THE CRYSTALLIZATION AND X-RAY CHARACTERIZATION OF THE PHOSPHOLIPASE A$_2$$\beta$
OF CROTALUS ADAMANTEUS

by

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21 July 1976 Date
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Studies of Catalytic Mechanism</td>
<td>5</td>
</tr>
<tr>
<td>Monomer Dimer Structure/Function Interrelationships</td>
<td>8</td>
</tr>
<tr>
<td>Phospholipase Membrane Interactions</td>
<td>10</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Enzyme</td>
<td>13</td>
</tr>
<tr>
<td>Crystallization</td>
<td>14</td>
</tr>
<tr>
<td>Isomorphous Heavy Atom Derivatives</td>
<td>14</td>
</tr>
<tr>
<td>Initial X-Ray Work</td>
<td>14</td>
</tr>
<tr>
<td>Computing</td>
<td>15</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>Crystallization and Characterization of Crotalus Adamanteus Phospholipase A2β</td>
<td>16</td>
</tr>
<tr>
<td>Ca++ Binding Experiments</td>
<td>20</td>
</tr>
<tr>
<td>Isomorphic Derivative Preparation</td>
<td>22</td>
</tr>
<tr>
<td>X-Ray Crystallographic Data</td>
<td>22</td>
</tr>
<tr>
<td>pH Studies</td>
<td>25</td>
</tr>
<tr>
<td>Crystal Spectra</td>
<td>26</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>28</td>
</tr>
<tr>
<td>APPENDIX A: Ca++ BINDING SITE CALCULATIONS</td>
<td>35</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>37</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phospholipase A subunit site of hydrolysis</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Comparisons of initial amino acid sequence of phospholipases A subunit</td>
<td>4</td>
</tr>
<tr>
<td>3.</td>
<td>Minimal structure of phospholipase A subunit active substrate</td>
<td>6</td>
</tr>
<tr>
<td>4.</td>
<td>Possible mechanism for methanolysis of phosphatidyl choline</td>
<td>9</td>
</tr>
<tr>
<td>5.</td>
<td>Susceptibility of ester bond in phospholipid condensed phase</td>
<td>12</td>
</tr>
<tr>
<td>6.</td>
<td>Space group of <em>Crotalus Adamanteus</em> phospholipase A subunit</td>
<td>17</td>
</tr>
<tr>
<td>7.</td>
<td>Centric projections of 15° precession diffraction pictures of <em>Crotalus Adamanteus</em> phospholipase A subunit</td>
<td>19</td>
</tr>
<tr>
<td>8.</td>
<td>Okl 15° precession diffraction picture of Ca++ bound crystal</td>
<td>21</td>
</tr>
<tr>
<td>9.</td>
<td>Comparison of palladium soaked crystal and parent Okl centric projections</td>
<td>23</td>
</tr>
<tr>
<td>10.</td>
<td>Colored gold and palladium crystals formed on binding of the heavy atom</td>
<td>27</td>
</tr>
<tr>
<td>11.</td>
<td>Water soluble substrate inhibitor for phospholipase A subunit</td>
<td>30</td>
</tr>
<tr>
<td>12.</td>
<td>Discontinuous boundary formed at transition temperature</td>
<td>32</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>1. Comparisons of phospholipase A₂</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
ABSTRACT

