Liu/Mamiya/Yasunobu, eds. Frontiers in Protein Chemistry

PHOSPHOLIPHASES A_2: STRUCTURE, FUNCTION AND EVOLUTION

ANNE RANDOLPH*, THOMAS P. SAKMAR, AND ROBERT L. HEINRIKSON
Department of Biochemistry, The University of Chicago, 920 East 58th Street, Chicago, Illinois 60637

ABSTRACT

As part of an ongoing study of crotalid venom phospholipases A_2, the amino acid sequence has been determined for the enzyme from western diamondback rattlesnake, *Crotalus atrox*. The *C. atrox* phospholipase sequence shows strong homology to that of the enzyme from the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) determined earlier in our laboratory [Heinrikson, R.L., Krueger, E.T. and Keim, P.S., J. Biol. Chem. (1977) 252, 4913]. In fact, the *C. atrox* phospholipase A_2 differs in only six amino acid substitutions from *C. adamanteus* α and in five substitutions from the β form. The fact that all of the substitutions involving ionizable residues lead to increased negative charge in *C. atrox* phospholipase A_2 accounts for the observed chromatographic and electrophoretic properties of the enzyme as contrasted with those of the *C. adamanteus* α and β forms. The single methionyl residue at position 10 in the *C. atrox* phospholipase A_2 has been alkylated at pH 2.6 by reaction with a 50-fold molar excess of iodoacetamide. The resulting derivative was separated from unmodified enzyme by ion-exchange chromatography and, like the native phospholipase, is a fully active dimer. Cleavage with cyanogen bromide at Met-10 yields two fragments, an amino-terminal decapeptide and a 112-residue C-terminal fragment which contains all of the seven disulfide bonds in the native molecule. Neither the individual fragments, nor a reconstituted mixture of the two showed enzyme activity. These findings are discussed relative to those obtained in studies of enzymes from several sources in an attempt to elucidate structure-function and evolutionary relationships among phospholipases A_2 in general.

INTRODUCTION

There exists in nature a variety of phospholipases, each specific for the hydrolysis of a particular acyl or phosphodiester bond in the phospholipid molecule. The specificities of phospholipases A_1, A_2, C and D are indicated in Fig. 1; phospholipases B, or lysophospholipases can degrade further the products of hydrolysis with enzymes A_1 or A_2.

Because of their relative abundance in mammalian pancreas and in the venoms of snakes and arthropods, phospholipases A_2 (EC 3.1.1.4) have been studied in

* Supported by predoctoral Training Grant 5T32 HL-7237 from the United States Public Health Service.

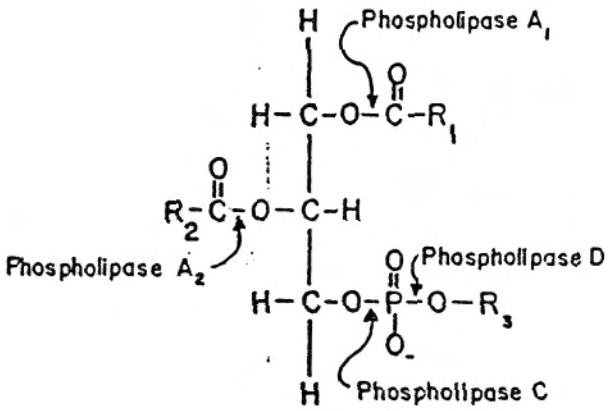

Fig. 1. Specificities of phospholipases in the cleavage of the acyl and phosphodiester bonds of a phospholipid.

greatest detail. These heat-stable, esterolytic enzymes catalyze the selective, calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides (Fig. 1). Phospholipases A_2 play a central role in lipid metabolism[1] and have been applied in probing the structural organization of phospholipids in membranes[2] and in lipoproteins.[3] Since the liberated C-2 acyl substituent may serve as a precursor for prostaglandins and thromboxanes,[4,5] phospholipases A_2 may be involved in processes of thrombosis. Moreover, since these enzymes act at solution-micelle interfaces they serve as useful models for studying heterogeneous catalysis[6] and lipid-protein interactions in general.

Phospholipases A_2 have been the subject of extensive structural investigation. Complete amino acid sequences have been reported for the enzyme from porcine,[7] equine,[8] and bovine[9] pancreas, from honeybee venom,[10,11] and from the venoms of numerous reptiles.[12-20] With the exception of the honeybee enzyme, all of these phospholipases show strong sequence homology.[20] Venom phospholipases A_2 appear to be secreted as the active enzyme, perhaps in the presence of an inhibitor.[21,22] The pancreatic enzymes, however, are secreted as very weakly active zymogens (prophospholipases) with a seven residue extension at the N-terminus that is removed during activation by trypsin. Phospholipases A_2 are relatively small (about 125 amino acid residues) and highly cross-linked with 6 to 7 disulfide bridges. Some venom enzymes have been shown to exist as stable dimers in solution.[23,24] The three-dimensional structures determined by X-ray crystallography have been reported for prophospholipase A_2 from porcine pancreas[25] and for the bovine pancreatic phospholipase A_2.[26] These structures have

been useful in interpreting the many results obtained by chemical modification of the phospholipases A_2.

In the present article, we describe our recent studies regarding the primary structural analysis and chemical modification of the venom phospholipase A_2 from *Crotalus atrox* (western diamondback rattlesnake). These findings are considered within the context of our current understanding of structure-function and evolutionary relationships among phospholipases A_2 in general.

STRUCTURAL ANALYSES

Primary structure

An earlier report from this laboratory[20] described the sequence analysis of phospholipase A_2-α from the venom of *Crotalus adamanteus* (eastern diamondback rattlesnake). The venom of this reptile contains two active forms of the enzyme, α and β, which are chromatographically and electrophoretically distinct,[27] but which are nevertheless indistinguishable in terms of specific enzyme activity, molecular weight, and amino acid composition.[19,27] Our studies of the β form of the enzyme indicate that it differs from α only in the amidation state of a single residue, Gln 117, which is Glu in phospholipase A_2-β.

Because of general interest in the crystallographic and kinetic properties of *C. atrox* venom phospholipase A_2 and our interest in homology relationships, we initiated sequence studies of this enzyme based upon strategies utilized with the *C. adamanteus* phospholipases A_2. To summarize briefly, our approach to the sequence analysis was based primarily upon automated Edman degradation of intact reduced and alkylated enzyme and of large fragments obtained therefrom by cleavage with cyanogen bromide and trypsin. Tryptic digestion was restricted to the 4 arginyl residues in the chain by prior citraconylation of lysyl amino groups in the protein derivative. A detailed description of fragment purification and analysis has been reported for *C. adamanteus* phospholipase A_2-α;[20] essentially the same results were obtained in the determination of the *C. atrox* enzyme sequence. The complete amino acid sequence of *C. atrox* venom phospholipase A_2 is given in Fig. 2. It differs from *C. adamanteus* A_2-α in six substitutions (residues 12, 14, 35, 56, 74, and 117), four of which lead to an increase in negative charge in the *C. atrox* enzyme (Lys-14 to Gly-14; Arg-35 to Leu-35; Asn-56 to Asp-56; and Gln-117 to Glu-117). Since the *C. atrox* enzyme has a Glu at 117, it differs in only 5 positions from *C. adamanteus* A_2-β. The sequence analyses of *C. atrox* and *C. adamanteus* α and β phospholipases A_2 have thus revealed charge characteristics in accord with the behavior of these enzymes during separation procedures by ion-exchange chromatography[27] and polyacrylamide gel electrophoresis.[19,27]

