Metal Ion Interactions with Bovine Prothrombin and Prothrombin Fragment 1. Stoichiometry of Binding, Protein Self-Association, and Conformational Change Induced by a Variety of Metal Ions†

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ABSTRACT: The interaction of metal ions with bovine prothrombin fragment 1 was investigated by examining two ion-induced phenomena: a conformational change measured by intrinsic protein fluorescence change or circular dichroic spectral changes and protein self-association. All metal ions tested induced the conformational change. In contrast, self-association varied depending on the metal ion. Magnesium caused essentially no self-association, calcium caused partial (50–75%) dimerization, manganese and cadmium led to a full dimer, and lanthanides caused hexamer formation. Despite these widely differing states of self-association, titration experiments indicated six ions bound per monomer in each case. That is, six magnesium or calcium ions were bound during the conformational change; three manganese ions caused the conformational change, but a total of six manganese ions were required to form a dimer; three lanthanide ions caused the conformational change, but a total of six ions were required to form the hexamer. On the basis of these results and previous data it is postulated that prothrombin fragment 1 contains six metal ion binding sites regardless of its state of aggregation. Two or three ions induce the conformational change, and these sites generally are filled with higher affinity than the sites causing self-association. However, the sequence in which the sites are filled depends on the metal ion, thereby causing overlap or separation of conformational change and self-association. The parent molecule, prothrombin, showed very little self-association in the presence of calcium or manganese. Lanthanides induced formation of a prothrombin trimer. The major difference between prothrombin and its fragment 1 segment is the failure of the former to dimerize. In this one respect it is concluded that fragment 1 is not an ideal model system for studying cation interaction with vitamin K dependent plasma proteins. Hexamers of prothrombin fragment 1 may be formed by dimerization of lanthanide-induced trimers.

The vitamin K dependent plasma proteins constitute a class of calcium-binding proteins which are dependent on γ-carboxyglutamic acid residues. These proteins contain several multivalent cation binding sites. Estimates for prothrombin range from 6 to 15 sites (Bensen & Hanahan, 1974; Stenflo & Ganrot, 1973; Bajaj et al., 1975, 1976; Nelsestuen et al., 1975; Henriksen & Jackson, 1975; Brittain et al., 1976). Ion binding induces at least two changes in the protein. A conformational transition which can be monitored by protein fluorescence (Nelsestuen, 1976; Prendergast & Mann, 1977; Marsh et al., 1979a) or circular dichroism spectra (Bloom & Mann, 1978; Marsh et al., 1979a) and a cation-induced self-association of prothrombin fragment 1[1] detected by changes in sedimentation velocity (Prendergast & Mann, 1977; Jackson et al., 1979).

Reports in the literature vary on the question of whether these two ion-induced phenomena are distinct. Nelsestuen (1976) initially reported that the rate constant for fluorescence change was unaffected by protein concentration and proposed a conformational change. Subsequently, Jackson et al. (1979) proposed that dimerization could account for the cooperativity observed in metal ion binding and protein fluorescence change. Jackson & Brenkle (1980) have presented data suggesting that the number of metal ion binding sites varies with the degree of protein self-association. This evidence appears to depend upon exceedingly precise radioisotope analysis. Some evidence does not concur with these latter proposals. For example, conformationally specific antibodies have detected a transition at protein concentrations where self-association is minimal (Tai et al., 1980). Furthermore, the change in sedimentation velocity observed in the presence of calcium was not characteristic of protein dimer formation. Recent light-scattering studies confirmed that calcium-induced dimerization of prothrombin fragment 1 is incomplete (Pletcher et al., 1980).

There have also been varying reports regarding self-association of the parent molecule, prothrombin. This protein has been reported to self-associate in the absence (Cox & Hanahan, 1970; Jackson et al., 1979) and presence (Jackson & et al., 1979) of calcium. At neutral pH the evidence supporting these conclusions is not strong. At pH 5.6, concentration-dependent molecular weight changes of bovine prothrombin best fit a K_A for dimerization of 0.45×10^2 mL/g (Cox & Hanahan, 1970) which corresponds to 50% dimer at 22 mg of prothrombin/mL. At pH 7.5 no significant concentration-dependent changes in sedimentation coefficient were observed (Cox & Hanahan, 1970). In contrast, subsequent reports (Jackson et al., 1979) show small changes in the sedimentation velocity (from 4.3 to 5 S) at neutral pH between 0 and 2.5 mg of prothrombin/mL. These changes have been attributed to self-association even though they are much less than expected for dimer formation. Observed increases in sedimentation velocity when calcium is added (Jackson et al., 1979) were barely greater than experimental error. Therefore, the calcium-induced self-association properties of prothrombin may be quite different from those of prothrombin fragment 1. Clearly, the number of ion-binding sites and the degree and effect of protein self-association on the number of sites are

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[1] Abbreviations used: QLS, quasielastic light scattering; prothrombin fragment 1 or fragment 1, residues 1–156 of prothrombin; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CD, circular dichroism.

0006-2960/81/0420-0351$01.00/0

important considerations for further investigation into the nature of the calcium-binding sites created by γ-carboxyglutamic acid. This study reexamines the self-association–conformational transition questions.

Some approaches to the question of γ-carboxyglutamic acid function have used model compounds. Small peptides containing γ-carboxyglutamic acid bind metal ions and offer a number of advantages over large proteins [cf. Marsh et al. (1980), Sperling et al. (1978), and Furie et al. (1979)]. The elegant studies of Furie et al. (1979) tentatively identified a metal-binding site in a peptide corresponding to residues 12–44 of prothrombin. This peptide also showed evidence of conformational changes in the presence of metal ions (Furie et al., 1978; Furie & Furie, 1979). These peptides, however, do not display the function of membrane binding (Nelsestuen et al., 1975). It therefore will be necessary to correlate peptide studies with intact protein studies. The smallest protein segment which retains the membrane-binding function is prothrombin fragment 1 (Nelsestuen et al., 1980). This peptide is amenable to analysis by NMR [e.g., Esnouf et al. (1980)] and X-ray crystallography (Aschaffenburg et al., 1977; Kung et al., 1980). Fragment 1, however, is another model system that must be compared to the parent molecule, prothrombin.

