Spectral Perturbations of *Crotalus adamanteus* Phospholipase A_2 Induced by Divalent Cation Binding†

Michael A. Wells

ABSTRACT: The binding of certain cations to *Crotalus adamanteus* phospholipase A_2 is accompanied by spectral perturbations of tryptophans in the protein. Alkaline earth cations such as Ca^{2+}, Ba^{2+}, and Sr^{2+} cause spectral perturbations which are interpreted as arising primarily from the removal of a charged group from the vicinity of the tryptophan. The spectral perturbations are pH dependent, and the pK of the group controlling the perturbation is estimated to be 7.6 in the presence of Ca^{2+}. There is also a pH-dependent spectral perturbation seen in the absence of cations, which has identical characteristics to that seen in the presence of cations, except the pK of the group is 8.9 in the absence of cations. Zn^{2+} and Cd^{2+} also give rise to spectral perturbations; however, the character of the perturbation is different than seen with alkaline earth cations. The interaction with Zn^{2+} and Cd^{2+} is interpreted as causing a conformational change in the binding site, which alters the exposure of the tryptophan to the solvent. No spectral effects are observed in the presence of Li^+, Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Co^{2+}. Other ions such as Al^{3+}, Cu^{2+}, Fe^{3+}, Ce^{3+}, Nd^{3+}, and Gd^{3+} precipitate the protein. When the protein is reacted with 2-hydroxy-5-nitrobenzyl bromide, two tryptophans are derivatized. This modified protein retains complete enzymatic activity, and also exhibits spectral perturbations in the presence of the alkaline earth cations, and Zn^{2+} and Cd^{2+}. The binding of the alkaline earth cations give spectral perturbations which are primarily charge related. The pK of the *p*-nitrophenol group is 8.15 in the presence of cations and 8.40 in the absence. Zn^{2+} and Cd^{2+} cause spectral changes which indicate a conformational change in the protein. Using equilibrium dialysis or spectral perturbations it was determined that there are two cation binding sites per mole of enzyme. The alkaline earth cations and Cd^{2+} have dissociation constants of approximately 5×10^{-3} M, whereas Zn^{2+} has a dissociation constant of 1×10^{-6} M. There are no detectable spectral perturbations caused by the substrate for the enzyme, phosphatidylcholine. The data are discussed in light of the possible role of Ca^{2+} in activating the enzyme, and the inhibitory effects of Ba^{2+} and Zn^{2+}.

It has been recognized for several years that phospholipase A_2 (EC 3.1.1.4) is activated by calcium. Long and Penny (1957) apparently first reported the requirement for calcium and noted an inhibitory effect of zinc. They suggested that the activating effect of calcium was a direct effect on the enzyme, and not a means of removing potentially inhibitory products. Roholt and Schlamowitz (1961) also suggested that the effect of calcium was directly on the enzyme, and from inhibition studies with barium, concluded that a metal ion–substrate complex was not involved in the reaction. Similar conclusions have been reached by many subsequent workers. Most recently de Haas *et al.* (1971) and Wells (1972) have demonstrated an absolutely specific requirement for calcium using the highly purified enzymes from porcine pancreas and *Crotalus adamanteus* venom, respectively. On the basis of kinetic studies de Haas *et al.* (1971) concluded that the addition of calcium and substrate to the porcine pancreatic enzyme was random, whereas Wells (1972) studying the *C. adamanteus* enzyme, concluded that the addition was ordered with calcium adding first and then the substrate. Possible origins of the difference in these results has been discussed previously (Wells, 1972).

Although kinetic studies have provided unequivocal evidence for the essential role of calcium, there is no information concerning the mechanism whereby calcium activates the enzyme, or the mechanism of inhibition by barium and zinc. Taking advantage of the spectral properties of *C. adamanteus* enzymes (Wells, 1971b), information has been gathered on some of the properties of the metal ion–protein interaction. These data and their possible interpretation are reported in this paper.

Materials and Methods

The preparation of the α and β forms of *C. adamanteus* phospholipase A_2, lecithins, enzyme assays, and other methods have been previously reported (Wells and Hanahan, 1969; Wells, 1971a,b, 1972). 2-Bromoacetamido-4-nitrophenol was from Eastman Kodak (Rochester, N. Y.), 2-hydroxy-5-nitrobenzyl bromide was from Calbiochem (La Jolla, Calif.), and ^{45}Ca from New England Nuclear (Boston, Mass.). All other chemicals were reagent grade and used without further treatment.

