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Spectral Perturbations of the Histidine and Tryptophan in Cobra Venom Phospholipase A$_2$ upon Metal Ion and Mixed Micelle Binding*

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Metal ions and nonionic surfactant/phospholipid mixed micelles are shown to affect the structure of pure cobra venom phospholipase A$_2$ (Naja naja naja). This small phospholipase, with 1 histidine and 1 tryptophan residue per molecule, has an absolute requirement for Ca$^{2+}$, although many divalent metal ions can bind to it. Inhibitory metals include Zn$^{2+}$, Ba$^{2+}$, and Sr$^{2+}$, whereas Mn$^{2+}$, Mg$^{2+}$, and Cd$^{2+}$ bind without altering enzyme activity. The pH rate profile of the reaction with phosphatidylcholine as substrate shows that Ca$^{2+}$ lowers the apparent pH, controlling the reaction (for 10 mM Ca$^{2+}$, pH$_{50}$ = 5.6; for 0.9 mM Ca$^{2+}$, pH$_{50}$ = 6.2). The binding of Ca$^{2+}$ to the enzyme is pH-dependent with pH$_{50}$ = 5.9 and K$_{a}$ = 0.15 mM for the unprotonated form of the enzyme as determined by both fluorescence emission and UV difference spectroscopy. The Ca$^{2+}$-induced difference spectra are characterized by a blue-shifted tryptophan perturbation at basic pH and a red-shifted tryptophan perturbation at acidic pH. The difference spectrum induced by Ca$^{2+}$ at acidic pH is similar to the titration difference spectrum observed with apoenzyme. The latter shows a pH dependence with pH$_{50}$ = 6.8 to 7.1. Gel filtration studies of the molecular weight of the enzyme suggest that at acidic pH and high enzyme concentrations, this conformational change involves dimerization of the enzyme.

These results suggest that when Ca$^{2+}$ binds to phospholipase A$_2$, it triggers a conformational change which lowers the pH of a critical residue, probably the histidine, and in so doing alters somewhat the monomer-dimer equilibrium of the enzyme. $^1$H nmr studies of the aromatic region of the enzyme are consistent with this interpretation. Furthermore, binding studies with substrate in mixed micelles and metal ions suggest that the reaction of phospholipase A$_2$ is ordered; enzyme must bind metal ion before it can bind to phospholipid in mixed micelles.

The mechanism of action of phospholipase A$_2$ (EC 3.1.1.4) is of considerable interest because in vivo, its substrate is always part of a lipid-water interface such as that which occurs in mixed micelles with bile salts, membranes, or lipoproteins. Previous work in our laboratory has concentrated on the characterization of the phospholipase A$_2$ from cobra venom (Naja naja naja) and kinetic studies on its action toward natural phospholipide in mixed micelles with the nonionic surfactant Triton X-100 (2, 3). This work has led to a general model for the action of lipolytic enzymes which allows for the quantitation of the involvement of phospholipase A$_2$ with the lipid-water interface (3). The cobra venom phospholipase A$_2$ has a native molecular weight of only 11,000 and contains 1 histidine and 1 tryptophan residue (1). This enzyme exhibits "half-site reactivity" in the chemical modification of its single histidine residue by p-bromophenacyl bromide and other reagents (4). This result suggested that the active form of the enzyme is an asymmetric dimer or higher order aggregate (4). Recent cross-linking experiments with dimethyl suberimidate support this postulate and together with gel filtration binding studies and "surface dilution" kinetics (2, 3) lead to a model whereby the phospholipid substrate induces the aggregation of monomeric enzyme to form asymmetric dimers (5). In these structures, one subunit binds to phospholipid in the lipid-water interface and the other binds to phospholipid in a catalytically productive manner.