The phospholipases A₂ are a widely distributed class of enzymes catalyzing the selective hydrolysis of 2-acyl groups from \textit{sn}-3-phosphoglycerides. Structural determination of \textit{Crotalus Adamanteus} phospholipase A₂β is in the initial stages. Crystals of the native enzyme have been grown from 60\% saturated unbuffered ammonium sulfate with 5\% by volume added dimethyl formamide. Under the above given conditions, the protein crystallizes in the orthorhombic space group \textit{P}2₁2₁2₁ with \( a = 35.49 \, \text{Å} \), \( b = 82.63 \, \text{Å} \), and \( c = 84.99 \, \text{Å} \). The crystallographic asymmetric unit contains 30,000 MW protein, corresponding to a single catalytically active dimeric molecular species per asymmetric unit. (It is of interest to note that no crystal forms have yet been observed having a monomer in the asymmetric unit.) Parent data have been collected to 2.2 Å resolution by diffractometer with less than 10\% loss of intensity in standard reflections. We currently have precession film evidence for five heavy atom derivatives: mersalyl, osmium ammonium iodide, palladium tetrachloride, gold chloride and platinum tetrachloride. Difference Patterson maps of the mersalyl, palladium tetrachloride and osmium ammonium iodide based on a 5 Å three dimensional data set have been solved giving three, five and four major sites, respectively.
CHAPTER 1

INTRODUCTION

The phospholipases A_2 are a widely distributed class of enzymes catalyzing the selective hydrolysis of 2-acyl groups from sn-3-phosphoglycerides (Fig. 1). Thus far, phospholipases A_2 (EC 3.1.1.4) have been isolated from mammalian pancreatic tissue (1), where they are synthesized as trypsin activated zymogens, the venoms of several snake species (2-5) and honeybees (6). The enzymes of all species studied share a number of properties including an absolute requirement for Ca^{++} as an enzymatic cofactor (7), remarkable heat stability (1,8), and a large number of disulfide bonds (Table 1). Indeed, comparison of the amino acid sequences of several species suggests that they are all members of a single protein family (9) (Fig. 2).

The most striking property of these enzymes is their capacity (in fact preference) to catalyze hydrolytic degradation of phospholipids in micelles, emulsions, monolayers, and membranes in general. This property has made them objects of considerable interest in studies concerning mechanisms of heterogeneous catalysis, as well as in investigations of the nature of biological membranes and the way in which proteins interact with them.
Figure 1. Phospholipase A2 site of hydrolysis.

\[ R_1 + R_2 = \text{fatty acid aliphatic side chains}, \]
\[ X = \text{ethanolamine, choline or serine}. \]
<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acids</th>
<th>Active Unit</th>
<th>Disulfide Bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. adamanteus</em></td>
<td>135</td>
<td>dimer</td>
<td>7</td>
</tr>
<tr>
<td><em>A. halysblomhoffi</em></td>
<td>126</td>
<td>monomer</td>
<td>7</td>
</tr>
<tr>
<td><em>B. gabonica</em></td>
<td>118</td>
<td>monomer</td>
<td>6</td>
</tr>
<tr>
<td>Porcine pancreas</td>
<td>123</td>
<td>monomer</td>
<td>6</td>
</tr>
<tr>
<td><em>A. mellifica</em></td>
<td>129</td>
<td>monomer</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 2. Comparisons of initial amino acid sequence of phospholipases A₂.
**Crotalus Adamanteus** phospholipase A$_2$$\alpha$: This enzyme, isolated from the venom of the Eastern diamondback rattlesnake, differs from those isolated from most other animal and insect species, in that it is functionally active only as a dimer consisting of two identical chains of MW $\sim$ 15,000. The amino acid sequence of this protein is being determined at the University of Chicago, although preliminary sequencing results indicate significant homology with other phospholipases A$_2$ (9). The catalytic and physical properties of this enzyme have been principally investigated by Michael Wells at The University of Arizona Biochemistry Department. What follows is a brief summary of the current physicochemical data relating to this enzyme.