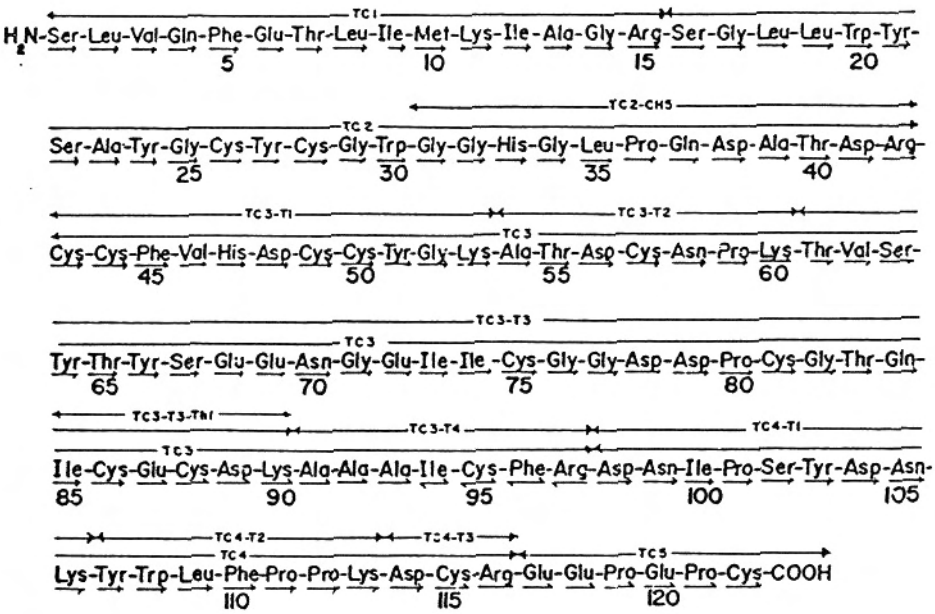

Fig. 2. The amino acid sequence of phospholipase A_2 from the venom of *C. atrox*. Peptide designations are as defined in an earlier publication concerning the sequence analysis of the enzyme for *C. adamanteus* venom.[20]

To date, complete amino acid sequences have been reported for about 20 phospholipases A_2 including those from three mammalian pancreatic sources,[7-9] and for snake venom enzymes from several elapids,[12-16] from a hydrophid,[28] from a viper, *Bitis gabonica*[17] and from three crotalids including two rattlesnake species (20 and the present work), and the Japanese water moccasin, *Agkistrodon halys blomhoffii*.[18] The comparative analysis of representative phospholipase A_2 sequences from the four categories, i.e., pancreatic, elapid (*Naja melanoleuca*), viperid (*B. gabonica*) and crotalid (*C. adamanteus* and *A. halys blomhoffii*) was the subject of relatively lengthy discourse in a recent publication from our laboratory.[20] The sequence of phospholipase A_2 from a member of the *Hydrophidae* (sea snakes), *Laticauda semifasciata*, determined recently by Tamiya et al.[28] provide a fifth category for comparison (Fig. 3) so that we now have phospholipase A_2 sequences representative of all four classes of venomous reptiles (Fig. 4). Several important points have emerged from these comparisons.

1. The sequence of these phospholipases A_2 are all very similar and are clearly homologous, with minimum base changes per codon (mbc/c) ranging from 0.05 to 0.95 (Table 1). Striking identities occur in most of the half-cystine,

TABLE 1

SEQUENCE SIMILARITY MATRIX FOR PHOSPHOLIPASES A_2 FROM VARIOUS SOURCES[a]

	C. atrox	*C. adamanteus*	*A. halys blomhoffii*	*B. gabonica*	*N. melanoleuca*	*L. semifasciata*
C. adamanteus	0.05					
A. halys blomhoffii	0.28	0.31				
B. gabonica	0.71	0.69	0.62			
N. melanoleuca	0.86	0.88	0.81	0.85		
L. semifasciata	0.83	0.81	0.77	0.72	0.53	
Bovine pancreas	0.88	0.88	0.89	0.95	0.65	0.70

[a]Sequences are aligned as shown in Fig. 3. The values shown are the minimum number of nucleotide base changes per codon for each comparison (cf. reference 63 for details).

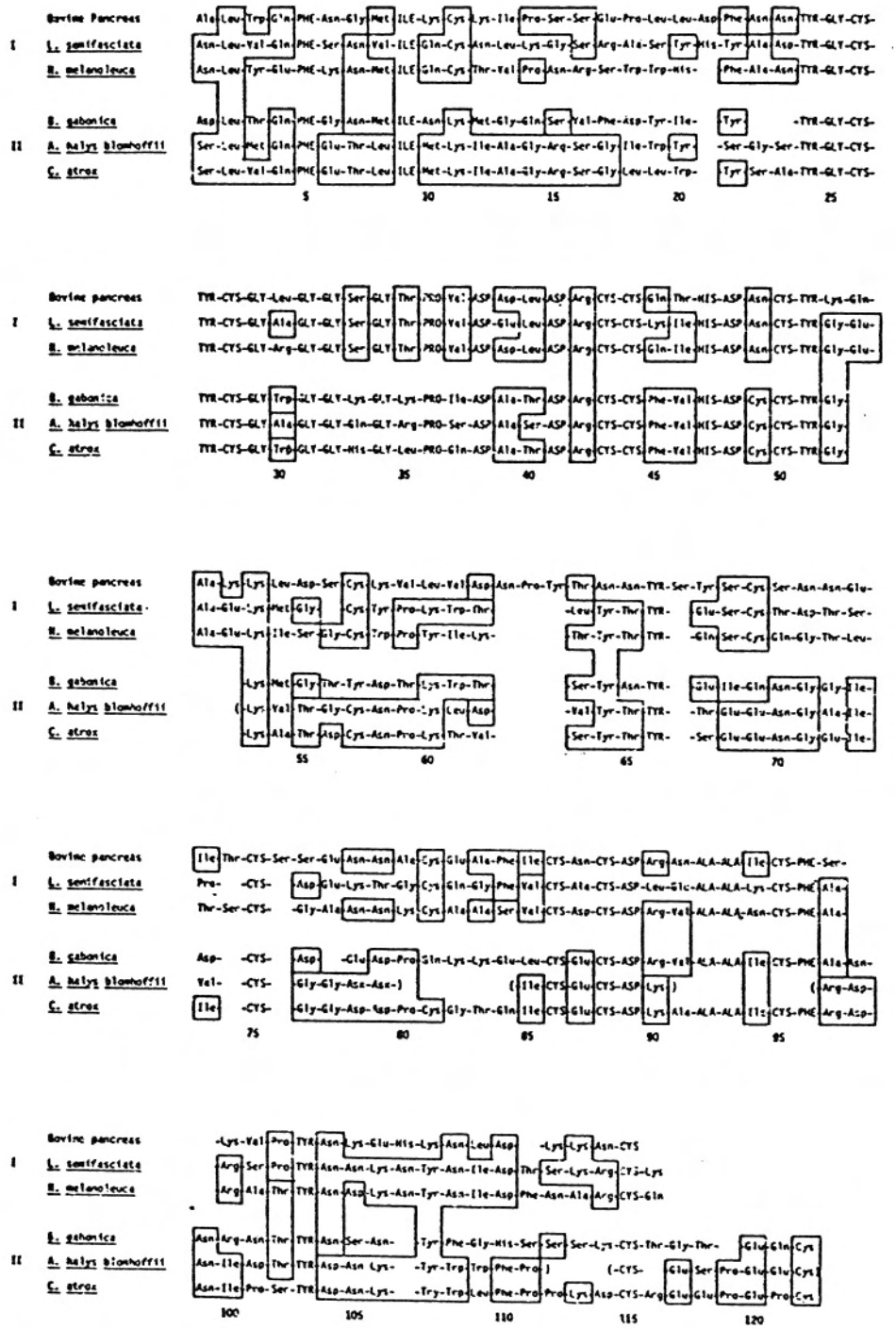
Bovine Pancreas
I L. sentifasciata
H. melanoleuca
B. gabonica
II A. halys blomhoffii
C. atrox
Bovine pancreas
I L. sentifasciata
H. melanoleuca
B. gabonica
II A. halys blomhoffii
C. atrox
Bovine pancreas
I L. sentifasciata
H. melanoleuca
B. gabonica
II A. halys blomhoffii
C. atrox
Bovine pancreas
I L. sentifasciata
H. melanoleuca
B. gabonica
II A. halys blomhoffii
C. atrox
Bovine pancreas
I L. sentifasciata
H. melanoleuca
B. gabonica
II A. halys blomhoffii
C. atrox

Fig. 3. Comparison of sequences for phospholipases A_2 from bovine pancreas and from the venoms of the elapid *N. melanoleuca* DE-II, the hydrophid, *L. semifasciata*, the viper *B. gabonica* and the crotalid *C. atrox*. Gaps are introduced to provide proper alignment of half-cystine residues and the greatest homology. Also included are segments of the sequence of phospholipase A-II from the crotalid *A. halys blomhoffii*, including sequence analysis of the intact chain performed in our laboratory. These segments are enclosed in *brackets* to designate that they are taken from regions of the molecule other than as published originally.[18] Residues invariant in all proteins are capitalized and residues at any position which occur in more than one enzyme sequence are enclosed in *boxes*.

