Effects of Chemical Modification on the Activity of *Crotalus adamanteus* Phospholipase A_2. Evidence for an Essential Amino Group†

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ABSTRACT: Oxidation of *Crotalus adamanteus* phospholipase A_2 with *N*-bromosuccinimide leads to rapid destruction of two tryptophans. The resulting protein has no enzymatic activity and no longer exhibits anomalous solvent-induced spectral perturbations or cation-induced spectral perturbations. These data are interpreted to indicate that these tryptophans are located close to the active site of the enzyme. Ethoxyformic anhydride inactivates the enzyme by acylation of one lysine per dimer. The modified protein has no enzymatic activity, although one cation binding site is normal. The other cation binding site has been modified as determined by spectral studies. These data indicate that the lysine is near the cation binding site, and are tentatively interpreted to indicate that the lysine is responsible for the cation induced spectral perturbations. After acylation of one lysine per dimer, the stability of the dimer is reduced. At pH 5.0 the modified dimer dissociated into subunits. One subunit is normal and reassociates to form native, active, dimer, while the modified subunit remains in the monomeric form. The rate of inactivation by ethoxyformic anhydride is influenced by various cations. Zinc increases the rate of inactivation by altering the exposure of the sensitive lysine to the solvent. Barium has a small protective effect, and calcium reduces the rate of inactivation markedly. These data indicate that calcium binding alters the conformation of the protein in such a way as to bury the sensitive lysine. In the presence of 4 M urea, which dissociates the enzyme into subunits, the rate of inactivation is very slow, indicating that the sensitive lysine is only present in the dimer. The substrate for the enzyme, phosphatidylcholine, has a small protective effect in the absence of added cations. In the presence of calcium and substrate the rate of inactivation is slowed tenfold. These data support the suggestion that calcium must add to the enzyme before the substrate can bind. In the presence of barium there was no protective effect of added substrate, indicating that barium inhibits the enzyme by causing a conformational change which prevents substrate binding. The enzyme was not affected by treatment with iodoacetate either at pH 3.0 or 6.0. Acetylation of tyrosines with *N*-acetylimidazole had no effect on the enzymatic activity. Photooxidation in the presence of Rose Bengal also had no effect on enzymatic activity.

Virtually no information is available concerning the nature of the groups essential to the activity of phospholipase A_2 (EC 3.1.1.4). There is general agreement that a sulfhydryl group is not involved based on studies with inhibitors and the absence of any free SH groups in the enzyme (Long and Penny, 1957; Saito and Hanahan, 1962; Kurup, 1965; de Haas *et al.*, 1968; Wells and Hanahan, 1969; Salach *et al.*, 1971; Shipolini *et al.*, 1971a). Sulfhydryl reagents have been reported to inhibit the *Crotalus atrox* enzyme (Brown and Bowles, 1966; Wu and Tinker, 1969) as well as the enzyme from bee venom (Munjal and Elliott, 1971), although the primary structure of the latter enzyme contains no free SH (Shipolini *et al.*, 1971b). The effect of sulfhydryl reagents in these enzymes needs to be clarified.

Organophosphorus compounds have generally been found to cause no inhibition of the enzyme from various sources (Saito and Hanahan, 1962; de Haas *et al.*, 1968; Salach *et al.*, 1971; Shipolini *et al.*, 1971a). Coupled with studies on ^{18}O exchange and fatty acid exchange (Wells, 1971a), these data would suggest that an active serine is not involved. Studies on the *C. atrox* enzyme, which report inhibition by diisopropyl fluorophosphate (Brown and Bowles, 1966; Wu and Tinker, 1969), again do not agree with observations on the enzyme from other sources.

Photooxidation leads to loss of activity (Kochalaty, 1966; Salach *et al.*, 1971), but the loss of activity has not been correlated to destruction of a particular type of amino acid. *O*-Methylisourea has been reported to inactivate the *C. atrox* enzyme (Brown and Bowles, 1966; Wu and Tinker, 1969).

The purpose of this work was to try to determine which amino acid residues are essential for catalytic activity and to identify the group involved in the cation-induced spectral perturbations reported in the previous paper (Wells, 1973).

Materials

The α and β forms of *Crotalus adamanteus* phospholipase A_2 were prepared by the method of Wells and Hanahan (1969). Substrates were prepared as indicated in the preceding publication (Wells, 1973). Iodoacetate was crystallized from petroleum ether (bp 30–60), *N*-acetylimidazole (Eastman Kodak) was purified as described by Riordan *et al.* (1965), ethoxyformic anhydride (Eastman Kodak) was distilled *in vacuo* (Melchior and Fahrney, 1970) and stored at −20°, *N*-bromosuccinimide was crystallized from water, and urea was crystallized from ethanol. Dioxane was purified by passage through a column of aluminum oxide (Merck) and distilled from CaH_2. Acetonitrile was dried by distillation from anhydrous $CaCl_2$. Both solvents were stored over molecular sieve 4A (Matheson, Coleman & Bell). Other materials were reagent grade and used without purification.

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Methods

Enzyme assays were carried out in diethyl ether using either hen's egg yolk lecithins or dioctanoyllecithin as substrate. Generally 1 ml of a 10 mM substrate solution in diethyl ether–methanol (95:5, v/v) was used. To this were added 16 μl of water and 2 μl of enzyme solution in 0.05 M $CaCl_2$. The reaction was allowed to proceed for 2 min at room temperature. After stopping the reaction with 3 ml of ethanol, the released fatty acids were titrated in a N_2 atmosphere with 0.01 N NaOH in methanol–H_2O (90:10, v/v) using Cresol Red as an indicator. The amount of enzyme used was adjusted to be equivalent to the activity of 0.1–0.2 μg of unmodified protein.