**Studies of Catalytic Mechanism**

Van Deenan and de Haas reported the substrate specificity of the **C. Adamanteus** enzyme using whole venom and substrates in aqueous emulsions (10). They established the basic structural requirements for substrates of the enzyme: (a) The enzyme is stereospecific and hydrolyzes only the fatty acids from 1,2 diacyl sn-glycerophospholipids. (b) $\beta$-Lecithins, with only one fatty acid chain attached, are also stereospecifically hydrolyzed. From this data, these authors deduced the minimum structural requirements for an active substrate (Fig. 3). It was subsequently shown (11)
Figure 3. Minimal structure of phospholipase A$_2$ active substrate.
in a study of the degradation of 1,2 dipalmitoyl-sn-glycero-3-phosphorylcholine that enzyme catalyzed ester hydrolysis proceeded by a mechanism involving O-acyl cleavage. Attempts to isolate an acyl-enzyme intermediate by various trapping experiments were unsuccessful, however, suggesting that the enzyme did not catalyze ester hydrolysis by a mechanism analogous to that proposed for amide and ester hydrolysis by serine proteases. Further studies demonstrated an absolute requirement for Ca$^{2+}$ for activity (7). Kinetic studies carried out upon 1,2-dibutyryl-glycero-3-phosphorylcholine, a monomeric substrate for the enzyme, suggested that catalysis proceeded by a mechanism involving the ordered addition of Ca$^{2+}$ and substrate to the enzyme. Product inhibition studies with butyramide suggested an ordered product release, with the fatty acid being released prior to the lysolecithin moiety. Studies of the dependence of Vm for this reaction upon pH suggested that a group with an apparent pK near 7.6 in the enzyme-substrate complex was involved in catalysis. Modification studies (12) subsequently indicated that the protein could be totally inactivated by acylation of a single lysine residue with ethoxyformic anhydride, whereas chemical modification directed towards histidine and tyrosine had no effect upon enzymatic activity. (This result is to be contrasted with that obtained with the monomeric porcine pancreatic enzyme,
where histidine was implicated in modification studies as being an active site catalytic moiety (13). From this work it was inferred that the group exhibiting the $pK_{app}$ of 7.6 was a lysine ε-amino group held in close proximity to the bound $Ca^{++}$ ion, which rendered the lysine amino group abnormally acidic. Support for this hypothesis was presented in a model system study (14), in which it was shown that $Ca^{++}$ enhanced the rate of methanolysis of phosphatidylcholine in octylamine. The observed enhancement was proposed to arise by interaction of $Ca^{++}$ with the methanol solvent which tended to lower its $pK$. This, in turn, was proposed to facilitate proton abstraction by the adjacent amine to generate a methoxide ion which attacked the carbonyl group of the phosphatidylcholine (Fig. 4).

Monomer Dimer Structure/Function Interrelationships

As pointed out above, C. Adamanteus phospholipase A$_2$ is distinguished from the mammalian pancreatic, cobra, and bee venom enzymes by the fact that it is active only as a dimeric species (15,16), composed of identical chains of 15,000 MW. The dissociation constant for the dimer is $K_d = 2.0 \times 10^{-9}$ M at pH 6.0 and is independent of $Ca^{++}$ concentration. The most interesting aspect of the dimeric nature of this enzyme is that while there are two $Ca^{++}$ binding sites per dimer, reaction of the active enzyme with ethoxyformic
Figure 4. Possible mechanism for methanolysis of phosphatidyl choline.
anhydride gives a totally inactivated product upon acylation of a single lysine ε-amino group per dimer (12). This modification decreases the stability of the dimer (which in the native state may be reversibly dissociated at pH 3.5-4) so that it dissociates into monomers at pH 5. Unreacted monomers, however, reassociate at this pH to regenerate 50% activity from the initially totally inactivated products. This phenomena is not dissimilar to that observed for the case of iodoacetate inactivated ribonuclease dimers (17).

