The Effects of Calcium Ions and pH on Bovine Prothrombin Fragment 1

INTRINSIC FLUORESCENCE STUDIES

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The effects of pH and Ca^{2+} on the intrinsic fluorescence of bovine prothrombin fragment 1 were investigated to deduce the nature of protein functional groups involved in Ca^{2+} binding to fragment 1. From pH values of 9 to 3, increasing the H_3O^+ concentration results in quenching of the fluorescence of fragment 1. Reversible pH-titration curves are obtained which appear to consist of two regions. From pH 4 to pH 6.5 a broad titration curve is obtained, whereas from pH 6.5 to 9 a more pronounced titration behaviour is evidenced by a group or groups on fragment 1 with an apparent pK_a of approx. 7.5. In contrast, the apparent association constant for Ca^{2+} and fragment 1 shows a sharp pH-dependence in the region between pH 7 and 8 with tighter Ca^{2+} binding at higher pH values. A pK_a of approx. 7.5 can be estimated for the group or groups on fragment 1 linked to the tight binding of Ca^{2+} . Both H_3O^+ and Ca^{2+} result in blue-shifts in the wavelengths of fragment-1 emission. These results are interpreted in terms of H^+ - and Ca^{2+} -induced changes in the conformation of fragment 1 as a result of surface-charge neutralization.

The presence of Ca^{2+} and phospholipid, along with Factor Va, are necessary for the optimal rate of conversion of prothrombin into thrombin by Factor Xa in the blood-coagulation process (Papahadjopoulos & Hanahan, 1964; Cole *et al.*, 1965; Gitel *et al.*, 1973; Henriksen & Jackson, 1975; Nelsestuen, 1976). At neutral pH, the formation of a functional prothrombin-membrane complex depends on the presence of γ -carboxyglutamic acid residues near the prothrombin *N*-terminus, and to some extent on intact protein tertiary structure. Within the first 156 amino acid residues from the *N*-terminus of prothrombin, known as the fragment-1 region, are all of the γ -carboxyglutamate residues present in prothrombin. It is appropriate and convenient to study fragment 1 as an analogue of prothrombin, since many properties of the two molecules, including their Ca^{2+} and phospholipid interactions, are very similar. Considerations of the role of Ca^{2+} in mediating the interaction of prothrombin with phospholipids are complex owing to potential

effects of these ions on both the lipid and the protein. It is clear, however, from the work of Esmon *et al.* (1975) that Ca^{2+} -mediated phase changes in the phospholipid phase are not sufficient in themselves to induce dicoumarol prothrombin (lacking a normal complement of γ -carboxyglutamate residues) to bind to phospholipid.

Nelsestuen (1976), Nelsestuen *et al.* (1976) and Prendergast & Mann (1977) have observed a relationship, at neutral pH, between Ca^{2+} -induced fluorescence quenching in prothrombin fragment 1 and the ability of fragment 1 to associate with phospholipid vesicles. Perhaps related to the lipid-binding processes discussed above and co-operativity noted for Ca^{2+} binding to fragment 1 (Henriksen & Jackson, 1975; Bajaj *et al.*, 1975) is the observed intrinsic and Ca^{2+} -mediated association of fragment-1 molecules (Prendergast & Mann, 1977; Agarwal *et al.*, 1977). Thus, whether it is itself sufficient to initiate phospholipid binding or not, a conformational change in the protein, monitored by fluorescence quenching in fragment 1, has been implicated in Ca^{2+} -mediated phospholipid binding in this system.

The studies reported here were undertaken to deduce the nature of the functional groups in fragment 1 involved in the protein's response to

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; fragment 1, *N*-terminal portion of prothrombin released upon treatment of prothrombin with thrombin; Gla, γ -carboxyglutamic acid; SDS, sodium dodecyl sulphate.

added Ca^{2+} . A significant dependence of fragment-1 fluorescence on pH is observed, involving the ionization of at least two groups, having apparent pK values of about 7.4 and 5.0. Also, a significant dependence of Ca^{2+} binding on pH is seen, reflecting the ionization of a group with an apparent pK of about 7.4.