Phospholipase A$_2$ has an absolute requirement for Ca$^{2+}$ and its role in the "dual phospholipid" model described above (5) must be elucidated. We have now examined the effect of Ca$^{2+}$ and other metal ions on phospholipase A$_2$ activity by kinetics, on the monomer-dimer equilibrium by gel chromatography, on the single tryptophan residue by fluorescence and UV difference spectroscopy, and on the single histidine residue by $^1$H nmr spectroscopy. The results which are reported here suggest that Ca$^{2+}$ binds at or near the active site of phospholipase A$_2$ and triggers a conformational change that lowers the pH$_{50}$ of the catalytically essential histidine, also altering somewhat the monomer-dimer equilibrium of the protein. The metal ion-induced conformational change must take place before phospholipid binding can occur. The tryptophan is in a very exposed environment and is quite sensitive to Ca$^{2+}$, substrate, and product binding, as well as the ionization state of the histidine. Preliminary results have been presented (6).

EXPERIMENTAL PROCEDURES

Phospholipase A$_2$ Purification — Lyophilized cobra venom, Naja naja naja (Pakistan), lot NNPOL and NNP-455, was obtained from the Miami Serpentarium. The phospholipase A$_2$ was purified accord-
ing to the procedure of Deems and Dennis (1). The enzyme was then passed through a DEAE-cellulose (Whatman DE11) column. In a typical experiment, 100 mg of protein (specific activity 530 pmol min⁻¹ mg⁻¹) were loaded on a column (1.5 × 40 cm) that had been pre-equilibrated in 5 mM phosphate buffer at pH 7.0. Either a continuous salt gradient (0 to 0.3 M NaCl) or a step gradient (0.1 M NaCl, 0.3 M NaCl) was applied to elute two major peaks. The first peak eluted in 5 mM phosphate, pH 7.0, containing 0.10 M NaCl. This peak represented about 20% of the protein and had a specific activity of 204 pmol min⁻¹ mg⁻¹. The second peak eluted with 0.20 M NaCl, contained about 80% of the protein, had a specific activity of 517 pmol min⁻¹ mg⁻¹, and is the pure phospholipase A₂ employed in the studies reported here. About 20% of the protein remained on the column. This pure phospholipase A₂ is extremely stable. Dialysis, repeated lyopholization, or limited heat treatment do not affect the specific activity of the enzyme over the pH range 3 to 9.

**RESULTS**

**Phospholipase A₂ Preparation** — Phospholipase A₂ prepared by the procedure of Deems and Dennis (1) was shown to be homogeneous by Sephadex G-100 chromatography and polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate (SDS). The sample used was comprised of 11,700 (M₉ = 13,700), and chymotrypsinogen (M₉ = 25,700) were used to calibrate the column.

**Thin Layer Chromatographic Assay** — At pH values below 5.0, the pH-stat technique is not a reliable method of determining enzyme activity; therefore, a thin layer chromatographic assay was employed in determining the pH rate profile. L-a-Dipalmitoyl(2-palmitoyl-sn-glycero-3-phosphatidylcholine) (Applied Science, specific activity 7 to 8 pmol/mg), was mixed with unlabeled dipalmitoyl phosphatidylcholine and assayed under the following conditions: 40 °C, 4 mM phospholipid, 12 mM Triton X-100, 0.02 mM EDTA, varying phospholipid concentrations of 0.1 to 10 mg/ml, and other divalent cations (1 to 5 mM) formed the mixed micelle system. Protein concentration was determined by the method of Lowery et al. (8) or by the absorbance at 280 nm (ε = 1.45).

**Thin Layer Chromatographic Assay** — At pH values below 5.0, the procedure of Deems and Dennis (1) contained varying amounts of neutral carbohydrate (1 to 4%) as measured by the phenol/H₂SO₄ test of Dubois et al. (11). This carbohydrate was first detected by sharp lines in the ¹H nmr spectrum of the enzyme (900 Hz downfield of 3(trimethylsilyl)tetradeutero sodium propionate) as shown in Fig. 1A. The carbohydrate could not be removed by dialysis, but could be removed specifically by passage of the enzyme through a concanavalin A-Sepharose column. Fig. 1B shows the ¹H nmr spectrum of enzyme treated in this fashion. The DEAE-cellulose column employed under "Experimental Procedures" also removes the contaminating carbohydrate from the enzyme; both protein peaks contain less than 0.4% by weight neutral carbohydrate as measured by the phenol/H₂SO₄ test and the ¹H nmr spectrum. Thus, phospholipase A₂ can be obtained in a form that is homogeneous by all of the usual biochemical criteria, and does not contain any carbohydrate.

