Effect of pH on the Kinetic and Spectral Properties of *Crotalus adamanteus* Phospholipase A_2 in H_2O and D_2O†

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ABSTRACT: The activity of *Crotalus adamanteus* phospholipase A_2 is controlled by a group with a pK = 7.65 in H_2O and 8.40 in D_2O. Inhibition by H^+ is uncompetitive with respect to Ca^{2+} or substrate and appears to involve an effect on the interconversion of the central complexes. At high pH there is no deuterium isotope effect. Ca^{2+} causes a spectral perturbation of phospholipase A_2 which is pH dependent. In H_2O the pK of the perturbing group is 7.6 and in D_2O 8.3. The spectral perturbation caused by Ca^{2+} is the same in H_2O and D_2O. These results suggest that a charge-induced spectral perturbation of tryptophan is relieved at high pH. The close correlation between the effect of D_2O and pH on the enzymatic activity and the Ca^{2+}-induced spectral perturbations strongly suggests that the same residue in the protein is involved in both processes.

Reports from this laboratory have provided some insight into the mechanism of action of phospholipase A_2 (EC 3.1.1.4) from *Crotalus adamanteus* venom. The reaction proceeds by *O*-acyl cleavage without a detectable acyl-enzyme intermediate (Wells, 1971a). Calcium is absolutely required and the addition of calcium must precede the addition of the substrate, phosphatidylcholine (Wells, 1972). Preliminary evaluation of the pH dependence of the reaction suggested that a group with a pK = 7.6 was involved in the catalytic step (Wells, 1972). Recently, it was shown that calcium binding induces a spectral change in the enzyme which was also pH dependent. Preliminary evidence suggested that the pK of the group involved in the spectral perturbation was also 7.6 (Wells, 1973a). Chemical modification studies showed that acylation of a single lysine leads to loss of enzymatic activity and to loss of the calcium-induced spectral perturbations (Wells, 1973b).

In order to gain further support for the proposal that the effect of pH on the enzymatic reaction and the calcium-induced spectral changes were through the same group, a more detailed study of these phenomena has been carried out. In addition the effect of D_2O on these two properties of the enzyme has been characterized.

Materials and Methods

Enzyme purification, substrate (dibutyryllecithin) preparation and assay, and methods of treating kinetic data have been described (Wells and Hanahan, 1969; Wells, 1972; Misiorowski and Wells, 1973). D_2O (99.87 mol %) was obtained from Bio-Rad Laboratories (Richmond, Calif.), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes)[1] from Pierce Chemical Co. (Rockford, Ill.), tris(hydroxymethyl)aminomethane (Tris) from Sigma Chemical Co. (St. Louis, Mo.), and standard buffers from Mallinckrodt Chemical Works (St. Louis, Mo.). Other chemicals were reagent grade.

pH was measured using a Radiometer (Copenhagen) pH meter with a scale expander. The pH of D_2O solutions was obtained by adding 0.41 to the measured pH (Covington *et al.*, 1968).

Samples for difference spectra were prepared by gravimetric dilution of a stock solution of protein (8.25 mg/ml) in either H_2O or D_2O with 10 vol of buffer in either H_2O or D_2O. The pH of the protein solution was measured prior to and after spectral measurements. The values agreed within 0.1 pH unit. Precautions used in difference spectroscopy have been described (Wells, 1971b, 1973a). In all cases the reference cell contained the protein in 0.01 M Tes–0.15 M KCl (pH 6.0) in either H_2O or D_2O. The sample solutions contained 1 mM $CaCl_2$. For pH 6–7.5 0.01 M Tes, 0.15 M KCl was used and for pH 7.5–9.5 0.01 M Tris, 0.15 M KCl was used. Spectra were recorded on a Perkin-Elmer Model 356 two-wavelength double beam spectrophotometer at a scan speed of 10 nm/min and a slit of 0.15 mM. Data are reported as Δa_m at 292 nm (Wells, 1973a). With the protein concentration used a ΔA_m of 1000 corresponds to ΔA_{292} = 0.025.

