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Metal Ion Induced Conformational Transitions of Prothrombin and Prothrombin Fragment 1†

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ABSTRACT: Circular dichroism experiments indicate that prothrombin fragment 1 undergoes essentially the same secondary structural change whether in the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+}. Titration with any of these metal ions results in a sigmoidal titration curve indicative of cooperative binding. Mg^{2+} and Ca^{2+} have nearly identical transition midpoints, while that for Mn^{2+} is an order of magnitude less. These results correlate well with the results of previous metal ion intrinsic fluorescence quenching experiments. Fragment 1 has previously been shown to undergo a second transition corresponding to dimerization at high calcium concentrations. The present circular dichroism experiments show that this transition does not result in a gross alteration of secondary structure in the fragment 1 molecule. Studies with prothrombin, similar to those with fragment 1, indicate a similar metal ion dependent conformational change but of smaller magnitude. As apparently only the fragment 1 portion of the molecule undergoes the transition, it would appear that the covalently linked fragment 1 is constrained from attaining the same conformation as the purified entity. This suggests that caution must be used in interpreting the results of metal ion binding studies using fragment 1 as an analogue for prothrombin.

Prothrombin fragment 1 has been shown to be the region of the prothrombin molecule responsible for phospholipid binding (Gitel et al., 1973). The 10 γ-carboxyglutamic acid residues in prothrombin all occur in fragment 1 (Fernlund et al., 1975; Howard et al., 1975), and, like prothrombin, fragment 1 binds Ca^{2+} in a highly cooperative manner (Stenflo & Ganrot, 1973; Henriksen & Jackson, 1975; Benarous et al., 1976; Bajaj et al., 1975; Mann et al., 1973). Besides Ca^{2+}, prothrombin and fragment 1 have been shown to bind other di- and trivalent cations (Prendergast & Mann, 1977; Bajaj et al., 1976; Furie et al., 1976; Nelsestuen et al., 1976).

Nelsestuen (1976) and Prendergast & Mann (1977) have shown that prothrombin fragment 1 undergoes a fluorescence transition in the presence of a variety of metal ions, which results in about a 40% quenching of the tryptophan fluorescence. In addition, Prendergast & Mann (1977) have shown that, only in the presence of metal ions (calcium) which stimulate prothrombin activation and prothrombin lipid binding, a second transition occurs which involves dimerization of prothrombin fragment 1 molecules. This second transition occurs at metal ion concentrations higher (approximately two times) than those which elicit the fluorescence change. The calcium ion concentration which is optimal for prothrombin conversion to thrombin is equivalent to that metal ion concentration which brings about the second transition.

Fluorescence quenching data alone do not allow one to decide between a minor local environmental change around a tryptophan residue or a more substantial change in the overall backbone of the protein. The technique of circular dichroism (CD) allows one to make this distinction. Circular dichroism studies available from the literature on prothrombin fragment 1 (Gabriel et al., 1975) permit the conclusion that calcium concentrations twice those required for both the aforementioned calcium-dependent fragment 1 transitions induce con-

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formational changes. In view of the fact that there are two distinct metal ion dependent transitions which occur at different concentrations of calcium, and these are metal ion selective, it is impossible from the data of Gabriel et al. to decide which transitions of the fragment 1 molecule resulted in the CD change they observed. Furthermore, these studies were conducted outside of the pH range (7.4–8.5) for maximum metal ion binding to prothrombin (Benarous et al., 1976) and far from the pH optimum of the calcium dependent zymogen activation. For these reasons the present studies were conducted explicitly under sets of conditions which allowed a direct comparison of changes in CD spectra with known calcium binding phenomena and known metal ion induced transitions appropriate for the fluorescence and dimerization transitions in prothrombin fragment 1.

All studies of prothrombin fragment 1 metal ion transitions are done under the assumption that equivalent transitions occur in the prothrombin molecule. There is some evidence to support this assumption. Immunochemical methods have been used to show that Ca^{2+} induces a conformational change in prothrombin (Stenflo & Ganrot, 1973; Stenflo, 1972) and the intrinsic fluorescence of prothrombin is quenched approximately 6% by the binding of Ca^{2+} (Prendergast & Mann, 1977). However, Bjork & Stenflo (1973) reported that the CD spectrum of prothrombin is altered in the presence of Ca^{2+}, but only in the aromatic region of the spectrum indicating little or no secondary structural change in the molecule. Again, this study was conducted at a calcium ion concentration which would not permit discrimination of the two calcium ion dependent transitions in prothrombin fragment 1.

The present CD metal ion study therefore was conducted under conditions of pH and metal ion concentrations for which metal ion binding, lipid binding, and zymogen activation data are available in order to: (1) discriminate which prothrombin fragment 1 transition is reflected in a change in secondary structure and to permit some interpretation with respect to differential site filling (based upon preceding work); (2) test the hypothesis that prothrombin fragment 1 is a legitimate model for prothrombin in metal ion binding experiments.

Materials and Methods

Calcium chloride, magnesium chloride, and manganese chloride were obtained as reagent grade anhydrous solids from Fisher Chemicals. It was previously shown in this laboratory that calcium contamination of solutions made from these reagents was $<10^{-6}$ M and was therefore ignored (Prendergast & Mann, 1977). Rabbit brain cephalin was obtained from Sigma.

Proteins. Prothrombin was purified from citrated bovine plasma and from human Cohn fraction 3 (a gift of Dr. Charles Heldebrant, Abbott Laboratories) or Red Cross factor IX concentrate (gift of Dr. Yu Lee Hao, The American National Red Cross) as previously described (Bajaj & Mann, 1973; Downing et al., 1975). Prothrombin fragment 1 and prethrombin 1 were prepared by proteolytic cleavage of prothrombin by thrombin and were purified as described elsewhere (Downing et al., 1975; Heldebrant et al., 1973). The protein preparations were greater than 95% homogeneous as evaluated electrophoretically by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969).

Protein concentrations were determined and spectrophotometrically corrected for Raleigh scattering using the equation

$$A_{280} = A_{280\ \mathrm{abs}} - 1.706A_{320\ \mathrm{abs}}$$

The extinction coefficients ($E^{1\%}_{280}$) used were: 14.7 for human prothrombin, 17.8 for human prethrombin 1, 11.9 for human prothrombin fragment 1 (Butkowski et al., 1977), and 10.5 for bovine prothrombin fragment 1 (Mann, 1976). The molecular weights and mean residue weights of the proteins were calculated from the primary structures (Butkowski et al., 1977; Elion et al., 1976; Walz et al., 1977; Magnusson et al., 1975): 71 600 and 113.1 for human prothrombin, 72 100 and 113.7 for bovine prothrombin; 21 700 and 114.6 for human prothrombin fragment 1.