**Phospholipase Membrane Interactions**

Although many studies upon the kinetic mechanism have been carried out utilizing soluble monomeric substrates in order to simplify interpretation of the kinetic results, it is the capacity of phospholipases A₂ to hydrolyze phosphoglycerides in condensed phases at much higher rates than those observed for monomeric substrates which constitutes their most interesting and unique functional property. There are two related structural/functional questions which present themselves in view of the preference shown by phospholipase A₂ for hydrolysis of substrates existing in condensed surfaces phases. The first is the question of how the susceptible ester bond is made accessible to the enzymatic active site, since the current conceptions of the nature of phospholipid condensed phases would suggest that the susceptible bond is not readily accessible to enzymes
interacting at the surface of the condensed phase (Fig. 5). The second question concerns the structural origin of the observed enhancement in catalytic rate of the enzyme when acting upon aggregated substrates. Current proposals regarding the catalytic specificity of the enzyme attribute it with capabilities for partially penetrating the "membrane" surface (18,19), while the activating affect of substrate aggregation has been proposed to arise from steric restrictions imposed upon the substrate in the aggregated state, such that only the reactive end of the substrate molecule is presented to the catalytic site of the enzyme (20,21). Such proposals, while logical, remain largely speculative in the absence of any detailed structural information concerning the nature of the interaction of the enzyme with the aggregated substrate.
Figure 5. Susceptibility of ester bond in phospholipid condensed phase.

- Site of phospholipase A$_2$ ester hydrolysis.
CHAPTER 2

MATERIALS AND METHODS

The operational steps involved in the crystallographic structure determination of a protein are summarized as follows: (a) growing crystals of suitable size and diffraction properties for data collection; (b) formation of isomorphous heavy atom derivatives of native parent crystals (usually three are required); (c) parent and derivative crystal diffraction data collection; (d) calculation of derivative difference Patterson maps and their solution for heavy atom sites; (e) calculation and refinement of parent phases utilizing heavy atom substitution data; (f) calculation of Fourier electron density map and model construction of native molecule; (g) structural refinement of the obtained structure (usually involving the collection of the highest possible resolution data); and (h) difference Fourier studies on enzyme-inhibitor, or enzyme-substrate abortive complexes to assist in the elucidation of catalytic mechanism.

Enzyme

Lyophilized *C. Adamanteus* venom was obtained and phospholipase A<sub>2</sub>β was purified from this source according to the procedures of Wells and Hanahan (2).
Crystallization

A batch type crystallization was performed where 10 mg of protein were placed in a 1-ml Technicon plastic container. The protein was dissolved in 120 μl of doubly distilled H₂O and 120 μl of 60% saturated ammonium sulfate unbuffered and 5% by volume dimethyl formamide. Three months after initial set-up, crystals formed and about one month later had grown to maximum size. These crystals are suitable for X-ray diffraction to 2.0 Å. Their stability on a diffractometer is in the range of two to three weeks.

Isomorphous Heavy Atom Derivatives

A crystal taken from 60% saturated ammonium sulfate unbuffered and 5% by volume dimethyl formamide was transferred to a container with the same solution plus an added solution of ammonium sulfate and a heavy atom salt such as K₂PdCl₄. This was allowed time to bind the heavy atom. A 15° precession photograph of the zero level was taken. The films were analyzed for changes in electron density in the Miller planes. Changes from parent films would indicate the presence of heavy atom binding within the crystal.

Initial X-Ray Work

Norelco X-Ray Generator and Supper Rotation Cameras were used to collect the initial film data. Diffraction data was collected on a Picker Diffractometer. Oscillation film data was collected on the Supper Oscam.
Computing

All computing was done on the University of Arizona CDC 6400/PDP10 via a terminal in the Chemistry Building.
Numerous attempts at crystallization of phospholipase A₂ have been made. The crystal structure of mammalian pancreatic phospholipases is in progress in the laboratory of Professor Jan Drenth at Gröningen University. Work on the crystal structure of *Crotalus Atrox* (Western diamondback rattlesnake) is being done by Dr. Paul Sigler at the University of Chicago. There are five reported crystal forms for *Crotalus Adamanteus* either by our laboratory or others (22).