Protein modification reactions were carried out as follows. Iodination (Azari and Feeney, 1961) was carried out in 0.1 M borate buffer (pH 9.5) in an ice bath. Aliquots of an ice cold solution of 0.025 M iodine in 0.24 M KI were added and allowed to react for 15 min, and then the reaction mixture was dialyzed in the cold against distilled water. The extent of modification was calculated as moles of diiodotyrosine produced per mole of protein, using the method of Edelhoch (1962). The reaction with *N*-acetylimidazole (Riordan *et al.*, 1965) was carried out in 0.05 M borate buffer (pH 7.5) in the presence and absence of 4 M urea, using a 60-fold molar excess of reagent. The reaction was allowed to proceed for 90 min at room temperature, and then the excess reagents were removed by dialysis against distilled water in the cold. The extent of modification was calculated as described by Riordan *et al.* (1965). The reaction with maleic anhydride (Butler *et al.*, 1969) was carried out in an ice bath in 0.1 M borate buffer (pH 9.5.). The protein in 1 ml of buffer was treated with six 5-μl aliquots of 0.1 M maleic anhydride in anhydrous dioxane at 15-min intervals. After dialysis against distilled water, the extent of modification was determined from the change in absorbance at 250 nm (Butler *et al.*, 1969).

Oxidation with *N*-bromosuccinimide[1] was carried out in 0.01 M Tes buffer (pH 6.0). One milliliter of a protein solution, which contained 0.02 μmol of enzyme, was treated with succesive 1-μl aliquots of a 20 mM solution of NBS in the same buffer. After each addition the change in absorbance at 280 nm was measured. The extent of modification of tryptophan was determined by the method of Patchornik *et al.* (1958).

Acylation with ethoxyformic anhydride was carried out at room temperature in 0.01 M Tes–0.05 M KCl (pH 6.0) with and without the addition of 4 M urea. The reaction time and concentration of EOFA were varied. EOFA was added from a stock solution which was 100 mM in dry acetonitrile or in some instances as undiluted reagent. The extent of histidine modification was calculated from the change in absorbance at 230 nm (Melchior and Fahrney, 1970). Deacylation of histidine was achieved by incubation in 0.1 M NH_2OH (pH 7.0) for varying periods of time at room temperature.

Photooxidation was carried out in 0.02 M phosphate buffer (pH 6.5) in an ice bath in the presence of 0.01 Rose Bengal. A 150-W spot light was placed 8 cm from the sample solution and the solution was stirred vigorously. Aliquots were removed at various time periods ranging from 15 sec to 16 min. The reagents were removed by dialysis in the cold.

Reaction with iodoacetic acid was carried out, either at pH 3.0 in 0.01 M citrate buffer or at pH 6.0 in 0.01 M phosphate buffer using 0.01 M iodoacetate and a reaction time of 20 hr at room temperature. Excess reagents were removed by dialysis against distilled water in the cold.

Cation-induced spectral measurements were performed as described in the preceding paper (Wells, 1973). Ultracentrifugation was carried out as described previously (Wells, 1971b). The Sephadex G-75 column used for molecular weight determinations was calibrated with phospholipase A_2 (mol wt 30,000) and ribonuclease (Calbiochem) (mol wt 13,700).

In order to determine the number of amino groups of lysine that might be reacting with ethoxyformic anhydride the following experiment was carried out. Samples of the enzyme, amounting to approximately 0.01 μmol, were reacted with 10 mM ethoxyformic anhydride at pH 6.0 (0.01 M Tes–0.05 M KCl) for a period of time predetermined to lead to approximately 50% inactivation or 100% inactivation. The samples were diluted 20-fold with ice–water and dialyzed extensively against distilled water in the cold. Samples of the dialysate were taken for measurement of enzymatic activity and protein. The remaining sample was lyophilized in a small test tube and dissolved in 0.25 ml of 1% triethylamine in water, and then 0.5 ml of a 5% solution of FDNB in 95% ethanol was added. The reaction was allowed to proceed for 3 hr in the dark, then 0.5 ml of water was added, and the sample was extracted with diethyl ether until the ether phase was colorless. The aqueous phase was heated in a steam bath to remove residual ether and then lyophilized. The samples were then hydrolyzed in 0.5 ml of constant-boiling HCl for 24 hr in tubes sealed under vacuum. After removal of HCl, the samples were analyzed for amino acids using a Beckman Model 120C with a 10× scale expander and the accelerated method of Spackman *et al.* (1958). This procedure can readily determine 0.002 μmol of amino acid. The number of lysine residues per mol of protein was calculated relative to the amount of arginine, assuming 12 arginines per mol (Wells and Hanahan, 1969).

Results

Treatment of the enzyme with iodoacetate for 20 hr either at pH 3.0 or 6.0 caused no loss of enzymatic activity. Acetylation at pH 7.5 with *N*-acetylimidazole in the absence of urea led to the acetylation of two to three tyrosines per mole, and in the presence of 4 M urea, four to six tyrosines were acetylated. In neither case was there a significant loss of enzymatic activity. Photooxidation did not lead to any loss in enzymatic activity.

