Biochimica et Biophysica Acta 860 (1986) 448–461
Elsevier

BBA 73235

Anchoring of phospholipase A_2: the effect of anions and deuterated water, and the role of N-terminus region

Mahendra K. Jain [a,*], Badri P. Maliwal [b], G.H. DeHaas [c] and A.J. Slotboom [c]

[a] *Department of Chemistry, University of Delaware, Newark, DE 19716,*
[b] *Department of Biological Chemistry, University of Maryland Medical School, Baltimore, MD 21201 (U.S.A.), and*
[c] *Department of Biochemistry, Rijksuniversiteit, Padualaan 8, Utrecht, (The Netherlands)*

(Received December 17th, 1985)
(Revised manuscript received May 7th, 1986)

Key words: Phospholipase A_2; Anion binding; Interfacial catalysis; Intravesicle scooting; Deuterium isotope effect; Lipid-protein interaction

The effect of anions and deuterated water on the kinetics of action of pig pancreatic phospholipase A_2 is examined to elaborate the role of ionic interactions in binding of the enzyme to the substrate interface. Anions and deuterated water have no significant effect on the hydrolysis of monomeric substrates. Hydrolysis of vesicles of DMPMe (ester) is completely inhibited in deuterated water. The shape of the reaction progress curve is altered in the presence of anions. The nature and magnitude of the effect of anions depends upon the nature of the substrate as well as of the anion. Substantial effects of anions on the reaction progress curve are observed even at concentrations below 0.1 M and the sequence of effectiveness for DMPMe vesicles is sulfate > chloride > thiocyanate. Apparently, anions in the aqueous phase bind to the enzyme, and thus compete with the anionic interface for binding to the enzyme. Binding of the enzyme to anionic groups on the interface results in activation and increased accessibility of the catalytic site possibly via hydrogen bonding network involving water molecule. In order to elaborate the role of the N-terminus region in interfacial anchoring, the action of several semisynthetic pancreatic phospholipase A_2s is examined on vesicles of anionic and zwitterionic phospholipids. The first-order rate constant for the hydrolysis of DMPMe in the scooting mode by the various semisynthetic enzymes is in a narrow range: 0.7 ± 0.15 per min for phospholipase A_2 derived from pig pancreas and 0.8 ± 0.4 per min for the enzymes derived from bovine pancreas. In all cases a maximum of about 4300 substrate molecules are hydrolyzed by each phospholipase A_2 molecule. If anions are added at the end of the first-order reaction progress curve, a pseudo-zero-order reaction progress curve is observed due to an increased intervesicle exchange of the bound enzyme. These rates are found to be considerably different for different enzymes in which one or more amino acids in the N-terminus region have been substituted. Steady-state and fluorescence life-time data for these enzymes in water, ^2H_2O and in the presence of lipids is also reported. The kinetic and binding results are interpreted to suggest that the N-terminus region of phospholipase A_2 along with some other cationic residues are involved in anchoring of phospholipase A_2 to the interface, and the catalytically active enzyme in the interface is monomeric.

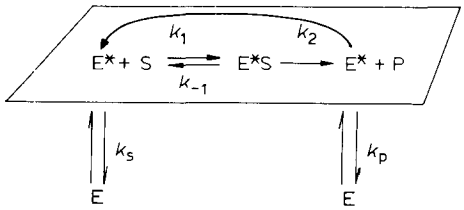
* To whom correspondence should be addressed.

Abbreviations: DHPMe (ether), 1,2-dihexylphosphatidylmethanol; DMPMe (ester), 1,2-dimyristoylphosphatidylmethanol; DTPMe (ether), 1,2-ditetradecylphosphatidylmethanol; DMPC (ester), 1,2-dimyristoylphosphatidylcholine; DTPC (ether), ditetradecylphosphatidylcholine; lysoPC, 1-palmitoyllysophosphatidylcholine; NATA, *N*-acetyl tryptophanamide; OPN, *cis*-octadec-9-enylphosphocholine; PNPG, octadecylphosphoglycerol; N7, the N-terminus heptapeptide from pig pancreatic phospholipase A_2; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Introduction

As indicated in Scheme I and elaborated in the preceding paper [1], binding of phospholipase A_2 to the substrate interface precedes the catalytic turnover by the bound enzyme (E*) in the scooting mode.