Results

Spectral Measurements. The pH dependence of the molar absorptivity change at 292 nm induced by Ca^{2+} binding in H_2O and D_2O is shown in Figure 1. There was no effect of the two buffers used since at pH 7.5 both gave identical results. The shapes of the difference spectra were identical for both H_2O and D_2O and are the same as previously reported (Wells, 1973a). For all samples the ratio Δa_m(286 nm)/Δa_m(292 nm) was 0.32 ± 0.05 (sd). Based upon an analysis reported before (Wells, 1973a)[2] and using all possible combinations of data pairs in Figure 1, the pK of the group affecting the spectral properties in H_2O is 7.58 ± 0.08 (sd) and in D_2O it is 8.28 ± 0.10 (sd).

D_2O is known to cause spectral perturbations of proteins (Herskovits and Sorensen, 1968). Wells (1971b) reported

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[1] Abbreviations used are: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; PC, phosphatidylcholine.

[2] $\Delta a_m\alpha = \Delta A_{292}$, where α represents the fraction of the amino group in the unprotonated form and Δa_m = molar absorptivity change when α = 1. The ratio of α at one pH to α at another pH is given by $\alpha_1/\alpha_2 = \Delta A(292)_1/\Delta A(292)_2$. Furthermore, $pH_1 - pH_2 = \log \alpha_1/\alpha_2 + \log (1 - \alpha_2)/(1 - \alpha_1)$. Simultaneous solution of these equations yields α_1 and α_2 which can be substituted in pH = pK + log $\alpha/(1 - \alpha)$ to determine pK.

TABLE I: Predicted and Observed Inhibition Patterns Caused by H^+ Binding to Phospholipase A_2.

Equilibrium Constants Used to Evaluate H^+ Inhibition[a]	Variable Substrate: Ca^{2+}, Not Saturated with PC	Ca^{2+}, Saturated with PC	Lecithin, Not Saturated with Ca^{2+}	Lecithin, Saturated with Ca^{2+}
K_1	Comp[b]	–	Comp	–
K_2	Uncomp	–	Comp	Comp
K_1 and K_2	Noncomp	–	Comp	Comp
K_1 and K_2 and K_3 and/or K_4	Noncomp	Uncomp	Noncomp	Noncomp
K_1 and K_3 and/or K_4	Noncomp	Uncomp	Noncomp	Uncomp
K_2 and K_3 and/or K_4	Uncomp	Uncomp	Noncomp	Noncomp
K_3 and/or K_4	Uncomp	Uncomp	Uncomp	Uncomp
Observed	Uncomp	Uncomp	Uncomp	Uncomp

[a] Refer to Figure 2; constants not considered are assumed to involve enzyme intermediates which are active in either the protonated or unprotonated form. The equilibrium constants were set equal to infinity. [b] Comp, competitive inhibition; uncomp, uncompetitive inhibition; noncomp, noncompetitive inhibition; –, no inhibition.

anomalous effects of D_2O on the spectral perturbations of phospholipase A_2. In the earlier studies, no attempt was made to correct the pH in D_2O. If, however, the difference spectrum between the protein in H_2O at pH 7.6 and D_2O at pH 8.3 (in the presence of Ca^{2+}) was recorded, the anomalous shifts noted before disappeared and the difference spectrum is now similar to other proteins (Herskovits and Sorensen, 1968). These pH values were chosen so that the extent of ionization of the perturbing group would be the same in H_2O and D_2O.

Kinetic Studies. pH can affect enzymatic activity through protonation or deprotonation of substrates and/or the enzyme. In the pH range over which phospholipase A_2 is active (Wells, 1972) it is unlikely that deprotonation or protonation of the substrate is important. Since the enzyme is inactive at low pH, the proton is considered to be an inhibitor. Protonation can lead to inhibition by (1) producing an inactive form of the enzyme or (2) producing a form with altered activity. The first case is mathematically the simplest, since one need only consider that protonation alters the distribution equations for the various enzyme forms as is the case for any inhibitor (Cleland, 1963).