One of the most powerful tools available to probe an ion-binding site is substitution of different metal ions. A preliminary study examined the function of a number of ions in causing the prothrombin conformational change (Nelsestuen et al., 1976). Subsequently, a number of metal ion substitutions have been followed by more sophisticated spectral analysis. Examples include manganese ESR (Bajaj et al., 1976), magnesium and calcium NMR (Marsh et al., 1979a; Robertson et al., 1979), fluorescence energy transfer to terbium (Brittain et al., 1976), europium luminescence decay (Marsh et al., 1980), and gadolinium, europium, praseodymium, and lanthanum effects on NMR (Furie et al., 1979). Interpretation of results obtained with these ions is dependent upon knowledge of their overall effects on the protein including conformational change, self-association, and site of metal binding (i.e., do they actually substitute at calcium-binding sites).

The present study was initiated to examine many of these questions. Among other observations, we found that six metal ion sites are present on prothrombin fragment 1 for a variety of metals, that the state of self-association varies with different metal ions from a monomer to hexamer, and that cation-induced dimerization of prothrombin fragment 1 is not observed with prothrombin.

Materials and Methods

Metal ions were purchased commercially and were of the highest available purity. Prothrombin (Ingwall & Scheraga, 1969; Nelsestuen & Suttie, 1973) and prothrombin fragment 1 (Heldebrant & Mann, 1973) preparations are described in the literature. These proteins were quantitated by using $E_{280nm}^{1\%}$ values of 14.4 for prothrombin(Cox & Hanahan, 1970) and 10.1 for fragment 1 (Heldebrant & Mann, 1973).

Intrinsic protein fluorescence was monitored on a Perkin-Elmer 44A fluorescence spectrophotometer at ambient temperature. Excitation was at 280 nm and analysis was at 344 nm. Fluorescence measurements in the presence and absence of metal ion were made in one of two ways. The first involved measurement of the apoprotein followed by addition of metal ion and measurement of fluorescence after a 15-min equilibration time. The other involved measurement of the fluorescence intensity of a metal ion–protein mixture followed by dissociating the complex with excess EDTA to generate the apoprotein which was measured. The maximum fluorescence decrease at saturating metal ion concentration was 53% and was essentially the same for all metal ions used. This provides evidence that the same conformational change is observed for all metal ions. Marsh et al. (1979a) have reached the same conclusion.

Solutions were buffered with 0.05 M Tris–0.1 M NaCl (pH 7.5) for calcium, magnesium, cadmium, and manganese studies. Cacodylate (0.05 M, pH 6.5)–0.1 M NaCl or piperazine (0.05 M, pH 6.5)–0.1 M NaCl were used for the lanthanide ions. These two buffers gave indistinguishable results for lanthanum. The cacodylate buffer was used for the remaining lanthanides. The pH range used has no effect on titration of protein fluorescence (Resnick & Nelsestuen, 1980). Control experiments also showed that manganese caused the same extent of self-association of prothrombin fragment 1 at these pH values. The lanthanide results therefore do not stem from pH effects.

Circular dichroism spectra were obtained with a Jasco-41J CD spectrometer equipped with scanning memory. Four scans were collected for each spectrum, and background (buffer alone) was subtracted when necessary. Protein concentrations were 0.126 mg/mL.

Molecular weights were estimated from light-scattering intensity measurements obtained as described elsewhere (Pletcher et al., 1980). Refractive index increments of 0.19 and 0.18 were used for prothrombin and fragment 1. The small change in this value for fragment 1 in the presence of metal ions (Pletcher et al., 1980) was not included in calculations. Monodisperse, monomeric prothrombin fragment 1 was obtained after chromatography of the purified protein on a 1.1 × 80 cm Sephadex G-100 superfine gel filtration column. Samples were also centrifuged at 10000*g* for 30 min before analysis. Monodisperse, monomeric prothrombin was more difficult to obtain. Chromatography on a column of Sepharose 6B (1.2 × 60 cm) eluted with 0.05 M Tris buffer (pH 7)–0.5 M NaCl was followed by chromatography on a similar column eluted with Tris (pH 7.5)–0.1 M NaCl. Ultraviolet absorption analysis of the column effluent showed a single symmetrical protein peak from both columns. Despite these efforts, the prothrombin still displayed a molecular weight which is 5–10% high. This is thought to be due to remaining dust or incorrect protein concentration estimates due to inaccuracies in the extinction coefficient. Small variations in the molecular weight of different preparations of prothrombin fragment 1 (22 700–24 000, see figure legends) are either within experimental error or result from minor dust contamination.

Quasielastic light scattering (QLS) was used to obtain diffusion constants and an estimate of the polydispersity of the sample. Diffusion constants were measured by published procedures (Bloomfield & Lim, 1978; Pletcher et al., 1980). The normalized second cumulant (quality factor) from the cumulant analysis represents an estimate of the maximum relative polydispersity of the sample. Quality factor is related to the square of the standard deviation of particle size (Koppel, 1972). A monodisperse solution of protein such as bovine serum albumin (Pletcher et al., 1980), gel chromatographed to remove dimers, gives a value of ~0.03. Samples thought to be contaminated by dust or in various stages of self-association gave quality factors as high as 0.3. Since light scattering provides a weight-average molecular weight, the quality factor term is important for distinguishing, for example, a pure dimeric protein solution from a mixture of monomers and trimers.