**Kinetic Studies** — The apparent Kₘ for Ca²⁺ under standard assay conditions was found to be 0.21 ± 0.04 mM. Several divalent metal ions (Mg²⁺, Mn²⁺, Ba²⁺, Sr²⁺, Zn²⁺, Cd²⁺) and trivalent metal ions (Gd³⁺, Tb³⁺, Eu³⁺) were examined for their metal ion and mixed micelle binding of phospholipase A₂.
their ability to act as metal ion activators for phospholipase A₂. Less than 1% activity was observed with these metal ions (5 to 10 μM) at both pH 6.5 and pH 8.0. In the presence of 0.25 mM Ca²⁺, 5 mM Mg²⁺, Mn²⁺, or Cd²⁺ gave only a 6 to 9% loss of enzymatic activity, while comparable concentrations of Ba²⁺ or Sr²⁺ gave 70 to 80% inhibition and 5 mM Zn²⁺ gave complete inhibition. A more detailed kinetic analysis showed that Ba²⁺ is competitive with respect to Ca²⁺ (Kᵢ = 0.54 ± 0.10 mM).

The pH rate profile for phospholipase A₂ was determined using the thin layer chromatographic assay. Since saturation with respect to phospholipid is not possible in the Triton X-100 mixed micelle system, Vₘₐₓ cannot be determined easily (3). For the purposes of a pH rate profile we have used standard assay conditions with 10 mM Ca²⁺ and 0.9 μM Ca²⁺. As seen in Fig. 2 for the higher concentration of metal ion, the enzyme activity has a broad plateau in the range pH 7 to 9.5 and decreases below pH 7. The titration curve suggests that activity is dependent on an amino acid with an apparent pKₐ of about 5.6. With the lower Ca²⁺ concentration, the apparent pKₐ is shifted to 6.3. Thus, the observed value for the pKₐ depends on Ca²⁺ binding. The range of the apparent pKₐ is suggestive of a histidine.

UV Difference Spectra Induced by Ca²⁺—Fig. 3A shows the Ca²⁺-induced difference spectrum of phospholipase A₂ at pH 8.0. The sign of the difference spectrum is negative, the minimum are 292 nm and 283 nm and indicate perturbation of a tryptophan residue (12). Titration of a protein solution with increasing amounts of Ca²⁺ showed a hyperbolic dependence of the absorbance at both minima on the amount of metal ion added. A double reciprocal plot of the absorbance at the minima versus Ca²⁺ concentration gave straight lines from which the difference extinction coefficients (Δεₕₐ₉₃⁰) were calculated. For Ca²⁺ binding at pH 8.0, these values are Δεₙ₉₃₃⁰ = 0.023 and Δεₕₐ₉₃₃⁰ = 0.009.

The difference spectrum induced by Ca²⁺ was investigated over a wide pH range. The sign and shape of the spectrum varied with pH (Fig. 3). Under acidic conditions (Fig. 3C), a positive spectrum with maxima at 280.5 nm and 282 nm, Δεₙ₉₃₃⁰ = 0.010 and Δεₕₐ₉₃₃⁰ = 0.008, was observed. A hybrid of the acidic and basic Ca²⁺-induced difference spectra was observed between pH 6 and 7.5 (Fig. 3B).

An apparent binding constant for Ca²⁺ at each pH can be calculated by following the magnitude of the maximum (or minimum) of the difference spectrum. In Fig. 4 is a Dixon plot...
showing the pH dependence of the apparent $K_a$ for Ca$^{2+}$. The intersection of the two lines gives the $pK_a$ of the group affected by Ca$^{2+}$ binding as 5.8 to 6.0. The slope at the pH-dependent linear section is 0.9. This equals the change of ionization of the protein that occurs when the complex dissociates to free enzyme and Ca$^{2+}$.