Iodination did lead to a loss of enzymatic activity as shown in Table I. It can be estimated from these data that 8–9 mol of diiodotyrosine would have to be produced, out of a possible 16, to lead to complete loss of enzymatic activity. Treatment with maleic anhydride led to a complete loss of activity. Spectral measurement indicated that 16.5 lysines (out of a possible 16.0) had reacted. Treatment at pH 3.5 for 24 hr (Butler *et al.*, 1969) restored 90% of the enzymatic activity. Although these experiments do destroy enzymatic activity, they can hardly be considered to represent the reaction of a specific group in the active site.

Somewhat more promising results were obtained using *N*-bromosuccinimide oxidation. The results of one such experiment are presented in Figure 1. This experiment was carried out at pH 6.0. The data indicated the presence of two tryptophans which react more readily with NBS than the rest. There is a good correlation between the loss in enzymatic activity and the oxidation of these two tryptophans. Protein samples which had been treated with NBS to the point where 1.9–2.2

[1] Abbreviations used are: NBS, *N*-bromosuccinimide; EOFA, ethoxyformic anhydride; FDNB, fluorodinitrobenzene; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

TABLE I: Effect of Iodination on the Activity of Phospholipase A_2.[a]

Mol of I_2Tyr/Mol of Protein	% Act. Remaining
0.0	100
0.5	98
1.0	88
2.0	75
4.7	46

[a] An ice-cold solution of the enzyme in 0.1 M borate buffer (pH 9.5) was treated with aliquots of 0.025 M I_2 in 0.24 M KI. After 15 min the solutions were dialyzed in the cold against distilled water. The extent of tyrosine modification was determined as moles of diiodotyrosine produced per mole of enzyme (Edelhoch, 1962). Aliquots of the iodinated protein were used for enzyme assays.

tryptophans had been oxidized showed the following characteristics. The molecular weight, as determined by sedimentation equilibrium studies, was 30,000, indicating that the molecules were still dimers. The samples had no detectable enzymatic activity. The peculiar solvent perturbations caused by methanol and dimethyl sulfoxide (Wells, 1971c) could not be detected. The cation-induced spectral perturbations reported in the previous paper (Wells, 1973) were no longer observed. These data suggested selective oxidation of those tryptophans whose spectral properties are influenced by cation binding (Wells, 1973) and which give rise to anomalous solvent-induced spectral perturbations (Wells, 1971c). NBS oxidation carried out in the presence of Ca^{2+} (1×10^{-3} M) or Zn^{2+} (5×10^{-5} M) gave results which were not significantly different than those reported in Figure 1.

The results of previous experiments (Wells, 1972, 1973) have been tentatively interpreted to indicate that either a lysine or a histidine might be important in the active site. Therefore a direct chemical modification of histidine was at-

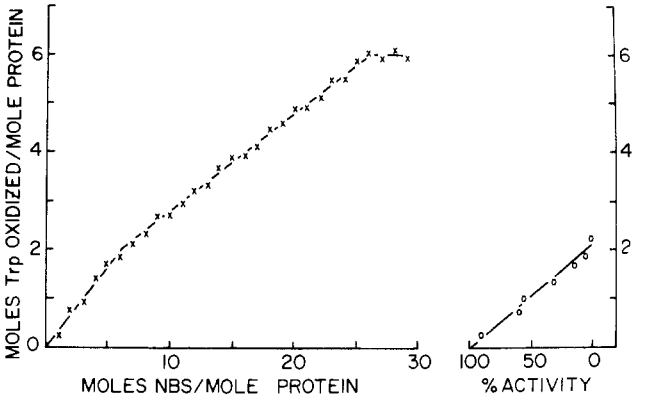

FIGURE 1: Oxidation of phospholipase A_2 by *N*-bromosuccinimide. A sample of protein in 0.01 M Tes–0.05 M KCl (pH 6.0) was treated with aliquots of a solution of NBS in the same buffer. After each addition, the moles of oxidized tryptophan were determined from the decrease in absorbance at 280 nm. Aliquots were also taken for enzyme assays. The left side of the figure shows the moles of tryptophan oxidized as a function of added NBS. The right side of the figure shows the decrease in enzymatic acitivity as a function of the moles of oxidized tryptophan.

TABLE II: Effect of Ethoxyformic Anhydride on the Activity of Phospholipase A_2.

Conditions[a]	Mol of Modified His/ Mol of Protein[b]	% Enzymatic Act. Remaining
1. 0.01 M Tes–0.05 M KCl (pH 6.0)	<0.2	<2
2. Product from step 1 after treatment with NH_2OH[c]	<0.2	<2
3. 0.01 M Tes–0.05 M KCl (pH 6.0) with 4 M urea	4.1	26
4. Product from step 3 after treatment with NH_2OH[c]	<0.2	30

[a] The reaction was carried out for 1 hr in the presence of 30 mM EOFA. Excess reagents were removed by dialysis. [b] Estimated from change in absorbance at 230 nm. [c] Treated with 0.1 M NH_2OH (pH 7.0) for 1 hr. This treatment did not affect the native enzyme.

tempted using ethoxyformic anhydride (Melchior and Fahrney, 1970). When the reaction was carried out at pH 6.0 using 30 mM EOFA, the results reported in Table II were obtained. These results indicated that EOFA could react with the protein and cause a loss of activity. However, it is unlikely that a reaction with histidine was involved since complete loss of activity occurred without apparent modification of histidine as determined by spectral measurements. In addition treatment with neutral hydroxylamine, which rapidly regenerates histidines (Melchior and Fahrney, 1970), did not restore activity. When the reaction was carried out in 4 M urea, there was also a loss of activity and in this case modification of histidine was observed. However treatment with hydroxylamine did not restore activity, although it did regenerate histidine. Control experiments showed that the native enzyme was not affected by treatment with hydroxylamine.