**Crystallization and Characterization of**

* Crotalus Adamanteus Phospholipase A₂β

The crystal form reported here has diffraction and X-ray stability properties markedly superior to those previously examined. Crystals of phospholipase A₂β were grown in 60% saturated unbuffered ammonium sulfate with 5% by volume added dimethyl formamide. These crystals are suitable for X-ray diffraction analysis to at least 2.0 Å. It crystallizes in space group \( P2_1 \bar{2} 2_1 \) (Fig. 6), \( a = 35.49 \, \text{Å} \), \( b = 82.63 \, \text{Å} \), and \( c = 84.99 \, \text{Å} \). The crystallographic asymmetric unit contains 30,000 MW protein, corresponding to a single catalytically active dimeric molecular species.
Figure 6. Space group of *Crotalus Adamanteus* phospholipase A2.
Figure 7 shows precession photographs of three centric zones of these crystals and illustrates the quality of diffraction which they exhibit.

Phospholipase $A_2$ from *Crotalus Adamanteus* exists in two chromatographically distinct forms, $\alpha$ and $\beta$ (2). The chemical, enzymatic, and physical properties are indistinguishable other than the electrophoretic mobility.

The results following are with phospholipase $A_2\beta$. Although with phospholipase $A_2\alpha$ the described crystallization protocol yields crystals that appear to be the same but are smaller in volume, no characterization of these crystals is available at this time.

Various attempts were made to improve the quality of the crystals and decrease the time for crystallization (which is approximately 3 months). Ammonium sulfate concentration, dimethyl formamide concentration, and pH were varied. The result was that as ammonium sulfate concentration increased the size of the crystals diminished, the amount of crystals increased, and the time for crystallization shortened. Similar results were noted with an increase in dimethyl formamide. A pH change within a range of $\pm .3$ pH units did not seem to affect the crystallization. Larger pH changes were not attempted.
Figure 7. Centric projections of 15° precession diffraction pictures of *Crotalus Adamanteus* phospholipase A<sub>2</sub>. 
Ca\textsuperscript{++} Binding Experiments

Since the enzyme shows a strict requirement for Ca\textsuperscript{++} for activity, and the observed Ca\textsuperscript{++} binding is independent of substrate binding ($K_d = 5 \times 10^{-5}$), it should be possible to locate the Ca\textsuperscript{++} binding sites by difference Fourier methods since the current crystals are obtained in the absence of Ca\textsuperscript{++}, and addition of Ca\textsuperscript{++} to the crystals appears to have no significant effect upon their diffracting quality (Fig. 8). We interpret this result positively, since the incorporation of two Ca\textsuperscript{++} molecules per dimer would, in the absence of large scale effects on protein conformation, be expected to have little visible effect on the diffraction intensities, although it might be argued that crystalline lattice packing has made the binding site inaccessible. The gold chloride derivative crystals are, however, very strongly colored, suggesting the formation of a specific complex of this metal ion with the protein. Such effects have been observed with a variety of metal ions (23) in the solution state, all of which are both inhibitors of Ca\textsuperscript{++} binding and enzyme activity. This would indicate the Ca\textsuperscript{++} binding site is accessible. Approximately 1/3 of the binding sites in the crystal should be occupied (see Appendix A for crystal site calculation).
Figure 8. 0kl 15° precession diffraction picture of Ca^{++} bound crystal.
**Isomorphic Derivative Preparation**

Attempts to form isomorphic derivatives of these protein crystals were carried out by soaking a few crystals in a 10- to 100-molar excess of heavy atom reagent in ammonium sulfate, buffered at various pH's. After 3 days to 2 weeks of soaking, a representative crystal was mounted in a Lindamann glass capillary in the conventional manner, and 15° precession photographs of the 0kl or h0l reciprocal lattice nets were obtained. The base plane projection photographs of potential isomorphic derivatives were compared with analogous projections obtained from the native parent protein crystal in order to ascertain whether or not a derivative had been formed (Fig. 9). After 140 attempts as previously mentioned a total of five film type derivatives were found: AuCl₄, K₂PtCl₄, mersalyl, OsNH₄I (1M KSCN), and K₂PdCl₄ (1M KSCN). At this point the last three have been collected on the diffractometer and all three have given as interpretable 5 Å resolution difference Patterson.