The reaction sequence for catalytic turnover occurs in the bilayer phase. In the presteady-state phase, binding of the enzyme in the aqueous phase (E) to the substrate interface is accompanied by significant changes in the spectroscopic properties of the protein, largely due to perturbation of Trp-3 residues in the interfacial recognition region (IRR) [2–5]. For example, binding of phospholipase A_2 to phospholipid dispersions is accompanied by an increase in the fluorescence quantum yield, and such changes provide a basis for quantifying the catalytically meaningful binding of the enzyme to the substrate interface [5]. However, the molecular changes in phospholipase A_2 that accompany interfacial binding, as well as the nature of the underlying interactions remain to be elaborated. An increase in the fluorescence emission intensity of a tryptophan residue of a membrane bound protein could result from a change in the dielectric properties of the microenvironment, or from a change in the mobility of the indole side chain, or from the release of intramolecular quenching. For phospholipase A_2 we have shown that the increase in the fluorescence intensity occurs without a significant increase in the fluorescence lifetime. Also, accessibility of Trp-3 residue in phospholipase A_2 to water soluble quenchers, like succinimide and 1-methyl nicotinamide chloride, is not appreciably altered when the enzyme binds to the interface [6]. This suggests that the polarity of the microenvironment of Trp-3 and its accessibility for collisional quenching by solutes in the aqueous phase does not change appreciably on binding of the protein to the interface. The Trp-3 fluorescence is not quenched by cationic quenchers, suggesting that the Trp-3 residue is somehow shielded from the aqueous phase by cationic group(s). Since Trp-3 is a part of the interfacial recognition region (IRR), and phospholipase A_2 readily hydrolyzes the substrate in anionic interfaces, it appears that ionic interactions are involved in the interactions of phospholipase A_2 with the substrate interface.



Scheme I. Scheme of interfacial catalysis.

In this paper we report catalytic properties of phospholipase A_2 on bilayer substrates as perturbed by anions. Water soluble anions promote desorption of the bound enzyme. It is postulated that an anion binding site on phospholipase A_2 is involved in interfacial anchoring of the enzyme during interfacial catalysis in scooting mode. The N-terminus of pancreatic phospholipase A_2 is believed to be a part of the interfacial recognition region that anchors the enzyme to the substrate interface [7–11]. This implies that the anion binding site could be somehow associated with the N-terminus region of the enzyme. In order to understand the exquisite coupling between the interfacial anchoring and the catalytic action of phospholipase A_2, it is necessary to identify the variables that modulate interfacial recognition region (IRR) on phospholipase A_2. One possible approach is to alter the amino acid residues which make up the IRR. Several semisynthetic phospholipase A_2 from pig and from bovine phospholipase A_2, with substitutions of amino acid residues at selected positions in the N-terminus region, have been described [11–14]. In this paper we report the catalytic and binding properties of these semisynthetic enzymes on the anionic and zwitterionic phospholipid bilayers. Fluorescence properties of the semisynthetic enzymes are also examined in order to understand the spatial characteristics of the N-terminus in the aqueous phase as well as in the interface.

Materials and Methods

All reagents were analytical grade. Source of lipids and the procedures for kinetic study are described in the preceding paper [1] or as mentioned in the text. All anions were added as sodium salts. A small change in the pH on addition of

salts was readily compensated in less than 10 s. This shift does not affect any of the results described here. Control experiments also showed that chlorides of Li, Na, K, Cs, and Rb exhibited essentially the same effect, even in the most sensitive regions of the reaction progress curves.

The semisynthesis of the various enzymes in amidinated form (AMPA) has been described elsewhere [11–14]. The amino acid residues in the positions 1 to 8 pancreatic phospholipase A_2 are:

Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-

and for the bovine pancreatic phospholipase A_2 are:

Ala-Leu-Trp-Gln-Phe-Asn-Gly-Met-

The substitutions in the positions 1, 4, 6, 7 and 8 were examined and the rationale for these substitutions are also discussed elsewhere [11–14]. A total of 12 proteins were used in this study: 1, pig pancreatic prophospholipase A_2; 2, pig pancreatic phospholipase A_2 (PLA); 3, amidinated pig PLA; 4, Gly-1-amidinated pig PLA; 5, D-Ala-1-amidinated pig PLA; 6, Ala-7-amidinated pig PLA; 7, Glu-6-Ala-7-amidinated pig PLA; 8, bovine pancreatic phospholipase A_2; 9, amidinated bovine PLA; 10, Glu-4-amidinated bovine PLA; 11, Asn-4-amidinated bovine PLA; 12, Arg-6-amidinated bovine PLA. The concentrations of these proteins were determined on the basis of their ultraviolet absorbance at 280 nm ($A = 13$, 1 cm for 1% solution).

Steady-state fluorescence measurements were made on a SLM4800S spectrofluorimeter with excitation at 290 nm, and emission spectra were scanned from 300 to 400 nm (slitwidths 4 and 4 nm). All measurements were done in stirred and thermostated 1 cm pathlength quartz cuvettes, and unless stated otherwise the aqueous phase contained 0.6 mM $CaCl_2$, 10 mM Hepes, 0.1 M KCl at pH 8.0 and 30°C.