A more detailed study of the inhibition by EOFA is presented in Figure 2. The loss of activity is a first-order reaction with respect to both enzyme and EOFA. As shown in Figure 2, the presence of 2.5×10^{-3} M Ca^{2+} significantly reduced the rate at which the enzyme was inactivated, whereas 1×10^{-4} M Zn^{2+} significantly increased the rate of inactivation. Barium at 2.5×10^{-3} M had a slight protective effect, although much less than Ca^{2+}. In the presence of 4 M urea, which causes dissociation of the dimer (Wells, 1971b), the rate of inactivation was slowed more than 10-fold. The observation that inactivation by EOFA was first order with respect to both enzyme and EOFA was not expected since the enzyme is a dimer at pH 6.0 (Wells, 1971b,c). Previous studies (Wells, 1973) showed that there were two identical Ca^{2+} binding sites, and so it was reasonable to assume that there were two active sites per dimer. The data presented in Figure 2 could be interpreted in two ways. First the active form of the enzyme is the dimer and there was two active sites, but the reaction with EOFA is an example of "half of the site reactivity" (Levitzki *et al.*, 1971). Alternatively the form of the enzyme which reacts with EOFA and which is enzymatically active is the monomer, although the markedly reduced rate of inactivation in 4 M urea would tend to rule this out. Several experiments were carried out which show that the first alternative is most likely.

TABLE III: Residual Lysine in DFNB-Treated Phospholipase A_2.[a]

Sample	% Inactivation by EOFA	Residual Lys after FDNB[b]	100 × Residual Lys[c]/% Inactivation
1. (Control)	0.0	0.12, 0.15	
2.	55.0	0.60, 0.65	0.89
3.	90.0	1.09, 1.10	1.06

[a] Samples were treated with FDNB as detailed in the text. Acid hydrolysates were analyzed for amino acids. [b] Calculated per mole of protein (mol wt 30,000) using the arginine content of the hydrolysate and assuming 12 arginines per mole (Wells and Hanahan, 1969). Results of two analyses. [c] After correction for residual lysine in the control sample.

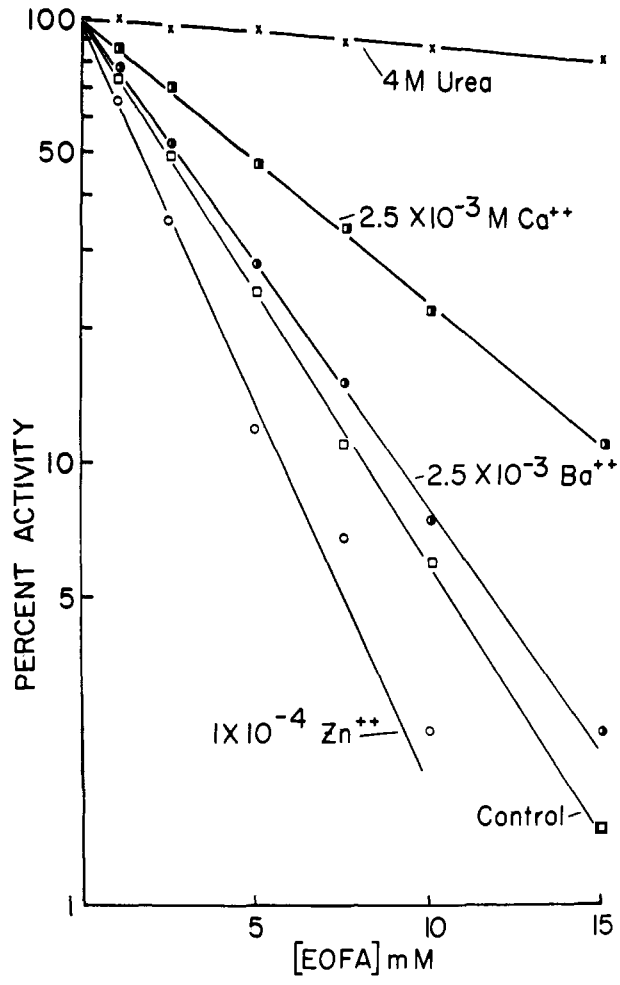

FIGURE 2: Inactivation of phospholipase A_2 by ethoxyformic anhydride. Protein samples in 0.01 M Tes–0.05 M KCl (pH 6.0) were incubated for 1 hr with the indicated amounts of EOFA. The sample labeled control contained no further additions. The other samples contained the indicated amount of the compound added. The residual activity was compared to samples that were incubated for 1 hr in the absence of EOFA.

At pH 6.0 EOFA would be expected to react either with histidine or amino groups (Melchior and Fahrney, 1970). The data presented in Table II appeared to rule out a histidine. Previous studies (Wells and Hanahan, 1969) indicated that the N-terminal amino group of the enzyme was blocked. It therefore seemed probable that EOFA was reacting with an ϵ-amino group of lysine. Experimental support for this suggestion is found in Table III. If EOFA had reacted with an amino group of lysine, then that amino group would be protected from derivatization by FDNB. Furthermore since the EOFA derivative of lysine is acid labile, there should be lysine found in an acid hydrolysates of EOFA-reacted protein after treatment with FDNB. The amount of lysine found should correlate with the extent of loss of enzymatic activity. Furthermore if EOFA reacted with one site per dimer there should be one lysine per mole (mol wt 30,000), whereas if the reaction occurred at one site per monomer there should be two lysines per mole (mol wt 30,000). The data presented in Table III show that inactivation correlates well with the assumption that inactivation by EOFA involves acylation of lysine, and that one lysine per mole has reacted. These data provide support for the "half of the sites reactivity" hypothesis.