---

and in many of the Gly and Asp positions as well as in apparently invariant lengths of sequence such as Tyr-Gly-Cys-Tyr-Cys-Gly-(——)-Gly-Gly-(——)-Gly-(——)-Pro- (residues 24-36); Cys-Cys-(——)-(——)-His-Asp-(——)-Cys-Tyr (residues 43-51); and Cys-(——)-Cys-Asp-(——)-(——)-Ala-Ala-(——)-Cys-Phe (residues 86-96). The high conservation in the latter three stretches of sequence would strongly imply some functional involvement, if not directly in catalysis, then in substrate binding or maintenance of the active site conformation. We have already commented[20] on the fact that the sequence of the phospholipase A_2 (A II) from *A. halys blomhoffii* venom reported by Samejima et al.[18] shows little homology to the structures depicted in Fig. 3. The partial structure for this enzyme given in Fig. 3 is based, in part, upon our own sequence analysis of the *A. halys blomhoffii* protein[29] and some fragments from the published sequence[18] which have been realigned on the basis of homology.

2. Perhaps even more interesting than the sequence similarities were the differences observed. These differences led to our classification[20] of phospholipases A_2 into two groups (Fig. 3). Group I comprises the enzymes from elapid and sea snake venom and pancreatic sources while crotalid and viperid venom phospholipases A_2 are classified in Group II. The basis for this classification is emphasized by the residues enclosed in boxes in Fig. 3. Moreover, Group I enzymes have a disulfide bond at 11-69 and a loop of structure between residues 52 and 53 (Fig. 3) not seen in Group II phospholipases. The latter enzymes appear to have a unique insertion of Asx-Asn at 98-99, but their most interesting feature is an extension of 6 or 7 residues at the C-terminus of the polypeptide. This extension terminates with Cys-122, a half-cystine residue unique to the Group II phospholipases. The only other residue of this kind is Cys-49 and, presumably, Cys 122 and Cys 49 are bonded in disulfide linkage. As will be mentioned later, His 47 has been implicated in catalysis and the close proximity of the C-terminal extension to this region of the enzyme might play some role in modulating the function of Group II phospholipases.

3. Finally, the results obtained from the sequence comparisons are in accord with phylogenetic relationships inferred from morphology. Hydrophids and elapids are considered to be more primitive than the vipers which have evolved a more complex venom secreting apparatus and hinged fangs. Vipers are, in turn, regarded as the progenitors of pit vipers (crotalids) which possess heat-sensitive facial pits in addition to the other features characteristic of vipers. Rattlesnakes, with their distinctive terminal appendage, represent the most highly evolved of venomous snakes. The tree presented in Fig. 4 was constructed from chemical information (Table 1) and is qualitatively the same as that published by Brattstrom,[30] who reported taxonomy of species within the *Viperidae* and *Crotalidae*. A sequence similarity matrix for phospholipases A_2 from C. *atrox* and C. *adamanteus* (pit vipers with rattles), A. *halys blomhoffii* (pit viper), B. *gabonica* (viper), N. *melanoleuca* (cobra, elapid), L. *semifasciata* sea snake, hydrophid) and bovine pancreas is presented in Table 1 based upon sequences given in Fig. 3. Values of mbc/c less than 1.00 demonstrate the strong homology among all these enzymes. The rattlesnake phospholipases are clearly very similar to one another and, as expected, bear a relationship of decreasing similarity to enzymes from, respectively, another pit viper, a viper, and phospholipases from the hydrophid, the elapid and a pancreatic source.

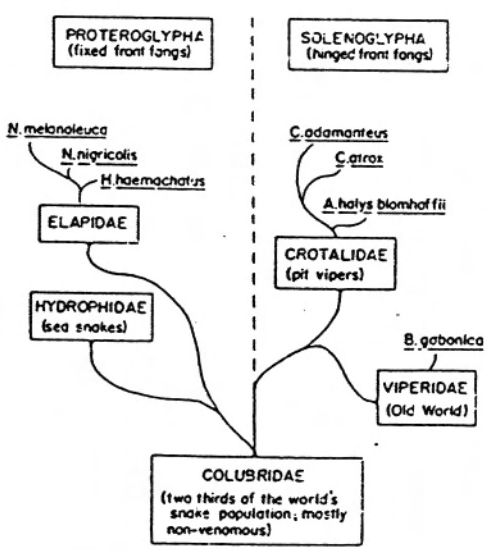

Fig. 4. Evolutionary relationships among elapid, viperid and crotalid venomous snakes as deduced from sequence analysis of venom phospholipases A_2.

Similarly, and in agreement with the taxonomy based upon morphology, one could easily classify the elapids and hydrophids together based upon the sequence similarity observed between their venom phospholipases (Fig. 4). This is all very satisfying in terms of reptilian evolution, but when one calculates the mbc/c in a comparison between the sequences of phospholipases from cobra venom and bovine pancreas, a value of 0.65 is obtained (Table 1). Therefore, one might be led to the amusing conclusion that cobras are more closely related to cows than to rattlesnakes! These findings suggest that although the elapid venom and pancreatic phospholipases A_2 have undergone parallel divergent evolution from the ancestral enzyme, their sequences have not diverged as much from the progenitor as have those of the enzymes from the vipers and crotalids. The more pronounced sequence changes in the Group II phospholipases may reflect some as yet unknown functional attribute of these enzymes. It was of interest to determine whether the rattlesnake with a Group II venom phospholipase might possess a Group I pancreatic enzyme. Analysis of rattlesnake pancreas homogenates failed to detect the presence of phospholipase A_2 activity; it may be that the digestive function is satisfied by the action of the venom enzyme subsequent to envenomation.

It should be noted at this juncture that the covalent structure of another phospholipase A_2, that from honey bee (*Apis mellifica*), has been determined.[10,11] It is an enzyme similar in size to those represented in Fig. 3 but is otherwise considerably different. The *A. mellifica* enzyme sequence shown in Fig. 5 shows strong homology to the corresponding region of the Group I phospholipases. It would seem highly unlikely, therefore, that the honey bee enzyme is an example of convergent evolution to a similar phospholipase A_2 function and we are inclined to believe that this enzyme, too, bears a divergent genetic relationship to those in Fig. 3.

Tertiary structure

Two X-ray crystallographic studies of phospholipases A_2 have been reported recently, both from the laboratory of Dr. Jan Drenth in the Netherlands. The first, that of the proenzyme from porcine pancreas,[25] was fraught with technical difficulties related to the quality of the crystals and diffraction pattern and the fact that the rather poor map was interpreted relative to a covalent structure which has since been revised.[31] More recently, the three-dimensional structure of the active enzyme from bovine pancreas has been reported as determined at 2.4 Å resolution.[26] Stereo diagrams of two views of the molecule are given in Figs. 6 and 7. It should be pointed out that the disulfide bond

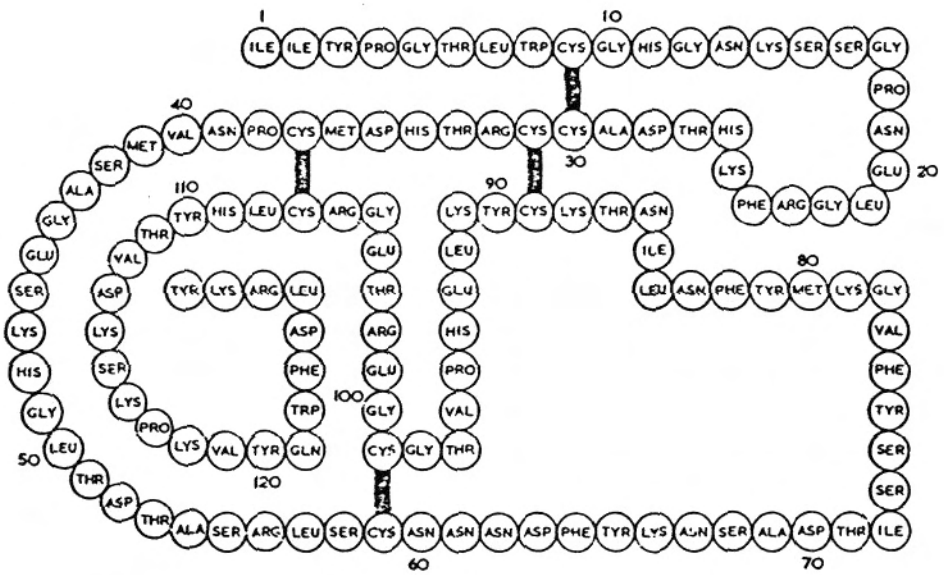

Fig. 5. The amino acid sequence of honey bee venom phospholipase A_2 showing the positions of four disulfide bridges. Taken from Shipolini et al.[11] with permission.

assignments in proteins with such a high degree of cross-linking are extremely difficult to determine chemically. Certainly one of the important contributions of the bovine enzyme X-ray analysis was the placement of all seven disulfide bridges in the molecule.