In preparation for analysis, the proteins were dialyzed in plastic containers, first against 5 mM EDTA in 0.01 M Tris, 0.10 M NaCl, pH 7.4, to ensure minimal Ca^{2+} contamination and then exhaustively against 0.01 M Tris, 0.10 M NaCl, pH 7.4, to ensure minimal residue EDTA.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were measured with a Jasco Model J-20A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at room temperature (25 °C). The far-ultraviolet spectra were recorded with protein concentrations of 0.3–0.6 mg per mL or 0.1 mg/mL in respectively 0.05-cm or 0.106-cm pathlength cuvettes. Titrations were performed in 3.0-mL volumes in 1-cm pathlength standard cuvettes containing approximately 0.1 mg/mL of protein by making 1.0- or 2.0-μL additions of titrant (metal ion in appropriate buffer). Corrections were usually not made for volume changes as only up to 15-μL additions were made in the titrations which is <0.5% volume change. Buffer baseline deviations were subtracted from all spectra. All spectra were measured in at least duplicate and the averaged values have been reported.

To study the effect of phospholipid on the CD changes, an amount of phospholipid equivalent to that used in the prothrombin assay (130 μM), as rabbit brain cephalin taken up in 0.01 M Tris, 0.10 M NaCl, pH 7.4, was added to the reaction mixture containing approximately 0.1 mg/mL of protein prior to the titration.

The results have been expressed in terms of mean residue ellipticity $[\theta']$, or molar ellipticity $[\theta]$:

$$[\theta]_\lambda \text{ (or } [\theta']_\lambda) = \frac{\theta_{\mathrm{obsd}} \times \mathrm{MW\ (or\ MRW)}}{10 \times d \times c}$$

where λ = wavelength; θ_{obsd} = observed ellipticity in degrees; MRW = mean residue weight; MW = molecular weight; C = concentration in grams per milliliter, and d = pathlength in centimeters. Ellipticity has the units of deg cm^2 dmol^{-1}.

Comparisons were made of the far-UV CD spectra of prothrombin and prothrombin fragment 1 in the absence and presence of metal ions. In an attempt to make the comparisons objective, the spectra were analyzed by a technique which assumes that only three types of fundamental protein conformations contribute to a protein's structure in solution: "α helix", "β sheet", and "random coil". The unique tertiary structure of a protein, however, may also contain distorted or extremely short segments of α or β chains, portions of which are "random" in the sense that there is no regular repeated structure, and side-chain interactions and optically active disulfide and aromatic groups which may absorb in the far-UV. Thus, although the analysis of the spectrum, solely in terms of α helix, β sheet, and random coil, may not delineate the "true" solution conformation of the protein, the justification for using such an analysis is in the objectivity it introduces to the comparison of different spectra.

Chen et al. (1972) have found that the CD spectrum of a protein can be expressed as:

$$X(\lambda) = f_{\mathrm{H}}X_{\mathrm{H}}(\lambda) + f_\beta X_\beta(\lambda) + f_{\mathrm{R}}X_{\mathrm{R}}(\lambda) \qquad (1)$$

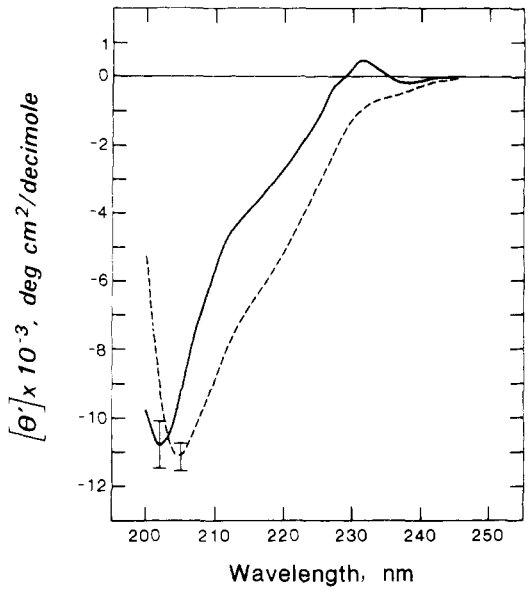

FIGURE 1: The far-ultraviolet CD spectra of human prothrombin fragment 1 in the presence (- - -) and absence (—) of 5 mM Ca^{2+}. The cuvette pathlength was 0.050 cm and the protein concentration 0.3 mg/mL. [θ'] is the mean residue ellipticity.

where $f_H + f_\beta + f_R = 1$ and all f's ≥ 0. $X(\lambda)$ is the ellipticity at any wavelength and the f's are the fractions of α helix (H), β sheet (β), and random coil (R). Chen et al. (1972) used f values empirically determined for five proteins whose structures were already known from X-ray studies to compute $X_H(\lambda)$, $X_\beta(\lambda)$, and $X_R(\lambda)$ at numerous wavelengths through a least-squares method. Using the tabular data of Chen et al. (1974) and the assumption that the five proteins used in his calculations of $X_H(\lambda)$, $X_\beta(\lambda)$, and $X_R(\lambda)$ are a representative sample, the contributions of α helix, β sheet, and random coil to the secondary structure of any protein can be computed from its CD spectrum. The solution of eq 1 is a special case of an overdetermined system of linear equations where the third variable can be expressed in terms of the other two. The least-squares approximation computer program used to solve eq 1 for each spectrum was written by Mr. Aloysius Chu and Dr. Barry K. Gilbert and solved on a CDC 3500 computer.

Fluorescence Measurements. The fluorescence intensities of a sample of bovine prothrombin fragment 1 were recorded on an Aminco-Bowman spectrofluorometer. Titrations of fluorescence intensity were performed in 3.0-mL volumes containing 0.111 mg/mL of protein by making 1.0- or 2.0-μL additions of Ca^{2+} in the appropriate buffer. Measurements were made at room temperature (25 °C) at 340 nm with excitation at 295 nm. After addition of titrant and careful mixing, the solution was allowed to equilibrate for 2.5 min before a reading was taken. At the end of the titration experiment, the solution was made 6 mM in EDTA and the fluorescence intensity was found to have returned to its initial (no Ca^{2+}) value.

