine-*p*-nitroaniline; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SUV, small unilamellar vesicle(s).

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specific sites on the protein (Cutsforth et al., 1989). Both the binding isotherms and the prothrombinase activity (Jones et al., 1985; Rosing et al., 1988) show specificity for phosphatidylserine- (PS) as compared with phosphatidylglycerol- (PG) containing membranes. Here, we report spectroscopic evidence that prothrombin undergoes a PS-specific conformational change upon binding to a membrane, a result consistent with the existence of specific lipid binding sites.

Fourier transform infrared spectroscopy has received increased attention for the study of protein secondary structure and lipid-protein interactions (Mendelsohn and Mantsch, 1986; Gendreau et al., 1986). Fourier self-deconvolution (Kauppinen et al., 1981*a*; 1981*b*) and derivative spectroscopy make it possible to assign the amide bands of proteins in aqueous solution in a reasonably quantitative manner (Byler and Susi, 1986). Because of problems of sensitivity, this method has not been widely applied to proteins the size of prothrombin. However, the difficulties associated with accurate circular dichroism measurements in the presence of high concentrations of membranes made FTIR a reasonable method of choice for testing for changes in prothrombin secondary structure associated with binding to a membrane.

EXPERIMENTAL

Materials

Calcium chloride was reagent grade from Fisher Chemicals. Deuterium oxide (99.8%), *Echis carinatus* snake venom, and 3-[*N*-Morpholino] propane-sulfonic acid (MOPS) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine brain phosphatidylserine (bov PS), 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) and 1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG) were purchased from Avanti Biochemicals (Birmingham, AL). The thrombin-specific substrate (phenylalanyl-pipecolyl-arginine-*p*-nitroaniline, S-2238) was purchased from Helena Laboratories (Beaumont, TX). All other chemicals were ACS reagent grade or the best available grade; all solvents were HPLC grade.

Protein isolation, purification, and characterization

Bovine prothrombin was isolated from a barium citrate precipitate prepared from freshly collected bovine plasma (Mann, 1976; Tendian and Lentz, 1990). The barium citrate precipitate was a gift from Dr. Richard Hiskey of the Chemistry Department of the University of North Carolina at Chapel Hill. Prothrombin obtained in this manner was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Kodavue (Eastman Kodak, Rochester, NY) to reveal only one major band and no more than two very minor bands. A final purification step was performed one day before an experiment by HPLC on a Perkin-Elmer Isopure LC system using a MonoQ HR 5/5 ion exchange column (Pharmacia Fine Chemicals, Norwalk, CT). This step removed small quantities (< 10%) of proteolysis products that occasionally formed during storage (Tendian and Lentz, 1990).

Bovine prothrombin fragment 1 was prepared by thrombin proteolysis of prothrombin induced by *Echis carinatus* venom, followed by chromatography on DEAE cellulose and P-100 gel filtration columns as described elsewhere (Downing et al., 1975). Fragment 1 preparations were evaluated by sodium dodecylsulfate polyacrylamide gel electrophoresis and by Ca^{2+}-induced tryptophan fluorescence quenching (Nelsestuen, 1976; Predergast and Mann, 1977) to test for a native conformation. Acceptable preparations were > 45% quenched by the addition of 5 mM Ca^{2+}.

Prothrombin and prothrombin fragment 1 concentrations were determined from the absorbance at 280 nm after correcting for Raleigh scattering using the absorbance at 320 nm. The extinction coefficients used were 1.44 ($ml \cdot mg^{-1} \cdot cm^{-1}$) for bovine prothrombin and 1.05 for bovine prothrombin fragment 1 (Mann, 1976).

Phospholipid vesicles

Small, unilamellar vesicles (SUV) were prepared from quantitative mixtures of stock solutions of bov PS, POPC, and DOPG in $CHCl_3$/CH_3OH. A small amount of 1,2-di[1-^{14}C]myristoyl-3-*sn*-phosphatidylcholine (< 0.0002 mol fraction; lot B0141; Amersham, Arlington Heights, IL) was included in the phosphatidylcholine stock solutions. The lipid mixture was dried in a thin film on the wall of a 5-ml ampoule, then lyophilized from a frozen benzene solution overnight. After dispersing in 2 ml of D_2O buffer (10 mM MOPS, 0.1 M NaCl, pD 7.4), the sample was sonicated in a Heat Systems Cup Horn (Heat Systems Ultrasonics, Inc., Plainview, NY) as described (Barrow and Lentz, 1980) and centrifuged for 25 min at 70,000 *g* in a Beckman TL100 centrifuge (TLA-100.3 rotor) to remove large vesicles (Barenholz et al., 1977). The composition of vesicles prepared from mixtures of two lipids was obtained by scintillation counting to measure the concentration of the phosphatidylcholine component and phosphate analysis to get the total phospholipid concentration.

FTIR measurements

For spectral measurements, the proteins were dialyzed against D_2O buffer for 48 h to complete deuterium exchange and then lyophilized overnight. Samples were generally prepared in a N_2 environment as D_2O solutions with 0.01 M MOPS, 0.1 M NaCl, 0.1 mM Na_2EDTA (pD 7.4) as a buffer, with a protein concentration in the range of 2–5% wt/vol. Completion of deuterium exchange was verified by monitoring the shift of the 1,550 cm^{-1} amide II band to 1,450 cm^{-1} (Byler and Susi, 1986). No further shift occurred between 48 and 72 h of dialysis. To evaluate the functionality of the lyophilized protein, prothrombin concentrations were also determined using a synthetic chromogenic substrate, S-2238, to assay for thrombin formation by the fully assembled prothrombinase (Jones et al., 1985). By this criterion, lyophilized prothrombin was still 85–90% active after the treatment outlined here.