Experimental

All chemicals used were reagent grade or better. EDTA (disodium salt), sodium citrate and KCl were Fisher Certified grade, Fisher Scientific Co., Fair Lawn, NJ 07410, U.S.A.; Tris was Ultrol grade from Calbiochem, La Jolla, CA 92037, U.S.A.; Mes was from Sigma Chemical Co., St. Louis, MO 63178, U.S.A.; CaCl_2 was ultrapure grade from Alfa Products, Danvers, MA 01923, U.S.A.; L-tryptophan was from Nutritional Biochemical Corp., Cleveland, OH 44128, U.S.A.

Protein preparation

Bovine prothrombin fragment 1 was prepared by the procedure of Mann (1976). The fragment 1 obtained exhibited a single Coomassie Blue-staining band on SDS/polyacrylamide-gel electrophoresis. From the mobility of this band relative to standards, a mol.wt. of 23 500 is obtained. Further, the fragment 1 yields satisfactory amino acid analyses. After purification the fragment 1 was stored for periods of as long as several months in 50% glycerol at -20°C . The pH values of solutions were measured with an Orion 601A digital pH-meter, using an Orion semi-micro combination pH electrode. The meter was calibrated at two pH values with Fisher Certified standard solutions.

Fragment 1 was prepared for fluorescence experiments by an initial dialysis against 0.02M-EDTA in either 5mM-Tris or 5mM-Mes at pH values of 7.5 or 6.0 respectively in 0.1M-KCl at 4°C . The EDTA was subsequently removed by exhaustive dialysis of the protein against the appropriate buffer without EDTA. The A_{290} after dialysis was recorded on a Unicam SP.1800 spectrophotometer utilizing a 1 cm path length and dialysis buffer in the reference cuvette. Appropriately diluted fragment-1 solutions were stored at 4°C and were never frozen. After dilution, fragment-1 samples were used or discarded within 1 week.

Fluorescence experiments

Uncorrected fluorescence spectra were obtained with an Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a strip-chart recorder for recording spectra. The cell compartment temperature was controlled at 24.5°C with a circulating water bath. An excitation wavelength of 290nm was used in all experiments reported here. A trypto-

phan standard was used to monitor and correct for instrumental instability.

pH titrations

The pH titrations were carried out by two different methods. The first method (direct addition) involved addition of microlitre amounts of 1.0M- or 0.1M-HCl or -KOH directly to rapidly stirred fragment-1 samples. The pH titrations were carried out in the presence and absence of added CaCl_2 . From the data presented below, it appears that this method of titration causes irreversible changes in the structure of fragment 1 and/or aggregation due to locally high concentrations of protons or hydroxides. Rapid stirring was employed during and after addition of acid or base to minimize such locally high or low pH values. The effects of varying the stirring rate were not pursued, since the irreversible behaviour could be eliminated by performing the titrations by an alternative method which involved dilution of fragment 1, buffered at neutral pH, into more concentrated buffers set at the desired final pH values.

The pH titrations of fragment 1 involving dilution into buffer solutions were organized as follows. The pH of the fragment-1 solution was adjusted by dilution of a more concentrated fragment-1 solution ($A_{290} = 0.5\text{--}0.9$) with an appropriate amount of a buffer of the desired pH, such that the final A_{290} was 0.05. This will be referred to as the dilution method of pH titration. The following buffers were used to cover the full pH range: 0.01M-sodium citrate/0.1M-KCl, for pH 3.0–6.0; 0.01M-Mes/

Table 1. λ_{max} values for pH titrations of fragment 1. Emission-maximum wavelength values (λ_{max}) obtained in pH-fluorescence titrations of bovine fragment 1. The pH was adjusted by dilution of a concentrated fragment-1 solution with buffers of the desired pH. The λ_{max} values are seen to depend on the particular buffer system used. An excitation wavelength of 290nm was used.