Results

Metal Ion Induced Prothrombin Fragment 1 CD Changes. Circular dichroism spectra, in the far-ultraviolet spectral region ($\lambda < 250$ nm), provide information about the secondary structures of the polypeptide backbones of proteins in solution. The far-UV CD spectra of human prothrombin fragment 1 in the presence and absence of 5 mM Ca^{2+} are shown in Figure 1. The calculated apparent α helix, β sheet, and random coil contents of fragment 1 in the presence and absence of 5 mM Ca^{2+} are given in Table I. The spectrum of human fragment 1 has a minimum at 202 nm with a mean residue ellipticity of $-10\,800 \pm 700$ and a maximum at 231–232 nm of 400 ± 200. In the presence of 5 mM Ca^{2+} the spectrum has a minimum at 205 nm with a mean residue ellipticity of $-11\,200 \pm 400$ and the spectrum maximum at 231 nm has decreased in magnitude to -1000 ± 200. This difference in the spectra corresponds to an apparent difference in secondary structure of 8% more "α helix", 6% more "β sheet", and 14% less "random coil" in the presence of 5 mM Ca^{2+} than in its absence. Gabriel et al. (1975) obtained the CD spectra of human fragment 1 in the presence and absence of 5 mM Ca^{2+} at pH 6.5 in the region 225–250 nm and they are similar to those presented here.

TABLE I: Calculated Apparent "α Helix", "β Sheet", and "Random Coil" Contents of Prothrombin and Fragment 1 in the Presence and Absence of Ca^{2+}.

	"α helix" (%)	"β sheet" (%)	"random coil" (%)
human prothrombin fragment 1	6	31	63
+ 5 mM Ca^{2+}	14	37	49
bovine prothrombin fragment 1	9	33	58
+ 5 mM Ca^{2+}	15	39	46
human prothrombin	8	15	77
+ 5 mM Ca^{2+}	9	17	74

The far-UV spectrum of bovine fragment 1 has a minimum at 202 nm with a mean residue ellipticity of $-11\,200 \pm 500$; thus, within experimental error, the bovine and human fragment 1 spectra have identical minima. The spectra for both species have shoulders centered around 215 nm with the bovine fragment 1 spectrum having a more negative value, -3500 ± 200 vs. -2700 ± 200 for human. The bovine fragment 1 spectrum has a maximum at 233–234 nm of -100 ± 200. In the presence of 5 mM Ca^{2+}, the spectrum has a minimum at 204 nm with a mean residue ellipticity of $-11\,800 \pm 500$ and the spectrum maximum at 233–234 nm has decreased in magnitude as compared with the spectrum without Ca^{2+} to a value of -700 ± 200. The calculated apparent α helix, β sheet, and random coil contents of bovine fragment 1 in the presence and absence of 5 mM Ca^{2+} are given in Table I. The difference in the spectra corresponds to an apparent difference in secondary structure of 6% more "α helix", 6% more "β sheet", and 12% less "random coil" in the presence of 5 mM Ca^{2+} than in its absence.

Circular dichroism spectra in the near-ultraviolet region ($\lambda > 250$ nm) predominantly contain absorption bands for phenylalanine, tyrosine, and tryptophan. The near-UV CD spectra of human prothrombin fragment 1 in the presence and absence of 5 mM Ca^{2+} are shown in Figure 2. The most prominent features of the spectra are maxima at 284 and 291 nm and shoulders centered at approximately 278 nm. The spectrum of fragment 1 in the presence of 5 mM Ca^{2+} differs from the spectrum of the protein in the absence of Ca^{2+} in that the amplitudes of the peaks are smaller. These spectra are similar to those published by Gabriel et al. (1975) for fragment 1 in the presence and absence of 5 mM Ca^{2+}. The spectra are also similar to the spectrum of *N*-acetyl-L-tryptophanamide (Adler et al., 1973) suggesting that tryptophan residues are the predominant chromophores contributing to the near-ultraviolet spectrum of fragment 1. Within experimental error, the near-UV spectra of bovine and human prothrombin fragment 1 in both the presence and absence of 5 mM Ca^{2+} are identical.

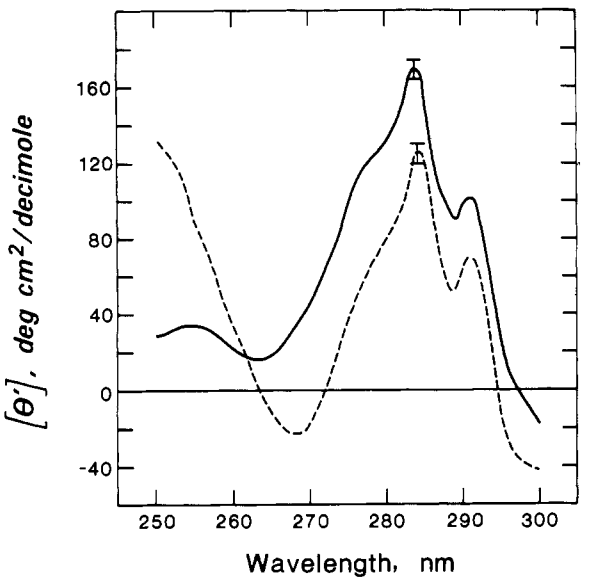

FIGURE 2: The near-ultraviolet CD spectra of human prothrombin fragment 1 in the presence (- - -) and absence (—) of 5 mM Ca^{2+}. The cuvette pathlength was 1.000 cm and the protein concentration 0.3 mg/mL. [θ'] is the mean residue ellipticity.

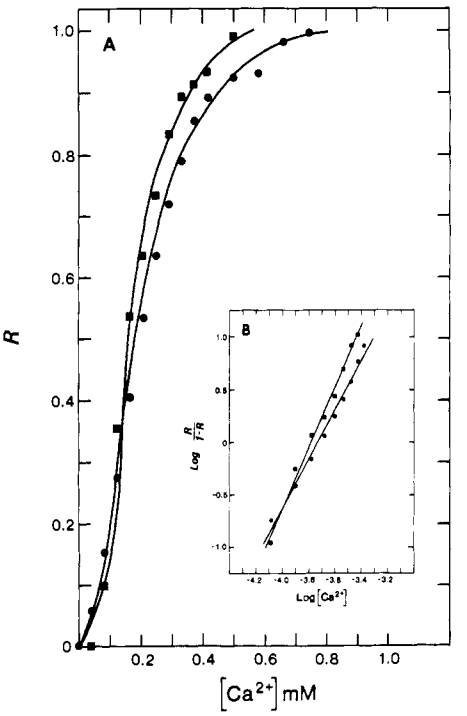

FIGURE 3: (A) Ca^{2+} titration by CD (■) at 231 nm and fluorescence quenching (●) of bovine prothrombin fragment 1 (0.11 mg/mL) in a 1.000-cm cuvette. For the CD experiment, R is the mean residue ellipticity at a given Ca^{2+} concentration divided by the value obtained when no further change in the spectrum occurs upon Ca^{2+} addition. For the fluorescence quenching experiment, R is defined as $(F - F_0)/(F_\infty - F_0)$ where F is the observed fluorescence, F_0 is the untitrated value, and F_∞ is the value obtained when no further quenching occurs upon Ca^{2+} addition. (B) Hill plots of the data from A. The intercepts and slopes obtained by CD and fluorescence are respectively: 0.17 mM, 2.9 and 0.19 mM, 2.4.