FTIR transmission spectra were obtained with a Mattson Instruments (Madison, WI). Polaris FTIR spectrometer equipped with a high sensitivity HgCdTe detector and interfaced with an IBM/AT computer. Samples for Fourier transform infrared spectroscopy were examined in a Harrick cell with a 56-μm teflon spacer equipped with CaF_2 windows (Harrick Scientific Corp., Ossining, NY). Temperature was controlled with a Neslab (Newington, NH) RTE-8 circulating water bath and monitored with a thermocouple sensor (Iron-constantan, Type-J; Omega Engineering, Inc., Stanford, CT) placed close to the windows. For each type of sample, spectra (to a resolution of 2 cm^{-1}) were obtained at 22°C from at least three independently prepared samples. 500–1,000 scans were collected per measurement to

obtain signal-to-noise ratios in the range of 1,000–2,000, which is more than adequate (>500) for self-deconvolution (Byler and Susi, 1986). The resulting spectra were solvent subtracted over the 1,400–1,900 cm^{-1} region by using the D_2O buffer spectrum recorded at the same temperature and with the same pathlength used for the sample under study. To minimize water vapor perturbation in the sample compartment, the instrument was purged continuously with dry nitrogen for at least 24 h before an experiment, for 0.5–1 h after loading a sample, as well as during and between experiments. Background spectra were taken under the same conditions and on the same day as buffer and sample spectra. Uncompensated water vapor fine structure still evident in background-corrected and solvent-subtracted spectra was removed computationally by subtracting a weighted water vapor spectrum, using an adjusted weighing factor chosen so as to eliminate this fine structure in the range of 1,600–1,900 cm^{-1} (Dong et al., 1990). After this procedure, our spectra all met the two criteria specified by Dong et al: a flat featureless baseline and no evidence of contributions at specific water vapor peak positions. In addition, we examined on an expanded scale several duplicate spectra of prothrombin, which, before correction, showed evidence of both over- and under-compensation for water vapor. After correction, these spectra all had identical spectral features, confirming that subtle spectral features in the amide I′ region were unaffected by the water vapor correction procedure.

Selfdeconvolution and spectral analysis

The intrinsically overlapped amide I′ spectral region was subject to resolution enhancement using the Fourier self-deconvolution (FSD) technique. The algorithm used for deconvolution was based on the one described by Kauppinen et al. (1981*a*). It assumed a Lorentzian line-shape function for the original unresolved components. Fourier deconvolution was accomplished using a software package written in Turbo C for an IBM/AT computer by Dr. D. J. Moffatt, National Research Council, Ottawa, Canada. This package includes a "smooth" function to reduce signal-to-noise ratio losses associated with FSD. This was not used, as our spectra had a sufficiently large signal-to-noise ratio to make this loss immaterial. The input data file consisted of 1,024 data points in the 1,400–1,900 cm^{-1} region spaced at 0.482 cm^{-1}. To optimize the resolution enhancement and to minimize spectral artifacts and distortion (Jaworsky et al., 1986) simultaneously, a bandwidth of 18 cm^{-1} (full width at half height FWHH) and a resolution-enhancement factor (*K* value; Kauppinen et al., 1981*a*) of 2.6 were used.

A semiquantitative estimation of the contributions of different protein secondary structures was obtained using curve-fitting procedures. The Fourier self-deconvolution procedure alters the shape of the resulting resolution-enhanced bands but not their integrated intensity (Kauppinen et al., 1981*a*). On an empirical basis, it has been shown (Levitt and Greer, 1977; Byler and Susi, 1986; Mantsch et al., 1988) that the sum of all the integrated peak intensities associated with a particular protein secondary structure (e.g., β-sheet structures, α-helical structures, "random coil", or all other structures), divided by the total integrated Amide I band intensity, provides a reasonable estimate of the fraction of amino acid residues participating in that structure. A Simplex nonlinear curve-fitting algorithm (Caceci and Cacheris, 1984) was used to fit the partially resolved Amide I′ band spectrum to a sum of Gaussian line-shape functions. To limit the number of adjustable parameters, an iterative procedure was used, as follows: (*a*) The peak positions of the partially resolved peaks were fixed at values determined by second derivative techniques and consistent with reported values (Byler and Susi, 1986). Band widths at half height were fixed at 18 cm^{-1}. Peak intensities were adjusted to minimize the mean-square deviation of calculated from observed spectra (χ^2). (*b*) Peak intensities and frequencies were fixed, and band widths were adjusted to reduce χ^2. (*c*) When obvious gaps between the data and the fitted curve were found, additional components were added (e.g., at 1,648 cm^{-1} for unordered segments). These components were maintained for subsequent iterations. (*d*) This procedure was repeated several times until a convergent fit was obtained.