Results

Equilibrium Studies. Two metal ion induced phenomena

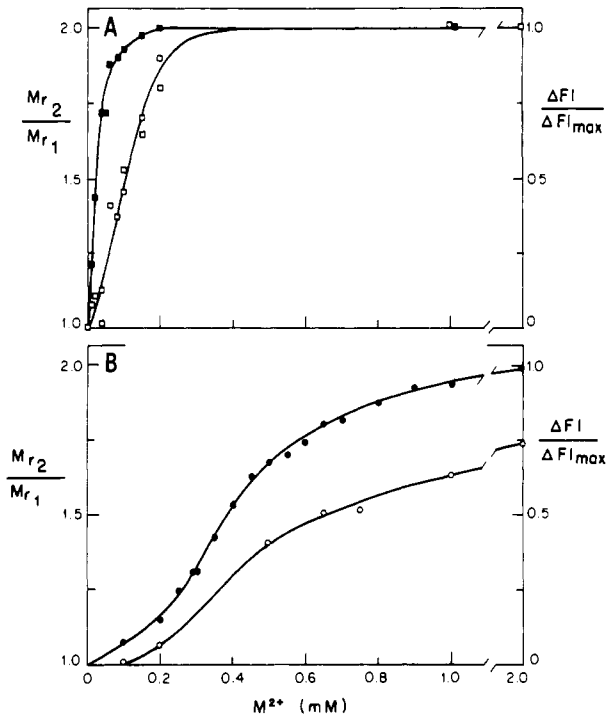

FIGURE 1: Metal ion titration of fragment 1 fluorescence change and self-association. (A) gives the results for manganese while (B) shows the results for calcium. Protein (0.162 mM) was equilibrated with cations of the appropriate concentration by dialysis against a large excess of the metal ion in the appropriate buffer. Fluorescence change (ΔFl, ●, ■) was determined by comparing protein fluorescence intensity in the presence of metal ion with fluorescence after addition of excess EDTA. The maximum fluorescence change at saturating metal ion concentration (ΔFl_{max}) was −53%. The molecular weight (○, □) of the protein in the presence (M_{r2}) and absence (M_{r1}) of metal ion was determined by light-scattering measurements as outlined in the text. M_{r1} was 24 000.

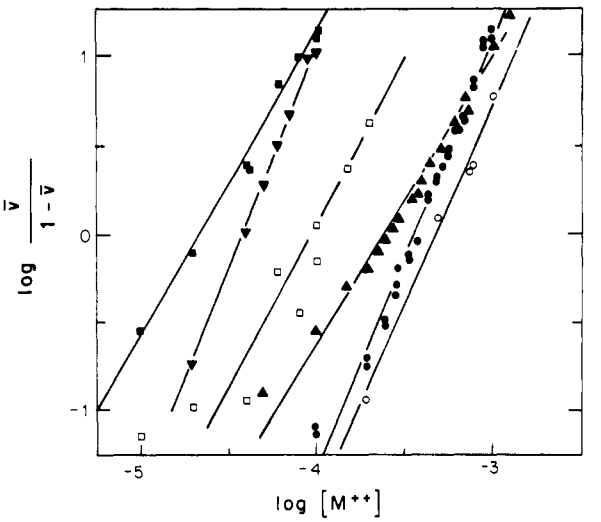

FIGURE 2: Hill plots of metal ion titrations. The data from Figure 1 are plotted along with results for magnesium- and cadmium-induced fluorescence change. $\bar{v}$ is the fraction of the maximum change observed. The metal ions and respective titration midpoints are as follows: manganese-induced fluorescence change (■, 0.022 mM); cadmium-induced fluorescence change (▼, 0.04 mM); magnesium-induced fluorescence change (▲, 0.25 mM); calcium-induced fluorescence change (●, 0.36 mM); manganese-induced self-association (□, 0.096 mM); calcium-induced self-association (○, 0.49 mM).

were routinely measured: quenching of intrinsic protein fluorescence and protein self-association. The former correlates with a conformational change in the protein. Equilibrium studies for both processes are shown in Figure 1 for manganese (Figure 1A) and calcium (Figure 1B). Hill plots of these data are given in Figure 2. The results for manganese most clearly demonstrate that fluorescence change and self-association are separable; fluorescence change required 0.022 mM manganese for half-reaction while dimerization was half-maximal at 0.096 mM manganese. The quality factor measured for fully self-associated fragment 1 (0.04) was indicative of a homogeneous population of molecules. It therefore appears that manganese-induced self-association is an actual dimerization of prothrombin fragment 1. Prendergast & Mann (1977) reported that manganese did not cause self-association while Jackson et al. (1979), in agreement with our findings, reported that it did. We estimate that the manganese to protein ratio originally used (Prendergast & Mann, 1977) was not sufficient to cause the self-association (see below).

Calcium (Figure 1B) differs from manganese in that self-association and fluorescence change occur concomitantly (this is best seen in the Hill plot of the data, Figure 2). Prendergast & Mann (1977) originally reported that calcium-induced self-association and fluorescence change had widely different calcium requirements. Their studies appear to report total rather than free calcium. We estimate that bound calcium in their experiment can account for the difference from our results.

A second difference between calcium and manganese is the failure to obtain a full dimer with calcium (Figure 1B). A more comprehensive recent study also showed that at 15 mg/mL protein the weight-average molecular weight of fragment 1 in the presence of calcium was still less than that of a dimer (Pletcher et al., 1980). These results agree well with changes in sedimentation velocity (Prendergast & Mann, 1977; Jackson et al., 1979) which were less than expected for dimer formation. We found that addition of manganese to the calcium–protein solutions increased the molecular weight to a full dimer (data not shown).

The failure to obtain a full dimer implies that preparations of fragment 1 used by several laboratories consist of two populations of molecules, one of which is not capable of self-association in the presence of calcium. This heterogeneity appears to be important only for in vitro studies using fragment 1 as a model compound. As shown below, dimerization is not displayed by the parent prothrombin molecule. Dimerization is therefore one reason that fragment 1 is not ideal for use as a model for metal ion interactions with γ-carboxyglutamic acid containing plasma proteins.