The 2-hydroxy-5-nitrobenzyl (HNB)[1] derivative of phospholipase A_2 was prepared essentially as described by Koshland *et al.* (1964), except that the reaction was carried out at pH 5.0 in a pH-Stat (Radiometer, Copenhagen), and the pH was maintained with 0.1 N NaOH. After the consumption of base had ceased (about 30 min), the excess reagents were removed by dialysis against distilled water at 4°. The extent of modification was calculated from the absorbance of the derivative at 410 nm in 0.1 N NaOH, using an extinction coefficient of 18,000 M^{-1} cm^{-1} (Koshland *et al.*, 1964). The reaction with 2-bromoacetamido-4-nitrophenol (BANP) (Burr

† From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724. *Received October 27, 1972.* Supported by grants from the National Science Foundation (GB-35527) and U. S. Public Health Service (HE NB 1552-05).

[1] Abbreviations used are: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Mes, 2-(*N*-morpholino)ethane sulfonic acid; HNB, 2-hydroxy-5-nitrobenzyl-; BANP, 2-bromoacetamido-4-nitrophenyl-.

and Koshland, 1964) was carried out in 0.1 M acetate buffer (pH 3.0) using a 5-fold excess of reagent (calculated from the methinione content of the protein). The reaction was allowed to proceed for 3 weeks at room temperature. The reagents were removed by extensive dialysis at 4°, first against 0.1 M acetate buffer (pH 3.0), and then against distilled water. The extent of modification was estimated as indicated above for the HNB derivative. The BANP derivative was used immediately after preparation to ensure minimal degradation (Burr and Koshland, 1964).

Direct and difference spectra were recorded on a Cary Model 15 with appropriate precautions for baseline corrections and linearity of response. Reproducibility of absorbance changes in difference spectra was ±0.002. Buffers were prepared using a Radiometer pH meter with a scale expander. The effect on the pH of buffers caused by the addition of various cations was checked using a pH meter 4 (Radiometer) and found to be less than 0.02 pH unit. All buffers contained 0.05 M KCl to eliminate possible effects of changes in ionic strength by addition of cations. Equilibrium dialysis was carried out at 25° using 10^{-4} M enzyme in 0.01 M Tris (pH 8.0) containing 0.15 M KCl, and various amounts of $CaCl_2$. ^{45}Ca (50 μCi) was added per 100 ml of buffer solution. The amount of calcium present in the solutions at equilibrium was determined by measurement of radioactivity using a scintillation counter. Sufficient counts were accumulated such that the expected counting error was ±0.2%. The data were treated in the usual manner (Scatchard, 1949). Binding constants were also determined from spectral perturbations by the method of Halfman and Nishida (1972). Ultracentrifugation was carried out as previously described (Wells, 1971a).

Results[2]

The perturbations of the ultraviolet spectrum of phospholipase A_2 caused by Ca^{2+} and Zn^{2+} at pH 8.0 are shown in Figure 1. The figure is presented such that the cell containing the protein and the divalent cation is in the reference position, and cell containing the protein without cation is in the sample position. In the presence of both cations there was a decrease in absorbance with peaks near 292 and 286 nm, and an increase in absorbance with a broad peak near 260 nm. There were, however, differences in the spectral perturbations caused by the two ions. In the case of calcium the decrease in absorbance had a broad shoulder near 305 nm, the ratio of the molar absorptivity change at 286 nm to that at 292 nm was 0.33, and there was a pronounced increase in absorbance in the 250–275-nm region. In the presence of zinc the shoulder near 305 nm was not as pronounced, the ratio of changes of molar absorptivities was 0.72, and the increase of absorbance in the 250–275-nm region was smaller.

Barium and strontium caused spectral perturbations which were identical to those caused by calcium. Cadmium caused perturbations identical with those found in the presence of zinc. The molar absorptivity changes caused by these ions at pH 8.0 are summarized in Table I. At pH 8.0 and at a concentration of 5×10^{-3} M the following ions caused no detectable spectral perturbations: Li^+, Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}. Other ions such as Al^{3+}, Cu^{2+}, Fe^{3+}, Ce^{3+}, Nd^{3+}, and Gd^{3+} precipitated the protein. In addition to calcium which is known to be required for activity, the other ions which caused spectral perturbations are known to be competitive inhibitors of the enzyme (Wells, 1972).