The bovine pancreatic phospholipase molecule is composed of about 50% α-helix (A), followed by a random coil conformation from 14 to 40. One of the two long antiparallel α-helices (C) extends from 40 to 58 and the other (E) comprises residues 90 to 108. These form the backbone of the molecule and could be quite rigid relative to one another due to their attachment by two disulfide bridges. Antiparallel ß strands are found between residues 74 to 78 and 80 to 85. It is noteworthy that the disulfide bond between Cys 11 and Cys 77 (11 to 69 in Fig. 3) is missing in Group II phospholipases. A salt bridge may serve this function in the crotalid enzymes since 11 is a Lys and 69 is a Glu (Fig. 3). It also would appear that the Group II phospholipase C-terminal extension of 6 to 7 residues making disulfide bond connection with the half-cystine at 49 (numbering in Fig. 3) could be easily accommodated. It will be of interest in this regard to examine the tertiary structure of *C. atrox* phospholipase A_2 currently under analysis in the laboratory of Dr. Paul Sigler at the University of

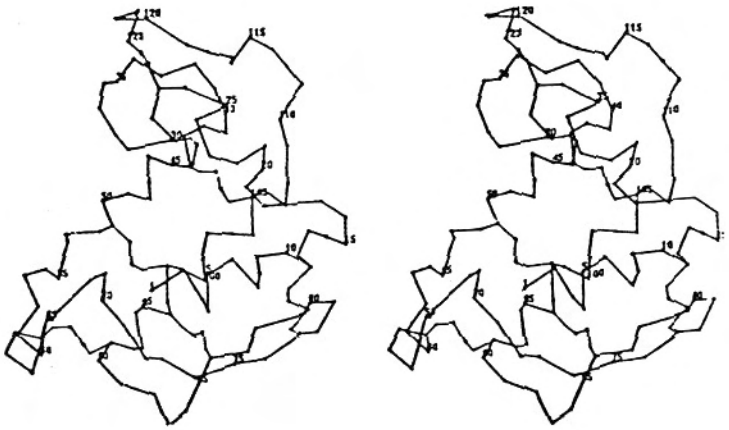

Fig. 6. Stereo diagram showing the $^{\infty}$C-atoms and disulfide bridges of the bovine pancreatic phospholipase A_2 molecule. Reproduced from Dijkstra et al.,[26] with permission.

Chicago. Unlike the pancreatic phospholipases A_2 which are monomeric, the rattlesnake venom enzymes appear to be isolated and functional as dimers and, in the case of the C. *adamanteus* enzyme, the dimer has a $K_d = 2.0 \times 10^{-9}$ M.[24] The structural analysis of C. *atrox* phospholipase A_2 at 5 Å resolution reveals a dimer in the asymmetric unit[32] so it would appear that the chemically identical monomer chains do not exist in identical conformations in the dimer crystal. The question as to whether phospholipases are functional as monomers or dimers (or both) will be addressed in a later section of this paper.

With this background of structural information relative to the sequence and conformation of the phospholipases A_2 we will proceed to examine and interpret results accumulated by other lines of investigation concerning the function of these enzymes. Implicit in these discussions is the assumption that the enzymes represented in Fig. 3 share a similar tertiary structure. Indeed, as pointed out by Dijkstra et al.[26] it is easy to accommodate the sequences of homologous phospholipases A_2 into the bovine enzyme structure since the positions of insertions and deletions are always at the enzyme surface.

Fig. 7. Stereo diagram showing C^{α}-carbon atoms of bovine pancreatic phospholipase A_2. The molecule is rotated by 90° about the vertical axis with respect to Fig. 6. Reproduced from Dijkstra et al.,[26] with permission.

STUDIES OF PHOSPHOLIPASE A_2 FUNCTION

Although the catalytic mechanism of phospholipase A_2 hydrolysis remains to be elucidated, some information is available relative to substrate specificity, the nature of the catalytic site, and a region of the molecule that appears to be involved with the recognition of organized lipid-water interfaces.[33] It might at first seem surprising that with so much detailed structural information in hand for the phospholipases A_2, we have only a rudimentary understanding of their mechanism of action. If one considers the well-characterized family of pancreatic serine proteases, however, it will be recalled that the essential features of the mechanism had been correctly deduced by kinetic analysis, prior to determination of the covalent or tertiary structures. In the case of the phospholipases A_2, kinetic studies have been hampered by the fact that the water-soluble enzymes are most efficient in the cleavage of substrates that are insoluble in aqueous media. The enzyme assays employed in many laboratories either do not lend themselves readily to kinetic interpretation or are difficult to manipulate experimentally. Chemical modification studies of phospholipases

A_2 must be interpreted with special care. Loss of activity attending modification of a particular functionality in any given enzyme could be due to the usual reasons, e.g., loss of the ability to bind or cleave the phospholipid substrate or structural alterations in native conformation unrelated to the substrate catalytic or binding sites (hereafter referred to as the active site). However, phospholipases A_2 possess, in addition, some means of recognizing, and modulating their catalytic efficiency with respect to, organized lipid-water interfaces. The residues or mechanisms involved in this interaction must be taken into account when assessing the results of any functional studies of phospholipases A_2. Nevertheless, kinetic analysis, chemical modification studies, comparative sequence analysis and various approaches based upon spectroscopic methods have provided results that can be considered relative to the structural information given in the section on structural analysis.

Substrate requirements

All naturally occurring glycerophosphatides are substrates for phospholipases A_2, regardless of the nature of the polar headgroup (R_3 in Fig. 1). These enzymes may vary considerably, however, in their preference for particular substrates depending upon whether the polar headgroup is choline, ethanolamine or serine. One of the requirements of substrates is that the acyl bond cleaved be on the carbon atom of the glycerol backbone that is adjacent to the carbon bearing the phosphate (or electronegative) group. For example, substrates with the fatty acyl substituents at positions 1 and 3, and the phosphodiester at position 2 are readily cleaved by phospholipases A_2 to yield a 3-acyl-2-sn-phosphoglyceride and fatty acid.[33] Thus, specificity is not necessarily displayed toward ester bonds with secondary alcohols. Phospholipases A_2 are highly stereospecific for 3-sn-phosphoglycerides (e.g., L-lecithins); the stereoisomeric 1-sn analogs (D-lecithins) are pure competitive inhibitors which bind with equal affinity to the enzyme but are not hydrolyzed. The electronegative group may be phosphate or sulfate[34] but it must possess at least one acidic hydroxyl group ionization. Therefore, a negative charge in the polar headgroup is an essential requirement of phospholipases A_2 substrates. Other pure competitive inhibitors in addition to D-lecithins include lysolecithin and L-lecithins in which the R_2 (Fig. 1) ester bond is replaced by an amide or ether linkage or in which R_2 itself contains bulky β substituents.

Phospholipase A_2--interface interaction

Most naturally occurring phosphoglycerides form emulsions composed of particles of varying size because of the presence of two aliphatic side chains of

14 to 18 carbon atoms. However, phosphoglycerides with shorter sidechains are distinctly soluble due to a reduced hydrophobicity. They yield monomers at low concentrations and micelles at higher concentrations exceeding the critical micelle concentration (CMC). One of the dramatic properties of phospholipases A_2 that is shared with pancreatic lipases and perhaps other enzymes that function in heterogeneous catalysis is that although they are somewhat active toward monomeric substrates, an exponential increase in activity is observed as the substrate reaches and exceeds its CMC. This phenomenon is exemplified by the curves given in Fig. 8[35] which describe the substrate concentration dependency of the rate of hydrolysis of 1,2-diheptanoyl-sn-glycero-3-phosphorylcholine by porcine pancreatic phospholipase A_2 (a) and its zymogen (b). The reactions appear to obey normal Michaelis-Menten kinetics in the concentration range below the CMC, indicating hydrolysis of monomers at low V_{max}. However, as the substrate aggregates to form micelles, the rate increases exponentially to a V_{max} 3-4 orders of magnitude higher than that observed in the monomer range. The zymogen, although moderately active, does not appear to recognize this structural transition in the substrate. A number of recent studies have attempted to explain "interface activation", and to determine whether it results from an enhanced reactivity of the substrate upon aggregation, or from an activation of the enzyme by a lipid-water interface.