The Hill plots (Figure 2) of the equilibrium data show that the Hill coefficients vary with different metal ions. Values of 1.5–2.6 are observed. These are in agreement with previous reports (Nelsestuen et al., 1976; Marsh et al., 1979a). The meaning of the Hill coefficient depends on the binding model. The simplest interpretation is that it represents the minimum number of metal ions involved in a given process. We would then conclude that two or more magnesium or manganese ions are involved in the fluorescence change while three or more calcium or cadmium ions are required. Jackson et al. (1979) have proposed that cooperative cation binding to prothrombin or fragment 1 is due at least in part to the self-association phenomenon. This cannot apply to cooperative calcium binding to prothrombin, which shows minimal self-association in the presence of calcium (see below). In the case of fragment 1, no consistent correlation between the Hill coefficient and the degree of self-association was observed (compare Mg, Ca, Mn, and Cd in Figure 2). Ultimate solution of this question for fragment 1 will require extensive further analysis.

CD Spectral Changes. Figure 3 gives CD spectra of prothrombin fragment 1 in the presence of no metals, manganese, or calcium. The question raised is whether CD spectral

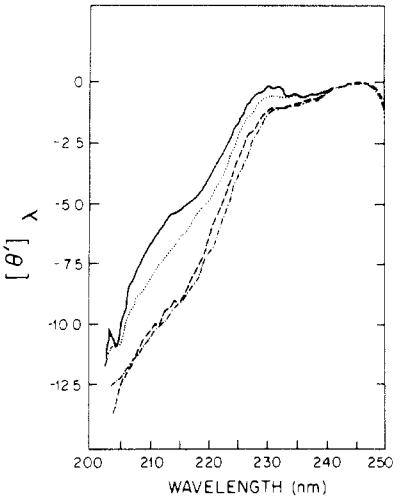

FIGURE 3: Circular dichroism spectra as a function of metal ion additions. Fragment 1 (0.126 mg/mL) was scanned from 250 to 205 nm, and the mean residue ellipticity ($[\theta']_\lambda$) in units of deg·cm^2/dmol was calculated. Spectra are shown in the presence of 2 mM EDTA (—), 0.01 mM manganese (···), 0.06 mM manganese (-·-), and 2 mM calcium (---).

changes correlate with the fluorescence change, self-association, or both of these processes. Bloom & Mann (1978) and Marsh et al. (1979a) showed close overlap of the calcium titration of fluorescence and CD spectral changes. Their results, however, are complicated by the close overlap of calcium-induced fluorescence change and self-association. The results in Figure 3, however, substantiate their conclusions. At 0.05 mM manganese where self-association is minimal but fluorescence change is complete, the CD spectral changes are complete. Manganese causes the same changes as calcium. The identity of fluorescence and CD changes is also supported by the report that magnesium, which causes very little self-association, induces an identical CD spectral change (Marsh et al., 1979a). In addition, the CD spectra are taken at protein concentrations where self-association is apparently minimal. The manganese results (Figure 3) therefore substantiate a number of other studies.

Stoichiometry of Manganese-Induced Changes in Fragment 1. Figure 4 shows results of fluorescence change and self-association as a function of manganese to protein ratio. These experiments were done at protein concentrations higher than the free metal concentrations required to induce the changes. The relatively small amounts of free metal ion present at various levels of change were estimated from the equilibrium studies (Figure 2); the extent of fluorescence change or dimer formation is used to estimate free metal ion concentration. Subtraction of this unbound metal ion gives an estimate of actual bound metal ion. Corrected values are given in solid symbols (Figure 4A,4B).

Simple linear extrapolation of the corrected fluorescence data (Figure 4A) indicates three manganese ions bound at full change. This value is an upper limit; we cannot determine if all of the sites are functional in fluorescence change. It is quite possible that the actual number of ions functioning in fluorescence change is two and that an apparent stoichiometry of three arises from partial filling of other ion sites (Figure 4B).

The slight upward curvature of this plot is anticipated for a noncooperative process of two overlapping phenomena or for a less than fully cooperative process such as

$$P + 2M \overset{1}{\rightleftharpoons} PM + M \overset{2}{\rightleftharpoons} PM_2$$

where fluorescence change is entirely associated with binding

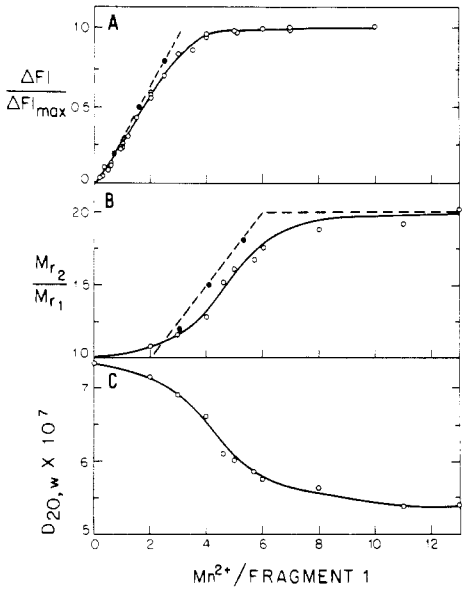

FIGURE 4: Manganese-induced changes in fragment 1. (A) gives the fluorescence change as a function of the manganese to fragment 1 ratio at 0.162 mM protein. The solid symbols are points from the smooth curve which have been corrected for free manganese as described in the text. The free manganese at these stages of fluorescence change was estimated from the equilibrium data (Figure 2). (B) shows the molecular weight change of the protein as a function of the manganese to fragment 1 ratio. M_{r2} is the molecular weight in the presence of metal while M_{r1} is in the absence of metal ion. M_{r1} is 22 800. The solid symbols represent points from the smooth curve which have been corrected for the free manganese (from Figure 2) present at this extent of self-association. (C) gives the diffusion constants.

the second ion (M). We were unable to fit the data to any combination of six ions bound by noncooperative processes. Attempts to fit the data to various cooperative metal-binding models are in progress.