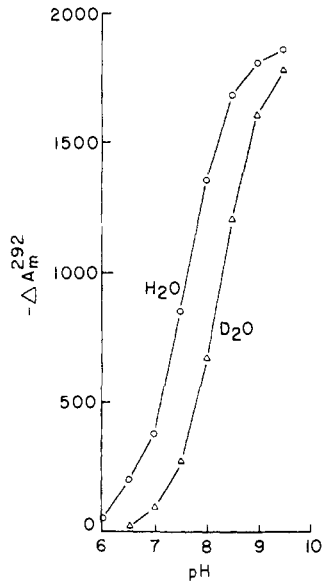

FIGURE 1: The effect of pH on the Ca^{2+}-induced spectral perturbation of phospholipase A_2 in H_2O and D_2O. The reference cell contained the enzyme in 0.01 M Tes–0.15 M KCl (pH 6.0). Perturbations were caused by 1 mM Ca^{2+}.

For a bi-ter mechanism, assuming one H^+ is involved and that protonation leads to complete inactivation, the general scheme shown in Figure 2 is obtained. Each enzyme form is supposed to have a different pK. Table I shows the predicted inhibition patterns of protonation for all possible combinations. Also included in Table I are the observed inhibition patterns. These data are consistent with protonation affecting the interconversion of the central complex and/or release of the first product.

Figure 3 shows that the H^+ is a linear inhibitor in both H_2O and D_2O. The pK determined in H_2O is 7.65 and in D_2O it is 8.40. It is important to note that V_m at high pH is the same in H_2O and D_2O.

Kinetic constants were evaluated by replotting slopes and intercepts from $1/V$ *vs.* 1/[Ca] plots at different fixed levels of dibutyryllecithin. The intercept replots as a function of the fixed levels of dibutyryllecithin in H_2O at various pH values are shown in Figure 4. The slope of this plot is K_{PC}/V_m and it is seen that pH does not affect this parameter. The intercept at infinite [phosphatidylcholine] is $1/V_m$. This parameter is clearly pH dependent. The intercept at $1/V = 0$ is $1/K_{PC}$, which is also pH dependent. The slope replots are shown in Figure 5. The slope of this plot is $K_{iCa}K_{PC}/V_m$. Since K_{PC}/V_m is pH independent, K_{iCa} must also be pH independent. These data are also consistent with H^+ inhibition occurring by an uncompetitive mechanism.

In D_2O data similar to those in Figures 4 and 5 were also obtained. Table II summarizes the kinetic constants obtained

TABLE II: pH Independent Kinetic Constants for the Hydrolysis of Dibutyryllecithin by Phospholipase A_2 in H_2O and D_2O.

	H_2O	D_2O
V_m (mol/sec per mol of enzyme)	2.6	2.6
K_{PC} (mM)	30	33
K_{iCa} (M)	4×10^{-5}	5×10^{-5}
K_{PC}/V_m (sec^{-1})	87	79
pK (kinetic)	7.65	8.40
pK (spectral)	7.58	8.28