RESULTS

Original spectra and data manipulations

Based on reported binding constants (Lim et al., 1977; Cutsforth et al., 1989), prothrombin should have been >95% bound to the membrane surface at the concentrations of membranes used to obtain these spectra. In addition, based on estimates of Ca^{2+} binding constants (Pollock et al., 1988), prothrombin's Ca^{2+} sites should have been >92% saturated and the protein should have been in its "Ca^{2+} conformation" (Prendergast and Mann, 1977; Nelsestuen, 1976) at the concentration of Ca^{2+} used (5 mM).

The original spectrum in the region of 1,900–1,400 cm^{-1} of PS/POPC-bound bovine prothrombin before any spectral manipulation is shown in Fig. 1*A*. Fig. 1*B* shows the spectrum of pure PS/POPC (25:75) SUV in the same D_2O buffer used for Fig. 1*A*. The asymmetric stretching vibration due to the free carboxyl group in the hydrophilic headgroup region of PS occurred at 1,623 cm^{-1}; the ester C=O stretching bands occurred in a broad band centered at ~1,730 cm^{-1} (FSD spectra showed resolved peaks at 1,725 and 1,742 cm^{-1}). These lipid peak positions agree with PS spectra shown by Dluhy et al. (1983). It is evident from comparing Figs. 1,

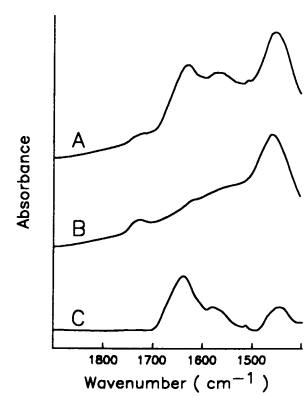

FIGURE 1 Raw FTIR spectra of (*A*) prothrombin (3.5% wt/vol) in the presence of 5 mM Ca^{2+} and 24 mM (25/75) PS/POPC SUV in a D_2O buffer; (*B*) (25/75) PS/POPC SUV in a D_2O buffer. Spectra were obtained as described under experimental procedure. Spectrum *C* is the difference between *A* and *B*.

A and *B* that the contribution of these lipid bands was small relative to the contribution of protein bands or of buffer background. The spectrum of PS/POPC-bound prothrombin (Fig. 1 *C*) was obtained by subtracting the concentration-scaled spectrum of lipid in D_2O (Fig. 1 *B*) from Fig. 1 *A* to eliminate the large lipid peaks in the range of 2,850–3,000 cm^{-1} and to obtain a flat baseline, according to standard procedures (Mendelsohn et al., 1984; Gendreau, 1986). This procedure effectively eliminated the less intense lipid peaks at 1,730 and 1,623 cm^{-1}, as can be judged from the flat and featureless 1,700–1,900 cm^{-1} region with near zero absorbance in Fig. 1 *C*.

Fig. 2 shows representative spectra, obtained as described above, for prothrombin alone, in the presence of Ca^{2+}, and in the presence of PS/POPC and DOPG/POPC membranes. As expected for protein infrared spectra in D_2O media, the region above 1,700 cm^{-1} was flat and featureless. The remainder of the spectrum showed two broad bands, one between 1,700 and 1,600 cm^{-1}, which is the amide I′ (C=O stretch in D_2O environment) band, and the other, known as the amide II′ band (CN stretch and ND bend), located, due to H-D exchange, in the region below 1,500 cm^{-1}. There are also some bands in the region between 1,600 and 1,500 cm^{-1} that are due to amino acid side-chain vibrations. Both amide bands are complex composites of several discrete bands that are characteristic of specific types of protein secondary structure, many of which can be revealed by Fourier self-deconvolution resolution enhancement (Yang et al., 1985; Byler and Susi, 1986).

Common sources of error in resolution-enhanced FTIR are side lobes adjacent to strong bands and enhanced noise, both of which can be mistaken for real spectral features (Mantsch et al., 1988; Surewicz and Mantsch, 1988). We examined a range of resolution enhancement factors (*k*; Kauppinen et al., 1981*a*) from 2.4 to 3.2 and chose a conservative value of 2.6 to insure that artifacts associated with resolution enhancement did not obscure real spectral features in the amide I′ region. In addition, too large a bandwidth parameter during FSD processing can amplify artifacts caused by noise, side lobes, and water vapor (Yang et al., 1985). We examined the effect of self-deconvolution bandwidth values from 14 to 24.5 cm^{-1} and chose a conservative value of 18 cm^{-1} as adequate to reveal amide I′ fine structure and at the same time to minimize noise amplification that could lead to misinterpretation of FSD spectra. Fig. 3 illustrates the effect of FSD on the spectrum of bovine prothrombin. Comparison of the featureless region from 1,700 to 1,900 cm^{-1} reveals the expected but not excessive decrease in the signal-to-noise in the FSD spectrum (Fig. 3 *B*) as compared with the undeconvolved spectrum (Fig. 3 *A*). The lack of significant sideband next to the amide I′ band in the FSD spectrum (Fig. 3 *B*) demonstrates further that our choice of FSD parameters did not introduce artifacts that would obscure interpretation of our spectra.

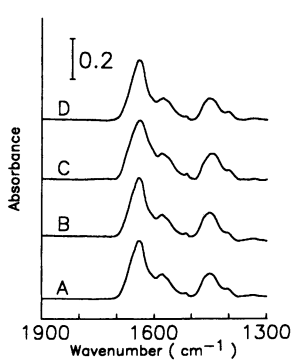

FIGURE 2 Undeconvolved FTIR spectra of bovine prothrombin in D_2O. (*A*) Bovine prothrombin (5% wt/vol); (*B*) prothrombin (5.2% wt/vol) in the presence of 5 mM Ca^{2+}; (*C*) prothrombin (3.5% wt/vol) in the presence of 5 mM Ca^{2+} and 24 mM (25/75) PS/POPC SUV; (*D*) prothrombin (2.8% wt/vol) in the presence of 5 mM Ca^{2+} and 24 mM 50/50 DOPG/POPC SUV.