As anticipated from the equilibrium studies (Figure 1A), fragment 1 dimerization occurs subsequent to the fluorescence change (Figure 4B). Linear extrapolation of the corrected data (dashed line, Figure 4B) indicates that six manganese ions are bound at full dimer formation.

Changes in the diffusion constant correlate closely with dimerization (Figure 4C) (e.g., compare the extent of fluorescence change, self-association, and diffusion coefficient change at 2 Mn^{2+}/fragment 1, Figure 4). Association of two spherical monomers ($D = 7.3 \times 10^{-7}$ cm^2/s) would be expected to give a dimer with $D = 5.5 \times 10^{-7}$ cm^2/s. These observations indicate that the conformational change measured by protein fluorescence does not cause substantial molecular shape changes.

Calcium and Magnesium. These metals show considerable similarity. For example, stoichiometric titrations of the fluorescence change (Figure 5A) are superimposable. Corrections for free metal ion are not significantly different for these metals, so that the number of metal ions bound at any stage of fluorescence change is similar for calcium and magnesium. Linear extrapolation of the corrected values indicates about six metal ions bound at complete fluorescence change (Figure 5A). It is not known if all six ions function in the fluorescence change. This stoichiometry could arise from similar binding affinities for different groups of metal ion sites.

This indirect documentation of six metal ion binding sites is important for purposes of comparison. It indicates a minimum of six sites on prothrombin fragment 1 regardless of the state of self-association; a full dimer formed with manganese (Figure 4B), a partial dimer formed with calcium (Figure 5B),

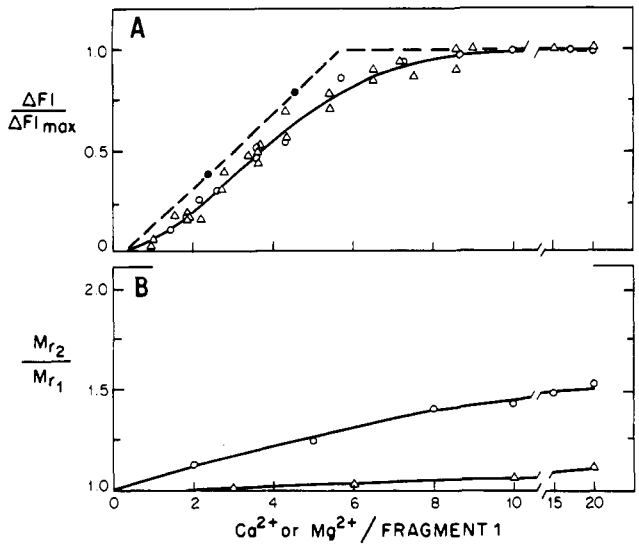

FIGURE 5: Calcium- and magnesium-induced changes in fragment 1. (A) shows the fluorescence change as a function of the metal ion to fragment 1 (0.78 mM) ratio. The data for the two metals are indistinguishable. The solid symbols represent points from the smooth curve which have been corrected for free metal ion as described for Figure 4A. (B) shows the molecular weight change as a function of the metal to fragment 1 (0.29 mM) ratio. M_{r2} is the molecular weight in the presence of metal while M_{r1} is in the absence of metal ion. M_{r1} is 24000. No correction for free metal ion was calculated. Data for calcium (O) and magnesium (Δ) are shown.

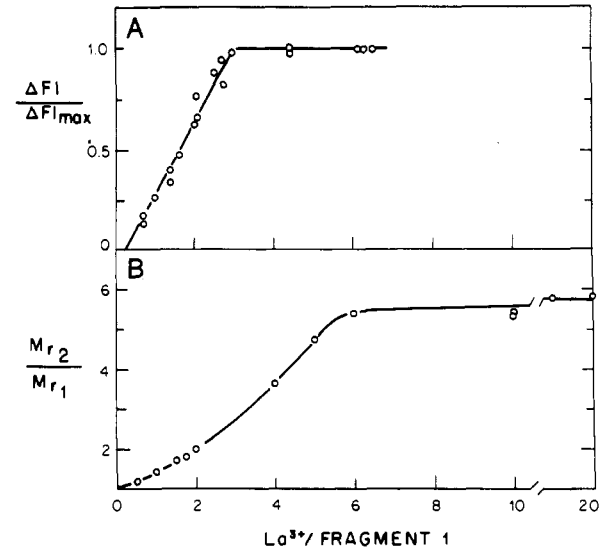

FIGURE 6: Lanthanum-induced changes in fragment 1. Fluorescence change (A) and molecular weight (B) as a function of the lanthanum(III) chloride to fragment 1 (0.165 mM) ratio are shown. Molecular weight is expressed as a ratio of M_{r2} (in the presence of metal ion) to M_{r1} (in the absence of metal ion). M_{r1} is 22700.