Metal ion titrations of prothrombin fragment 1, using Ca^{2+}, Mg^{2+}, and Mn^{2+}, were now undertaken using the circular dichroism technique in order to answer two questions. (1) Is the fluorescence spectral change previously observed (Prendergast & Mann, 1977; Nelsestuen, 1976) the result of only a perturbation of the local environment of a chromophore, or is it the result of a gross structural change in the molecule? (2) Fragment 1 has been observed by sedimentation studies (Prendergast & Mann, 1977) to undergo dimerization at calcium concentrations higher than those required for the fluorescence transition. Is this dimerization the result of a secondary structural change in the fragment 1 molecule? To answer these questions, prothrombin fragment 1 was titrated with metal ions by following the change in the CD spectrum maximum at 231 or 232 nm. These wavelengths were chosen primarily because of the relatively large metal ion induced change and because of the high signal-to-noise ratio in this region of the CD spectrum. The spectra were recorded in the region 225–240 nm and the mean residue ellipticity at 231 or 232 nm was determined. The titration curves were plotted in terms of R vs. metal ion concentration, where R is the mean residue ellipticity at a given metal ion concentration divided by the value obtained when no further change in the spectrum occurs upon metal ion addition. Since the titration curves were sigmoidal, they could be expressed as Hill plots. The Hill equation (Gutfreund, 1972) can be written for the present experiment as

$$\log K + n \log C = \log \frac{R}{1 - R} \qquad (2)$$

where K and n are constants, C is the metal ion concentration, and R is as defined above. The Hill coefficient, n, is related to the number of metal ion binding sites in that n cannot exceed the number of sites. Even though Hill plots may appear to be linear, they very seldom are; nor do they give a single Hill coefficient (Cornish-Bowden & Koshland, 1975). It is possible, however, to obtain a single coefficient for the region near the reaction midpoint (Nelsestuen, 1976) and that is the approach taken here. The metal ion concentration at the reaction midpoint intercept of a Hill plot is equivalent to the midpoint of the transition of the R vs. metal ion concentration plot. This value, designated T_m, is presented here as the average of the numbers obtained from the two plots.

The human prothrombin fragment 1 calcium ion titration curve is sigmoidal indicating cooperative binding and the concentration of Ca^{2+} at which the midpoint of the transition is achieved (T_m) is 0.25 mM and the Hill coefficient is 2.8. This value of T_m is in good agreement with the value of 0.22 mM obtained by fluorescence measurements (Prendergast & Mann, 1977).

Bovine prothrombin fragment 1 also had a sigmoidal titration curve with a Hill coefficient of 2.3 and a T_m of 0.20 mM as compared with fluorescence T_m values of 0.35 mM (Prendergast & Mann, 1977) and 0.40 mM (Nelsestuen, 1976). In contrast to the T_m values obtained for human fragment 1, the bovine values obtained by CD and fluorescence techniques are not in good agreement. Nelsestuen (1976) has observed that bovine fragment 1 exhibits reduced fluorescence quenching in the presence of calcium after storage for a few weeks at 4 °C (pH 7.5). In fact some preparations stored for several months lost all calcium-dependent fluorescence quenching properties. In order to determine if the disparities in CD and fluorescence T_m values were the result of fragment 1 "denaturation", the following experiment was undertaken. A fresh preparation of bovine fragment 1 was titrated with Ca^{2+} using both fluorescence and CD detection techniques. The results are presented in Table II and the titration curves and Hill plots are shown in Figure 3. The fluorescence data were analyzed in a manner analagous to that used for the CD data to allow direct comparison. Here R is defined as $(F - F_0)/(F_\infty - F_0)$, where F is the observed fluorescence, F_0 is the untitrated value, and F_∞ is the value obtained when no further quenching occurs

TABLE II: T_m Values and Hill Coefficients for Metal Ions Which Induce Circular Dichroism Transitions in Human and Bovine Prothrombin Fragment 1.[a]

metal ion	$T_{m(CD)}$	n(CD)	$T_{m(fluorescence)}$	n(fluorescence)	K_{dissoc}
Ca^{2+}	0.25 mM (H)	2.8 (H)	0.22 mM (H)[a]		
	0.20 mM (B)	2.3 (B)	0.35 mM (B),[a] 0.40 mM (B)[b]		0.63 mM[c]
	0.17 mM (B)[d]	2.9 (B)[d]	0.19 mM (B)[d]	2.4 (B)[d]	
Ca^{2+} + phospholipid	0.20 mM (B)	2.0 (B)	0.22 mM (B),[a] 0.24 mM (B)[b]		
Mg^{2+}	0.23 mM (H)	2.1 (H)	0.22 mM (H)[a]		
	0.21 mM (B)	1.9 (B)	0.45 mM (B)[a]		
Mn^{2+}	22.8 μM (H)	2.9 (H)	12.6 μM (H)[a]		22 ± 10 μM[e]

[a] The circular dichroism T_m values and Hill coefficients were determined as described in the text. The letters H and B in parentheses refer to human and bovine prothrombin fragment 1, respectively. The T_m values obtained by fluorescence techniques were obtained from published data, the sources being indicated by superscripts a and b equivalent to Prendergast & Mann (1977) and Nelsestuen (1976), respectively. Values labeled by superscript d were obtained in this laboratory on a fresh preparation of bovine fragment 1. Values for the dissociation constants (K_{dissoc}) were obtained from published data, the sources being indicated by superscript c and e equivalent to Bajaj et al. (1975) and Bajaj et al. (1976).

upon metal ion addition. The T_m and Hill coefficients obtained by CD and fluorescence are respectively: 0.17 mM, 2.9 and 0.19 mM, 2.4. Thus the T_m is essentially the same whether measured by CD or fluorescence. The intrinsic fluorescence of the fresh bovine fragment 1 preparation was quenched approximately −46% as compared with a value of −35% previously observed (Prendergast & Mann, 1977; Nelsestuen, 1976). These results suggest that the bovine fragment 1 used previously (Prendergast & Mann, 1977; Nelsestuen, 1976) contained a measurable amount of "denatured" fragment 1.