Analysis of prothrombin amide I′ FSD spectra and secondary structural shifts

After Fourier self-deconvolution in the region of 1,600 to 1,700 cm^{-1}, three major spectral features were resolved in the prothrombin spectrum: a peak at 1,642 cm^{-1}, a shoulder at ~1,658 cm^{-1}, and another peak at 1,632 cm^{-1} (Fig. 4 *A*). According to published FTIR spectra for more than 20 different proteins of known secondary structure (Byler and Susi, 1986; Yang et al., 1985), these correspond to "random-coil", α-helical and

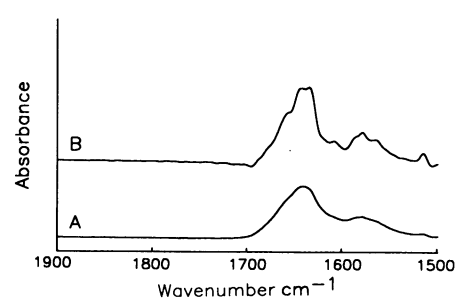

FIGURE 3 Illustration of the effects of Fourier self-deconvolution on the spectrum of bovine prothrombin. (*A*) Before deconvolution; (*B*) after deconvolution with parameters $K = 2.6$ and FWHH = 18 cm^{-1}.

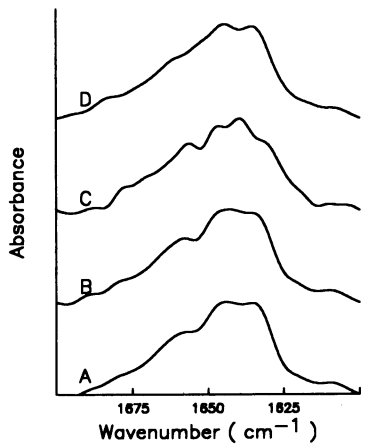

FIGURE 4 Fourier self deconvolved ($K = 2.6$; FWHH = 18 cm^{-1}) versions of the raw spectra shown in Fig. 2 plotted on an expanded scale to show the details of the amide I′ region. Labeling is as in Fig. 2.

β-strand structures, respectively. In addition to these three main assignments, several other weak features, as summarized in Table 1, could be detected in the self-deconvolved spectra or in the derivative spectra (not shown). These weak features apparently arise from higher frequency vibrations of some extended chain and beta turn structures (Byler and Susi, 1986), although the assignment of the band at 1,648 cm^{-1} was less evident. From the compilation of Byler and Susi, this band was assumed to be most reasonably assigned to a "random" or unordered conformation of the amide bond.

Fig. 4 summarizes the spectra of prothrombin under different conditions of ligation. Even after resolution enhancement, the effect of CA^{2+} binding to prothrombin was barely perceptible (compare spectrum *B* to *A*). However, the effect of membrane binding was evident by comparing spectrum *C* (PS/POPC bound) with spectrum *B* (*unbound*); an increase in the α-helical peak at 1,658 cm^{-1} as well an alteration in the random coil peaks (1,642 and 1,648 cm^{-1}) was detected. By contrast, the spectrum of DOPG/POPC-bound prothrombin (*D*) was qualitatively more similar to that of unbound prothrombin (*B*) than it was to that of PS/POPC-bound prothrombin (*C*).

Quantitative spectral analysis was obtained by the curve-fitting procedure described in Methods. The peak frequencies of the overlapping component bands needed to fit the amide I′ contour are summarized in Table 1. The data shown in Table 1 represent a composite of spectral assignments from two to five independent experiments (as indicated) with different samples, with all spectra collected under the same experimental conditions (i.e., protein concentration, buffer, temperature, instrument settings, etc.) and deconvolved with the same parameters. Spectra from such independent experiments were completely reproducible, to the extent of being nearly superimposible.

The effects of Ca^{2+} and membrane binding to whole prothrombin can be described in a semi-quantitative fashion in terms of the spectral resolution procedure. Our results are summarized in Table 2, along with an indication of the goodness of fit of the observed amide I′ contour by the spectral resolution procedure. An illustration of the success of our spectral decomposition is given in Fig. 5 for the case of prothrombin alone. Note that we did not attempt to fit the data below 1,625 cm^{-1}, as this region contains bands associated with amino acid side chain vibrations as well as with the C=O stretch of PS. At least two independent samples were examined to check for reproducibility in the case of each class of sample (see Table 1). In each case, however, the result of only one typical experiment is reported in Table 2. For the case of bovine prothrombin, however, four independent curve-fitting trials were performed, using the same set of assumptions about peak positions for each trial. These produced values of 6.7 ± 0.8%, 23.2 ± 1.2%, and 70.0 ± 0.7% for α-helix, β-structure, and random structure, respectively. This provided an estimate of the reproducibilities associated with the values in Table 2. These reproducibilities were consistent with values reported in the literature for another membrane associated protein, cytochrome b_5 (Holloway and Mantsch, 1989). Some idea of the accuracy (or more properly the consistency of secondary structural information obtained from different procedures) can be gleaned from comparison of our FTIR analysis with literature reports derived from both circular dichroism and x-ray diffraction studies of prothrombin and prothrombin fragment 1 (see Table 2). Except for the poor agreement between FTIR and circular dichroism results for whole prothrombin, the agreement between values reported by different laboratories using different techniques is reasonably good, especially considering the assumptions inherent in such structural analyses by any technique. The consistency between the two spectroscopic techniques and the x-ray scattering method for the case of fragment 1 plus Ca^{2+} is particularly satisfying. Two recently proposed alternative methods for analysis of FTIR spectra in terms of reference spectra have been shown (Dousseau and Pézolet, 1990; Lee et al., 1990) to predict known x-ray structure less well than the simple method of Byler and Susi (1986), as used here.