pH	λ_{max} (nm)	Buffer system
7.47	342	0.01 M-Tris/0.1 M-KCl
7.36	340	0.01 M-Tris/0.1 M-KCl
7.00	339	0.01 M-Tris/0.1 M-KCl
6.50	340	0.01 M-Citrate/0.1 M-KCl
6.00	338	0.01 M-Citrate/0.1 M-KCl
5.50	337	0.01 M-Citrate/0.1 M-KCl
5.04	337	0.01 M-Citrate/0.1 M-KCl
4.51	335	0.01 M-Citrate/0.1 M-KCl
4.00	334	0.01 M-Citrate/0.1 M-KCl
3.52	337	0.01 M-Citrate/0.1 M-KCl
3.00	339	0.01 M-Citrate/0.1 M-KCl
9.04	341	0.01 M-Mes/0.01 M-Tris/0.1 M-KCl
7.94	340	0.01 M-Mes/0.01 M-Tris/0.1 M-KCl
6.97	335	0.01 M-Mes/0.01 M-Tris/0.1 M-KCl
6.02	334	0.01 M-Mes/0.01 M-Tris/0.1 M-KCl

0.01-M-Tris/0.1-M-KCl, for pH 6.0–9.0. A separate fragment-1 sample was used for each pH value. Reversibility of the fluorescence changes on reverse titration were checked by the following procedure. Small amounts of concentrated fragment-1 solutions were diluted halfway, i.e. A_{290} was still larger than 0.05, each with a buffer of a different pH. After equilibrating for several minutes at a given pH, these samples were further diluted to a final A_{290} of 0.05, with 0.05-M-Tris/0.1-M-KCl, pH 7.5. The final pH value was between 7.2 and 7.5, although each sample had been subjected to a lower pH between 3.0 and 7.5.

Calcium titrations

Calcium titrations of fragment 1 were carried out on samples whose pH values were adjusted by both methods. In either case microlitre amounts of 1.0-mM- or 0.10-M- CaCl_2 titrant were added to the fragment-1 sample. After about 10 min equilibration time after each CaCl_2 addition, a fluorescence spectrum of the fragment 1 was recorded. In the direct-addition method the fragment 1, which was buffered only by 5-mM-Tris/0.1-M-KCl, was first rapidly adjusted to the desired pH, between 3.0 and 8.0, by addition of 1.0-M- or 0.1-M-HCl or -KOH. The solutions were held at constant pH during the calcium titration by monitoring the pH before and after CaCl_2 additions and adding, if necessary, small amounts of HCl or KOH sufficient to maintain the desired pH. In the dilution method each fragment-1 sample was well buffered at each particular pH value. Only buffers that would not bind Ca^{2+} appreciably were used. Calcium titrations by the dilution method were carried out between pH values of 5.0 and 9.0. The buffer system used in these latter calcium titrations was 0.01-M-Mes/0.01-M-Tris/0.1-M-KCl.

Data analysis

Graphs of normalized (I/I_0) fluorescence intensity versus pH were constructed for the pH titrations. Data from calcium titrations were plotted by the method of Stern–Volmer (1919), i.e. $(I_0/I) - 1$ versus total Ca^{2+} concentration, where I and I_0 are fluorescence intensity in the presence and absence of quencher respectively. In these plots the ordinate corresponds essentially to degree of quenching, which is inversely proportional to fluorescence intensity. Increasing degree of quenching will therefore appear as increasing values along the ordinate. Emission maximum wavelengths (λ_{max}) were obtained for all fluorescence spectra by manually subtracting from each the emission spectrum obtained from a buffer blank. The Raman emission from the aqueous solvent occurred close enough in wavelength to the fragment-1 emission to distort slightly

the shape of the fluorescence peak and prevent in many cases, a direct reading of the λ_{max} . The error in reading the λ_{max} values is estimated to be 1–2 nm.