Additional support for this hypothesis was obtained when the cation-induced spectral perturbations (Wells, 1973) of the modified protein were examined. When the reaction was carried out to complete inactivation at pH 6.0, there were still detectable cation-induced spectral effects as illustrated in Figure 3. Figure 3-1 shows the spectral perturbations caused by calcium at pH 8.0. The upper curve was obtained with native enzyme and the lower curve with the same concentration of modified protein. In the case of the modified enzyme the molar absorptivity change caused by calcium binding was one-half that seen with the native protein. The shapes of the perturbation curves were identical. Increasing the Ca^{2+} concentration did not produce any further change in the molar absorptivity change of the modified protein. Analogous results were obtained when the perturbation was caused by zinc (Figure 3-2). The conclusion is that there is one binding site left in the modified protein which reacts normally with divalent cations and one site which does not react, at least as judged by this criteria. The difference spectra of the native and modified protein is shown in Figure 3-3. The modified protein had a lower absorbance than the native protein. The spectra is consistent with a conformational change having occurred in one site of the native protein. The spectrum is identical in all important respects to that seen when the dimer is converted to monomer (Wells, 1971c) and that caused by Zn^{2+} (Wells, 1973). However this protein is still in the dimeric state (see below).

Further data to substantiate the hypothesis that EOFA was reacting with a single lysine per dimer were obtained when the products of the reaction were examined by gel filtration. A sample of enzyme which had been completely inactivated by EOFA was subjected to gel filtration on Sephadex G-75 in the same buffer in which the reaction had occurred. A single protein peak of mol wt 30,000 was observed which had no detectable enzymatic activity, Figure 4. If, however, the gel filtration were carried out in 0.01 M acetate–0.05 M KCl, at pH 5.0, two protein peaks were observed (Figure 4). The first peak, mol wt 30,000, accounted for one-half of the protein, and was enzymatically active with a specific activity equal to that of the native enzyme. The second peak, mol wt 15,000, also accounted for one-half the protein, but had no detectable enzymatic activity. If the reaction with EOFA was allowed to proceed to 50% inactivation, and the products separated by gel filtration as outlined above, the following results were obtained. At pH

TABLE IV: Flow Sheet for the Analysis of the Products Formed by Inactivation of Phospholipase A_2 by EOFA at pH 6.0.

	Phospholipase A_2							
	Treated with EOFA to 50% inactivation at pH 6.0				Treated with EOFA to 100% inactivation at pH 6.0			
	Gel filtration at pH 5.0		Gel filtration at pH 6.0		Gel filtration at pH 5.0		Gel filtration at pH 6.0	
	peak I	peak II	peak I	peak II	peak I	peak II	peak I	peak II
Molecular weight	30,000	15,000	30,000	15,000	30,000	15,000	30,000	15,000
Protein (%)	75	25	100	0	50	50	100	0
Relative specific activity	1.0	0.0	0.5	0.0	1.0	0.0	0.0	0.0

6.0 a single protein peak of mol wt 30,000 was observed which had a specific activity one-half that of the native enzyme. At pH 5.0 two protein peaks were observed. The first, mol wt 30,000, accounted for 75% of the protein and had a normal specific activity. The second peak, mol wt 15,000, accounted for 25% of the protein and had no activity. The results of these experiments are summarized in Table IV.

If the reaction with EOFA involves acylation of a single lysine per dimer, these data can be explained in the following manner. The acylation leads to a loss of enzymatic activity and also reduces the stability of the dimer. Thus at pH 5.0 some of the modified protein dissociates into subunits. One subunit is acylated, but the other is normal. Previous studies (Wells, 1971b,c) have shown that the native dimer does not dissociate to a detectable extent at pH 5.0, so the native monomers reassociate to form native, active, dimer. The modified monomers apparently cannot reassociate. After equilibrium, all the unmodified subunits have reassociated to form native dimer, and all the modified subunits are left as monomers. Further studies on factors influencing the stability of the native and modified proteins are under way.

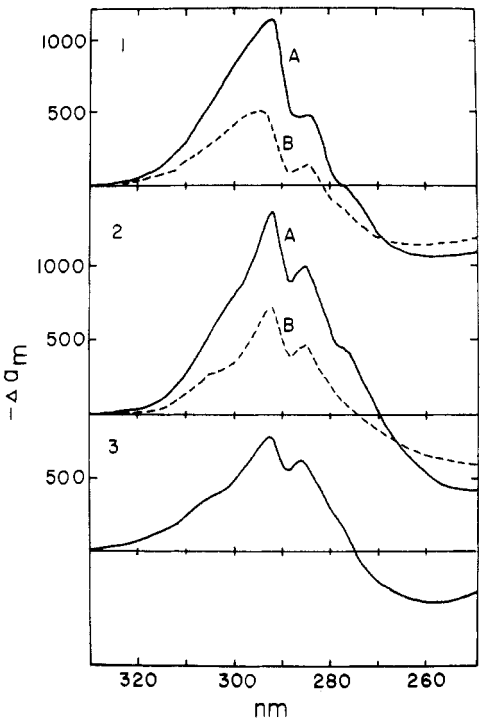

FIGURE 3: Comparison of spectral properties of phospholipase A_2 and EOFA-inactivated phospholipase A_2. All measurements were made in 0.01 M Tris–0.05 M KCl (pH 8.0). (1) Spectral perturbations caused by 1×10^{-3} M $CaCl_2$. Curve A represents the unmodified enzyme and curve B the modified enzyme. (2) Spectral perturbations caused by 5×10^{-5} M $ZnCl_2$. Curve A represents the unmodified enzyme and curve B the modified protein. (3) Difference spectrum of modified and unmodified protein. The sample cell contained the modified protein and the reference cell the unmodified protein. Protein concentration was 0.60 mg/ml.