The positive difference spectrum induced by Ca$^{2+}$ at acidic pH is similar to the difference spectrum observed when comparing apoenzyme at basic pH with that observed at acidic pH (Fig. 5A). Enzyme at pH 8.5 in the sample cuvette and at acidic pH values in the reference cuvette yielded a positive difference spectrum with maxima at 290.5 and 283 nm. The magnitude of the 290 nm peak followed a typical titration curve in the range pH 4 to 9, with $pK_a$, 6.8 to 7.1, again suggestive of a histidine. It should be noted that the pH-dependence of the rate of inactivation of the cobra venom phospholipase A$_2$ with p-bromophenacyl bromide (which modifies 0.5 histidine) is due to a group with $pK_a$, 6.8 to 7.1 (4).

The same pH titration of enzyme saturated with Ca$^{2+}$ produced a strikingly different spectrum (Fig. 5B). The maximum is now 287 nm with shoulders at 293 and 279 nm. This is the shape that would be expected if one or more tyrosine residues as well as the tryptophan is perturbed. Because of the difficulty in saturating enzyme with Ca$^{2+}$ at acid pH, the maximum spectral change upon titration ($\Delta_{max}$, 0.03) may have been underestimated. Use of this value gives a $pK_a$ of about 6.3 for the group controlling this spectral change.

UV Difference Spectra Induced by Other Divalent Cations – At pH 8.0, difference spectra induced by Ba$^{2+}$ and Sr$^{2+}$ were similar to those produced by Ca$^{2+}$ at that pH. Mg$^{2+}$ and Mn$^{2+}$, although not kinetic inhibitors, also produced similar difference spectra, but the minima were shifted to 288 and 280 nm, suggesting that the binding of these metal ions perturbs tyrosine residues. Cd$^{2+}$ and Zn$^{2+}$ induced positive difference spectra with maxima suggesting tryptophan perturbation. Adding 1 mM Ca$^{2+}$ to enzyme solutions containing Mg$^{2+}$, Mn$^{2+}$, or Cd$^{2+}$ changed these difference spectra to the typical Ca$^{2+}$ pattern. Several trivalent cations (Al$^{3+}$, Gd$^{3+}$, Pr$^{3+}$, La$^{3+}$, Dy$^{3+}$, Eu$^{3+}$, Tb$^{3+}$) at 1 mM were examined for production of difference spectra at pH 7.0, but in each case, these metal ions caused extensive aggregation of the enzyme as detected by light scattering. Even at pH 6.5, lower concentrations of lanthanides (0.1 mM) caused some aggregation, although a positive difference spectrum very much like that of Zn$^{2+}$ was observed. A comparison of the various classes of metal ion-induced difference spectra is shown in Fig. 6. The values of $\Delta_{max}$, $\lambda_{max}$, or $\lambda_{min}$, and $K_a$ for various metal ions at pH 8.0 are shown in Table I. In calculating binding constants, only a single binding site for divalent metals was observed. Ba$^{2+}$ and Sr$^{2+}$, which mimic Ca$^{2+}$ at pH 8.0, do not give rise to positive difference spectra at 5.0. Unlike Ca$^{2+}$, their difference spectra were the same shape and sign in the range pH 5 to 8; only the magnitude varied with pH.

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**Table I**

| Metal ion | $\lambda_{max}$ or $\lambda_{min}$ | $\Delta_{max}$ (%) | $K_a$ (mM) |
|-----------|----------------------------------|--------------------|------------|
| Ca$^{2+}$ | 292                              | -0.023             | 0.15       |
|           | 283                              | -0.009             |            |
| Ba$^{2+}$ | 292                              | -0.011             | 0.4        |
|           | 283.5                            | -0.008             |            |
| Sr$^{2+}$ | 292                              | -0.013             | 0.6        |
|           | 282                              | -0.010             |            |
| Mg$^{2+}$ | 288                              | -0.013             | 2.2        |
|           | 279.5                            | -0.008             |            |
| Mn$^{2+}$ | 287.5                            | -0.014             | 0.2        |
|           | 280                              | -0.009             |            |
| Cd$^{2+}$ | 291                              | +0.016             | 0.6        |
|           | 283.5                            | +0.011             |            |
| Zn$^{2+}$ | 292                              | +0.020             | 0.3        |
|           | 283.5                            | +0.011             |            |