Dissociation constants, K_d, for ligand–substrate interactions are determined by measuring the free ligand concentration. In CD and fluorescence experiments, however, only the total ligand (metal ion) concentration is known. For metal ions with large dissocation constants, the free ion concentration is not appreciably different from the total ion concentration and T_m is nearly identical with K_d. The average K_d for the five or six bovine prothrombin fragment 1 Ca^{2+} binding sites has been determined to be 0.63 mM by equilibrium dialysis (Bajaj et al., 1975), while the T_m for a fresh fragment 1 preparation is approximately 0.18 mM. The large difference between the dissociation constant observed by equilibrium dialysis and T_m values observed by fluorescence and circular dichroism is consistent with the idea that fewer than five or six Ca^{2+} binding sites are involved in the Ca^{2+}-induced conformational change.

In order to determine whether the Ca^{2+}-induced fragment 1 transition was reversible, the following experiment was performed. The far-UV CD spectrum of bovine fragment 1 was recorded in the absence and presence of 5 mM Ca^{2+}. The calcium containing sample was then dialyzed against 5 mM EDTA in 0.01 M Tris, 0.10 M NaCl, pH 7.4, and then back into the same buffer without EDTA. The protein concentration (0.31 mg/mL) was unchanged by dialysis. The CD spectrum was recorded and found to be the same, within experimental error, as the fragment 1 spectrum taken before the addition of Ca^{2+}. Thus, the Ca^{2+}-induced conformational change of fragment 1 is completely reversible. This reversibility was also observed in the fluorescence experiment.

The human prothrombin fragment 1 Mg^{2+} titration curve is sigmoidal and the midpoint of titration, T_m, occurs at 0.23 mM and the Hill coefficient is 2.1, while the T_m obtained by fluorescence was 0.22 mM (Prendergast & Mann, 1977). Bovine prothrombin fragment 1 has a T_m for Mg^{2+} of 0.21 mM and the Hill coefficient is 1.9, while the fluorescent T_m value was 0.45 mM (Prendergast & Mann, 1977). This difference between the CD and fluorescence T_m values for the bovine molecule is probably due to "denaturation" as in the Ca^{2+} case.

The human prothrombin fragment 1 Mn^{2+} titration curve is sigmoidal and the T_m value occurs at 22.8 μM, an order of magnitude less than Mg^{2+} or Ca^{2+}, suggesting that Mn^{2+} binds with a greater affinity to fragment 1 than Ca^{2+} or Mg^{2+}. The Hill coefficient is 2.9. Bajaj et al. (1976) have shown, using EPR and NMR techniques, that fragment 1 possesses two high affinity Mn^{2+} binding sites with a K_d of 22 ± 10 μM and at least two lower affinity sites with a K_d of approximately 0.25 ± 0.1 mM. Thus the T_m value determined by CD agrees very well with the K_d of the high affinity Mn^{2+} binding sites. The T_m value for Mn^{2+} binding to fragment 1 determined by fluorescence measurements was 12.6 μM (Prendergast & Mann, 1977). In general, fluorescence quenching experiments with paramagnetic metal ions such as Mn^{2+} must be viewed with caution since a fraction of the Mn^{2+} quenching effect may be due to paramagnetic quenching of tryptophyl fluorescence (Prendergast & Mann, 1977).

The T_m values and Hill coefficients obtained by the titration of prothrombin fragment 1 with Ca^{2+}, Mg^{2+}, and Mn^{2+} using circular dichroism are summarized in Table II and compared with the results obtained from fluorescence measurements. The similarity in the T_m values obtained by the two different techniques suggests that the same molecular transformation is being observed. In addition, all three ions cause essentially the same alteration in the 228–240-nm region of the CD spectrum of fragment 1. Thus, the fluorescence changes previously observed (Prendergast & Mann, 1977; Nelsestuen, 1976) can be attributed to a gross alteration in the secondary conformation of the fragment 1 molecule, and not just to a perturbation of the local environment of a tryptophan residue.

The question of prothrombin fragment 1 dimerization in the calcium concentration range 1–2 mM was now investigated. The magnitudes of the 231-nm maxima in the fragment 1 CD spectra are −908 ± 25 in the presence of 0.79 mM Ca^{2+} and −900 ± 200 in the presence of 5 mM Ca^{2+}. Thus, little or no change has occurred in the magnitude of the spectral maximum in the Ca^{2+} concentration region of 0.79 to 5 mM Ca^{2+}. The same experiment done with bovine fragment 1 gave similar results: in the absence of Ca^{2+} the mean residue ellipticity at 232 nm was −100 ± 200; in the presence of 0.75 mM Ca^{2+}, −580 ± 20; in the presence of 1.97 mM Ca^{2+}, −640 ± 20; and in the presence of 5 mM Ca^{2+} was −700 ± 200. Both human

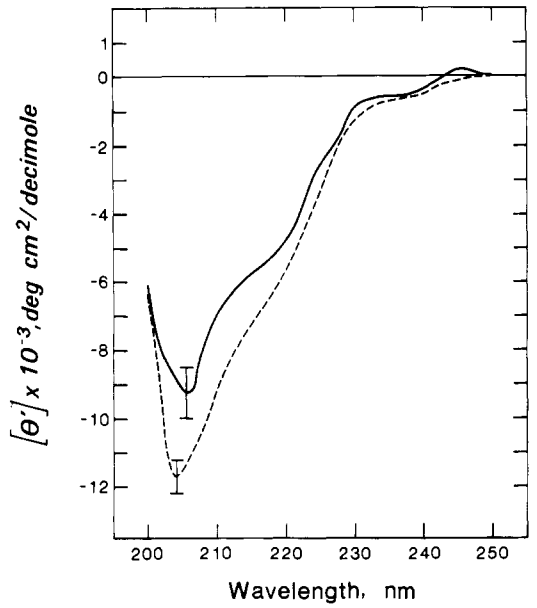

FIGURE 4: The far-ultraviolet CD spectra of bovine prothrombin fragment 1 plus 5 mM Ca^{2+} in the presence (—) and absence (- - -) of phospholipid. The cuvette pathlength was 0.106 cm and the protein concentration 0.11 mg/mL while the phospholipid was 130 μM. [θ'] is the mean residue ellipticity.

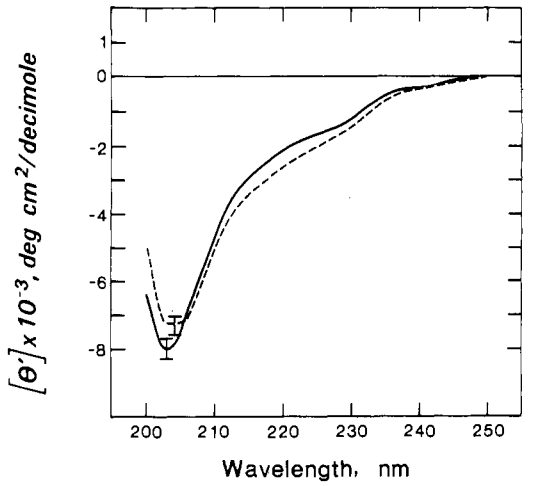

FIGURE 5: The far-ultraviolet CD spectra of human prothrombin in the presence (- - -) and absence (—) of 5 mM Ca^{2+}. The cuvette pathlength was 0.050 cm and the protein concentration 0.58 mg/mL. [θ'] is the mean residue ellipticity.

and bovine prothrombin fragment 1 then, within experimental error, undergo relatively little or no structural change in the titration range 1–5 mM Ca^{2+}. As this is the Ca^{2+} concentration region of the titration curve in which fragment 1 undergoes dimerization (Prendergast & Mann, 1977), dimerization is not related to a gross secondary structure conformational change in addition to that observed to occur in the Ca^{2+} concentration range 0–1 mM.