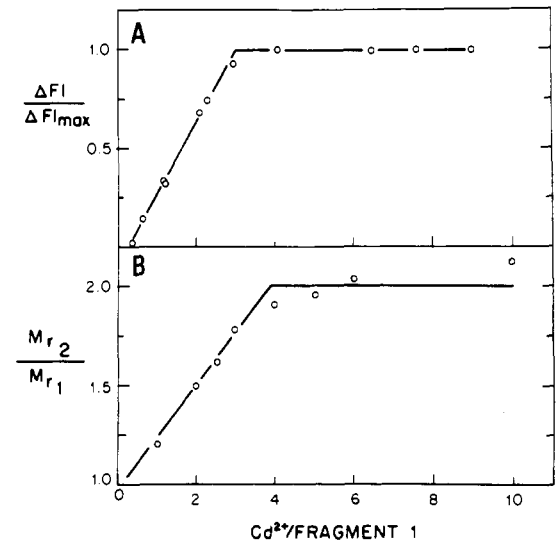

FIGURE 7: Cadmium-induced changes in fragment 1. Fluorescence change (A) and molecular weight (B) as a function of the cadmium chloride to fragment 1 (0.24 mM) ratio are shown. M_{r2} is the molecular weight in the presence of metal while M_{r1} (22700) is in the absence of metal.

or very little dimer formed with magnesium (Figure 5B). While this approach cannot fix the total number of sites, a stoichiometry of about six total sites was found in several direct binding studies (Bajaj et al., 1975, 1976; Prendergast & Mann, 1977; Brittain et al., 1976). Our own calcium-binding measurements also indicated a total of six binding sites (Nelsestuen et al., 1975; unpublished data). The observation of six sites regardless of the state of protein self-association argues strongly against the proposal that some metal ion binding sites are created at the protein–protein interface (Jackson et al., 1979; Jackson & Brenckle, 1980; Jackson, 1980).

Magnesium induces very little self-association (Figure 5B). We do not know if the small change observed represents a self-association with very low affinity or if it arises as an artifact of adding solutions containing high metal ion concentrations. The extent of self-association caused by magnesium is therefore interpreted as a maximum value.

Lanthanide-Induced Changes in Prothrombin Fragment 1. Lanthanides are important tools in probing calcium-binding sites. It is therefore important to understand the effects of these metal ions on the protein. Figure 6 shows stoichiometric titrations of fluorescence change and self-association induced by lanthanum. The binding of gadolinium to fragment 1 was found to be very tight (Furie et al., 1976). Correction for free lanthanide ion should therefore be negligible in the experiments described. As seen in figure 6A, the fluorescence change was complete when three lanthanide ions were bound per protein molecule. This is very similar to the manganese results (Figure 4A). A major difference is observed in the extent of protein self-association where the weight-average molecular weight suggests a hexamer (Figure 6B). The quality factor obtained from QLS analysis of this sample (0.02) was indicative of a monodisperse scattering species. We therefore conclude that lanthanum causes true hexamer formation.

Other lanthanide ions were also studied including europium(III), gadolinium(III), praseodymium(III), and terbium(III). Although not as complete as the lanthanum results, the data were superimposable on the plots in Figure 6. All of the lanthanides bind to prothrombin fragment 1, cause a conformational change, and induce hexamer formation.

An interesting observation is that about six lanthanum ions are required per protein molecule for complete hexamer formation (Figure 6B). These studies therefore document six metal ion binding sites in monomeric fragment 1 as observed with magnesium, six metal ion binding sites in dimeric fragment 1 as observed with manganese, and six metal ion binding sites in hexameric fragment 1 as observed with the lanthanides. A total of six sites per protein molecule agrees with studies on terbium–fragment 1 interaction (Brittain et al., 1976). It therefore appears likely that six sites are present on the protein monomer and that no new sites are generated during self-association.

Cadmium-Induced Changes in Prothrombin Fragment 1. Cadmium was included in this study because two isotopes of cadmium (^{111}Cd and ^{113}Cd) have nuclear magnetic moments and are of potential use as probes for calcium-binding sites [e.g., Chlebowski et al. (1977)]. Stoichiometric titration of

Table I: Cation-Induced Protein Associations

protein	concn (mg/mL)	total metal	$\bar{M}_r$	M_{r2}/M_{r1}[a]	$D_{20,w} \times 10^7$ (cm^2/s)	Stokes radius (Å)
fragment 1	6.7	0	22 700	1.0	7.34	29
	6.0	4 mM Mg	25 800	1.13	7.20	29.5
	6.3	4 mM Ca	34 900	1.54	6.19	34.5
	6.78	2.5 mM Mn	46 700	2.06	5.39	49.6
	3.7	2 mM La	138 000	6.06	3.86	111
prothrombin	2.00	0	80 600	1.0	4.86	44
	4.01	0	78 000	1.0	4.81	44
	2.00	4 mM Ca	83 700	1.04	4.80	44.5
	4.01	4 mM Ca	83 400	1.07	4.73	45.1
	1.14	0.9 mM Mn	90 900	1.14	4.65	46

[a] M_{r2}/M_{r1} is the ratio of molecular weight in the presence of the indicated metal ion (M_{r2}) to the molecular weight in the absence of metal ion (M_{r1}).

the cadmium-induced fluorescence change and self-association are shown in Figure 7. Equilibrium studies (Figure 2) indicate that corrections for free cadmium would be similar to those for manganese (Figure 4). These small corrections are not shown. As seen in Figure 7A, the fluorescence change requires about three cadmium ions. This is similar to manganese and the lanthanides. Self-association (Figure 7B) attains the level of a dimer. The quality factor from QLS analysis (0.05) was indicative of only slight polydispersity. It therefore appears that cadmium functions in a manner similar to manganese in causing complete fluorescence change with three metal ions and full dimerization of prothrombin fragment 1.

Cadmium differs from manganese in two respects. First of all, separation of self-association and fluorescence change was not observed with cadmium; the concentration of cadmium which induces self-association "overlaps" the concentration of metal ion which causes fluorescence change (Figure 7). Secondly, only four cadmium ions are required for full dimerization of fragment 1. This result suggests that all six metal ions are not required for self-association. Therefore, cadmium may be a valuable probe for identifying metal ion sites which actually function in the self-association.

Self-Association of Prothrombin. For the parent molecule, prothrombin, it is still unclear whether the protein self-associates in either the presence or absence of metal ions. Cox & Hanahan (1970) reported self-association in the absence of calcium at pH 5.6; 50% dimer required a protein concentration of 22 mg/mL. Yet, a pH 7.5 the sedimentation velocity coefficient showed no concentration dependence, which suggests no protein self-association. Subsequently, Jackson et al. (1979) reported a small increase in sedimentation coefficient (from 4.3 to ~5 S) between 0 and 2.5 mg of prothrombin/mL and interpreted this as evidence for self-association. While this observed change appears greater than experimental error, it is much less than anticipated for protein dimerization.