Results

pH titrations

Changes in bovine fragment-1 fluorescence, in the presence and absence of added Ca^{2+} , as a result of direct addition of microlitre amounts of 1.0-M- or 0.1-M-HCl or 1.0-M-KOH (back titrations), are illustrated in Fig. 1. Titrations were carried out from pH 7.4 downward to 3.0 and finally back to pH 7.4 (Fig. 1a). The direction of titration is indicated by the arrow heads in the Figure. Titration from a pH value of 7.4 to 6.0 required 10 μl of 1.0-M-HCl, whereas titration from pH 6.0 to 4.5 required only 5–7 μl of 0.1-M-HCl. Titration from pH 4.0 to 3.0 required about 10 μl of 1.0-M-HCl. Fluorescence quenching is pronounced in the region between pH 7 and 8 and between pH values of 5.0 and 3.0. However, striking hysteresis is observed on back-titration of the sample to the initial pH. Even after 24 h at the final pH value only 14% of the original fluorescence intensity is recovered in the absence of Ca^{2+} . The same titration-curve morphology was obtained in the presence of added Ca^{2+} (5 or 20 mM); however, the magnitude of the observed fragment-1 fluorescence decrease was considerably smaller and the recovery of the original fluorescence intensity on back-titration was increased to 25% (see Fig. 1). The intensities of fragment-1 fluorescence in the absence and presence of Ca^{2+} have been normalized to the same value at their respective initial pH values. This was done to facilitate comparison of the relative magnitudes of decrease in intensity for the two titration curves. The actual relative fluorescence-intensity change observed in the presence of Ca^{2+} was 35–40% lower at the initial pH than that of the corresponding curve obtained in the absence of added Ca^{2+} . In the absence of added Ca^{2+} , a fragment-1 solution was titrated from pH 7.4 to 6.5 and back to 7.4, then from 7.4 to 6.1 and back to 7.4, then from 7.4 to 6.0 and back to 7.4, and finally from 7.4 to 2.0 and back to 7.4. In each case, the fragment-1 fluorescence decreased when the pH was lowered and remained unchanged by return to the initial pH value, indicating that the irreversible process is cumulative and is not a function of final solution pH, but rather of the addition of HCl itself.

A second set of pH titrations was carried out by the direct-addition method in the acid-to-base direction (Fig. 1b). These were performed by quickly lowering the pH of the fragment-1 solution to 3.0 with about 15 μl of 1.0-M-HCl, and then titrating from pH 3.0 up to 8.0 by addition of 1.0-M- and 0.1-M-KOH. Thus Fig. 1(b) illustrates the titration

Fig. 1. pH-dependence of the fluorescence-emission intensity of bovine fragment 1

(a) The buffer was 5mM-Tris/0.1M-KCl and the fragment-1 concentration was about 0.05mg/ml. The pH was adjusted by direct addition to the fragment-1 solution of 1.0M- or 0.1M-HCl (acid direction) and 1.0M- or 0.1M-KOH (base direction). Arrow heads indicate direction of titrations. Each curve represents the titration of a single fragment-1 sample. —, No CaCl_2 present; ----, 5mM- CaCl_2 present. An excitation wavelength of 290nm was used. Emission λ_{max} varied with pH (see the text). (a) The curves have been normalized to the same intensity at their respective initial pH values. The actual relative fluorescence intensity of curve ---- at the initial pH was 35–40% lower than that of curve —, due to Ca^{2+} -induced quenching. (b) Fragment 1 at an initial pH of 7.4 was quickly adjusted to pH 3.0 by addition of 10–15 μl of 1.0M-HCl. The curves were normalized to the same intensity at pH 3.0 (see the text for further details).