**X-Ray Crystallographic Data**

Parent X-ray intensity data was collected by the use of a Picker four circle automatic diffractometer.

Diffractometer data was collected by the peak scanning technique described by Wyckoff (24). Typically, counts were accumulated for nine 10-second intervals on 0.054° increments of θ. The sum of the counts over the peak during
Figure 9. Comparison of palladium soaked crystal and parent 0k1 centric projections.
the nine steps at 10-second intervals was considered proportional to the reflection intensity. Background corrections were applied from an empirically determined curve of diffuse background scatter versus 2θ for each crystal. An absorption curve (25) was also empirically determined for each crystal. Full sets of unreplicated data containing about 7,000 and 14,000 observations to 2.8 Å and 2.2 Å resolution, respectively, were taken on individual crystals. The maximum duration of X-ray exposure during data collection was about 400 h for an unreplicated 2.2 Å data set. A small set of reflections was continuously monitored at intervals throughout data collection. A 15% decrease in intensity of these reflections due to crystal damage was considered the maximum allowable.

Two sets of diffractometer data to 2.8 Å and 2.2 Å (5,477 observations of 4,959 reflections) with a weighted R of 5.4% and (12,365 observations of 10,925 reflections) with a weighted R of 13.2%. The overall scaling R-factor for combined parent data (14,577 observations of 10,714 reflections) was 7.6%.

The three heavy atom derivatives collected to date were isomorphic with the parent crystals to within 0.8 percent on all unit cell axes. All isomorphic derivative data used in refinement was collected on the automatic diffractometer. Data on mersalyl was collected to 3.5 Å. Data on
palladium tetrachloride and osmium ammonium iodide were collected to 2.8 Å and 3.0 Å respectively. Overall residual scaling and R-factors for the derivatives range from 4.8 to 6%.

**pH Studies**

Crystal pH was studied in order to enhance Ca$^{++}$ and substrate analog binding. The crystals which are grown at a pH of 5.2 were studied through the pH range 5-8. The results were the crystals are stable from pH 5-7. At pH 7.5 they are stable for a limited time of 3-7 days. Between pH 7.5 and 8.0 a change in the crystal is noted immediately; fissures appear and within minutes the crystal dissolves. This could be due to a change in ionic strength of solution leading to a greater solvent-protein interaction, favoring it over a protein-protein interaction. It might also be due to a change in protein structure between pH 7.5 and 8.0, or possibly a combination of both. The crystals are stable in the area of physiological pH and hence can be studied in this area.

Crystals were studied in solutions of polyethylene glycol (average MW 400) and H$_2$O. They appear to be stable down to a 50% by volume PEG solution. This may be important in floating-in substrate analogs that would be insoluble in the crystallization solution.
Crystal Spectra

As mentioned previously, gold chloride derivative crystals are strongly colored. This phenomenon occurs with two other heavy atoms, palladium tetrachloride and osmium ammonium iodide. A single crystal transmission spectra of the three were taken: \( \text{AuCl}_4 \) peaks at 6640 nm, \( \text{K}_2\text{PdCl}_4 \) peaks at 6400 nm and \( \text{OsNH}_4\text{I} \) peaks at 6520 nm. The change in the color of the \( \text{AuCl}_4 \) in solution from yellow to red in the crystal is probably due to a concentration effect. The other two solutions have the same wavelength peaks as the crystal. The crystal is just more intensely colored than the solution, indicating a concentration effect in the crystal. The crystal site is more likely to be a charge interactive site between the protein and the heavy atom in all cases. These heavy atoms are not known to ligand well to any of the groups in the crystal and, since the solution and crystal color have not changed, one would not expect an electronic change as in changing ligands to occur. In the case of \( \text{AuCl}_4 \), either the gold is changing to \( \text{Au}^0 \) which would cause this red color, or yellow solutions when concentrated sometimes, and up-shifting the wavelength of transmittance to the red end of the spectrum (Fig. 10).
Figure 10. Colored gold and palladium crystals formed on binding of the heavy atom.
CHAPTER 4