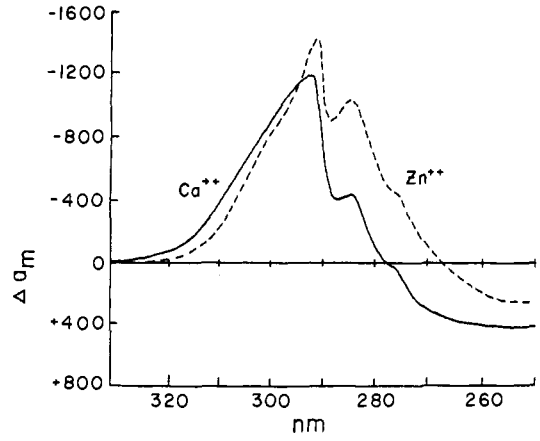

FIGURE 1: Spectral perturbations of phospholipase A_2 induced by divalent cations. Molar absorptivity changes (Δa_m) as a function of wavelength caused by 5×10^{-5} M Zn^{2+} or 1×10^{-3} M Ca^{2+} in 0.01 M Tris–0.05 M KCl (pH 8.0). The cell in the reference position contained the added cation. A change in absorbance of 0.035 = Δa_m of 1000.

The pH dependence of the spectral perturbations caused by calcium was determined by measurements made at pH 6.0 and 7.0 (0.01 M Tes–0.05 M KCl), 8.0 and 9.0 (0.01 M Tris–0.05 M KCl). At higher pH values the ionization of tyrosines with concomitant increases in absorbance in the 280-nm region complicated the interpretation of the spectra. The molar absorptivity changes determined from these studies are presented in Table II. There was a clear pH dependence of the spectral perturbations, which showed a maximal value at pH 8.0. An increase in the magnitude of the zinc-induced spectral changes were also noted, however, measurements could only be made up to pH 8.0 due to precipitation of $Zn(OH)_2$ at pH 9.0.

The decreased effect of calcium at pH 9.0 compared to pH 8.0 suggested that the protein might be undergoing a pH-dependent spectral change in the absence of calcium. In order to investigate this possibility the pH dependence of the spectral perturbations was determined in a different manner. A cell containing the protein in 0.01 M Tes–0.05 M KCl (pH 6.0) was compared to a cell containing the same concentration of protein in buffers of different pH: pH 7.0 (Tes), pH 8.0 and

TABLE I: Molar Absorptivity Changes (Δa_m) Induced by Divalent Cations at pH 8.0.[a]

	Ca^{2+}	Sr^{2+}	Ba^{2+}	Zn^{2+}	Cd^{2+}
Δa_m at 292 nm	−1200	−1100	−1300	−1450	−1000
Δa_m at 286 nm	−400	−400	−450	−1050	−700
Δa_m at 286 nm/ Δa_m at 292 nm	0.33	0.36	0.35	0.72	0.70

[a] Protein samples were in 0.01 M Tris–0.05 M KCl (pH 8.0). The concentration of Ca^{2+}, Sr^{2+}, Ba^{2+}, and Cd^{2+} was 1×10^{-3} M. The concentration of Zn^{2+} was 5×10^{-5} M. A change in absorbance of 0.035 corresponds to a $\Delta a_m = 1000$.

[2] As has been noted in other studies on these proteins (Wells, 1971a,b, 1972), no difference was detected between the α and β forms of *C. adamanteus* phospholipase A_2 in these experiments. No distinction will be made as to which results reported were obtained with which form of the enzyme.

TABLE II: pH Dependence of Molar Absorptivity Changes (Δa_m) Caused by Calcium.[a]

pH	Δa_m at 292 nm	Δa_m at 286 nm	Δa_m at 286 nm/ Δa_m at 292 nm
6.0	−50[b]		
7.0	−500	−150	0.30
8.0	−1200	−400	0.33
9.0	−900	−360	0.39

[a] Tes buffers were used at pH 6.0 and 7.0, Tris buffers were used at pH 8.0 and 9.0. All buffers contained 0.05 M KCl. Perturbations were caused by 1×10^{-3} M $CaCl_2$. [b] Discernible peak, but too small to measure accurately.

9.0 Tris (all buffers were 0.01 M and contained 0.05 M KCl). In one series of experiments 1×10^{-3} M $CaCl_2$ was present in all solutions including the pH 6.0 sample, and in the other experiments no calcium was present. The observed molar absorptivity changes are presented in Table III.

In the presence or absence of calcium there was a pH-dependent spectral perturbation, although the perturbation occurred at a lower pH in the presence of calcium. In all these experiments the shapes of the spectra were identical. The ratio of the molar absorptivity change at 286 nm to that at 292 nm was approximately the same in all cases. This was taken to indicate that cause of the spectral perturbation is the same whether calcium is present or not. The spectral perturbations caused by pH alone were clearly different than those caused by zinc or by dimer dissociation (Wells, 1971b).