The results presented in Figure 2 showed that Ca^{2+} protected the enzyme from inactivation of EOFA. It was therefore of interest to determine what effect the substrate might have on the inactivation by EOFA. The substrate chosen for these experiments was dihexanoyllecithin for the following reasons. It is water soluble and its critical micelle concentration has been determined to be 11 mM (Roholt and Schlamowitz, 1961). Therefore it was possible to carry out experiments where the substrate was monomeric, 10 mM, and predominantly micellar, 50 mM. Previous studies (Wells, 1972) showed that the micellar phase was a much better substrate than the monomeric form. Therefore any effect of the substrate on the inactivation by EOFA, which was related to an enzymatically relevant binding of the substrate, should be much more pronounced in the presence of the micellar form of the substrate.

The results of these experiments are presented in Table V as the first-order rate constants for inactivation of the enzyme. The results can be summarized as follows. There is a small, but probably significant, effect of Ba^{2+} as noted in Figure 2. Ca^{2+} protects the enzyme from inactivation and the protection is concentration dependent. At 5×10^{-5} M Ca^{2+}, there is still significant protection. This concentration equals the dissociation constant of Ca^{2+} from the enzyme (Wells, 1972, 1973) and indicates that the protection may be related to the enzymatically essential role of Ca^{2+}. There is a small effect of substrate although the difference between 10 and 50 mM substrate does not reflect the expected higher affinity of the enzyme for the micellar phase.

When Ba^{2+} and subsrate are both present, there was not a significant increase in protection over that observed when these compounds were present individually. In the presence of Ca^{2+} and 10 mM substrate there is a significant decrease in the rate of inactivation compared to Ca^{2+} alone. When the substrate concentration was increased to 50 mM, the rate of in-

TABLE V: Inactivation of Phospholipase A_2 by Ethoxyformic Anhydride under Various Conditions.

Conditions[a]	First-Order Rate Constant for Inactivation (hr^{-1})
1. No further additions	8.9
2. $+1 \times 10^{-3}$ M $BaCl_2$	8.4
3. $+1 \times 10^{-3}$ M $CaCl_2$	4.6
4. $+5 \times 10^{-5}$ M $CaCl_2$	7.0
5. +10 mM DiC_6[b]	8.5
6. +50 mM DiC_6	8.1
7. $+1 \times 10^{-3}$ M $BaCl_2$ and 50 mM DiC_6	7.9
8. $+1 \times 10^{-3}$ M $CaCl_2$ and 10 mM DiC_6	3.4
9. $+1 \times 10^{-3}$ M $CaCl_2$ and 50 mM DiC_6	0.8

[a] All reactions were carried out in 0.01 M Tes–0.05 M KCl (pH 6.0) in the presence of 10 mm EOFA at 25°. Samples were removed at zero time and after 15, 30, 45, and 60 min for enzyme assays. The apparent first-order rate constant was calculated from the decay of enzyme activity. [b] DiC_6 refers to dihexanoyllecithin.

activation was decreased markedly. The extent of this latter protection is greater than could be accounted for by a five-fold increase in substrate concentration. This was taken to indicate that the protection was greater because of interaction of the Ca^{2+} enzyme complex with the micellar phase. This observation strongly suggested that the protection afforded by the substrate in the presence of Ca^{2+} was related to substrate binding to the enzyme. Under the conditions of these experiments the amount of substrate hydrolyzed was less than 10%.

Discussion

The data reported in this paper support the conclusion that two tryptophans and two lysines are important to the catalytic activity of *C. adamanteus* phospholipase A_2. Since the enzyme is composed of two identical subunits (Wells, 1971b), it is assumed that each subunit contains one each of these essential residues. These data in conjunction with earlier studies (Wells, 1971a–c, 1972, 1973) provide the basis for some reasonably firm conclusions regarding the role of calcium in the catalytic process, the order of addition of calcium and substrate to the enzyme, the identification of the group involved in cation induced spectral perturbations, some factors influencing the stability of the dimeric form of the enzyme, and evidence that the dimer is the active form of the enzyme.

While it is improbable that tryptophan participates directly in the catalytic process, considerable evidence suggests that the environment of one tryptophan per subunit is intimately related to the active site. The environment of this tryptophan confers upon it certain unique spectral properties which can be used to monitor different states of the active site. (1) The cation induced spectral perturbations reported in the accompanying paper (Wells, 1973) allow one to distinquish two types of binding. One type of binding which involves alkaline earth cations gives rise to spectral changes which are interpreted as being primarily charge related. The other type of

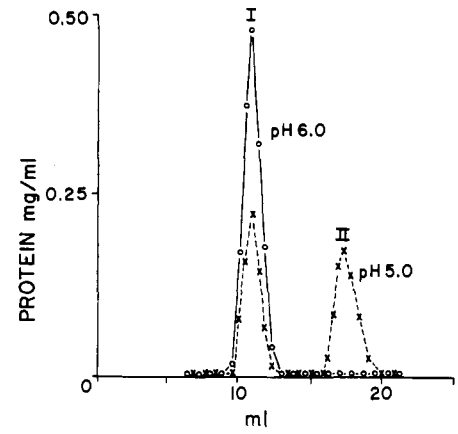

FIGURE 4: Gel filtration of EOFA-inactivated phospholipase A_2 on Sephadex G-75. (O) Gel filtration at pH 6.0. (X) Gel filtration at pH 5.0. (I) Molecular weight of 30,000; (II) molecular weight of 15,000.