Ca^{2+}-Induced Prothrombin Fragment 1 CD Changes in the Presence of Phospholipid. As prothrombin fragment 1 binds to phospholipid in the presence of Ca^{2+} (Nelsestuen, 1976), the possibility that phospholipid binding might alter the secondary structure of fragment 1 was examined by the use of circular dichroism. The far-UV CD spectra of bovine prothrombin fragment 1 in the presence and absence of phospholipid were nearly identical. However, the far-UV CD spectra of fragment 1 plus 5 mM Ca^{2+} differ in the presence and absence of phospholipid as shown in Figure 4. The greatest difference in the spectra occurs at the minima: 204 nm with a mean residue ellipticity of $-11\,800 \pm 500$ in the absence of phospholipid and 206 nm with an ellipticity of -9300 ± 700 in the presence of lipid. In the region 228–240 nm, however, the spectra are identical within experimental error. Comparisons between the CD spectra of fragment 1 in the presence and absence of phospholipid are made difficult by the turbidity introduced into the protein solution by the phospholipid. In addition Ca^{2+} has been shown to induce fusion in pure phospholipid membranes (Papahadjopoulos, 1976) which can be expected to lead to a further increase in turbidity. Turbidity has been shown to cause a flattening of the absorption spectrum which is maximal at the peak in absorption (Schneider, 1973). The fact that the CD spectra of fragment 1 are nearly identical in the presence and absence of phospholipid in the absence of Ca^{2+} but differ in the presence of Ca^{2+} is likely to be the result of absorption flattening caused by solution turbidity rather than a fragment 1 secondary structural change upon phospholipid binding. Thus, it is not possible to determine unequivocally from our circular dichroism data whether phospholipid binding alters the secondary structure of fragment 1.

The Ca^{2+} titration curve of bovine prothrombin fragment 1 in the presence of phospholipid is sigmoidal with a T_m of 0.20 mM, a value identical with that determined in the absence of phospholipid (Table II). The Hill coefficients are 2.3 in the absence and 2.0 in the presence of phospholipid. These results are in contrast to the fluorescence studies (Prendergast & Mann, 1977; Nelsestuen, 1976) in which the T_m decreases upon phospholipid addition. As discussed earlier, the bovine fragment 1 used in the fluorescence experiments probably contained a measurable amount of "denatured" fragment 1 which may account for the disparity. The presence of phospholipid then does not appear to alter the Ca^{2+} binding properties of fragment 1 as measured by CD.

Ca^{2+}-Induced Human Prothrombin CD Changes. Since prothrombin fragment 1 is used as a model for prothrombin in Ca^{2+} binding studies, it is important to investigate whether the two molecules undergo similar conformational changes in the presence of Ca^{2+}. The far-UV CD spectra of human prothrombin in the presence and absence of 5 mM Ca^{2+} are shown in Figure 5. The calculated apparent α helix, β sheet, and random coil contents of human prothrombin in the presence and absence of 5 mM Ca^{2+} are given in Table I. The spectrum of human prothrombin has a minimum at 203 nm of -8000 ± 300, while in the presence of Ca^{2+} the spectrum has a minimum at 204 nm of -7300 ± 300. The two spectra have broad shoulders between about 215 and 230 nm centered at approximately 220 nm with an ellipticity of -2100 ± 100 in the absence and -2600 ± 100 in the presence of 5 mM Ca^{2+}. This difference in the spectra corresponds to an apparent difference in secondary structure of 1% more "α helix", 2% more "β sheet", and 3% less "random coil" in the presence of 5 mM Ca^{2+} than in its absence. The far-UV spectra of bovine prothrombin in the presence and absence of Ca^{2+} were also recorded and found to be similar to the human prothrombin spectra.

The difference between the spectra and calculated apparent secondary structures of prothrombin in the presence and absence of 5 mM Ca^{2+} is small. In fact, Bjork & Stenflo (1973) reported that there is no difference between the spectra. When looked at in closer detail in the region 225–240 nm, however, a small but real difference is apparent between human prothrombin in the presence and absence of 0.62 mM Ca^{2+}. The

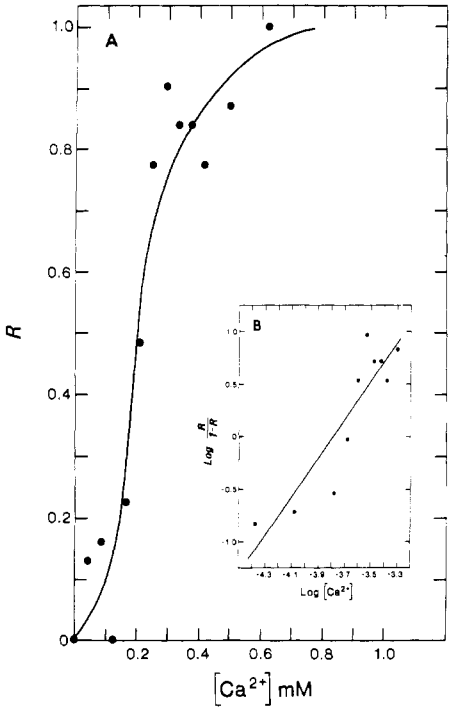

FIGURE 6: (A) Ca^{2+} titration by CD of human prothrombin (0.10 mg/mL) in a 1.000-cm cuvette at 231 nm. R is the mean residue ellipticity at a given Ca^{2+} concentration divided by the value obtained when no further change in the spectrum occurs upon Ca^{2+} addition. (B) Hill plot of the data from A (the straight line drawn is a least-squares fit). The intercept is 0.16 mM and the slope is 1.8.