Without ascribing the phenomenon to any specific group, it is clear that the pH dependence of the spectral perturbations must involve the titration of some group in the protein, $RH^+ \rightarrow R + H^+$. In the usual notation where

$$pH = pK + \log \frac{\alpha}{1 - \alpha} \quad (1)$$

α refers to the fraction of the group in the form R and $1 - \alpha$ to the fraction in the RH^+ form. The magnitude of the spectral perturbation is proportional to α. If it were possible to carry out experiments at high enough pH, the maximal value of the molar absorptivity change could be determined. It is then a simple manner to calculate α at intermediate pH values and determine pK. However, as indicated above, the ionization of tyrosines precludes making measurements at pH's greater than 9.0. It is, however, possible to obtain an estimate of pK in the following manner. There are two unknowns in eq 1, namely, α and pK. However it is known that $\Delta a_m \cdot \alpha = \Delta A_{292}$, where Δa_m is the molar absorptivity change when $\alpha = 1$. If measurements at two different pH values are combined, then the following information is available. The ratio of α at one pH to α at another pH is given by

$$\frac{\alpha_1}{\alpha_2} = \frac{\Delta A_{(292)_1}}{\Delta A_{(292)_2}} \quad (2)$$

Simultaneous solution of eq 1 for two different pH's gives

$$pH_1 - pH_2 = \log \frac{(\alpha_1)}{(\alpha_2)} + \log \frac{(1 - \alpha_2)}{(1 - \alpha_1)} \quad (3)$$

Using eq 2 and 3, α_1 and α_2 can be determined and hence the value of the pK can be calculated from eq 1. Applying this method to the data in Table III, the value of the pK of RH^+ in the presence of Ca^{2+} can be estimated to be 7.6 and in the absence of Ca^{2+} to be 8.9. The method of calculating these pK's does not imply that Δa_m need be the same in the presence and absence of calcium. It should be noted that Wells (1972) has reported evidence from kinetic studies that a group with a pK = 7.6 in the enzyme–substrate complex is important in catalysis. This analysis must be considered tentative since possible electrostatic interactions have been ignored. A more detailed investigation of these pH effects is in progress.

Wells (1971a,b) has presented evidence that the phoshopholipase A_2 from *C. adamanteus* venom are dimers. Treatments which lead to disaggregation, such as low pH or the presence of 4 M urea, caused spectral perturbations. There is a decreased absorbance in the monomer when compared to the dimer, and the spectra were identical with that induced by zinc (Figure 1). It was therefore important to determine whether cation binding caused dissociation of the dimer. The molecular weight of the enzyme was determined from low-speed sedimentation equilibrium measurements made at a protein concentration of 0.1 mg/ml in 0.01 M Tris–0.05 M KCl (pH 8.0) containing 1×10^{-3} M $CaCl_2$, or 5×10^{-5} M $ZnCl_2$, or no added divalent cations. In all three cases the molecular weight was 30,000 ± 1000, indicating that the proteins were still dimers in the presence of the added cations. Further support for the necessity of the dimeric form of the enzyme to detect divalent cation induced spectral changes was derived from the fact that there were no detectable perturbations caused by either Zn^{2+} or Ca^{2+} when the experiments were carried out at pH 8.0 in the presence of 4 M urea. It is assumed that urea itself does not complex these cations.

Spectral Perturbations of Protein Derivatives. In order to gain further insight into the origin of the spectral perturbations caused by divalent cation binding, two derivatives of the protein were prepared which introduced the *p*-nitrophenol group into the protein. The first of these was prepared by the reaction of 2-hydroxy-5-nitrobenzyl bromide with tryptophan in the protein (Koshland *et al.*, 1964). The reaction was carried out at pH 5.0 to avoid possible complications due to formation

TABLE III: Effect of pH on Molar Absorptivity Changes (Δa_m) in the Presence and Absence of $CaCl_2$.[a]

	pH 7.0	pH 8.0	pH 9.0
1. With 1×10^{-3} M $CaCl_2$			
Δa_m at 292 nm	−400	−1400	−1800
Δa_m at 286 nm	−120	−450	−600
Δa_m at 286 nm/Δa_m at 292 nm	0.30	0.32	0.33
2. Without $CaCl_2$			
Δa_m at 292 nm	~0[b]	−200	−1000
Δa_m at 286 nm	~0[b]	−50[b]	−350
Δa_m at 286 nm/Δa_m at 292 nm			0.35

[a] Spectra were measured using protein in 0.01 M Tes–0.05 M KCl (pH 6.0) as the reference. In the cases of $CaCl_2$ effects, calcium was also added to the reference. Tes buffers were used at pH 6.0 and 7.0, and Tris buffers at pH 8.0 and 9.0. [b] Discernible peak, but too small to measure accurately.