behaviour of fragment 1 only after the pH has already been adjusted to 3.0. In one set of experiments the pH of fragment 1, in the absence of Ca^{2+} , was quickly taken from 7.4 to 3.0 by the addition of 15 μl of 1.0M-HCl. The fluorescence intensity at pH 3.0 remained unchanged from that at the initial pH of 7.4. In another set of experiments the pH of fragment 1, in the absence of Ca^{2+} , was taken from 7.4 to 2.9–3.0 by addition of 10–15 μl of 1.0M-HCl. The fluorescence intensity at pH 2.9–3.0 was in the order of 18% less than it was at the initial pH 7.14. Several different experiments were carried out in which the pH of fragment 1 in the presence of 5mM- CaCl_2 was taken from 7.14 to 3.0. Results ranging from a 24% to a 35% increase in fluorescence

intensity were obtained when the pH was lowered. The irreproducibility of the results obtained from carrying out what appear to be sets of identical experiments further demonstrates the extreme sensitivity of fragment 1 to pH adjustments in this manner. pH titrations from low to high pH values show an apparent pH-dependence of the fluorescence intensity opposite to that previously described. The fluorescence intensity decreased steeply from pH 3.0 to 6.0, levelled off from 6.0 to 7.0, and then increased somewhat from 7.0 to 8.0. Upon reverse titration with HCl the fluorescence intensity continued to decrease in a manner very similar to the previous base-to-acid titration, although the magnitude of the decrease was much diminished. The fragment-1 fluorescence intensity at the initial pH of 3.0 in the presence of 5mM- CaCl_2 has been normalized to the same value as that of fragment 1 in the absence of Ca^{2+} , pH 3.0, to aid in comparison of the two. In the presence of 5mM- CaCl_2 the fluorescence intensity decreased more steeply than in the absence of added Ca^{2+} from pH 3.0 up to pH 5.5, then levelled off from pH 5.5 to 7.0 and increased slightly from pH 7.0 to 8.0.

Because of the disturbing irreversibility of the direct-addition titration curves reported above, pH titrations of fragment 1 were also carried out in a manner avoiding possible local extremes of pH as described in the Experimental section.

The pH titrations carried out in this manner indicate that there is still a pronounced pH-dependence of the fluorescence intensity of fragment 1 (see Fig. 2). However, in this case the fluorescence intensity of fragment 1 exposed to the various pH values was fully reversible when the protein, incubated at each pH, was taken back to pH 7.4. Overlapping pH values were used in separate experiments involving different buffers in order to cover a wide variety of pH values and allow normalization of the results. Control experiments indicate that direct quenching of fragment-1 fluorescence by buffer molecules was not occurring.

The pronounced reversible pH-dependence of the fluorescence emission of fragment 1 (Fig. 2) appears to consist of two distinct regions. From pH 4 to pH 6.5 a broad titration curve is obtained which is typical of the titrations of multiple carboxy groups with differing pK_a values. From pH 6 to 9 a more pronounced titration behaviour is evidenced by a group or groups with an apparent pK_a of approx. 7.5. Thus from pH 9 to 3 increasing the H_3O^+ concentration results in quenching of the fluorescence of fragment 1.

A pH-dependence of the λ_{max} of the fragment-1 emission is also observed. The λ_{max} value for any given pH varied, depending on the buffer system used (see Table 1). The general trend for any one experiment, however, is that the λ_{max} blue-shifts as the pH

Fig. 2. pH-dependence of fluorescence intensity of bovine fragment 1

The buffers were 0.1 M-KCl and either 0.01 M-citrate (pH 3.0–6.5) or 0.01 M-Mes/0.01 M-Tris (pH 6.5–9.0). The fragment-1 concentration was about 0.05 mg/ml. The pH was adjusted by dilution of a concentrated fragment-1 solution in dilute Tris buffer, pH 7.4, with a concentrated (0.01 M) buffer at the indicated pH values. A separate fragment-1 sample was used for each pH value in the titration. The titration curve was reversible back to the original pH 7.4. Excitation wavelength was 290 nm and emission $\lambda_{\max}$ varied with pH (see the text).

is lowered in the region from pH 9.0 down to 4.0. From pH 4.0 to 3.0 the $\lambda_{\max}$ red-shifts back toward the values at pH 6.0 to 7.0.

The fact that the pH-induced fluorescence changes were reversible when the pH was adjusted by the dilution method indicate strongly that the results from the direct-addition pH titrations were, in large part, due to the method of pH adjustment.