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**Fig. 5.** Ultraviolet difference spectra of phospholipase A$_2$ upon going from a neutral or acid pH to a more basic pH (A) in the presence of metal ions with 0.57 mg ml$^{-1}$ of enzyme in 0.1 M NaCl (the reference cell is at pH 6.5, the sample cell at pH 8.5); and (B) in the presence of 20 mM Ca$^{2+}$ with 0.43 mg ml$^{-1}$ enzyme in 0.1 M NaCl (reference cell at pH 4.5, sample cell at pH 8.5).

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**Fig. 6.** Spectral changes of phospholipase A$_2$ in the presence of divalent cations. The sample cuvette contains enzyme in 0.025 M Tris/HCl, 0.10 M NaCl, pH 8.0, with (A) Mg$^{2+}$ (10 mM), enzyme (0.79 mg ml$^{-1}$); (B) Ba$^{2+}$ (10 mM), enzyme (0.62 mg ml$^{-1}$); (C) Cd$^{2+}$ (10 mM), enzyme (0.54 mg ml$^{-1}$).
Gel Filtration Molecular Weight Studies—The subunit structure of phospholipase A$_2$ is pH dependent and concentration-dependent as shown in Table II. At a concentration of 0.2 to 0.5 mg ml$^{-1}$ the enzyme exists as a dimer at pH 8 and a monomer at pH 5. Addition of Ca$^{2+}$ shifts this equilibrium so that the enzyme exists as a dimer at pH 5.4. Zn$^{2+}$ at this pH also causes dimerization, while Ba$^{2+}$ has no effect on the molecular weight. As the enzyme concentration is lowered by a factor of 10 at pH 8, the enzyme dissociates into monomers. Saturation of the enzyme with Ca$^{2+}$ alters the apparent molecular weight somewhat, but Ca$^{2+}$ does not completely convert it to the dimer form.

Two surfactants below the critical micelle concentration were examined for their effect on the apparent molecular weight of dilute phospholipase A$_2$ at pH 8. Triton X-100 did not affect the enzyme, while n-dodecyl octaethylene ether, which has a long alkyl chain similar to that of a fatty acid, increases the apparent molecular weight of the enzyme slightly.

Fluorescence Emission of Phospholipase A$_2$—Only tryptophan fluorescence is observed for proteins with both tryptophan and tyrosine residues (13). The intensity of the fluorescence depends on the environment of the indole group. Studies with model compounds have shown that the maximum of the emission spectrum of the indole group is 350 nm in aqueous solutions (14). The fluorescence spectrum of phospholipase A$_2$ (pH 8.0) exhibited a maximum of 350 nm with excitation at 283 nm. The quantum yield of the single tryptophan was measured as 0.24, consistent with tryptophan located in an exposed environment as opposed to the interior of a protein (10). The accessibility of the tryptophan is also supported by its extremely rapid rate of oxidation by N-bromosuccinimide (4).

The fluorescence did not change significantly (less than 10%) in the pH range 5 to 8, suggesting that the group with pK$_a$, 6.8 to 7.1 in the apoenzyme, is not in an appropriate environment to quench the fluorescence of the tryptophan (15). With further decreases in pH (below 5), the intensity of emission decreased dramatically, but the maximum wavelength did not shift. When 1 mM Ca$^{2+}$ was added to the enzyme at pH 8.0, an 18% decrease in fluorescence intensity was observed. The emission maximum was not shifted. The quenching phenomenon can be used to determine Ca$^{2+}$ binding constants at different pH values. The binding constants calculated from fluorescence studies agree with those calculated from UV difference experiments which show that n-dodecyl octaethylene ether does not bind to phospholipase A$_2$ (5).