Ca^{2+} binding produced only a 1% increase in the band characteristic of α-helical (1,658 cm^{-1}) conformations and only a 1% increase in the β-structure content of

TABLE 1 Spectral resolution of the amide I′ band of bovine prothrombin and fragment 1

Protein (# of independent experiments)	α-Helix frequency (cm^{-1}) intensity linewidth (cm^{-1})	Unordered frequency (cm^{-1}) intensity linewidth (cm^{-1})		β-Sheet and Turns frequency (cm^{-1}) intensity linewidth (cm^{-1})					
Prothrombin (5)	1659	1642	1648	1632	1666	1672	1678	1688	
	0.183	0.543	0.037	0.112	0.179	0.120	0.148	0.068	
	8.8	28.5	8.0	6.8	7.5	6.2	9.7	10.9	
Prothrombin (3) +5 *mM* Ca^{2+}	1659	1642	1648	1632	1666	1672	1679	1688	
	0.178	0.463	0.040	0.089	0.178	0.128	0.119	0.070	
	8.3	27.0	5.3	6.5	7.7	7.0	7.6	11.0	
Prothrombin (3) +5 *mM* Ca^{2+} and PS/PC	1659	1642	1648	1632	1666	1672	1679	1689	
	0.292	0.563	0.092	0.130	0.267	0.172	0.200	0.121	
	7.5	23.6	2.9	4.3	6.8	5.9	8.1	7.5	
Prothrombin (2) +5 mM Ca^{2+} and PG/PC	1654	1641	1646	1633	1666	1672	1683	1688	1662
	0.150	0.478	0.063	0.091	0.041	0.170	0.090	0.030	0.168
	8.5	28.0	4.8	5.8	7.4	11.7	8.3	5.9	8.3
Fragment 1 (2)	1658	1642		1632	1666	1672	1678	1687	
	0.240	0.886		0.095	0.330	0.036	0.067	0.112	
	12.4	20.5		8.8	18.6	7.9	8.3	8.5	
Fragment 1 (2) +5 mM Ca^{2+}	1654	1642	1646	1632	1666	1672	1678	1684	1661
	0.148	0.260	0.019	0.091	0.080	0.076	0.026	0.040	0.103
	9.3	17.5	4.3	9.3	7.4	8.5	6.5	6.7	6.7
Fragment 1 (2) +5 mM Ca^{2+} and PS/PC	1654	1642	1646	1632	1666	1672	1678	1684	1661
	0.066	0.114	0.007	0.035	0.033	0.040	0.008	0.020	0.051
	9.1	17.5	4.3	8.5	6.5	10.0	5.9	6.9	6.5
Fragment 1 (2) +5 mM Ca^{2+} and PG/PC	1654	1644		1634	1661	1666	1672	1680	1685
	0.141	0.269		0.145	0.119	0.079	0.060	0.027	0.012
	7.4	13.6		8.1	5.6	5.2	7.0	5.9	3.7

Protein concentration: 5% wt/vol
Lipid concentration: 24 *mM*

Buffer: D_2O, 0.01 M mops, 0.1 M NaCl, 0.1 mM EDTA, pD 7.4

prothrombin (Table 1). This is not significantly outside of the uncertainty in reproducibility of our measurements, so we cannot conclude with reasonable certainty that Ca^{2+} binding increases the ordered secondary structure of prothrombin. This is not surprising because Ca^{2+} is reported to produce a change of only 5% in the α-helical and β-structure content of the fragment 1 region (the *N*-terminal 30%) of prothrombin. This makes it difficult to detect changes in the whole molecule (Nelsestuen, 1976; Predergast and Mann, 1977; Bloom and Mann, 1979; Marsh et al., 1979; see also Table 2 and below).

Binding of prothrombin in its Ca^{2+} conformation to bovPS/POPC membranes produced a somewhat larger increase in the integrated intensities of bands associated with ordered secondary structure (2% α-helix and 3% β-structure; Table 2) and a corresponding decrease in the integrated intensity of bands associated with "random" or unordered structure (5%; Table 2). Although these changes were small, they were sufficiently larger than our estimated uncertainties in reproducibility to suggest a shift in prothrombin conformation on binding to PS/POPC membranes. On the other hand, binding to DOPG/POPC vesicles did not alter prothrombin secondary structure significantly relative to that observed for the Ca^{2+} conformation of prothrombin (Table 2), as expected from visual examination of the spectra (Fig. 4, *B* and *D*).