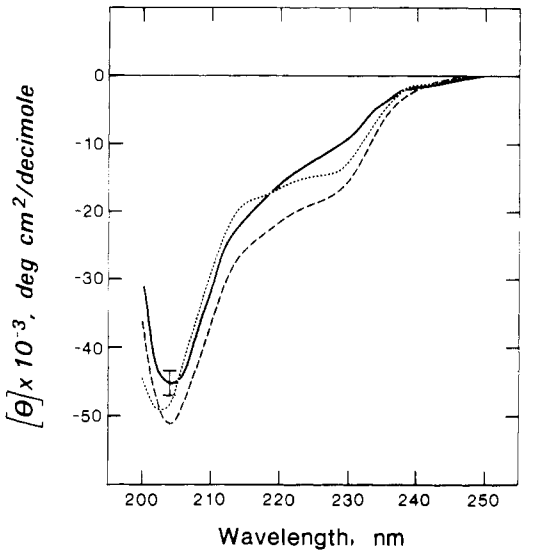

FIGURE 7: CD spectrum of human prothrombin plus 5 mM Ca^{2+} (—). (- - -) Represents the sum of the individual CD spectra of human prethrombin 1 (Bloom & Mann, unpublished experiments) and human prothrombin fragment 1 plus 5 mM Ca^{2+}. (• • •) Represents the sum of the individual CD spectra of human prethrombin 1 and human prothrombin fragment 1. [θ] is the *molar* ellipticity.

mean residue ellipticity at 231 nm in the absence of Ca^{2+} is -1620 ± 50 and -1980 ± 50 in the presence of 0.62 mM Ca^{2+}. The Ca^{2+} titration curve is sigmoidal (Figure 6A) and the resulting Hill plot is shown in Figure 6B (the straight line drawn is a least-squares fit). The average T_m from the titration curve and Hill plot is 0.18 mM and the Hill coefficient is 1.8. Thus prothrombin undergoes a CD transition in the presence of Ca^{2+} similar to the prothrombin fragment 1 transition in that the T_m values are similar: 0.18 mM for human prothrombin and 0.25 mM for human fragment 1.

It has been shown by circular dichroism studies that the prothrombin activation components, fragment 1 and prethrombin 1, exist as "domains" within the prothrombin molecule (Bloom & Mann, unpublished experiments). That is, there are no gross alterations in the secondary structures of the molecules upon activation and purification. The far-UV CD spectrum of prethrombin 1 is unaltered by the addition of 5 mM Ca^{2+}; thus the change in the prothrombin spectrum in the presence of Ca^{2+} must be due solely to the fragment 1 portion of the molecule. To further test this hypothesis, the CD spectra of human prethrombin 1 and fragment 1 plus 5 mM Ca^{2+} were summed in terms of *molar* ellipticity, and compared with the *molar* ellipticity spectrum of prothrombin plus 5 mM Ca^{2+} (Figure 7). The spectrum of the sum of the spectra of prethrombin 1 plus fragment 1 in the absence of Ca^{2+} is included for comparative purposes (Bloom & Mann, unpublished experiments). Although qualitatively similar the spectrum of the sum of the components in the presence of Ca^{2+} and the spectrum of prothrombin in the presence of Ca^{2+} differ in the region 215–235 nm. The differences in the calculated secondary structures, derived from CD spectra, of human fragment 1 and the covalently linked fragment 1 portion of the prothrombin molecule in the presence and absence of 5 mM Ca^{2+} were also determined. As reported earlier, human fragment 1 has an apparent difference in secondary structure of 8% more "α helix", 6% more "β sheet", and 14% less "random coil", while human prothrombin has 1% more "α helix", 2% more "β sheet", and 3% less "random coil" in the presence of 5 mM Ca^{2+} than in its absence. As the prethrombin 1 secondary structure is unaltered by the addition of Ca^{2+}, the change in the structure of fragment 1 in the intact prothrombin molecule can be calculated by multiplying the apparent prothrombin secondary structural difference by the ratio of the molecular weights of prothrombin and fragment 1 (3.3). When calculated in this way, the human fragment 1 portion of the intact prothrombin molecule has an apparent difference in secondary structure of 3% more "α helix", 7% more "β sheet", and 10% less "random coil" in the presence of 5 mM Ca^{2+} than in its absence. Thus human fragment 1 covalently linked in the prothrombin molecule can be contrasted with human fragment 1 as a distinct entity. The apparent secondary structure of human fragment 1 as a distinct entity has 5% more "α helix", 1% less "β sheet", and 4% less "random coil" than the covalently linked fragment 1 portion of the prothrombin molecule in the presence of 5 mM Ca^{2+} than in its absence. Thus it appears that the covalently linked fragment 1 portion of the prothrombin molecule is subject to constraints in the secondary structural change allowed upon Ca^{2+} addition.

Within experimental error, the near-UV CD spectra of human prothrombin in the presence and absence of 5 mM Ca^{2+} are identical. Similar results were obtained with bovine prothrombin. Bjork & Stenflo (1973), however, observed a marked difference in the near-UV spectra of bovine prothrombin in the presence and absence of 5 mM Ca^{2+}. A possible explanation for the difference in the results of the two laboratories is that Bjork & Stenflo's prothrombin preparation was contaminated with fragment 1.

Discussion

The effects of Ca^{2+}, Mg^{2+}, and Mn^{2+} on the secondary structure of prothrombin fragment 1 have been examined by circular dichroism and compared with the results of intrinsic fluorescence experiments. It was shown that fragment 1 undergoes a CD spectral change which is essentially identical,

no matter which ion is present. The metal ions bind cooperatively and Mg^{2+} and Ca^{2+} have nearly identical transition midpoints. The T_m for Mn^{2+} is an order of magnitude less, however, indicating that Mn^{2+} binds with a greater affinity to fragment 1 than Ca^{2+} or Mg^{2+}. These results are quantitatively similar to metal ion binding experiments using fluorescence quenching as a probe of fragment 1 conformational changes (Prendergast & Mann, 1977; Nelsestuen, 1976). Thus, the fluorescence quenching of tryptophyl residues is not solely the result of a local perturbation of the chromophore's environment upon metal ion binding but is primarily a perturbation of the secondary structure.

Fragment 1 has been observed by sedimentation studies (Prendergast & Mann, 1977) to undergo dimerization in the presence of Ca^{2+} at a higher Ca^{2+} concentration than that required for the alteration in secondary structure as observed by fluorescence and CD techniques. Since there is no additional apparent CD spectral change at these high Ca^{2+} concentrations, fragment 1 dimerization is not the result of an additional transition in the secondary structure. It was also found that the presence of phospholipid does not appear to alter the Ca^{2+} binding properties of fragment 1.

Furie et al. (1976) and Bajaj et al. (1976) found that fragment 1 possesses two high affinity Gd^{3+} and Mn^{2+} binding sites, respectively. The high affinity Mn^{2+} sites have a K_d identical within experimental error with the T_m values obtained by CD and fluorescence Mn^{2+} titrations. As Mn^{2+} produces a secondary conformational change in fragment 1 identical with that produced by Mg^{2+} and Ca^{2+}, it is reasonable to hypothesize that the two high affinity Mn^{2+} binding sites also bind Mg^{2+} and Ca^{2+}.