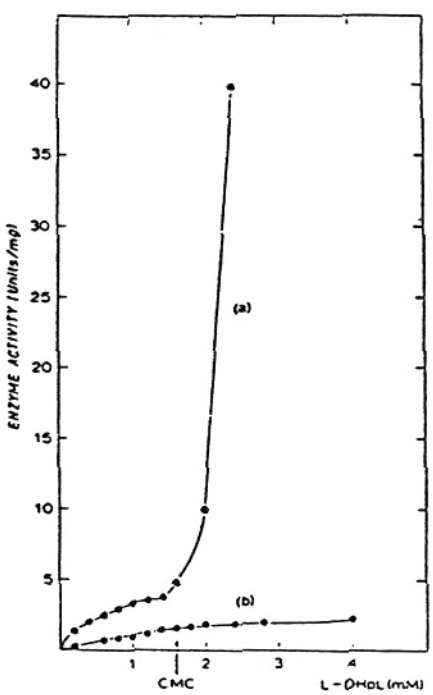

Fig. 8. Concentration dependency of rate of hydrolysis of 1,2-diheptanoyl-sn-glycero-3-phosphorylcholine (L-DHpL) by porcine pancreatic phospholipase A_2 (a) and its zymogen (b). Taken from Pieterson et al.[35] with permission.

Enhanced substrate reactivity

Several hypotheses have been proposed which implicate enhanced substrate reactivity as an explanation for interface activation. According to Brockerhoff and Jansen,[36] the hydration state of the substrate is a key factor regulating the rate of hydrolysis of lipolytic enzymes. The more soluble the substrate, the thicker the water shell surrounding the ester groups, and the lower the enzymatic activity. For the case of pancreatic lipase, Brockman et al.[37] showed that hydrolysis of a soluble substrate was increased 10^3-fold by the addition of siliconized glass beads. They proposed that the high local concentration of substrate molecules near the active sites of adsorbed enzyme molecules could account for interface activation. This hypothesis would imply that V_{max} for the hydrolysis of a monomeric substrate should be identical to that of the same substrate in aggregated form. However, the V_{max} for aggregated substrates has been shown to be higher than that for monomers with lipase[38] and phospholipase[35] from porcine pancreas. Furthermore, Chapus et al.[39] showed that deacylation of the monoacyl derivative of lipase, an intermediate in the catalytic mechanism, was accelerated by adsorption to siliconized glass beads in the absence of substrate. They concluded that, in addition to the substrate concentration effect proposed by Brockman et al.,[37] the catalytic properties of the enzyme were enhanced upon adsorption.

Enhanced catalytic activity--interfacial recognition site

A model emphasizing the regulation of enzyme catalysis by lipid water interfaces has been proposed by Dr. Gerard de Haas and coworkers at the State University of Utrecht in The Netherlands.[33] Their interfacial recognition site (IRS) hypothesis is based upon studies of pancreatic phospholipases A_2 and their zymogens. This system is nicely suited for chemical modification studies because reactions can be carried out on the zymogen and the functional consequences can be assessed prior to and following activation by tryptic cleavage of the Arg_7-Ala_8 bond in the zymogen. The IRS is defined as an exposed three-dimensional region of the enzyme involved in the formation of the enzyme-interface complex. This site is lacking in the zymogen and is induced following activation by trypsin. It is composed of residues 1-8 in the active enzyme, i.e., [Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-] and Tyr-69 (the Tyr preceding position 63 in Fig. 3), and is assumed to be topographically and functionally distinct from the active site. It is proposed that this region penetrates lipid water interfaces and anchors the enzyme. This process is facilitated by negative charges in the interface.

The IRS hypothesis is based upon a number of elegant chemical modification

studies. The importance of the α-amino group of Ala-1 has been indicated by the pH-dependency of the enzyme-micelle interaction. This interaction is controlled in the absence of Ca^{++} by a group of pK = 8.4 identified as the $\alpha\text{-}NH_3^+$.[40] As the Ca^{++} ion concentration is increased, both the pK of the $\alpha\text{-}NH_3^+$ group and the pH above which the enzyme fails to bind to the interface appear to shift from 8.4 to 9.3. Slotboom et al.[41] attribute this effect to Ca^{++} binding to a low affinity site close to Trp-3. Pieterson et al.[35] proposed that induction of the IRS was consequent to the formation of a stabilizing salt bridge between the newly liberated $\alpha\text{-}NH_3^+$ group of Ala-1 and a buried carboxylate group. Several specific chemical alterations in the N-terminal region of porcine pancreatic phospholipase A_2 have been described by Slotboom and de Haas[42] all of which point to the importance of maintaining the spatial integrity of the charge on the $\alpha\text{-}NH_3^+$. Treatment of the proenzyme with methylacetimidate gave a fully ε-amidinated product which, upon hydrolysis with trypsin, yielded *active* ε-amidinated phospholipase A_2 (AMPA). This derivative, AMPA, was then subjected to numerous chemical alterations in structure. Derivatives of AMPA in which Ala-1 is replaced by Gly or β-Ala or in which Trp-3 is replaced by Phe are similar to AMPA in enzymatic activity and show enhanced rates in the presence of micellar substrate. Chain shortening or elongation at the N terminus, blocking the $\alpha\text{-}NH_3^+$ group of Ala-1, and replacing L-Ala-1 with D-Ala all yield proteins which no longer show activity towards micellar substrates. Des-Ala-AMPA, Ala-Ala-AMPA, and D-Ala-AMPA are, however, active to varying degrees on substrate monolayers[40] which allow a change in the lipid packing density, but they show a weaker capacity for interaction with the interface than AMPA. The zymogen and the phospholipase A_2 derivatives with a blocked $\alpha\text{-}NH_3^+$ group are unable to hydrolyze monolayers even at very low surface pressures.[33] Specific iodination of Tyr-69[43] produces a protein that can hydrolyze monolayers at high surface pressures where native phospholipase is no longer active.[44] Thus, modifications of residues in the so-called IRS can enhance, diminish, or completely abolish the enzyme's ability to interact with interfaces.

The functional importance of the N terminal region has also been demonstrated in studies of a Group II snake venom enzyme. Recently Huang and Law[45] have described the synthesis of a photolabile phosphatidylethanolamine (PE) analog and its use in the inactivation of *C. atrox* phospholipase A_2. Irradiation of the enzyme in the presence of the photolabile PE analog of the pure L-configuration resulted in a modification restricted primarily to the N-terminus and subsequent analysis of the derivative showed that modification was within the first 10 residues. If racemic reagent was employed, another site of reaction was observed in addition to that described above, this being in the vicinity of the

active site His-47. Thus it would appear, in agreement with studies mentioned earlier, that the D-reagent can bind, albeit nonproductively, to a region near the active site. The difference in site modified may reflect this nonproductive binding.

The methionyl residue at position 8 of the pancreatic enzyme appears to be the limit of the IRS as defined thus far in the N-terminal region. Carboxymethylation of this residue leads to complete inactivation of the enzyme towards both monomer and aggregated substrate.[46] Spectroscopic studies indicate that the derivative still binds Ca^{++} and monomeric substrate with affinities equal to those of native enzyme. It is appropriate to consider here experiments carried out in our laboratory that were designed to evaluate the possible functional involvement of the single Met-10 in *C. atrox* phospholipase A_2. Carboxamidomethylation of this residue was accomplished by reaction of the native enzyme (8×10^{-4}M) with a 50-fold molar excess of iodoacetamide in 1% acetic acid, pH 2.6, for 24 to 48 hours. The alkylated derivative was purified by ion-exchange chromatography and was shown to be fully active both in bulk phase and monolayer assay systems. Thus, conversion of the methionyl side chain at 10 to a positively charged sulfonium derivative has no apparent effect upon enzyme activity. Reactions with iodoacetate, in agreement with the findings of Wells[47] gave no loss of activity during alkylation at pH 6.0 or 8.5. Incorporation of reagent was documented, however, and it would appear that Met-10 is the primary site of reaction with these reagents over the whole pH range.