TABLE 2 **Fractional areas of the amide I′ bands of bovine prothrombin and fragment 1**

Protein	α-helix percent	β-sheet and turns percent	Random coil percent	χ^2
Bovine prothrombin	7,10*	23,15*	70,75*	1.10
Bovine prothrombin +5 *mM* Ca^{2+}	8	24	68	1.97
Bovine prothrombin +5 *mM* Ca^{2+} and PS	10	27	63	1.71
Bovine prothrombin +5 *mM* Ca^{2+} and PG	7	25	68	1.20
Bovine fragment 1	10, 9‡	30, 33‡	60, 58‡	1.04
Bovine fragment 1 +5 *mM* Ca^{2+}	15,15,‡13§	36,39,‡40§	49,46,‡47§	1.45
Bovine fragment 1 +5 *mM* Ca^{2+} and PS	15	36	49	0.85
Bovine fragment 1 +5 *mM* Ca^{2+} and PG	14	38	48	2.24

*Bloom and Mann (1979).
‡Bloom and Mann (1978).
§X-Ray diffraction; Soriano-Garcia et al. (1989).
χ^2 is defined as the sum of squares of residuals between observed and calculated spectra divided by the square of the uncertainty in each data point and by the number of degrees of freedom. An adequate description of the data is indicated by a value near 1.

Analysis and difference spectroscopy with undeconvolved prothrombin spectra

To assure that the important conclusion of a membrane-induced change in prothrombin secondary structure could not be obscured by potential artifacts of the FSD procedure, the original, undeconvolved spectra were also analyzed by the curve-fitting procedure. The spectral curve-fitting procedure was not as successful in fitting the observed spectra (χ^2 on the order of 6 to 7) as it had been for the FSD spectra in Fig. 4, mainly because there was no basis for choosing individual peak widths without the additional spectral resolution provided by the FSD procedure. While this made semiquantitative analysis less reliable, a small increase (~3%) of ordered secondary structure and a corresponding decrease of "random" structure was still associated with binding to PS/POPC membranes, consistent with the analysis of FSD spectra summarized in Table 2. In addition, no change was detected for binding to DOPG/POPC membranes.

Finally, if buffer- and water-vapor-corrected spectra of prothrombin and Ca^{2+} and prothrombin and Ca^{2+} and PS/POPC membranes were overlayed, it was always possible to discern an increase in the 1,650–1,680-cm^{-1} region and a decrease in the 1,635–1,655-cm^{-1} region associated with the presence of PS/POPC membranes. These differences were reproducible between spectra taken on different days with independently prepared samples. To better illustrate this, difference spectra were obtained and are presented in Fig. 6. The difference between the original spectrum of prothrombin in the presence of Ca^{2+} plus PS/POPC membranes and the spectrum of prothrombin and Ca^{2+} was obtained by matching the baseline above 1,700 cm^{-1} and scaling the

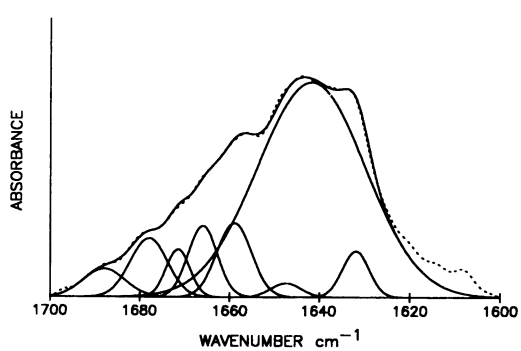

FIGURE 5 Spectral resolution (see text) of the Fourier self-deconvolved spectrum of bovine prothrombin (Fig. 4 *A*) in the amide I′ band region (1,700–1,600 cm^{-1}). Components below 1,625 cm^{-1}, which are due to side-chain vibrations, were not included in the spectral fitting procedure. The dashed curve shows the original data whereas the solid curve is the sum of the component contributions (also solid).

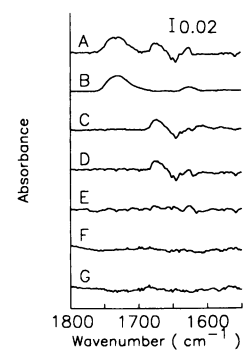

FIGURE 6 FTIR difference spectra of prothrombin. (*A*) Prothrombin in the presence of Ca^{2+} subtracted from prothrombin in the presence of PS/POPC membranes and Ca^{2+}; (*B*) PS/POPC membrane spectrum after buffer subtraction (the absorbance has been scaled to have the same value at 1,730 cm^{-1} as spectrum *A*); (*C*) difference between spectra *A* and *B* after water vapor correction; (*D*) difference between *A* and *B* before water vapor correction; (*E*) difference spectrum showing the effect of prothrombin and Ca^{2+} binding to DOPG/POPC membranes (obtained in a manner analogous to frame *C* for PS/POPC membranes); (*F*) difference between spectra of two independent samples of prothrombin and Ca^{2+} after water vapor correction; (*G*) difference between spectra of two independent samples of prothrombin and Ca^{2+} and PS/POPC membranes after water vapor correction.