Our results, which measure molecular weight (Table I), show no concentration-dependent molecular weight changes. The observed molecular weight of prothrombin is 5–10% higher than that calculated for monomeric prothrombin (72 500; Magnusson et al., 1975). This error could arise from dust contamination or from incorrect estimates of protein concentration. A systematic error in protein concentration estimates could arise from inaccurate extinction coefficients. Some variation in the $E_{280nm}^{1\%}$ values has been reported including 14.4 (Cox & Hanahan, 1970), 15.5 (Jackson et al., 1979), and 16.5 (Ingwall & Scheraga, 1969). At the present time we cannot distinguish between these two sources of error but feel that a few large protein aggregates or dust particles were the source of difficulty. Further efforts to resolve this minor discrepancy are in progress.

The important observation is that no concentration-dependent change in molecular weight of prothrombin is observed (Table I). Therefore, prothrombin does not self-associate at neutral pH and concentrations up to several milligrams per milliliter. The nonaggregation of bovine prothrombin is also consistent with a number of previous reports which obtained a monomeric molecular weight for this protein based on its physical properties (Lamy & Waugh, 1953; Tishkoff et al., 1968; Ingwall & Scheraga, 1969; Harmison et al., 1961).

As shown here and previously (Prendergast & Mann, 1977; Jackson et al., 1979), prothrombin fragment 1 displays calcium-induced self-association. It has been assumed that this is also relevant to prothrombin. Jackson et al. (1979) reported that prothrombin showed increased sedimentation velocity in the presence of calcium. The changes however were approximately equal to experimental error and much less than anticipated for dimerization. The molecular weight studies shown in Table I indicate that calcium caused 10% or less dimerization of prothrombin. The small change observed could arise from handling of the protein. Occasionally, the addition of small volumes of concentrated calcium solutions produced prothrombin aggregation. This was avoided by mixing dilute solutions. Other experiments (not shown) examined prothrombin concentrations of 0.5–10 mg/mL. The weight-average molecular weight of 95 000 and the quality factor (0.25) indicated greater dust contamination. Nevertheless, no change in molecular weight was observed over this concentration range and calcium had no detected effect on the observed molecular weight. This data also indicated that protein dimerization was not significant.

The results with manganese also show little self-association of prothrombin (Table I) at metal concentrations sufficient to cause fragment 1 dimerization (cf. Figure 1) and molar protein concentrations which allow extensive fragment 1 dimerization (Jackson et al., 1979). Therefore, a clear distinction between manganese-induced prothrombin and prothrombin fragment 1 dimerization is apparent. While higher manganese concentrations appeared to cause more extensive prothrombin aggregation, we did not determine if this represented a dimerization equilibrium or aggregation due to handling procedures.

We conclude that prothrombin is quite different from its isolated fragment 1 region with respect to calcium- or manganese-induced self-association. In this respect, fragment 1 is not a good model for ion-induced phenomena in γ-carboxyglutamic acid containing plasma proteins.

The lanthanides have a very interesting effect on bovine prothrombin (Figure 8). The protein self-associates to the apparent level of a trimer. The quality factor from QLS (0.07) indicated a fairly homogeneous solution of scattering particles

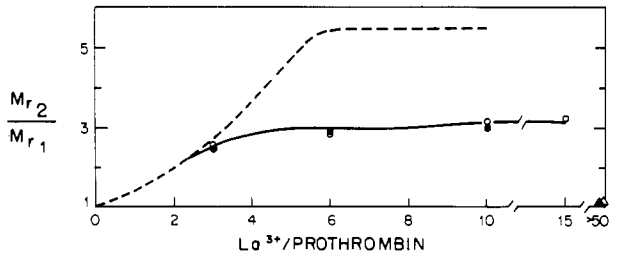

FIGURE 8: Lanthanum(III)-, europium(III)-, calcium(II)-, and manganese(II)-induced self-association of bovine prothrombin. M_{r2} is the molecular weight in the presence of metal ion while M_{r1} is the molecular weight in the absence of metal. M_{r1} is 80 200. Data for calcium (▲), manganese (△), lanthanum (○), and europium (●) are shown. The dashed line shows the results obtained for fragment 1 (from Figure 6B).

so that actual trimerization was indicated. Although limited, the data suggest that self-association follows lanthanum-induced fragment 1 self-association (dashed line, Figure 8) but terminates at a trimer rather than reaching a hexamer. This observation can be explained by two types of self-association: lanthanide-induced trimerization of either prothrombin or prothrombin fragment 1 followed by dimerization of prothrombin fragment 1 trimers; prothrombin lacks the dimerization sites and does not form the hexamer. This explanation is consistent with the observation that fragment 1 will dimerize in the presence of calcium or manganese but prothrombin will not.

On the basis of the values in Table I, a value of 0.66 cm^3/g for specific volume (Pletcher et al., 1980), and an assumed monolayer of hydration (0.41 g of H_2O/g of protein), we calculated the axial ratio of prothrombin and prothrombin fragment 1 to be 7.4 and 6.9, respectively. The axial ratios were not very sensitive to the choice of the degree of hydration between 0.3 and 0.5 g of H_2O/g of protein. A value of 6.9:1 for fragment 1 agrees with previous reports by Bloom & Mann (1979) but differs from Jackson et al. (1979) who reported axial ratios of less than 2:1. Nevertheless, the more prolate elliptical structure is consistent with gel filtration studies. Fragment 1 has an elution position characteristic of a globular protein of ~50 000 molecular weight (data not shown). This behavior is best explained by an elongated ellipsoidal shape. In the case of prothrombin, these calculations are tentative. Since a monodispersed species was not obtained, the diffusion constants reported in Table I represent minimum values and the true diffusion constant is somewhat larger.