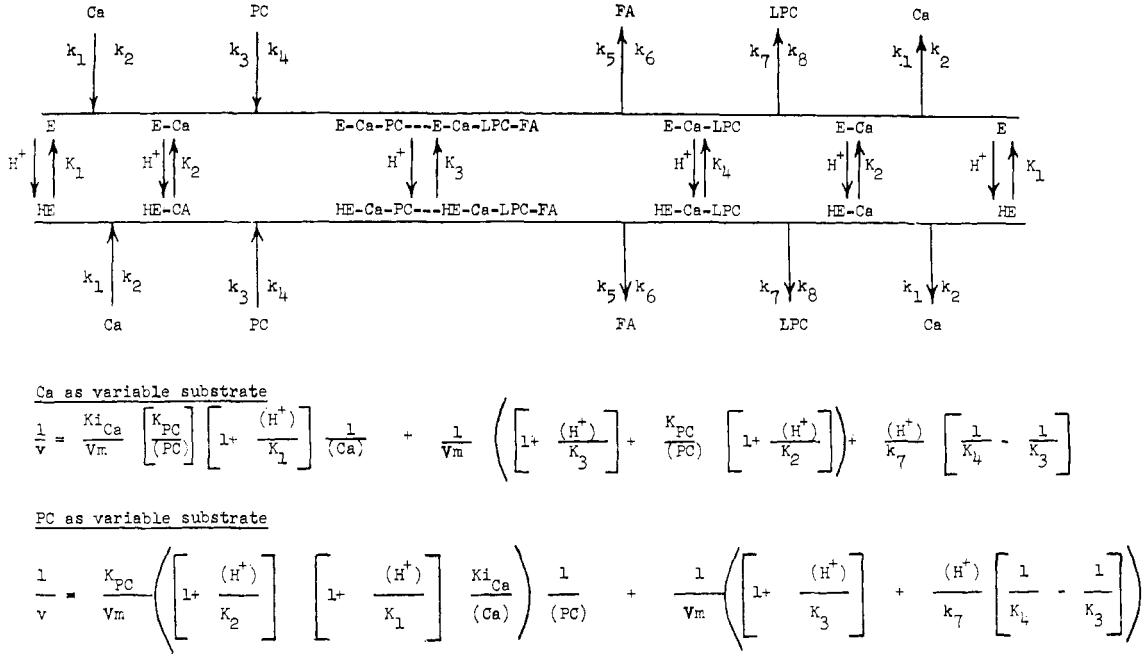

FIGURE 2: Inhibition caused by protonation in a bi-ter mechanism. k's refer to rate constants and K's refer to equilibrium constants for protonation.

in D_2O and H_2O. The inhibition caused by D_2O results from a change in pK of the H^+ sensitive step in the reaction. The change in pK observed is consistent with changes reported for the pK values of weak acids in D_2O by Bunton and Shiner (1961).

The close correspondence between the pK determined kinetically and that determined spectrally suggests that the same group may be involved. Figure 6 compares the effect of pH and D_2O on the spectral perturbations induced by Ca^{2+} binding and V_m. The solid line is calculated assuming both processes are controlled by a group with a pK = 7.6 in H_2O and 8.3 in D_2O.

Discussion

The results show that protonation of phospholipase A_2 does not inhibit the enzyme through an effect on substrate binding, but rather through an effect on the interconversion of the central complexes and/or release of fatty acid. The most appealing interpretation is that the reactive nucleophile in the enzyme must be unprotonated in order for the catalytic step to occur. Unequivocal kinetic support for this proposal is not possible to obtain. For the mechanism formulated in Figure 2 the kinetically determined pK will only equal the true pK if pK_3 = pK_4 and protonation effects both the interconversion of the central complexes and release of fatty acid. An alternate mechanism in which the interconversion of the central complex is rate limiting does not contain K_4 in the rate equation, and in this case the kinetically determined pK would equal the true pK. Also if the release of fatty acid is rate limiting then K_3 does not appear in the equation. There are not sufficient data available at the present time to choose between these alternatives.

The close correlation between the kinetically determined pK and the effect of pH on the Ca^{2+}-induced spectral perturbation of the enzyme strongly supports the conclusion that the same group is involved in both processes. The identical effect of D_2O on the pK of the group determined kinetically and spectrally further supports this conclusion.

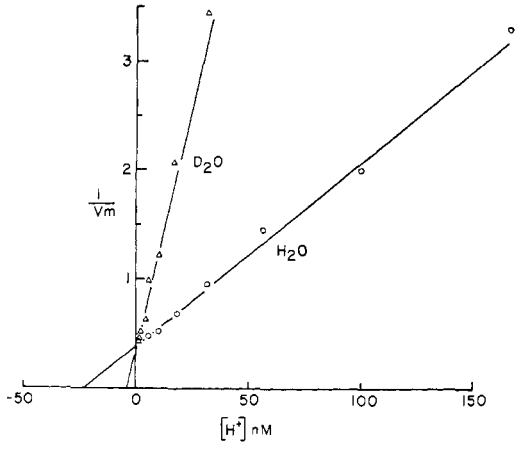

FIGURE 3: Inhibition of the phospholipase A_2 catalyzed hydrolysis of dibutyryllecithin by H^+ in H_2O and D_2O. V_m is determined from extrapolation to infinite Ca^{2+} and lecithin concentrations.