peak heights of the amide I′ band. This difference spectrum is shown in Fig. 6 *A*. Fig. 6 *B* shows the buffer subtracted spectrum of PS/POPC membranes. Spectrum *B* was then subtracted from *A* to obtain Fig. 6 *D*, which, after water vapor correction, gave Fig. 6 *C*. The clear, positive band between 1,655 and 1,685 cm^{-1} in Fig. 6 *C* can be taken as indicative of an increase in ordered structures. The negative peak between 1,630 and 1,655 cm^{-1} indicates a complementary decrease of random structure. The integrated areas of these negative and positive difference peaks were nearly equal, as expected. The lack of an obvious positive peak near 1,630 cm^{-1} corresponding to β-structure probably reflects overlap with the edge of the negative "random" structure peak at 1,642 cm^{-1}. The negative "blips" at ~1,620 cm^{-1} likely are due to imperfect subtraction of the lipid spectrum. This made interpretation of the difference spectrum less reliable for wavenumbers <1,630 cm^{-1}. The results for the spectrum that had not been water-vapor corrected (Fig. 6 *D*) were essentially the same, except that the positive and negative peaks were somewhat less clear due to the interference from the small water bands. These difference spectra make it evident that the conclusion of a PS/POPC-induced conformational shift in prothrombin is independent of ambiguities that might be associated with the FSD procedures or with uncompensated water vapor bands. The same difference methods were applied to the spectrum obtained in the presence of DOPG/POPC membranes, with the result (Fig. 6 *E*) that no clear difference could be detected, in agreement with the spectral analysis.

For detecting changes in prothrombin secondary structure associated with changes in its liganding state, the appropriate criterion for judging the credibility of observed changes is the reproducibility of experiments. For this reason, additional spectra of independently prepared prothrombin and Ca^{2+} and prothrombin and Ca^{2+} and PS/POPC membranes were analyzed, with a result similar to that shown in Fig. 6 *C*. To illustrate this reproducibility, we present in Figs. 6 *F* and 6 *G* the difference spectra between spectra obtained on different preparations of prothrombin and Ca^{2+} and prothrombin and Ca^{2+} and PS/POPC membrane samples. As can be seen, these are essentially flat and featureless except for noise. These difference spectra demonstrate that the difference peaks seen in Fig. 6 *C* are real, reproducible, and reflect secondary structural changes associated with binding of prothrombin specifically to PS/POPC membranes. Next, we show that these effects were not observed with the *N*-terminal domain of prothrombin fragment 1.

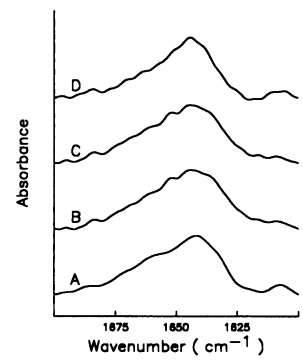

FIGURE 7 Fourier self-deconvolved FTIR spectra of (*A*) fragment 1 (2.5% wt/vol); (*B*) fragment 1 (2.5% wt/vol) in the presence of 5 mM Ca^{2+}; (*C*) fragment 1 (3.3% wt/vol) in the presence of 5 mM Ca^{2+} and 24 mM 25/75 PS/POPC SUV; (*D*) fragment 1 (3.3 wt/vol) in the presence of 5 mM Ca^{2+} and 24 mM 50/50 DOPG/POPC SUV.

Secondary structural changes in prothrombin fragment 1

Deconvolved spectra of bovine prothrombin fragment 1 alone and in the presence of Ca^{2+} or Ca^{2+} and membranes are shown in Fig. 7. As can be seen, the shape of the Amide I′ region was essentially unaltered by binding to membranes (compare Fig. 7, *C* and *D* to Fig. 7 *B*). Quantitative spectral analysis confirmed that there was no detectable change in secondary structure associated with binding to these membranes (Table 2).

By contrast, a clear measurable shift in secondary structure was observed when prothrombin fragment 1 bound Ca^{2+}, as can be seen qualitatively from the spectra (Fig. 7 *A* compared with Fig. 7 *B*) or quantitatively from our spectral analysis (Table 2). These results confirm the ability of Fourier self-deconvolved FTIR spectra to detect the well documented (Bloom and Mann, 1978; Marsh et al., 1979) increase in α-helix and β-structure associated with Ca^{2+} binding to fragment 1. We note that the small and inconclusive secondary structural change associated with Ca^{2+} binding to whole prothrombin (Table 2 and Fig. 4) is consistent with the clear change we have detected in the N-terminal 30% of prothrombin. This lends credibility to the spectral analysis procedure that we have used to detect small ligand-induced changes in the secondary structure of prothrombin.

DISCUSSION

The role of membranes in the prothrombinase complex is to increase by roughly a thousand-fold the turnover number for thrombin generation by the membrane-

assembled prothrombinase relative to the rate of catalysis by the prothrombinase components in solution (Rosing et al., 1980; Mann, 1987). It is generally accepted that negatively charged phospholipids accomplish this enhancement of catalysis at least in part by assembling the prothrombinase complex. At present, it is not known by what molecular mechanism membrane binding might contribute, if it does contribute, to the catalytic process. However, it has been realized lately that certain acidic phospholipids promote thrombin production much more effectively than others (Jones et al., 1985; Rosing et al., 1988). In particular, we have shown that PS-containing membranes support thrombin formation at a thousandfold lower concentration than do PG-containing membranes (Jones et al., 1985). The molecular mechanism for this specificity in the lipid component of the prothrombinase complex is also incompletely understood. We examine here the possibility that the substrate prothrombin might have a conformation on PS-containing membranes different from and more conducive to proteolysis than the conformation that occurs on PG-containing membranes.