binding, as exemplified by zinc, gives rise to spectral changes which indicate a conformational alteration in the binding site. As pointed out before, this difference can be utilized to understand the inhibition caused by zinc and cadmium. (2) Dissociation of the dimer leads to spectral changes (Wells, 1971c), a loss of enzymatic activity, a loss of anomalous solvent-induced spectral perturbations, and a loss of the cation spectral perturbations. This suggests that the peculiar state of the tryptophan in the active site arises from subunit interactions attendent on dimer formation. (3) Oxidation of this tryptophan leads to a loss of enzymatic activity, a loss of anomalous solvent induced spectral perturbations, and a loss of cation-related spectral changes. Although these properties would suggest that oxidation had led to dissociation, the modified protein is still a dimer. These observations lend further support to the suggested relationship between this tryptophan and the active site. Possible additional effects of NBS have not been excluded.

Studies on the inhibition of the enzyme by EOFA provide a considerable amount of information about the enzyme. The data presented in Tables II and III strongly suggest that the inhibition caused by EOFA is through acylation of one lysine per dimer. The consequences of this acylation are many fold. First, of course, is a loss of enzymatic activity. At pH 6.0 the inactivation occurs with acylation of one lysine per dimer. This conclusion is supported by the following observations. (1) One lysine per dimer is protected from reaction with FDNB. (2) The inactivated enzyme still shows cation dependent spectral changes, but the molar absorptivity change is one-half that of the native enzyme. (3) The inactivation is first order with respect to both enzyme and EOFA, suggesting a single site per dimer is reacting. (4) If the inactive dimer is dissociated at pH 5.0, one-half of the protein forms an active, native dimer. Thus the reaction with EOFA at pH 6.0 exhibits "half of the sites reactivity" (Levitzki *et al.*, 1971). The acylation of this lysine leads to alterations in the active site as determined by spectral measurements, and also to decreased stability of the dimer at low pH. These observations again emphasize the relationship between the peculiar environment of the tryptophan, the activity of the enzyme, and the dimeric form of the enzyme.

Several lines of evidence suggest that the lysine which is reactive toward EOFA is the group which is involved in the cation perturbations reported in the previous paper. In order

for the lysine to react with EOFA selectively it must have a lower pK than other lysines. The pK of the group involved in cation spectral changes was estimated as 8.9 in the absence of cations. This would be consistent with the higher reactivity toward EOFA. In accord with previous studies, the lysine would be expected to be more reactive in the presence of cations, since the pK is lowered even further. The higher reactivity of the lysine in the presence of zinc conforms with this expectation. However, the lower reactivity in the presence of calcium was not expected. If, however, the binding of calcium not only causes the deprotonation of the amino group, but also buries the NH_2, these results are understandable. The observation that acylation of the lysine abolishes the cation-induced spectral perturbations at one site in the dimer, while at the same time causing spectral changes which indicate a conformational change in the site, provides strong support for the suggestion that the lysine is the group responsible for the cation-induced spectral perturbations.

Previous kinetic studies (Wells, 1972) have been interpreted to indicate that the addition of calcium and substrate to the enzyme is ordered, with calcium adding before the substrate. Further support for this proposal is derived from the observation that substrate strongly depresses the rate of inactivation of the enzyme by EOFA only when calcium is present. That this protection of the enzyme by substrate is related to a catalytically relevant process is indicated by the observation that the micellar substrate protects the enzyme to a greater extent than the monomeric substrate. This is consistent with previous studies which showed that the micellar substrate was hydrolyzed more rapidly than the monomeric substrate (Wells, 1972).

It had previously been postulated that calcium was directly involved in substrate binding (Wells, 1972). The data presented in this and the previous paper clearly show that this is an oversimplification. The calcium-induced spectral perturbations reported in the preceding paper, taken with the observations reported here that calcium itself can protect the enzyme from inactivation by EOFA, strongly suggest that the binding of calcium causes a conformational change in the enzyme, which permits the substrate to bind. The fact that both calcium and calcium plus substrate protect the enzyme at pH 6.0, where catalytic activity is quite low, suggests that the effect of pH on the enzymatic activity is not related to binding of either of these compounds. As noted previously, barium binds to the enzyme and causes the same spectral alterations as calcium, although barium is a competitive inhibitor. Since barium does not protect the enzyme nearly as well as calcium, and since there is no protection by substrate in the presence of barium, it is suggested that barium alters the conformation of the active site in such a way that the substrate cannot bind.

Several lines of evidence suggests a very exacting conformational requirement for the active site of phospholipase A_2. With regard to cation requirements: (1) Mg^{2+} does not bind to the enzyme as judged by kinetic experiments and the lack of spectral effects of this ion; (2) Ca^{2+} and Ba^{2+} seem to bind in a similar manner as determined by spectral studies and have very similar dissociation constants. Ba^{2+} seems to prevent the substrate from binding, at least as judged from inhibition studies with EOFA; (3) zinc binding seems to alter the conformation of the active site leading to the exposure of the essential lysine and tryptophan to the solvent as indicated by spectral studies and enhanced reactivity with EOFA. In addition to cation requirements it was shown that modification of the essential lysine or tryptophan leads to complete loss of activity. As determined by spectral measurements the acylation of the essential lysine leads to a conformational change in the active site. Spectral studies support the conclusion that the active site is formed upon interaction of the subunits to form the dimer. Although the dimer can bind two cations in an apparently independent manner, the acylation of one lysine per dimer alters the subunit interaction in such a way that the other lysine cannot be acylated. Alterations in the active-site lysine leads to reduced stability to the dimer.