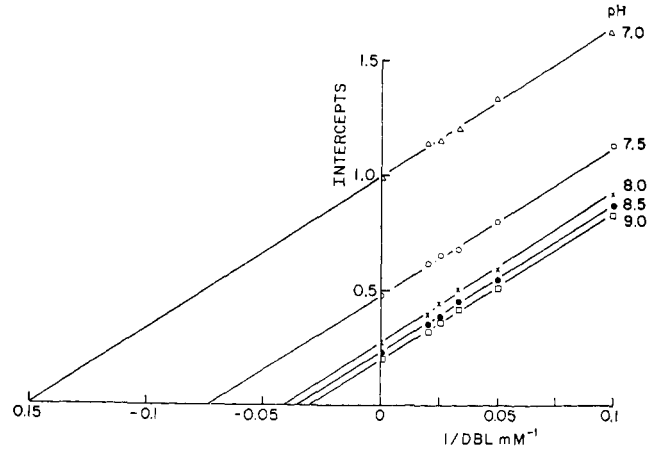

FIGURE 4: pH dependence in H_2O of the effect of dibutyryllecithin concentration on the intercepts of double reciprocal plots of velocity as a function of Ca^{2+} concentration.

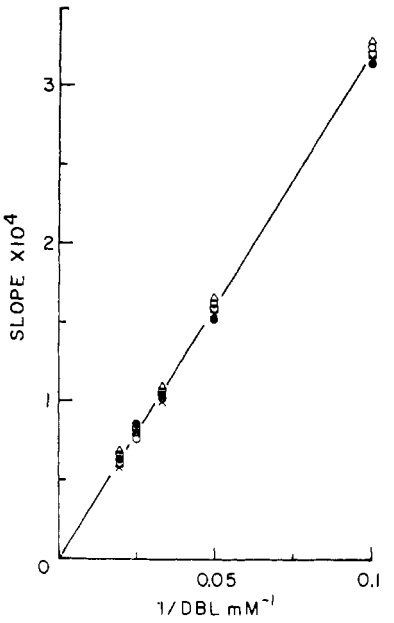

FIGURE 5: pH dependence in H_2O of the effect of dibutyryllecithin concentration on the slopes of double reciprocal plots of velocity as a function of Ca^{2+} concentration. The symbols refer to pH values shown in Figure 4.

The data collected in D_2O also provide two additional pieces of information. Since D_2O does not alter the pH-independent rate constant, it is concluded that proton transfer is not involved in the rate determining step of the enzymatic reaction. The identical spectral effects of Ca^{2+} in H_2O and D_2O show that the affected tryptophan does not change its environment upon Ca^{2+} binding and strongly support the earlier conclusion that the spectral effects of Ca^{2+} binding are due to relief of a charge perturbation of tryptophan (Wells, 1973a).

The lack of a deuterium isotope effect in the enzymatic reaction and the apparent necessity for an unprotonated amine in the catalytic step provide further support for consideration of the mechanism proposed in the previous paper (Wells, 1974). While there are obvious limitations to the data currently available and it is risky to push the model system too far, the results of these studies suggest that the catalytic role for calcium in the mechanism of phospholipase A_2 warrants further investigation.

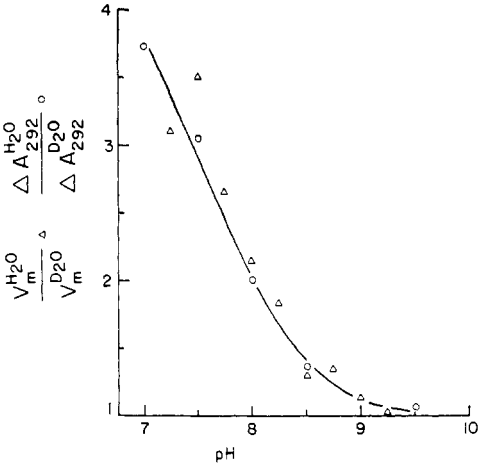

FIGURE 6: pH dependence of the apparent isotope effect of D_2O on the maximal velocity of the phospholipase A_2 catalyzed hydrolysis of dibutyryllecithin (open triangles) and on the magnitude of the Ca^{2+}-induced spectral perturbation of phospholipase A_2 (open circles). The solid line is calculated assuming the spectral effect is controlled by a group with $pK = 7.6$ in H_2O and $pK = 8.3$ in D_2O.

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