FTIR spectroscopy, even though difficult to use in aqueous suspensions, can yield direct information about protein secondary structure and conformational changes in solution (Mantsch et al., 1988; Surewicz and Mantsch, 1988). It has the added advantage for our measurements of not being obscured by the substantial light scattering encountered in membrane suspensions, as are optical rotatory dispersion measurements. The disadvantage of protein FTIR spectroscopy is that artifacts caused by noise, water vapor, and/or FSD-induced side lobes may interfere with the interpretation of spectral features. For this reason, reliable data and a cautious approach are necessary. The present FTIR investigations present such carefully collected data and provide direct spectroscopic evidence for conformational changes in bovine prothrombin associated with binding to PS-containing membranes. Although the changes in integrated peak intensities were small, the changes were reproducibly observed in the deconvolved spectra (Fig. 4 and Table 1) and were detected as well in the undeconvolved spectra by spectral analysis and difference methods (Fig. 6). Especially noteworthy was the lack of a significant membrane-induced conformational shift in the fragment 1 region of prothrombin (Fig. 7 and Table 2). This argues strongly that the spectral changes we have recorded reflect PS-induced changes in the conformation of the nonfragment 1 region of the prothrombin molecule.

It is commonly accepted that the amino-terminal region of the prothrombin molecule (fragment 1) contains the vitamin *K*-dependent modification, γ-carboxyglutamic acid, and that this moiety permits the binding, in the presence of Ca^{2+}, of this region to membranes that contain negatively charged phospholipid (Nelsestuen, 1976). It has also been known for some time that Ca^{2+} binding induces in prothrombin (in particular, in the fragment 1 portion of prothrombin) a special "Ca-conformation" necessary for tight binding to membranes (Nelsestuen et al., 1976; Prendergast and Mann, 1977; Bloom and Mann, 1978; Borowski et al., 1986). Ploplis et al. (1981) showed that fragment 1 responded to Ca^{2+} as a separate structural domain of prothrombin, whereas the remainder of the molecule (prethrombin 1) was little affected by Ca^{2+} binding. We have observed recently by differential scanning calorimetry that the denaturation profile of bovine prothrombin was altered substantially when this protein was bound to PS-containing membranes, but altered less significantly when prothrombin was bound to PG-containing membranes (Lentz et al., 1991). The most significant change in the denaturation profile was not in the fragment 1 domain but in the prethrombin 1 domain. The spectroscopic results presented here are consistent with and complement this recent calorimetric study of protein denaturation. The consistency of the results obtained by two different techniques lends credence to our conclusion that binding to a PS-containing membrane alters in some way the structural organization of the prothrombin molecule.

The sum total of our results suggest a tentative model for the conformational changes in the prothrombin molecule associated with binding of Ca^{2+} and PS-containing membranes. While fragment 1 is the region of prothrombin traditionally viewed as being responsible for membrane binding (Nelsestuen, 1976; Dombrose et al., 1979), it must be that the prethrombin 1 domain of prothrombin either interacts directly with the membrane or "feels" the presence of the membrane through the fragment 1 domain. It is not possible from our current results to distinguish between these two possibilities. It appears that Ca^{2+} binding puts the fragment 1 domain of prothrombin into a "Ca-conformation" appropriate to membrane binding, which event induces a "membrane-bound conformation" in the prethrombin 1 domain. This is illustrated in Fig. 8.

Finally, we note a correlation between the conformational change in the prethrombin 1 domain induced by a particular membrane and the ability of that membrane to support thrombin formation in an in vitro assay system. It is intriguing that the prethrombin 1 domain contains the two peptide bonds that must be broken to form thrombin. Other results from our laboratory have demonstrated a tenfold lower k_{cat} for human thrombin generation by the prothrombinase assembled on PG-containing membranes as compared to PS-containing membranes (G. Pei, D. Powers, and B. Lentz, unpublished results). Despite the fact that this structure-

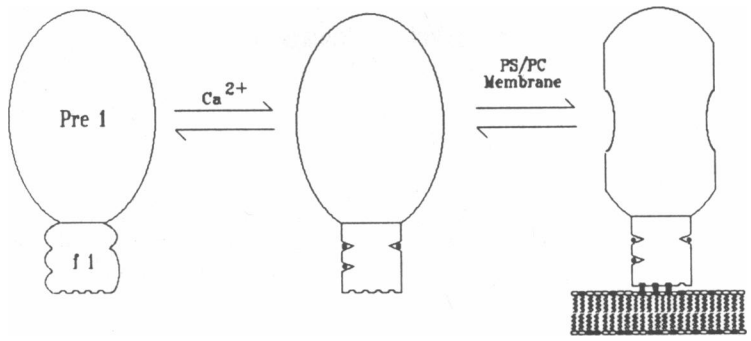

FIGURE 8 Schematic diagram of the effect of Ca^{2+} ion and membrane surface on prothrombin conformation. Ca^{2+} ion induces a conformational change in the fragment 1 (f1) domain of the molecule. A PS-containing membrane surface induces a conformational change in the prethrombin 1 portion of the molecule. A PG-containing membrane has little effect on prethrombin secondary structure.

function correlation crosses species lines, it is very tempting to suggest that a specific interaction with PS at a membrane surface might alter the conformation of prothrombin in such a way as to make it a better substrate for proteolysis to thrombin.

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