Within the active site a tryptophan and a lysine have been identified as being essential to the enzymatic activity. The lysine which reacts with EOFA has been tentatively identified as the group involved in cation-induced spectral perturbations and evidence has been presented supporting the suggestion that Ca^{2+} binding not only leads to deprotonation of the lysine, but also induces a conformational change which buries the NH_2 group out of contact with the solvent. Substrate binding limits solvent access to this NH_2 group even further. An NH_2 group buried in this manner might be expected to be an excellent nucleophile, which could promote base-catalyzed hydrolysis of the ester bond in the substrate. This mechanism would be consistent with the observed ^{18}O studies, the lack of exchange reactions, and the irreversibility of the reaction (Wells, 1971a). It is of course possible that the role of the essential lysine is only in controlling the conformation of the active site, and that it is not involved directly in catalysis. If the lysine must be deprotonated before the active site can be generated, and if the catalytic nucleophile only becomes active after this conformational change has occurred, it may not be possible to detect it unless the active site has been generated. This latter proposal explains the essential role of Ca^{2+} and the lysine, but leaves open the question as to the nature of the catalytic process. Studies are now under way to design active-site-directed inhibitors which will only react after the proposed conformational change has occurred. Such studies should allow distinction between the above proposed alternative mechanisms.

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Cobaltous Ion Complex of Reduced Lipoamide Dehydrogenase†

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ABSTRACT: Lipoamide dehydrogenase is a dimeric flavoenzyme with no metal requirement. Nicotinamide adenine dinucleotide reduced lipoamide dehydrogenase under anaerobic conditions at pH 10 complexes with Co(II) generating a unique absorption band at 650 nm with molar absorptivity of 980 based on flavine adenine dinucleotide (FAD^+) concentration. Optically active bands appear at 465 and 395 nm. Complex formation is stoichiometric with respect to FAD^+ and has a $pK = 8.25$. The $Co(II)_2$LipDH complex, when returned to pH 7, is stable in air with minimal native reductase activity. 1,10-Phenanthroline reverses cobalt binding at higher pH. Of the common divalent metals only cobalt exhibits these properties. This stable and specific complex appears to be the first demonstration of its kind, based on chemical reactivity. The cobalt-binding site of this enzyme is probably distinct from the arsenite- and cadmium-sensitive sites, because the diaphorase activities of the modified enzymes are quite different, and since complexation properties of the NADH-reduced enzyme are quite different from those of the dihydrolipoate-reduced enzyme, the equilibrium states of the two reduced enzymes are not the same.

The spectroscopic usefulness of cobaltous ion as a structural probe for enzymes has been documented by Latt and Vallee (1969) for carboxypeptidase, by Simpson and Vallee (1968) and Applebury and Coleman (1969) for alkaline phosphatase, by Lindskog and Nyman (1964) for carbonic anhydrase, and by Drum and Vallee (1970) for liver alcohol dehydrogenase. All these enzymes possess a site tailored to metals and require no other cofactors. As far as we know, there is no published attempt to induce a nonmetalloflavoenzyme to bind cobaltous ions uniquely and specifically and to investigate the resultant complex as a probe of active-site structure. The principal reason is that nonmetalloproteins are not expected to bind metals in a uniquely liganded fashion. Certain proteins are known to bind a spectrum of metals nonspecifically, one example being casein (Minato and Tanaka, 1955). These do not generate colored complexes and may simply be the result of salt linkages.

The affinity of cobaltous ion for amino acids, especially the imidazole group of histidine and dipeptides, is well established (Morris and Martin, 1970). Colored tetrahedral complexes form in basic solution anaerobically after deprotonization of the liganding imino nitrogen. Free flavines, particularly the semiquinone form, also possess metal binding capacity (Hemmerich *et al.*, 1965). We therefore sought to use cobalt as a structural probe for the flavoprotein lipoamide dehydrogenase. As with a few other transition metals having unfilled d shells, cobalt (d^7) possesses the ability to generate colored complexes, wherein the d–d transitions become susceptible to spectral analysis. Under appropriate conditions, cobalt can be expected to interact with either the semiquinone flavine or vicinal amino acid residues or both. The nature of the complex may be interpretable on the basis of either one of the above models. Formation of such a flavoprotein–metal complex may also shed light on the nature of metal binding in metalloflavoproteins. At this time, only iron and molybdenum are known to be essential in any metalloflavoenzyme, and the strong affinity of the free semiquinone flavine for metals is apparently lost once it is bound to a nonmetalloflavoprotein. Beinert and Hemmerich (1965) have used the loss of metal affinity for protein-bound semiquinone to suggest that the free semiquinone–metal complex is probably of no significance in metal binding in metalloflavoenzymes. They have given evidence based on epr studies for the forced interaction of Fe(III) and Ni(II) with glucose oxidase, but only with a 100-fold excess of paramagnetic ion.

We wish to present here evidence for the reaction of pig heart lipoamide dehydrogenase, a nonmetalloflavoprotein, with cobalt to form a specific complex with unique absorption and circular dichroic spectra.

Materials and Methods

Pig heart lipoamide dehydrogenase, type III, was obtained from Sigma Chemical Co., St. Louis, Mo. Beef heart lipo-

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