Discussion

These studies have indirectly documented six multivalent ion binding sites on prothrombin fragment 1. Six sites are present regardless of the state of protein self-association, from a monomer to a hexamer. Although some reports have indicated more than six total sites (Bensen & Hanahan, 1975; Henriksen & Jackson, 1975), several reports are in agreement with a total stoichiometry of six sites. Bajaj et al. (1975) reported about seven calcium-binding sites on prothrombin fragment 1. Prendergast & Mann (1977) and our own studies (Nelsestuen et al., 1975) also indicated a total of six or seven calcium sites. Bajaj et al. (1976) reported about five manganese-binding sites on prothrombin fragment 1, but their data do not rule out six sites.

Lanthanides have also been studied. Brittain et al. (1976) studied terbium binding to fragment 1 by fluorescence energy transfer and observed a total of six or seven ions bound such that they accepted fluorescence excitation energy from amino acids. Furie et al. (1976) reported about four sites for gadolinium, but their results do not rule out a larger number of sites filled at lower affinity. Marsh et al. (1980) studied europium binding to prothrombin fragment 1 by luminescence decay and observed different groups of bound ions. Two or three ions bound with loss of eight water ligands from the inner coordination sphere of europium, an intermediate number of water ligands were lost from europium up to six bound ions per fragment 1, and, above six ions per fragment 1, the data indicated binding with low coordination to the protein. The latter group may simply constitute adventitious interactions such as may occur with any protein. This suggestion of different classes of binding sites is in agreement with binding of gadolinium (Furie et al., 1976) and manganese (Bajaj et al., 1975). In both of these cases Scatchard plots of the data suggested two or three tight binding sites followed by filling of other sites. Prendergast & Mann (1977) demonstrated that the two or three tight manganese-binding sites correlate well with the manganese-induced fluorescence change.

The results which we present here are in agreement with different groups of metal ion sites and indicate that at least two, but probably three, metal ions are required for a protein conformation change monitored by protein fluorescence. These constitute a subgroup of high-affinity sites for most ions. Filling these sites with lanthanide ions appears to cause protein trimer formation. The second group of metal ion sites which will accept at least three metal ions is generally filled with lower affinity and, for most ions, causes fragment 1 dimerization. Not all metal ions segregate these groups of binding sites; cadmium shows considerable overlap of fluorescence change and dimerization; calcium shows essentially full overlap of these phenomena.

Thus, our results as well as a number of previous studies are consistent with six metal ion binding sites on prothrombin fragment 1. The simplest explanation is that all sites will accept any of the ions, the number of sites is not affected by protein self-association, and the sequence in which the sites are filled varies from one cation to another.

This model does not evoke ion-specific sites. However there is some evidence suggesting that ion-specific sites exist on prothrombin fragment 1. Utilizing ^{25}Mg and ^{43}Ca NMR, Marsh et al. (1979a) reported that calcium did not competitively remove all magnesium from fragment 1 and vice versa. These studies were performed at high metal ion concentrations ($\geq$20 mM Mg^{2+} and $\geq$18 mM Ca^{2+}) which are far removed from the other studies discussed here. For this reason it is possible that the NMR experiments are not entirely relevant to the current studies. Bajaj et al. (1976) reported that calcium did not entirely displace manganese from prothrombin. On the basis of their data, it is possible that the ion-specific site is not associated with the fragment 1 region of prothrombin. For fragment 1, we observed six sites for every metal ion. The proposal of ion-specific sites therefore requires, for example, that the number of siies specific for magnesium must equal the number of sites specific for calcium. This seems unlikely, and we prefer the former model. Nevertheless, we cannot totally rule out the existence of ion-specific sites. For example, it is possible that the fragment 1 molecules which do not dimerize with calcium contain sites which no longer accept calcium and are therefore ion specific.

As given above, we find little evidence of bovine prothrombin dimerization in the presence or absence of physiological levels of calcium. Any aggregation we observe is not of an equilibrium dimerization nature. These observations may also relate to human prothrombin. Agarwal et al. (1977) reported extensive self-association corresponding to 25 and 60% dimer at 2 and 5 mg of protein/mL, respectively. In contrast, a

recent study at concentrations of 4.1–57 mg of human prothrombin/mL obtained monomeric molecular weights in the presence and absence of calcium (Osterberg et al., 1980). Although the authors extrapolated their data to zero protein concentration, the results of Agarwal et al. (1977) indicate ~50% dimer at the lowest protein concentration used (Osterberg et al., 1980). Also, the lowest molar concentration of protein used (Osterberg et al., 1980) is sufficient for nearly maximum calcium-induced self-association of bovine prothrombin fragment 1 (Pletcher et al., 1980; Jackson et al., 1979). Consequently, reports of self-association of human prothrombin appear inconsistent and do not suggest calcium-induced self-association at all.

A critical question for future studies relates to the use of fragment 1 as a model for intact γ-carboxyglutamic acid containing plasma proteins. This peptide is easily obtained in large amounts and is subject to analysis by NMR (Esnouf et al., 1980) and X-ray crystallography (Aschaffenburg et al., 1977). In many respects, fragment 1 appears to be a good model. For example, both prothrombin and fragment 1 display a slow conformational change upon calcium addition, and both bind to phospholipid (Nelsestuen, 1976). Marsh et al. (1979b) have proposed that the conformational change is related to cis–trans isomerization about proline-22. Prothrombin contains ~10 calcium-binding sites (Stenflo & Ganrot, 1973; Bajaj et al., 1975; Nelsestuen, 1976), and it seems reasonable that the six ion sites on fragment 1 represent sites present on the parent molecule. The major difficulty is that calcium induces self-association unique to fragment 1. Some caution must be exercised in the use of fragment 1 as a model compound.

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