A further study undertaken in our laboratory involving Met-10 concerned the isolation and assay of fragments generated by cleavage of the *C. atrox* enzyme with cyanogen bromide. Both the N-terminal decapeptide and the C-terminal fragment of 112 residues were totally inactive in monolayer assays and activity was not regenerated in mixtures of the separated fragments. Gel filtration of the C-terminal fragment in the concentration range 10^{-5}–10^{-6}M indicated that it was monomeric and no longer possessed the dimeric character of the native enzyme. Since the disulfide bridges and, presumably most of the native structure of the enzyme are preserved in the C-terminal fragment, one must ascribe some functional role to the N-terminal region of the *C. atrox* phospholipase A_2. It is not clear from the X-ray model (Figs. 6 and 7) which aspect of the enzyme function would be most likely impaired by removal of the N-terminal 10 residues. The lack of activity of the C-terminus could be due solely to its inability to dimerize since it has been postulated that *C. atrox* is only active as a dimer;[24] alternatively, this lack of activity could be attributed to the loss of the N-terminal part of the IRS. Volwerk[48] speculates that the hydrophobic nature of the IRS may be responsible for the dimerization of the snake venom enzymes.

A complete model explaining the action of lipolytic enzymes must account for three main steps in heterogeneous catalysis: 1) the binding or adsorption of the enzyme to the interface, 2) interfacial activation or the ability of the enzyme to hydrolyze aggregated substrates faster than monomeric substrates, and 3) catalysis proper. The IRS hypothesis for pancreatic phospholipase addresses the first two steps. De Haas et al.[3] assume that the IRS is a relatively hydrophobic domain at the surface of the enzyme which is stabilized by an internal salt bridge involving the amino terminal group. However, the X-ray crystallographic analysis of the bovine pancreatic phospholipase[26] failed to discern such a salt bridge. Rather, it was shown that the α-NH_3^+ group of Ala-1 is located within H-bond distance of a water molecule possibly liganded to the active site calcium ion. Moreover, the actual penetration and depenetration of a lipid bilayer by the enzyme would be likely to constitute an energetically unfavorable situation. The IRS hypothesis also attributes interfacial activation to a conformational change after penetration of the IRS domain. Any conformational change in the enzyme resulting from interaction with an interface which enhances catalytic efficiency by 3-4 orders of magnitude must involve the active site proper. The IRS, rather than a distinct topographical region, may be a portion of the active site. Such an interpretation would appear to be consistent with the X-ray crystallographic model. Moreover, interfacial activation is probably a combination of effects on the enzyme and on the substrate. In any case, several lines of evidence point to the functional involvement of the N-terminal region in phospholipase catalyzed hydrolysis, and this continues to be an important focus for future research concerning the enzyme mechanism.

Half-site reactivity and the functional dimer hypothesis

In a study of the effects of a variety of reagents toward the activity of *C. adamanteus* phospholipase A_2, Wells[47] found, among other things, that total loss of activity was observed upon modification of one amino group per dimer. This observation was the first indication of a possible "half-site" reactivity associated with phospholipases A_2, and was not so surprising, perhaps, in view of the fact that under the experimental conditions employed in the modification, the enzyme exists as a dimer. Moreover, there is kinetic evidence that the rattlesnake enzymes are functional *only* as dimers.[24] A more recent case of half site reactivity has been reported by Roberts et al.[49] for the phospholipase A_2 from the venom of a cobra (*Naja naja naja*). In this instance, nearly complete inactivation by p-bromphenacyl bromide was accomplished following the modification of 0.5 residues of the lone histidine (presumably His-47) per monomer. Although the *N. naja naja* enzyme can dimerize at high concentrations, it is

monomeric under the conditions employed for alkylation and assay. Therefore, if the half-site reactivity is correct, the formation of an asymmetric dimer with nonequivalent histidines must be induced not only by substrate, but by the reagent p-bromophenacyl bromide as well. Moreover, since no activity is regenerated following dilution of the inactive enzyme to monomer concentrations prior to assay, one must assume that unmodified monomers have a much greater affinity for alkylated monomers than for themselves. In the model put forth by Roberts et al.[50] one phospholipase monomer binds Ca^{++}, and then to a phospholipid at the interface. This binding causes a conformational change that leads to dimerization to form the asymmetric dimer with one catalytic subunit. If this would be generally true for all phospholipases, then it would constitute an alternative to the IRS hypothesis to explain the enzyme-interface interaction. A recent publication by Zhelkovskii et al.[51] reports that inactivation of the venom phospholipase from *N. naja oxiana* results from modification of 0.5 mole of aspartic acid per monomer chain, a finding that would serve to reinforce the half-site reactivity model. However, it must be stressed that the half-site reactivity reported for the *N. naja naja* phospholipase A_2 has not been observed for other Group I enzymes including the pancreatic phospholipases[52] (always seen as monomers) and two elapid venom enzymes, notexin, from *N. scutatus scutatus*[14] and the phospholipase from *N. nigricollis*,[53] all of which were inactivated by p-bromophenacyl bromide only when His-48 was *totally* alkylated. Similar results have been reported for the Group II phospholipase A_2 from *B. gabonica*.[54] At present, therefore, the question as to whether phospholipases A_2 are generally, or necessarily, active as monomers or dimers remains unresolved and the case for the dimer hypothesis rests upon evidence from studies of the *N. naja naja*[49,50] and crotalid phospholipases A_2.[23,24,47] The enzyme from *C. atrox* is dimeric in solution at concentrations as low as 10^{-10}M, is apparently active only as a dimer,[24] and crystallizes as a dimer in the asymmetric unit.[32] The complete tertiary structural analysis of this protein may reveal some important insights regarding the possible function of phospholipases A_2 as dimers.

The active site

Initially, it was believed by many that the phospholipases A_2 might be typical serine esterases and that their mechanism would, therefore, involve formation of an intermediate acyl enzyme. Although O-acyl cleavage during the course of hydrolysis has been documented by Wells,[55] no evidence for an acyl enzyme intermediate could be demonstrated. Organophosphorus compounds have generally been found to have no inhibitory effect on phospholipases A_2[47] and the observation that diisopropylphosphofluoridate inhibits the *C. atrox* venom enzyme[56] must be

considered highly questionable. One of the strongest arguments against the direct participation of a serine in catalysis is based upon the fact that comparative analysis of the sequences of 20 phospholipases A_2 does not reveal the existence of an invariant serine residue (Fig. 3).

Histidine is always a likely candidate as a component of the machinery involved in hydrolytic catalysis and His-47 (Fig. 3) together with Asp-48 constitutes an invariant couplet present in *all* phospholipases A_2 sequenced to date, including that of the disparate honeybee enzyme (Fig. 5). In fact, alkylation of His-47 by p-bromophenacyl bromide leads to inactivation of phospholipases A_2. These findings, first reported by Volwerk et al.[52] in studies of porcine phospholipase A_2 and its zymogen, have been corroborated in studies of Group I phospholipases A_2 from *N. scutatus scutatus*[14] and *N. nigricollis*[53] venoms, and of a Group II enzyme from *B. gabonica*.[54] Thus it is clear that the C-terminal extension on the Group II phospholipases does not necessarily exert an inhibitory influence on the modification of His-47 by p-bromphenacyl bromide as we speculated earlier.[20] Since neither iodoacetate nor iodoacetamide reacts with His-47 it would seem that the modification by the analogous p-bromophenacyl bromide is facilitated in some way, perhaps by binding in a hydrophobic pocket. Both Ca^{++} ions and competitive inhibitors such as D-lecithin protect both the porcine enzyme and its zymogen from inactivation. These findings from the work of Volwerk, et al.[52] suggest that the molecular architectures of the porcine phospholipase A_2 and its zymogen are essentially the same and that the active site is largely preexistant within the zymogen.

Many chemical modification studies have been undertaken in attempts to assess the possible functional involvement of side chains other than that of His-47. The possible involvement of Trp-30 (numbering in Fig. 3) in the active site of *B. gabonica* was inferred from its selective modification during inactivation by N-bromosuccinimide.[57] Substrate protection studies suggested that Trp-30 is involved in substrate binding. We earlier[20] questioned this interpretation since position 30 is the only variable residue within a highly invariant sequence in phospholipases A_2 (residues 24 to 32, Fig. 3). Our suggestion that inactivation could have been due to a structural perturbation within this highly conserved sequence appears to have been borne out by X-ray crystallographic analysis[26] which indicates binding of the essential Ca^{++} to the peptide backbone in this region.