Calcium titrations

In addition to H_3O^+ ions, Ca^{2+} ions themselves have a pronounced effect on fragment-1 fluorescence. Furthermore the effects of H^+ and Ca^{2+} on fragment-1 fluorescence are interrelated.

Calcium-fluorescence titrations of fragment-1 solutions whose pH had been adjusted by the dilution method were carried out over the CaCl_2 concentration range from 0 to 90 mM. The Stern-Volmer curves for these calcium titrations form a family of curves whose initial slopes increase with increasing pH, except for pH 5.0, whose initial slope was somewhat higher than expected (see Fig. 3). The data shown in Fig. 3 are plotted up to a CaCl_2 concentration beyond which no further decrease in fluorescence intensity was seen. An apparent association constant for Ca^{2+} binding to fragment 1 may be

obtained from the Ca^{2+} -dependent fluorescence quenching, assuming that K_a equals $1/[\text{Ca}^{2+}]$, at half-maximal quenching (Prendergast & Mann, 1977). A plot of apparent K_a versus pH illustrates the pH-dependence of Ca^{2+} quenching and apparent Ca^{2+} binding to fragment 1 (Fig. 4). The apparent association constant for Ca^{2+} and fragment 1 shows a sharp pH-dependence in the region between pH 7 and 8, with tighter Ca^{2+} binding at higher pH values. Thus Ca^{2+} binding to fragment 1 shows a marked dependence on the ionization state of a group or groups on the protein with an apparent $\text{p}K_a$ of approx. 7.5. The broadly titrating groups between pH 7 and 3, observed in the pH titration of fragment 1 (Fig. 2), are not evident in the Ca^{2+} -binding curve. The emission $\lambda_{\max}$ values were measured from the fluorescence spectra at each Ca^{2+} concentration in the calcium titrations. In general, the $\lambda_{\max}$ blue-shifts on addition of CaCl_2 . For low CaCl_2 concentrations the shift in $\lambda_{\max}$, for a given CaCl_2 concentration, was larger as the pH of the fragment-1 solution was increased. The wavelength shifts at the different fragment-1 pH values were typically as follows, from zero to high CaCl_2 concentration: pH 7.93, 339–334 nm; pH 7.41, 338–334 nm; pH 6.89, 336–334 nm; pH 5.92, 336–334 nm; pH 4.98, 336–334 nm. By a concentration of 4 mM- CaCl_2 the $\lambda_{\max}$ had levelled off at 334 nm for all pH values tested. Also, in the pH titrations, 334 nm was the lowest

Fig. 3. Stern-Volmer plots for Ca^{2+} -induced quenching of bovine fragment 1 as a function of pH

The buffer used was 0.10 M-KCl/0.01 M-Mes/0.01 M-Tris and the fragment-1 concentration was about 0.05 mg/ml. The pH was adjusted by dilution of a concentrated fragment-1 solution in dilute Tris buffer, pH 7.4, with buffer solution of the desired pH. I_0 and I represent intensity of fluorescence in the absence and presence of quencher respectively, and $(I_0/I) - 1$ represents degree of quenching. Excitation wavelength was 290 nm and emission $\lambda_{\max}$ varied with pH and CaCl_2 concentration (see the text).

Fig. 4. Apparent K_a for Ca^{2+} binding to bovine fragment 1 as a function of pH

The buffer was 0.01 M-Mes/0.01 M-Tris/0.1 M-KCl and the fragment-1 concentration was about 0.05 mg/ml. The pH of fragment 1 was adjusted by dilution of a concentrated fragment-1 solution with buffer of the desired pH. K_a was calculated from calcium-quenching data for fragment 1 by the method of Prendergast & Mann (1977), namely $K_a = 1/[\text{Ca}^{2+}]$, at half-maximal quenching.

emission wavelength measured for any pH. Again, in the calcium titrations, the absolute value of λ_{max} seemed to depend on the particular buffer system used, but the change in λ_{max} for a given change in CaCl_2 concentration, or pH, was consistent throughout the experiments. Control experiments established that direct quenching of free tryptophan fluorescence by Ca^{2+} at neutral pH does not occur.