When enzyme is titrated with mixed micelles of n-dodecyl octaethylene ether and dipalmitoyl or dimyristoyl phosphatidylcholine in the presence of EDTA, a difference spectrum identical with that of monomer n-dodecyl octaethylene ether is observed, but the addition of an inhibitory metal such as Ba$^{2+}$ increases the magnitude of the difference spectrum by a factor of about two. Micelles of lysophosphatidylcholine and fatty acid induce the same shape and approximately the same magnitude as this mixed micelle Ba$^{2+}$-enzyme complex. When Ba$^{2+}$ is added to enzyme and surfactant alone, there is no increase in the magnitude of the difference spectrum.

$^1$H NMR Spectroscopy—The small size of phospholipase A$_2$ (Naja naja naja) makes its $^1$H nmr spectrum at 220 MHz partially resolvable as shown in Fig. 1B. The enzyme aggregates at nmr concentrations (1), resulting in line widths broader than would be expected for a protein with a molecular weight of 11,000. Fig. 7 shows the aromatic region of the spectrum (6 to 8 ppm downfield from 3(trimethylsilyl) -tetradeutero sodium propionate), which contains protons from 6 tyrosines, 3 phenylalanines, 1 tryptophan, and 1 histidine. The spectrum was examined for the effect of metal ions. Particular attention was paid to the small peak at 7.7 ppm downfield from internal 3(trimethylsilyl)tetradeutero sodium propionate. This peak could be due to the C-2 proton of the single histidine, although it is further upfield than is typically observed for protonated histidines (16). As shown in Fig. 7A, at pH 2.7, the line width of this peak was about 30 Hz making it difficult to determine its precise intensity. At pH 9, where other experiments suggest that the histidine is unprotonated, the 7.7 ppm peak has disappeared. Presumably, it has shifted into the bulk of the aromatic envelope. Histidine should be

| Table II |
| Molecular weight of phospholipase A$_2$ determined by Sephadex G-100 chromatography |
| Enzyme concentration (mg ml$^{-1}$) | pH | Ligand | Molecular weight |
| 0.25 | 5.1 | 12,000 |
| 0.27 | 5.5 | 15,000 |
| 0.28 | 5.4 | 10 mM Ca$^{2+}$ | 21,500 |
| 0.27 | 5.4 | 10 mM Ba$^{2+}$ | 15,000 |
| 0.25 | 5.4 | 10 mM Zn$^{2+}$ | 21,500 |
| 0.27 | 6.0 | 17,000 |
| 0.25 | 7.2 | 19,500 |
| 0.19 | 8.0 | 22,000 |
| 0.04 | 8.0 | 13,500 |
| 0.03 | 8.0 | 10 mM Ca$^{2+}$ | 16,000 |
| 0.03 | 8.0 | 0.06 mM Triton X-100 | 13,000 |
| 0.03 | 8.0 | n-dodecyl octaethylene ether | 15,000 |
parameters are: 1000 transients, 1.6-s acquisition time, 90° flip angle. (A), apparent pH 2.7, (B), apparent pH 5.9 (----) apoenzyme and (-- -) enzyme with 5 mM CaCl₂, (C), the nmr difference spectrum. Ca²⁺ enzyme minus apoenzyme.

half-protonated in the apoenzyme at pH 7.0. When saturating amounts of Ca²⁺ were added to the enzyme at this pH, the 7.7-ppm peak was either shifted upfield by 20 to 30 Hz into the main aromatic region or sufficiently broadened to be undetectable. Similarly, at pH 6, where histidine is protonated in the apoenzyme, addition of Ca²⁺ shifted or broadened (or both) part of this peak as detected by an nmr difference spectrum (Fig. 7, B and C). In both cases, there were also complicated changes in the rest of the aromatic region. Whether they were caused by tyrosine, phenylalanine, tryptophan, or a combination of these could not be determined, although these changes clearly suggest that Ca²⁺ causes a major conformational change of the enzyme.