DISCUSSION

From the preceding brief summary of the properties of \textit{C. Adamanteus} phospholipase A\textsubscript{2}, it can be seen that the enzyme possesses several functional properties whose mechanistic elucidation would be greatly facilitated by a knowledge of the tertiary structure of the enzyme molecule. These include: (a) the precise nature of the catalytic site of the enzyme, which is of interest with regard to the apparently novel catalytic mechanism carried out by this enzyme; (b) the apparent "half-site reactivity" pattern evidenced in the inhibition studies of this enzyme which is of interest in the general context of structure/function interrelationships in multimeric enzymes; and (c) the ability of this enzyme to catalyze the hydrolytic degradation of substrates in the aggregated state.

Clearly, the structural elucidation of last enumerated property is one which might be judged potentially inaccessible by crystallographic techniques, since it is in general difficult to ascertain the mode of enzyme-substrate interaction in the absence of suitable crystallographic (e.g., enzyme-inhibitor complex) structural data. While it appears feasible to structurally study either the enzyme
complexed with suitable monomeric inhibitors (Fig. 11), or abortive enzyme-substrate complexes, it is unlikely that sufficient phospholipid could be introduced into the protein crystalline phase to approximate the biologically significant condensed phases which normally constitute the substrate for this enzyme. Nevertheless, it is not unreasonable to suppose that a knowledge of the structure of the enzyme-"substrate" complex, coupled with some reasonable assumptions concerning the structural nature of the bulk condensed phase could lead to a mechanistic understanding of the catalytic activation exhibited when this enzyme degrades aggregated substrates.

The elucidation of this general problem of the interaction of proteins with spatially extended recognition sites (either membranous or other proteins) has been one of the primary objectives of the structural investigations in our laboratory, which thus far have focussed upon interactions of reversibly bound electron transfer proteins with their physiological oxidoreductases. From this work have emerged what we believe to be some general structural principles pertaining to many biological associations made between proteins and spatially extended recognition sites. Briefly stated, the available data support the contention that ionic-strength dependent protein-protein/membrane interactions arise due to complementary charge interactions
Figure 11. Water soluble substrate inhibitor for phospholipase A₂.

\[ K_i = 0.1 \mu m \]
formed between proteins having extended binding regions composed of ionic groups of like charge only. Thus, it was proposed (26-28) that it is the inability of the bulk water to optimally solvate the like charges structurally constrained in close proximity in the binding regions, which drives the ionic association of the two oppositely charged spatially extended surfaces.

There is a growing body of data which suggests that this principle may be applicable to the interaction of phospholipase A₂ with extended substrates. These include: (a) the observed inhibition of phospholipase A₂ catalytic activity at high ionic strengths (2), and (b) the dependence of the hydrolytic rate upon the degree of protein and phospholipid hydration (20,29,30).

Further studies of interest in this connection concern the various degrees of susceptibility of aggregated substrates to degradation by phospholipases A₂ of various species as a function of the "fluidity" of the condensed surface phase. Thus, while the C. Adamanteus enzyme will degrade surface monolayers with rates independent of monolayer surface pressure (16), the monomeric porcine pancreatic enzyme will degrade aggregated substrates only at their transition temperature (31). Figure 12 shows a possible interpretation of these results in light of the proposals made above. In Figure 12A, B, and C is shown the state of
Figure 12. Discontinuous boundary formed at transition temperature.
the lipid membrane above, at, and below the membrane phase transition temperature. Consistent with current structural conceptions of the nature of this transition (32,33), the membrane below the transition temperature is relatively ordered in a regular two-dimensional array with tight hydrocarbon chain packing (12A), whereas above the transition temperature, thermal energy disorders the fatty acid side chain packing to produce a thinner, more "fluid" membrane (12C). At the transition temperature, both the crystalline and disordered phases coexist. The discontinuous boundary between the coexisting phases at the transition temperature (12B) can be seen to provide a means by which the pancreatic enzyme may gain access to the susceptible phospholipid bond, which would be inaccessible in the absence of the phase boundary at higher or lower temperatures.