Discussion

A number of important characteristics of the interaction of bovine prothrombin fragment 1 with H_3O^+ and Ca^{2+} are indicated by the results presented here. First, due care must be exercised in carrying out pH titrations of bovine prothrombin fragment 1 in order to avoid potentially misleading results. Second, fragment-1 fluorescence is reversibly quenched upon protonation of groups on the fragment-1 molecule titrating between pH values of 9 and 3. Pronounced fluorescence quenching occurs concomitantly with titration of a group with a $\text{p}K_a$ of approx. 7.5. Below pH 7, fluorescence quenching appears to be the result of the overlapping titration of a number of ionizing groups with the result of a

broad titration profile extending down to pH 3. Third, Ca^{2+} binding to fragment 1, determined via Ca^{2+} -induced fluorescence quenching, depends on the ionization of a group on the protein with an apparent $\text{p}K_a$ of 7.3 or higher. The possible relationships between the H^+ and Ca^{2+} effects on fragment-1 fluorescence at neutral pH will be considered below.

The interpretation of titration curves involving the observation of tryptophan fluorescence in proteins and peptides containing multiple emitters requires caution. Conformational changes leading to quenching processes may be highly localized and relatively insensitive to conformational processes elsewhere in the macromolecule. Furthermore, such conformation changes can conceivably affect the apparent $\text{p}K_a$ values of involved groups. Fluorescence changes induced by Ca^{2+} in fragment 1, at neutral pH values, have already been correlated with phospholipid binding by this molecule (Nelsestuen, 1976). Thus the potentially highly localized reports obtained through observation of tryptophan emission may allow the discrimination of specific effects of Ca^{2+} from less specific effects not directly related to phospholipid binding. This is important because of the complex Ca^{2+} -binding properties of fragment 1 as determined from equilibrium-dialysis measurements (Bajaj *et al.*, 1975; Henriksen & Jackson, 1975; Benson & Hanahan, 1975) and fluorescence-quenching measurements (Nelsestuen, 1976). Studies carried out by Nelsestuen (1976) indicate that at neutral pH and using protein concentrations comparable with those used in this study, the slope of a Hill (1910) plot at the half-saturating ligand concentration (for Ca^{2+} , 0.5 mM) is 2.6. It has been demonstrated that a Hill coefficient for positively co-operative binding processes is unlikely to exceed the largest number of ligand-binding sites in the system (Dahlquist, 1978). Thus the observed positive co-operativity frequently observed in equilibrium-dialysis studies is also evident in fluorescence-quenching studies. This serves to establish the relevance of the results obtained here to physiologically important structural transitions induced by the presence of Ca^{2+} .

Two different experimental methods for carrying out pH titrations on isolated bovine prothrombin fragment 1 were used in these studies. We conclude that adjustment of the pH of fragment-1-containing solutions by direct addition of acid or base to stirred protein solutions leads, in some unknown manner, to irreversible changes (over the time scale of these experiments) in the protein or its aggregate state, as reflected by its fluorescence. The remainder of this discussion concerns only titration experiments carried out in a manner yielding reproducible and reversible fluorescence changes, i.e. pH adjustment by dilution of the protein into buffers of the desired pH.

The curves obtained for the pH-dependent quenching of fragment-1 fluorescence and for the blue-shift in the fluorescence-emission wavelength parallel one another. Both reveal a broad titration curve between pH values of 3 and 7. Above pH 7, however, the emission wavelength appears to level off at approximately 341 nm, whereas pronounced titration behaviour persists through this region, as monitored by fluorescence-emission intensity. The most straightforward interpretation of the observed emission λ_{max} changes would involve removal of the emitting tryptophan residue(s) from stabilizing interactions with the solvent lattice as pH is lowered (i.e. there is a greater difference in energy between the tryptophan Frank-Condon excited state and the ground state at pH 4 than at pH 8). On the other hand, since tryptophan-41 is located on a presumably relatively exposed region of the fragment-1 molecule and the remaining two tryptophan residues occupy positions adjacent to a disulphide bond in a region that may not be freely accessible to solvent, the observed emission-maximum shifts may be due to changing contributions of these three tryptophan residues to the total emission as one or more are quenched in these experiments. Whatever the detailed nature of the process involved in generating the observed emission maximum shifts, it is clear that they report the effects of pH and Ca^{2+} . Studies carried out in this laboratory on the pH- and Ca^{2+} -dependence of tryptophan oxidation (M. E. Scott, J. L. Gerth & J. Swofford, unpublished observations) are consistent with the hypothesis of changing exposure of tryptophan as a function of these parameters.