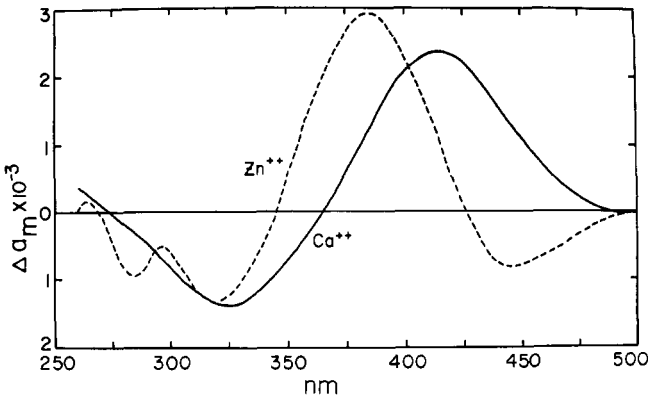

FIGURE 2: Spectral perturbations of the 2-hydroxy-5-nitrobenzyl derivative of phospholipase A_2 caused by divalent cations. Molar absorptivity changes (Δa_m) as a function of wavelength caused by 5×10^{-5} M Zn^{2+} or 1×10^{-4} M Ca^{2+}, in 0.01 M Tris–0.05 M KCl (pH 8.0). The cell in the sample position contained the added cation.

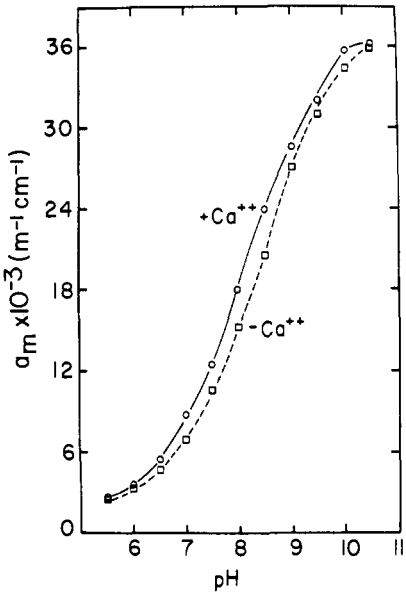

FIGURE 3: pH dependence of the molar absorbance (a_m) of the 2-hydroxy-5-nitrobenzyl derivative of phospholipase A_2. The molar absorbance was measured at 410 nm in the presence or absence of 1×10^{-3} M Ca^{2+} in 0.01 M buffers containing 0.05 M KCl.

of the monomer, which occurs at lower pH's. In several preparations the isolated derivative had a molar absorptivity of 36.5 (range 34.2–37.8) $\times 10^3$ M^{-1} cm^{-1} in 0.1 N NaOH. This indicates that 2.02 (range 1.9–2.1) tryptophans had been derivatized under these conditions. This derivative retained complete enzymatic activity within the variability of the assay (95–105% the specific activity of the native enzyme).

The derivative also showed divalent cation induced spectral perturbations as shown in Figure 2. In this figure the sample cell contained the divalent ion. Therefore there was an increase in absorbance in the presence of the divalent cation. As in the case of the native protein there was a clear distinction between the spectral perturbations caused by calcium and zinc. Also in agreement with the results presented for the native protein, Ba^{2+} and Sr^{2+} caused identical spectral perturbations to those caused by calcium, and cadmium acted similar to zinc. All the other ions discussed above had no effect on the spectra or precipitated the derivative.

The molar absorptivity was measured as a function of pH in the presence or absence of 1×10^{-3} M Ca^{2+} in order to determine the apparent pK of the *p*-nitrophenol group. All buffers contained 0.05 M KCl and the following solutions were used: pH 5.5–7.0, 0.01 M Mes; pH 7.0–8.5, 0.01 M Tes; pH 8.5–10.5, 0.01 M Tris. The difference between the pH of the solution containing calcium and that which did not contain calcium was less than 0.02. The results of these experiments are presented in Figure 3. Analysis of these data (eq 1) showed an apparent pK of 8.15 in the presence of calcium and 8.40 in the absence of calcium. The difference in pK could not be ascribed to variation in the pH of the buffers and must reflect some alteration in the electrostatic environment of the *p*-nitrophenol group caused by calcium binding. If it is assumed that the apparent pK of the *p*-nitrophenol group is sensitive to the local electrostatic environment, and that calcium binds to COO^- groups in the same environment, then binding of calcium will change the net charge in the environment by 2+. As a consequence the apparent pK of the *p*-nitrophenol group will be lowered.