Comparative sequence analysis provides another approach to the identification of residues that are essential for the proper structure and function of proteins. When the number of sequences compared is large, as is the case for the phospholipases A_2, conservation of residues at particular positions

constitutes strong evidence for some kind of functional requirement and serves as a basis for testing by chemical modification. Inspection of invariant residues among the nearly 20 phospholipase A_2 sequences determined to date (Fig. 3) reveals two hydrophobic sidechains within the proposed IRS (Phe-4 and Ile-9), Pro-36, and numerous residues of tyrosine (positions 24, 27, 51, 66, and 103), glycine (residues 25, 29, 31, 32, and 34), aspartate (residues 38, 41, 48, and 89) and half-cystine (positions 26, 28, 43, 44, 50, 75, 86, 88, 95 and 115). It was mentioned earlier that disulfide assignments were established largely by crystallographic analysis[26] and it might be useful here to note the pairings relative to the alignments in Fig. 3: 11-69, 26-115, 28-44, 43-95, 49-122, 50-88, 57-81, and 75-86. Invariance of half-cystine residues is the rule among homologous proteins. The high conservation of Gly and Pro residues could be due to the requirement in proteins such as these which are highly constrained by disulfide cross-links of maintaining certain bend structures in which Gly and Pro are prominent.

Tyrosyl and aspartyl residues could be functionally as well as structurally essential. Tyrosine modifications performed with C. *adamanteus* phospholipase A_2[47] did not appear to alter enzyme activity significantly. Recently, our laboratory has attempted to assess the role of carboxyl groups in phospholipase A_2 activity by chemical modification studies. Preliminary results indicate that reaction of the enzyme from C. *atrox* venom with a carbodiimide-nucleophile reagent under mild conditions leads to loss of activity. The enzyme was treated with 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide·HCl (EDC) and taurine at pH 6.5. Loss of activity was correlated with taurine incorporation and an analysis of inactivation kinetics implied the presence of at least one essential carboxyl group. These findings are consistent with chemical modification studies which employed a carboxyl group-specific diazo compound.[58] Phospholipase A_2 from *Naja naja oxiana* venom was reacted with N-diazoacetyl-N'-(2,4-*dinitrophenyl)ethylenediamine, leading to complete loss of activity and incor*poration of only one-half mole of substituent per mole of enzyme monomer.[51] Aspartic acid was identified as the site of modification.

Chemical modification of the amino groups in phospholipases A_2 appears almost always to be accompanied by inactivation of the enzyme; unfortunately the residues modified have usually not been identified. The alignments in Fig. 3 reveal no lysyl residues invariant among all phospholipases A_2. The experiments of Slotboom and deHaas[42] cited earlier demonstrated that all of the ε-amino groups can be amidinated in an active derivative of porcine pancreatic phospholipase A_2 as long as the α-amino group of Ala-1 is unaltered. Of course, amidination preserves the charge in the vicinity of the ε-amino groups, so that their

structural or functional involvement may not be impaired. We have found that citraconylation of the C. *atrox* phospholipase A_2 inactivates the enzyme and that complete reactivation occurs upon exposure of the derivative to acidic conditions. This may offer a useful means of partially and reversibly opening up the native structure in probing the functional consequences of other modes of derivitization. Wells[47] reported inactivation of C. *adamanteus* phospholipase A_2 with ethoxyformic anhydride by modification of one amino group per dimer. His assumption that the reaction had occurred at an ε-amino group was based upon the incorrect notion that the α-amino group was blocked and therefore it may well be that inactivation followed acylation of Ser-1. Conservation of Lys or positive charge is observed at residues 11, 35, 42, 53, 60 and 90 in Group II enzymes. The inactivation of B. *gabonica* phospholipase A_2 by incorporation of one mole of pyridoxal phosphate per mole of enzyme[54] is especially interesting in this regard because four sites of modification, i.e., 11, 35, 60 and 114, were documented. Since these modified residues occur at positions that appear to be far removed from the catalytic site it must be that they each are important in perhaps different ways in maintaining enzyme structure or contact with the interface. At the present time, however, there exists no evidence for direct implication of an ε-amino group in the catalytic function.

Proposed mechanisms

Although several hypotheses have been put forth to explain the phospholipase A_2 mechanism, most have been based upon an incomplete or incorrect understanding of the residues involved directly in catalysis. Therefore, we consider here only those proposed recently through inspection of primary and tertiary structures and chemical modification data. The tertiary structural analysis of bovine pancreatic phospholipase A_2[26] shows that His-47 is in the middle of one of the long central α-helices and that it is in a cavity at the molecular surface. Two carboxyl groups, one from Asp-48 and one from Asp-89, point towards the imidazole side chain from above and below, respectively. Some electron density attributed to Ca^{++} is in the vicinity of His-47 and Asp-89, and the phenolic group of Tyr-51 is in contact with this density. All four residues mentioned above are invariant among structures given in Fig. 3, but only His-47 and Asp-48 are observed in the bee venom phospholipase A_2 (Fig. 5).

A mechanism based in part upon the structural analysis of porcine prophospholipase and in part upon consideration of known esterase mechanisms was suggested by Drenth et al.[25] in which Asp-48 is the nucleophile, His-47 serves a stabilizing role similar to that of the amide NH groups in the serine proteinases, and Tyr-27 is the proton donor. Although specific modified residues were not

identified, Wells[47] showed that tyrosine modifications did not significantly alter the activity of C. *adamanteus* phospholipase A_2, and the replacement of Tyr-27 with a Trp residue in the honeybee enzyme would argue against direct participation of a phenolic side chain in catalysis. Drenth et al.[25] proposed that Arg-90 could be involved in binding the phosphate moiety of the substrate, a function that could be fulfilled by Lys-90 in the Group II enzymes and by His-109 in the bee venom phospholipase (Fig. 5). However, there is not strict conservation of a positive charge at this position (Fig. 3). As of now, none of these speculations is supported by evidence from chemical modification studies.

An intriguing mechanism proposed recently by Drenth[59] based upon the bovine pancreatic structure (Figs. 6 and 7) is that a His---Asp couple reminiscent of that seen in the serine proteases might exist in phospholipases between Asp-89 and His-47. In this model, Ser-195 is replaced by H_2O as the nucleophile and calcium ion is liganded through interactions with Asp-48 and the carbonyl groups of the peptide bonds joining residues 30 through 32. A calcium binding site so defined would correlate nicely with the observed inactivation by modification of Trp-30[57] and the spectral perturbations of tryptophan accompanying Ca^{++} binding to C. *adamanteus* phospholipase A_2.[60] However, it is noteworthy that in an earlier study,[26] the Ca^{++}-ion was reported to be bound in the vicinity of Asp-89. Limited comparison of the bovine phospholipase A_2 sequence in this vicinity with the sequences of Ca^{++} ion binding sites in other proteins indicates similarities. For example, the sequence Asp-Arg-Asn-Ala (residues 89-92) in bovine phospholipase A_2 is identical to a tetrapeptide region in Ca^{++} binding site III in rabbit skeletal muscle troponin C[61] and nearly identical to the sequence Asp-Lys-Asn-Ala in site III of bovine cardiac troponin C.[62] Since, at the time of this writing the various catalytic components, including the enzyme side-chains, substrate and Ca^{++} have not been placed definitively with respect to one another, it is an intriguing possibility that Asp-89 not Asp-48 is the site of Ca^{++} binding. Although arguments based upon entropic considerations might be raised against water as the nucleophile, it could well be that the H_2O molecule involved is ordered through interactions with the nearby Ca^{++}-Asp-48 complex. In any event, these various proposed mechanisms provide interesting subjects of investigation for evaluation by independent experimental approaches.

CONCLUDING REMARKS

It is clear that, despite an abundance of information concerning the primary and tertiary structures of a considerable number of phospholipases A_2, much remains to be done in clarifying the enzyme mechanism. X-ray crystallographic

analysis[64] has provided a structural framework for interpreting results from solution studies and, in general, a satisfying agreement exists between the structural model and functional predictions based upon kinetic analysis and chemical modification. Nevertheless, it would be very useful to examine models derived from crystallographic analysis of enzyme complexes with substrates or inhibitors. Assessment should be made of the possible participation in catalysis of aspartyl residues either by general carboxyl-labeling methods or by the development of chromogenic substrates[48] and/or poor substrates that might permit study of intermediate steps or kinetic analysis at low temperatures. Elucidation of the enzyme mechanism is certainly a top priority area of research.