Since the C. Adamanteus enzyme apparently does not require that the membrane preexist in a state where the crystalline and fluid phases coexist, it is interesting to speculate that this enzyme induces the formation of a phase boundary upon binding, consequently rendering the catalytically susceptible bond accessible to the enzyme. An extended complementary ionic charge interaction between the C. Adamanteus enzyme and the membrane phospholipid charged head groups might serve as one means of achieving the desired creation of a local phase boundary.
While it is unlikely that we can make a direct crystallographic observation of this extended interaction, it is clear that the establishment of the nature of the groups which form the surface perimeter of the catalytic site, as well as the general surface conformation of the enzyme molecule, can tell us if the proposed mechanism is structurally reasonable.
A saturated solution of CaSO₄ was made in the mother liquor of 3 M NH₄SO₄. A crystal was then placed in the solution for a period of 2 weeks. The sites the Ca⁺⁺ occupied were calculated in the following manner:

$$E + Ca^{++} \xrightarrow{K_d} E-Ca^{++}$$

$$K_d = \frac{[E][Ca^{++}]}{[E-Ca^{++}]}$$

$$K_d = 4 \times 10^{-5} \quad \text{kinetic studies}$$
$$= 5.3 \times 10^{-5} \quad \text{dialysis (equilibrium)}$$
$$= 8 \times 10^{-5} \quad \text{spectral perturbation}$$

$K_d$ used: $5 \times 10^{-5}$; Ca⁺⁺ source: CaSO₄

$$K_{sp} = [Ca^{++}][SO_4^{2-}] = 2.4 \times 10^{-5}$$

$$[SO_4^{2-}] \approx [SO_4^{2-}] \text{ from } (NH_4)_2SO_4, \text{ 3 molar } = 60\% \text{ saturated}$$

$$[Ca^{++}] = \frac{2.4 \times 10^{-5}}{3} = 8 \times 10^{-6}$$

$$\frac{[E][Ca^{++}]}{[E-Ca^{++}]} = 5.0 \times 10^{-5}$$
\[
\frac{[E]}{[E-Ca^{++}]} = \frac{5.0 \times 10^{-5}}{[Ca^{++}]} = 6.3
\]

\[ [E] = 6.3 [E-Ca^{++}] \]

Crystal volume = \(5 \times 10^2\) \(\text{A}^3\)
Volume unit cell = \(2.4 \times 10^5\) \(\text{A}^3\)
2 \(\times 10^{15}\) unit cells/crystal
4 asymmetric units/unit cell
2 binding sites/asymmetric unit
8 binding sites/unit cell
1.6 \(\times 10^{16}\) binding sites/crystal

1 ml solution = \(10^{24}\) \(\text{A}^3\)

solution contains 8 \(\times 10^{-9}\) moles/ml \(\text{Ca}^{++}\)

4.8 \(\times 10^{15}\) ions \(\text{Ca}^{++}\) in solution

1.6 \(\times 10^{16}\) sites in unit cell

1.6 \(\times 10^{16}\) sites - .48 \(\times 10^{16}\) ions = 1.12 \(\times 10^{16}\) sites open

\[ \frac{.48 \times 10^{16}}{1.6 \times 10^{16}} = \frac{1}{3.3} \] sites are occupied
REFERENCES