In order to test the possibility that the effect of the calcium was due to general charge effect on the protein or due to some interaction with the *p*-nitrophenol group, another derivative was prepared with the same chromophore, but with a different functional group, such that it would react with another residue in the protein. 2-Bromoacetamido-4-nitrophenol has been reported to react slowly with methionine groups in proteins that do not contain free SH groups (Burr and Koshland, 1964). After reaction of phospholipase A_2 with this reagent, a product was obtained which had approximately 1.5 *p*-nitrophenol groups (out of 2.0 possible based on the methionine content). This derivative retained complete enzymatic activity. No detectable spectral perturbations of the *p*-nitrophenol group were observed in the presence of calcium or zinc. This is taken as evidence that the effects of zinc and calcium are restricted to the local environment of their binding which also happens to contain the *p*-nitrophenol group attached to tryptophan, but not that presumed to be attached to methionine.

Number of Ion Binding Sites. The analysis of the number of ion binding sites was carried out by equilibrium dialysis or by analysis of the spectral perturbations induced by ion binding (Halfman and Nishida, 1972). Figure 4 presents the results of two experiments. Figure 4A represents the binding of zinc at pH 8.0 in 0.01 M Tris, containing 0.05 M KCl, determined from spectral measurements. There are apparently two identical and independent binding sites with a dissociation constant[3] of 1.0×10^{-6} M. Figure 4B shows the results of equilibrium dialysis studies of calcium binding at pH 8.0 in 0.01 M Tris containing 0.15 M KCl. Again there are apparently two independent and identical calcium binding sites with a dissociation constant of 5.3×10^{-5} M. In Table IV are reported the binding constants determined by various methods for calcium, zinc, barium, and strontium. In all cases the number of binding sites determined was 2. There is reasonable agreement between the constants determined by kinetic and physical studies on the native protein as well as those derived from spectral measurements using the 2-hydroxy-5-nitrobenzyl derivative. Zinc was determined to be a competitive inhibitory with re-

[3] Although the data presented in Figure 4 give association constants, the cation binding has been characterized by dissociation constants in order to allow ready comparison with kinetically determined constants.

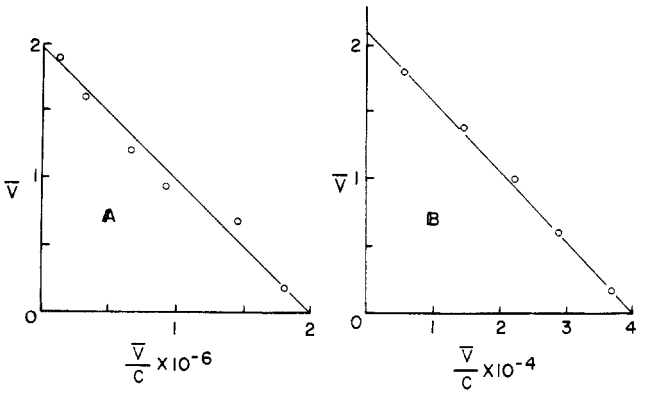

FIGURE 4: Scatchard plots of ion binding to phospholipase A_2. $\bar{\nu}$ represents the moles of cation bound per mole of protein. c refers to the unbound cation. (A) Zn^{2+} binding determined by spectral measurements at pH 8.0. (B) Ca^{2+} binding determined by equilibrium dialysis at pH 8.0.

spect to Ca^{2+} using dibutyryllecithin as a substrate (Wells, 1972) with a $K_i = 3 \times 10^{-6}$ M.

Effect of Dihexanoyllecithin. In the presence of 50 mM dihexanoyllecithin in 0.01 M Tris–0.05 M KCl (pH 8.0) without added Ca^{2+} no detectable spectral perturbations were observed. At the enzyme concentrations used for difference spectra measurements the reaction was too fast with both dihexanoyllecithin and Ca^{2+} present to allow measurements to be made.

Discussion

In the following discussion an attempt will be made to interpret the spectral perturbations reported here in terms of the possible mechanism whereby calcium activates phospholipase A_2 and zinc and barium inhibit the enzyme. Before proceeding, it is worthwhile to summarize the salient experimental observations.