The question must be resolved as to whether the requirement of a dimeric structure in the activity of certain phospholipases A_2 is 1) correct, 2) applies to just these select proteins, or 3) is a property of phospholipases A_2 in general. Unifying concepts are always sought after in science and in the case of these enzymes it is tempting to suppose that, because of the strong structural homology which they all share, they will all function essentially the same. Yet, it is known that phospholipases A_2 may or may not have toxic or anti-coagulant activities in addition to their esterase function. This multiple activity is, at present, impossible to predict based upon comparative sequence analysis and yet these functions must be coded within the primary structure. And so it is not unreasonable to imagine that different phospholipases A_2 will provide unique solutions to the ultimate confrontation with the interface whether it be through an interfacial recognition site or via the generation of an asymmetric dimer with a binding subunit.

ACKNOWLEDGMENTS

This work is supported in part by Grant PCM 75-23506 AOI from the National Science Foundation.

REFERENCES

1. Shen, B.W. and Law, J.H. (1979) in The Biochemistry of Atherosclerosis, Scanu, A.M., Wissler, R.W., and Getz, G.S., eds., Marcel Dekker & Co., New York, p. 275.
2. Dawson, R.M.C. (1973) in Form and Function of Phospholipids, Ansell, G.B., Hawthorne, J.N., and Dawson, R.M.C., eds., Elsevier, Amsterdam, p. 97.
3. Aggerbeck, L.P., Kézdy, F.J., and Scanu, A.M. (1976) J. Biol. Chem., 251, 3283.
4. Kunge, H., and Vogt, W. (1972) Ann. N.Y. Acad. Sci., 180, 123.
5. Schoene, M.W., and Iacono, J.M. (1976) in Advances in Prostaglandin and Thromboxane Research, Vol. 2, Samuelsson, B. and Paoletti, R., eds., Raven Press, New York, p. 763.
6. Bonsen, P.P.M., deHaas, G.H., et al. (1972) Biochim. Biophys. Acta, 270, 364.

7. deHaas, G.H., Slotboom, A.J., et al. (1970) Biochim. Biophys. Acta, 221, 31.
8. Evenberg, A., Meyer, H., et al. (1977) J. Biol. Chem., 252, 1189.
9. Fleer, E.A.M., Verheij, H.M., and deHaas, G.H. (1978) Eur. J. Biochem., 82, 261.
10. Shipolini, R.A., Callewaert, G.L., et al. (1971) FEBS Lett., 17, 39.
11. Shipolini, R.A., Callewaert, G.L., et al. (1974) Eur. J. Biochem., 48, 465.
12. Joubert, F.J. (1975) Biochim. Biophys. Acta, 379, 329.
13. Joubert, F.J. (1975) Biochim. Biophys. Acta, 379, 345.
14. Eaker, D. (1975) in Peptides: Chemistry, Structure, Biology, Walter, R., and Meienhofer, J., eds., Ann Arbor Science Publishers, Inc., Ann Arbor, p. 17.
15. Joubert, F.J. (1975) J. Biochem., 52, 539.
16. Halpert, J., and Eaker, D. (1975) J. Biol. Chem., 250, 6990.
17. Botes, D.P., and Viljoen, C.C. (1974) J. Biol. Chem., 249, 3827.
18. Samejima, Y., Iwanaga, S., and Suzuki, T. (1974) FEBS Lett., 47, 348.
19. Tsao, F.H.C., Keim, P.S., and Heinrikson, R.L. (1975) Arch. Biochem. Biophys., 167, 706.
20. Heinrikson, R.L., Krueger, E.T., and Keim, P.S. (1977) J. Biol. Chem., 252, 4913.
21. Braganca, B.M., Sambray, Y.M., and Sambray, R.Y. (1970) Eur. J. Biochem., 13, 410.
22. Vidal, J.C., and Stoppani, A.O.M. (1971) Arch. Biochem. Biophys., 147, 66.
23. Wells, M.A. (1971) Biochemistry, 10, 4074.
24. Shen, B.W., Tsao, F.H.C., et al. (1975) J. Am. Chem. Soc., 97, 1205.
25. Drenth, J., Enzing, C.M., et al. (1976) Nature, 264, 373.
26. Dijkstra, B.W., Drenth, J., et al. (1978) J. Mol. Biol., 124, 53.
27. Wells, M.A., and Hanahan, D.J. (1969) Biochemistry, 8, 414.
28. Tamiya, N., Nishida, S., and Kim, H.S. (1980) In Frontiers in Protein Chemistry, Liu, D.T., and Yasunobu, K.Y., eds., Elsevier-North Holland, New York, this volume.
29. Keim, P.S., Wells, M.A., and Heinrikson, R.L., unpublished observations.
30. Brattstrom, B.H. (1964) Trans. San Diego Soc. Nat. Hist., 13, 185.
31. Puijck, W.C., Verheij, H.M., and deHaas, G.H. (1977) Biochim. Biophys. Acta, 492, 254.
32. Keith, C.H. (1979) Ph.D. Thesis, University of Chicago.
33. deHaas, G.H., Slotboom, A.J., and Verheij, H.M. (1977) in Cholesterol Metabolism, Lipolytic Enzymes, Proc. Int. Cong. Biochem. Lipids, 19th, p. 191.
34. Bonsen, P.P.M., deHaas, G.H., and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta, 270, 364.
35. Pieterson, W.A., Vidal, J.C., et al. (1974) Biochemistry, 13, 1455.
36. Brockerhoff, H., and Jansen, R.G. (1974) in Lipolytic Enzymes, Academic Press, New York, p. 3.
37. Brockman, H.L., Law, J.H., and Kézdy, F.J. (1973) J. Biol. Chem., 248, 4965.
38. Sarda, L., and Desnuelle, P. (1958) Biochim. Biophys. Acta, 30, 513.
39. Chapus, C., Sémériva, M., et al. (1975) FEBS Lett., 58, 155.
40. Pattus, F., Slotboom, A.J., and deHaas, G.H. (1979) Biochemistry, 18, 2698.
41. Slotboom, A.J., Jansen, E.H.J.M., et al. (1978) Biochemistry, 17, 4593.
42. Slotboom, A.J., and deHaas, G.H. (1975) Biochemistry, 14, 5394.
43. Slotboom, A.J., Verheij, H.M., et al. (1978) FEBS Lett., 92, 361.
44. Pattus, F., Slotboom, A.J., and deHaas, G.H. (1979) Biochemistry, 18, 2703.
45. Huang, K.-S., and Law, J.H. (1978) in Enzymes of Lipid Metabolism, Gatt, S., Freysz, L. and Mandel, P., eds., Planum Publishing Corp., p. 177.
46. van Wezel, F.M., Slotboom, A.J., and deHaas, G.H. (1976) Biochim. Biophys. Acta, 452, 101.
47. Wells, M.A. (1973) Biochemistry, 12, 1086.
48. Volwerk, J.J. (1979) Ph.D. Thesis, State University of Utrecht.

49. Roberts, M.F., Deems, R.A., et al. (1977) J. Biol. Chem., 252, 2405.
50. Roberts, M.F., Deems, R.A., and Dennis, E.A. (1977) Proc. Natl. Acad. Sci. USA, 74, 1950.
51. Zhelkovskii, A.M., D'yakov, V.L., et al. (1978) Bioorg. Khim., 4(12), 1665.
52. Volwerk, J.J., Pieterson, W.A., and deHaas, G.H. (1974) Biochemistry, 13, 1446.
53. Yang, C.C., and King, K. (1979) Int. Cong. Biochem., Montreal, Canada, abstract 03-4-S38, p. 185.
54. Viljoen, C.C., Visser, L., and Botes, D.P. (1977) Biochim. Biophys. Acta, 483, 107.
55. Wells, M.A. (1971) Biochim. Biophys. Acta, 248, 80.
56. Wu, T.W., and Tinker, D.O. (1969) Biochemistry, 8, 1558.
57. Viljoen, C.C., Visser, L., and Botes, D.P. (1976) Biochim. Biophys. Acta, 428, 424.
58. Zhelkovskii, A.M., Apsalon, U.R., et al. (1977) Bioorg. Khim., 3(10), 1430.
59. Drenth, J., personal communication.
60. Wells, M.A. (1973) Biochemistry, 12, 1080.
61. Collins, S.M., Potter, J.G., et al. (1973) FEBS Lett., 36, 368.
62. van Eerd, J.P., and Takahashi, K. (1976) Biochemistry, 15, 1171.
63. Dayhoff, M. (1979) in Atlas of Protein Sequence and Structure 5, Suppl. 3, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C.
64. Dijkstra, B.W. (1980) Ph.D. Thesis, University of Groningen, The Netherlands.