The results of these experiments support the "two-class-of-sites" hypothesis for metal ion binding by fragment 1 (Prendergast & Mann, 1977) and suggest the model shown in Figure 8. This model is consistent with 10 γ-carboxyglutamic acid residues and 5 Ca^{2+} binding sites per prothrombin fragment 1 molecule. Prothrombin fragment 1 is represented as a prolate ellipsoid with an axial ratio of 6/1. This axial ratio was calculated using the known physical properties of fragment 1 (Mann & Elion, 1977) and assuming 0.2 g of bound water/g of protein (Oncley, 1941). The symbols Y in the model represent γ-carboxyglutamic acid residues.

Both metal ion dependent transitions are represented in Figure 8. The first transition occurs on filling of two high affinity sites which are relatively nonselective as to the particular metal ion bound. The filling of these high affinity sites results in a fluorescence change and a configurational change in the molecule as manifested by the circular dichroic spectral changes and has a T_m of about 0.2 mM for calcium ions. A second set of three sites of low affinity are formed at high concentrations of protein as a result of site sharing between two fragment 1 molecules; calcium bridging results in dimer formation. No conformational changes are observed on filling of this second group of sites. These shared sites are either already available on the fragment 1 molecule or are newly formed as a result of the conformational change which occurs on filling of the high affinity sites. T_m for the filling of the low affinity sites is 1 mM of calcium (Pendergast & Mann, 1977). The weighted average of the calcium affinities of these two classes of binding sites is 0.68 mM, a value very close to the K_d value of 0.63 mM for five sites determined by equilibrium dialysis experiments (Bajaj et al., 1975). The equilibrium dialysis experiments were conducted under conditions of high concentration, such that both types of transitions would occur and the K_d observed for the total process of calcium binding by equilibrium dialysis would be the weighted average of all sites. This

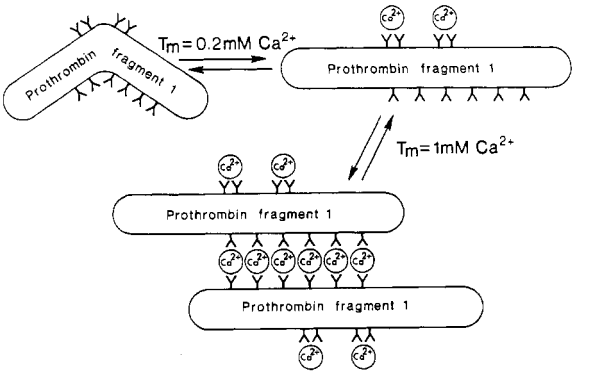

FIGURE 8: Model for prothrombin fragment 1 metal ion binding. Prothrombin fragment 1 is represented as a prolate ellipsoid with an axial ratio of 6/1. The symbols Y represent γ-carboxyglutamic acid residues.

model is thus consistent with all the known properties of the calcium binding sites of the prothrombin fragment 1 molecule.

Prothrombin fragment 1 is the amino-terminal activation product of prothrombin and the assumption is made that fragment 1 is a valid model for prothrombin in metal ion binding studies. Bjork & Stenflo (1973) have reported that there is no difference in the peptide bond region of the CD spectra of prothrombin in the presence and absence of Ca^{2+}. This study, however, was conducted at a calcium ion concentration which would not permit discrimination of the two calcium ion dependent transitions in prothrombin fragment 1, and, further, since the authors did not conduct a titration study of the spectra using multiple points, they were unable to see the small, but real, change in the spectra which suggests a secondary structural change for prothrombin in the presence of metal ions. The observation that this change in the CD spectra for prothrombin parallels the prothrombin fragment 1 transition, but is of smaller magnitude, strengthens, on the one hand, model studies of metal ion binding conducted with prothrombin fragment 1, but at the same time introduces a caveat since the metal ion dependent transition of the secondary structure of prothrombin is only qualitatively similar to prothrombin fragment 1 in that, while it is smaller in magnitude, it occurs over the same range of metal ion concentration.

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Proximity Relationships of Tryptophanyl Residues and Oxygen Binding Site in *Levantina hierosolima* Hemocyanin. A Fluorimetric Study†

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ABSTRACT: The effect of iodide on the fluorescence of *Levantina hierosolima* hemocyanin was studied. About half of the tryptophanyl fluorescence yield of the apoprotein was found to be accessible to quenching by iodide. In contrast, no quenching of the fluorescence of oxyhemocyanin by iodide could be detected. It was concluded that the residual fluorescence of oxyhemocyanin emanates exclusively from buried tryptophans. A comparison of the polarization of the fluorescence of deoxy- and oxyhemocyanin, as well as of apohemocyanin in the absence and presence of iodide, does not provide evidence for significant tryptophan-tryptophan energy transfer. The fluorescence decay of apohemocyanin could be fitted to a biexponential decay function with lifetimes of 4.6 and 1.2 ns, with amplitudes 0.30 and 0.70, respectively. The fluorescence decay of deoxyhemocyanin was virtually identical to that of the apoprotein, showing that the introduction of copper did not affect the emissive properties of the protein. Analysis of the decay curve of oxyhemocyanin showed two lifetimes, 3.8 and 0.71 ns with amplitudes of 0.17 and 0.83, respectively. Comparison of the quantum yields calculated from the decay kinetics with the ones measured directly showed that 0.55 of the tryptophanyl emission in deoxyhemocyanin was totally quenched by the introduction of oxygen. Our findings indicate that the copper-binding site in hemocyanin is located by the solvent-accessible tryptophans, near the exterior of the molecule.

Hemocyanin is a multisubunit protein that functions as an oxygen carrier in molluscs and arthropods (Van Holde and Van Bruggen, 1971). Each oxygen-binding site involves two copper ions and in the molluscs is associated with a unit of 50 000 daltons containing seven tryptophanyl residues (Ghiretti-Magaldi et al., 1966). A previous study of the luminescence properties of hemocyanin from the snail *Levantina hierosolima* showed that oxygenation brings about a marked quenching of the fluorescence of the protein tryptophans (Shaklai and Daniel, 1970). The observed similarity in the optical rotatory properties of oxy- and apohemocyanin (Cohen and Van Holde, 1964) ruled out an explanation of the quenching in terms of a conformational change in the protein as a result of oxygenation. The quenching was attributed to long-range nonradiative energy transfer from the tryptophans acting as donors to the Cu···O complex acting as acceptor (Shaklai and Daniel, 1970).

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