With regard to the native enzyme: (1) there are two independent binding sites per dimer, which can bind any of the following cations, Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, or Cd^{2+}. Since calcium is absolutely required for activity and the others are competitive inhibitors, it is reasonable to assume that there is only one type of binding site for all the cations. Since the sites are identical and the subunits are identical, it is also reasonable to assume that there is one binding site per subunit. (2) There are two types of spectral perturbations caused by cation binding. The first is found in the presence of the alkaline earth cations, Ca^{2+}, Sr^{2+}, and Ba^{2+} (Figure 1). The second is found in the presence of zinc and cadmium (Figure 1). (3) The magnitude of the spectral perturbation caused by calcium depends on the pH at which the experiment is conducted (Tables II and III). There is also a pH-dependent perturbation of the protein spectra found in the absence of added cation. These data indicate that a titratable group in the protein is somehow involved in the expression of the perturbation. As a first approximation the apparent pK of the group in the presence of calcium is 7.6 and in the absence of calcium it is 8.9. Wells (1972) has presented evidence for a catalytically essential group with an apparent pK of 7.6 in the enzyme–substrate complex. (4) The value of the binding constants determined by kinetic and physical methods (Table IV) strongly suggest that binding to the same site is being studied in all cases. (5) The binding of cations does not cause dissocation of the dimers, although the spectral perturbation caused by zinc is identical with that observed upon dissocation. (6) The cation-induced spectral perturbations are not observed when the dimers are dissociated by 4 M urea at pH 8.0. (7) There are no spectral perturbations caused by the substrate alone.

With regard to the spectral perturbations observed with the HNB–protein derivative: (1) there are two independent binding sites which can interact with Ca^{2+}, Ba^{2+}, Sr^{2+}, Cd^{2+}, or Zn^{2+}. (2) There are two tryptophans which have reacted with HNB and since there are two ion binding sites, it is reasonable to assume that one tryptophan per subunit has reacted. (3) There are two types of spectral changes produced. One caused by the alkaline earth ions and the other caused by zinc and cadmium (Figure 2). (4) The fact that the derivative on tryptophan, but not on methionine, is perturbed by cations suggests strongly that the same tryptophans are involved in the HNB–protein as in the native protein. (5) The effect of calcium on the HNB–protein can be explained as an effect on the *p*-nitrophenol groups' pK, most likely through electrostatic interaction. (6) The HNB–protein retains full catalytic activity and the binding constants determined are the same as found with the native protein. This suggests that the cation-induced spectral perturbations observed with the derivative arise from the same source as in the native protein.

The spectral perturbations seem to arise from tryptophanyl residues with little contribution from tyrosyl residues. Andrews and Forster (1972) and Strickland *et al.* (1972) have reported studies on the ultraviolet absorption spectra of model indole compounds, which can be used to tentatively describe the origin of the spectral perturbations observed here. Strickland *et al.* (1972) reported that hydrogen bond formation to the indolyl NH can cause a red shift in the ^1L_a absorption band amounting to 3–10 nm. Calculated difference spectra from the data presented by Strickland *et al.* (1972) indicates that the largest spectral changes associated with this type of interaction

TABLE IV: Dissociation Constants of Divalent Cations from Phospholipase A_2.

Cation	Method
Native Enzyme	
Ca^{2+}	
5.3×10^{-5} M	Equilibrium dialysis
8×10^{-5} M	Spectral perturbations
4×10^{-5} M	Kinetic studies[a]
Ba^{2+}	
9×10^{-5} M	Spectral perturbations
3×10^{-5} M	Kinetic studies[a]
Zn^{2+}	
1×10^{-6} M	Spectral perturbations
3×10^{-6} M	Kinetic studies
2-Hydroxy-5-nitrobenzyl Derivative	
Ca^{2+}	
3×10^{-5} M	Spectral perturbations
Ba^{2+}	
7×10^{-5} M	Spectral perturbations
Sr^{2+}	
8×10^{-5} M	Spectral perturbations
Zn^{2+}	
1.3×10^{-6} M	Spectral perturbations

[a] Taken from Wells (1972).

would occur at wavelengths between 300 and 305 nm, with progressively smaller changes near 292 and 286 nm. The observed changes (Figure 1) do not conform to these predictions.