Since Ca^{2+} ions are not directly quenching tryptophan fluorescence in this system, we interpret the non-linear Stern-Volmer plots obtained for Ca^{2+} quenching of fragment-1 fluorescence (Fig. 3) in terms of Ca^{2+} binding itself rather than in terms of differing accessibility of tryptophan residues to Ca^{2+} . Thus it appears likely that a conformational change is induced in the fragment-1 molecule in response to Ca^{2+} binding. This conclusion is consistent with the results of Nelsestuen (1976), Prendergast & Mann (1977) and Gabriel *et al.* (1975). It is necessary to hypothesize further that this Ca^{2+} -induced conformational change has as an immediate consequence the approximation of an intrinsic quencher with the emitting tryptophan.

Assuming then that the observed fluorescence quenching is an indirect reflection of Ca^{2+} binding to fragment 1, it is convenient to investigate the pH-dependence of the Ca^{2+} -fragment-1 average association constant. On varying sample pH from 6 to 8 the apparent association constant changes from approx. 500 to 9500M^{-1} .

The addition of Ca^{2+} to fragment-1 solutions leads to blue-shifts in the fragment-1 emission maximum of approximately the same magnitude as

those induced by variation of pH, in the absence of Ca^{2+} , from pH 7 to 4.

The effects of H_3O^+ and Ca^{2+} concentration on the fluorescence emission of fragment 1 appear to be related, but not simply. First, a direct relationship appears to exist between the ionization of a group(s) in fragment 1 with an apparent pK_a of at least 7.5 and Ca^{2+} binding. Below pH 7 it is apparent that Ca^{2+} ions affect the quenching of fragment-1 fluorescence in a concentration-dependent manner. Similarly, below pH 7 and in the absence of Ca^{2+} , broad titration behaviour of fragment 1 is noted (Fig. 2). Thus, although effects of Ca^{2+} and H^+ parallel each other in all cases, as reflected in fluorescence quenching, two processes appear to be involved. The first is apparent above pH 7 and involves tight Ca^{2+} -fragment-1 interactions and a relatively sharp fragment-1 fluorescence pH-dependence. On the other hand, the second process involves less tightly bound Ca^{2+} and more general titration behaviour of fragment 1, perhaps reflecting titration of surface carboxylic acid groups in general and relatively non-specific Ca^{2+} -anion interactions. It is striking that the tight Ca^{2+} binding to fragment 1 involves groups in fragment 1 with apparent pK_a values as high as 7.5 in view of the observed pK_a values for glutamate- and γ -carboxyglutamate-containing peptides in solution (Marki *et al.*, 1977). Of course the data presented here do not allow unambiguous identification of the involved residues or their number. Local environmental effects are known to have profound effects on the pK_a values of carboxylic acid groups in proteins.

The parallelism between the effects of H^+ and Ca^{2+} , supported by the observation by Gabriel *et al.* (1975), suggests that the ionizing groups observed in the pH titrations may be the same groups that are involved in Ca^{2+} binding, and further that charge neutralization in some portion of the fragment-1 molecule may be an event initiating conformational changes related to observed fluorescence quenching and ultimately to the ability of fragment 1 to engage in phospholipid binding. However, the high specificity of the prothrombin-phospholipid system for Ca^{2+} indicates a further role of this ion in mediating phospholipid binding itself.

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