**DISCUSSION**

Interpretation of Ca²⁺ and Surfactant-induced UV Difference Spectra — Only the aromatic side chains of tyrosine, phenylalanine, and tryptophan contribute significantly to protein UV difference spectra in the region 270 to 300 nm. For cobra venom phospholipase A₂, the difference spectrum induced by Ca²⁺ at pH 8 has minima consistent with a blue shift tryptophan perturbation. The results of Andrews and Forster (17) on model indole compounds may be applied to describe the origin of the spectral perturbation. The ratio of the magnitude of the 283 nm peak to the 292 nm peak is less than 0.4, suggesting a charge perturbation. Charge effects in the form of deprotonation of a titratable group close to tryptophan may contribute to this blue shift as has been suggested by Wells for Ca²⁺ binding to the enzyme from Crotaulus adamanteus (18).

At acid pH, the Ca²⁺-induced difference spectrum is positive and exhibits a maximum at 290.5 nm, which is intermediate between that expected for tyrosine (usually 296 to 299 nm) and tryptophan (282 to 294 nm). The perturbation is probably due to tryptophan, although tyrosine as well could be contributing to the difference spectrum. Using the empirical rules of Andrews and Forster (17) to distinguish between solvent-induced and charge-induced spectral perturbations, one finds that since the ratio of the magnitude of the 283 nm peak to the 290.5 nm peak is greater than 0.5, the perturbation is most likely solvent-induced. The red shift implies transfer of the group(s) to an environment of lower polarity. Calculation of the Ca²⁺ binding constants at each pH (which does not depend on the identity of the residue(s) perturbed) suggests that a pH-dependent conformational change with pKₐ 5.8 to 6.0 strongly affects Ca²⁺ binding.

The difference spectrum observed in going from acidic to basic pH also appears to be solvent-induced. It is interpreted as a red shift of tryptophan as the enzyme is deprotonated. The group in the apoenzyme that is responsible for the conformational change detected by the difference spectrum has a pKₐ of about 7. The similarity of this difference spectrum to that induced by Ca²⁺ at acid pH suggests that Ca²⁺ may function in part by lowering the pKₐ of a reactive group on the enzyme. Furthermore, the gel filtration studies strongly suggest that the positive difference spectrum observed in both cases correlates with dimerization of the enzyme.

The long alkyl chain surfactant n-dodecyl octaethylene ether binds as a monomer to phospholipase A₂. At pH 8, it induces a difference spectrum qualitatively similar to that induced by Ca²⁺ at acid pH. There is no evidence that pure surfactant micelles can bind to the enzyme. If the enzyme is titrated with lysophosphatidylcholine, palmitic acid, or the Ba²⁺/mixed micelle system, the magnitude of the UV difference spectrum is increased 2-fold. These experiments suggest that the Ca²⁺ dimer at acid pH and the dimer of apoenzyme at pH 8 do not represent exactly the same structures. With substrate or product binding to the dimer enzyme at pH 8, a conformational change occurs such that the new dimer is now similar to the Ca²⁺-induced dimer at acid pH. Previous chemical modification experiments with p-bromophenacylbromide were interpreted in terms of an asymmetric dimer for phospholipase A₂ (4). The Ba²⁺/mixed micelle result can also be explained in terms of asymmetric dimer formation. When surfactant alone is present or when no metal ions are present with mixed micelles, half of the asymmetric dimer binds monomer surfactant very tightly. The addition of Ba²⁺ causes the subunit to bind phospholipid, but now both subunits are involved in the binding hence the doubling of the difference spectrum. It is interesting to note that in studies on the dimeric phospholipase A₂ from C. adamanteus, it was observed that chemical modification of 1 lysine per dimer had an effect on dimer formation and metal binding to one subunit (19).

The effect of inhibitory metals is understandable if Ca²⁺ has two functions. Ca²⁺ interacts with the enzyme at a catalytic site, but also is important structurally via the monomer-dimer equilibrium of the phospholipase. Ba²⁺ binds to the enzyme at the catalytic site, but it does not alter the monomer-dimer equilibrium. Zn²⁺ on the other hand cannot fulfill the catalytic role of Ca²⁺ but fulfills the structural role. In the fluorescence emission experiments, where apoenzyme is a monomer, Ca²⁺ quenches the tryptophan fluorescence. It also shifts the monomer-dimer equilibrium toward the dimer as determined by gel filtration. Ba²⁺ does not quench the tryptophan, although it binds tightly at pH 8.0. Thus, the quenching of the enzyme tryptophan by Ca²⁺ reflects increased dimer content.