Based on their studies with model compounds Andrews and Forster (1972) suggest the following empirical rule to distinguish between solvent-induced and charge-induced spectral perturbations. If the ratio of the molar absorptivity change at 286 nm to that at 292 nm is greater than 0.5, the perturbation is most likely solvent induced, and if the ratio is less than 0.4, the perturbation is most likely charge induced. Application of this rule suggests that the calcium- (and other alkaline earth ions) induced spectral changes and those caused by pH in the absence of cations are primarily charge induced. The sign of the spectral change would require that calcium binding and increased pH remove a charged group from close proximity to the perturbable tryptophan. The spectral perturbations induced by calcium binding to the HNB–protein are also explicable by assuming that calcium binding changes the local electrostatic environment of the *p*-nitrophenol group which is attached to the perturbable tryptophan.

On the other hand, the spectral perturbations both of the native protein and the HNB–protein caused by zinc are more consistent with an ion-induced conformational change in the protein which alters the solvent environment of the tryptophan and its associated *p*-nitrophenol group. Dissociation of the dimer either at low pH or in the presence of 4 M urea (Wells, 1971b), which might reasonably be expected to alter the environment of the tryptophan, gives spectral perturbations closely resembling those caused by zinc. This is taken as additional support for the suggestion that zinc binding induce a conformational change in the dimer.

Based on the above observations and discussion, a plausible model can be proposed for the activating role of calcium and the inhibitory action of zinc and barium. In this model it is assumed that the group in the protein whose p*K* controls the expression of the spectral perturbations is also the charged group which is responsible for the perturbations at low pH and in the absence of cation. The pH dependence of the perturbation requires that the acid form of the group be charged. Within the pH range of the perturbation this would most likely be an ammonium ion or an imidazolium ion. If RH^+ is an ammonium group, then its p*K* in the presence of Ca^{2+} is unusually low, whereas if an imidazolium group its pK in the absence of Ca^{2+} is unusually high.

The model interprets the experimental observations as follows. An increase in pH causes RH^+ to lose its proton, thereby relieving the perturbation of the tryptophan. The binding of Ca^{2+}, on the other hand, not only causes RH^+ to lose a proton, but also induces a conformational change in the site which allows the substrate to be bound. Ca^{2+} must do something in addition to relieving the tryptophan perturbation, otherwise calcium would not be required at high pH. As judged by kinetic studies (Wells, 1972) and spectral measurements reported here, the substrate cannot bind to the enzyme in the absence of added calcium. When barium is bound in the site, RH^+ loses its proton, but the larger size of barium changes the conformation of the site such that substrate either cannot bind or the orientation of the groups in the active site precludes activity. Mg^{2+} being smaller than Ca^{2+} apparently cannot bind at all. The binding of zinc discharges the proton from RH^+, but in addition binds R as a ligand, thereby causing a different conformational change in the site which precludes activity and also changes the exposure of the tryptophan to the solvent.

There are several variations of the model which are experimentally indistinguishable at the present time. The apparent correspondence of the p*K* for RH^+ reported here and that of a catalytically essential group reported from kinetic studies (Wells, 1972) might suggest either that R is the active nucleophile in the reaction or that the conformational change induced by calcium binding brings the active nucleophile into proper orientation in the active site. The spectral perturbation can be interpreted as arising directly from the proximity of RH^+ to the tryptophan or that the conformation of the protein when RH^+ is present places a charged group near the tryptophan, and when R is deprotonated, the conformational change removes the charged group from the vicinity of the tryptophan. Experiments are now in progress to identify RH^+ and to attempt to differentiate between the above suggestions and other possible explanations

Acknowledgments

Dr. L. Forster provided helpful discussions and Mrs. N. Hewlett provided expert technical assistance.

References

Andrews, L. J., and Forster, L. S. (1972), *Biochemistry 11*, 1875.

Burr, M., and Koshland, D. E., Jr. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1017.

de Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., and VanDeenen, L. L. M. (1971), *Biochim. Biophys. Acta 239*, 252.

Halfman, C. J., and Nishida, T. (1972), *Biochemistry 11*, 3493.

Koshland, D. E., Jr., Karkhanis, Y. D., and Latham, H. G. (1964), *J. Amer. Chem. Soc. 86*, 1448.

Long, C., and Penny, I. F. (1957), *Biochem. J. 65*, 382.

Roholt, O. A., and Schlamowitz, M. (1961), *Arch. Biochem. Biophys. 94*, 364.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Strickland, E. H., Billups, C., and Kay, E. (1972), *Biochemistry 11*, 3657.

Wells, M. A. (1971a), *Biochemistry 10*, 4074.

Wells, M. A. (1971b), *Biochemistry 10*, 4078.

Wells, M. A. (1972), *Biochemistry 11*, 1030.

Wells, M. A., and Hanahan, D. J. (1969), *Biochemistry 8*, 414.