Furthermore, UV difference spectra studies show that the formation of Ba²⁺-enzyme mixed micelle complex is ordered. Metal ion must bind to the enzyme before phospholipid can bind. In the dual phospholipid model (4, 5), the enzyme is active as an asymmetric dimer and the aggregation is presumably substrate induced. At enzyme concentrations of 0.10 to 0.1 mg ml⁻¹, Ca²⁺ demonstrably alters the monomer-dimer equi-
librium in favor of dimers, but alone it does not cause dimers to form at assay concentrations. Thus, if a dimer or higher order aggregate is the active species of the enzyme at assay conditions, then these studies show that its formation requires prior binding of both Ca\(^{2+}\) and phospholipid.

**Identification of Histidine As Residue Triggering Conformational Change—**Phospholipase A\(_2\) activity depends on a group (or strongly interacting combination of groups) whose apparent \(pK_a\) is influenced by Ca\(^{2+}\) binding. Histidine is the single residue in proteins whose ionization state usually varies in the pH range 5.6 to 7.0. Nmr studies have shown that histidine \(pK_a\) values range from as low as 4 (\(\alpha\)-lytic protease) to as high as 8.1 (2 hemoglobin histidines (20)). Phospholipase A\(_2\) from cobra venom contains only 1 histidine/molecule, and this histidine has been implicated at the active site (4).

The pH dependence of calcium binding can most easily be explained by competition between Ca\(^{2+}\) ions and protons. The enzyme system can be described by the following equilibria:

\[
ECa^{2+} \rightleftharpoons K_{p} E + Ca^{2+} \quad K_{p} = 1.5 \times 10^{-4} M
\]

\[
EH^+ \rightleftharpoons K_{s} E + H^+ \quad pK_s = 7
\]

If one assumes that Ca\(^{2+}\) does not bind significantly to the protonated enzyme form, then the observed \(pK_s\) for enzymatic activity will depend on the binding constant for Ca\(^{2+}\), the concentration of metal ion, and the intrinsic \(pK_s\) in the absence of metal ion. From the data in Fig. 2, one can calculate the intrinsic \(pK_s\) for the group to be 7.2. The essential ionizable group is tentatively identified as a histidine. It may be kept in mind that what appears to be a histidine \(pK_s\) could be kept in mind that what appears to be a histidine \(pK_s\) could in detail result from another residue.

\(^{1}H\) nmr experiments of metal ion binding to phospholipase A\(_2\) are consistent with histidine involvement if the 7.7-ppm peak is in fact the C-2 proton. Saturating amounts of Ca\(^{2+}\) added to the enzyme at pH 7 shift the low field resonance upfield which would be consistent with deprotonation of the histidine. The nmr results are not definitive because of the ambiguity in the assignment of the 7.7-ppm peak to the histidine proton. Tryptophan protons from the single tryptophan of the enzyme would be expected to be observed in the region 7.2- to 7.6-ppm. For a typical protein, the tryptophan resonances would be expected to be broader than the histidine C-2 proton (23), and the resonance observed at 7.7 ppm is fairly broad, although broadening could reflect enzyme aggregation. For histidine in other proteins, the chemical shift of the C-2 proton is usually 8 to 9 ppm when histidine is protonated and 7.2 to 8 ppm for unprotonated histidine (20). Therefore a chemical shift of 7.7 ppm for the protonated form of histidine is unusual.

No other resonance was observed downfield of the aromatic region of the spectrum even under a wide variety of conditions (4 \(M\) guanidine HCl, pH 2, and temperatures up to 90°C). The unusual chemical shift could reflect a buried conformation of histidine in the native enzyme, or the proximity of histidine to an aromatic ring. There was little exchange of \(^{1}H\) into the 7.7-ppm peak over a period of 2 months and this would be expected for a nonaccessible or buried histidine residue. Thus, while the assignment of the 7.7-ppm peak to the single histidine can be rationalized, further work is clearly required to confirm the assignment of this peak.

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