Fluorescence life-times were measured from the fluorescence intensity decay curves recorded on a time-correlated single photon counting fluorimeter (Edinburgh Instrument, Model 199s). The protein samples were excited at 292 nm and the emission was observed through a 1 cm liquid filter containing 5% potassium phthalate solution (cut-off below 310 nm). Typically about 0.2 to 0.4 million photons were collected for each measurement. The light source was a coaxial hydrogen flash lamp operating at 30 kHz repetitive rate. The stop rate was maintained at about 2% of the lamp repetitive rate. The FWHM was typically about 1.2 to 1.3 ns [15]. For deconvolution, a lamp profile was obtained with a scatterer solution before each measurement. The deconvoluted data were analyzed assuming an approximate multiexponential decay law

$$s(t) = \sum \alpha_i \exp(-t/\tau_i)$$

where α_i is the relative weight and τ_i is the decay constant of the ith component. The data were analyzed by a nonlinear least-square reconvolution program which included iterative time shift of the fitted function, which corrects for any transit time wavelength shift between the measurements of the lamp and the fluorescence emission profiles [16]. For all the samples of phospholipase A_2, we needed at least three lifetime components to obtain an acceptable value of chi squared (χ^2). It may be pointed out that as the α_i and τ_i are correlated parameters, there is some uncertainty in the recovered parameters.

Results

Effect of anions and deuterated water on the hydrolysis of monomeric substrate

Hydrolysis of 2,3-bis(hexanoylthio)propylphosphocholine (0.25 mM) by phospholipase A_2 was monitored at 412 nm in the presence of 1 mM bis(dinitrothiobenzoic acid) at pH 8.0 in the presence of 0.2 M sodium salts according to the protocol described by Volwerk et al. [17]. Results not shown here demonstrate that sulfate, chloride and thiocyanate had little (< 10% in extreme cases) effect on the pseudo-zero-order steady-state rate of hydrolysis. Similarly, in ^2H_2O the rate of hydrolysis was the same as in water under identical conditions. These observations therefore show that anions or deuterated water do not have any significant effect on the hydrolysis of monomeric substrate, that is the catalytic site of phospholipase A_2 is not perturbed.

The effect of anions on the catalytic action of phospholipase A_2 on the bilayer form of substrates

According to the Scheme I, the quality of the substrate interface determines the kinetics of interfacial catalysis by phospholipase A_2 by regulating formation of E*S. In order to elaborate ionic interactions between the enzyme and the interface, we have examined the effect of water-soluble anions on four different types of bilayer vesicles as substrate interface. The rationale for these experiments is that if the anchoring of phospholipase A_2 to the interface is ionic, the kinetics of hydrolysis will be modulated by salts. Also if a specific binding interaction is involved, the effect of ions would exhibit considerable selectivity.

As shown elsewhere the action of phospholipase A_2 on bilayer form of vesicles can be monitored under a variety of conditions such as: in the absence of any additive [5,18], in the presence of freshly added lysoPC [18], and in the ternary codispersions of DMPC with lysoPC and fatty acid [19]. In all of these cases (data not shown), in the presence of anions a change in the shape of the reaction progress curve is observed, which is consistant with the hypothesis that the binding of the enzyme to the interface is reduced in the presence of anions. The effect of anions is concentration dependent, and their effectiveness is in the order: sulfate > chloride > thiocyanate.

Effect of anions on the hydrolysis of DMPMe vesicles was studied under a variety of conditions. As shown in Fig. 1 (and also in Ref. 1), in the absence of any added salt (other than 0.3 mM $CaCl_2$) a first-order reaction progress curve is obtained, and even in the presence of excess substrate vesicles the reaction ceases when 4300 ($= A$) substrate molecules have been hydrolyzed by each enzyme molecule. If at the end of this first-order reaction progress curve anions are added, hydrolysis of the excess vesicles starts immediately and proceeds until all the excess accessible substrate has been hydrolyzed. It may be noted that different anions promote the rate of hydrolysis to different extents and there is a short (less than 1 min) lag period before a steady-state phase of hydrolysis is established.

The ability of anions to promote hydrolysis is different whether the vesicles contain the products or not. When salt is present before initiating the reaction, the progress curve has an inflexion; the rate of hydrolysis is slower before the inflexion, and faster after the inflexion. Such a behavior would be expected if phospholipase A_2 bound to vesicles is desorbed in the presence of anions and allowed to equilibrate with excess substrate vesicles. Thus anions increase the aqueous phase concentration of phospholipase A_2 and therefore promote the rate of intervesicle transfer of the bound enzyme in the order: $SO_4^{2-} > Cl^- > CNS^-$.

Desorption of the phospholipase A_2 bound to DTPMe vesicles by anions is shown in Fig. 1B. The fluorescence intensity of phospholipase A_2 increases in the presence of DTPMe vesicles, and this increase is completely abolished by increasing concentration of anions. The efficacy of the anions is in the order sulfate > chloride > thiocyanate, and at high anion concentration the fluorescence intensity decreases to the level that is observed with the enzyme in the absence of DTPMe vesicles but in the presence of the anions. These results demonstrate that essentially all the bound enzyme can be desorbed in the presence of high salt concentrations. Intervesicle transfer of the bound enzyme is not due to fusion of vesicles but by desorption of the bound enzyme. Elsewhere [20] we have shown that conditions that favor the action of phospholipase A_2 on DMPC vesicles also favor fusion of vesicles, and both of these processes are inhibited by the same lipid-soluble amphipathic solutes. Therefore, it is of interest to examine the effect of anions on the rate of fusion of DMPMe vesicles. As shown in the preceding paper [1], under the conditions used for the kinetic studies the rate of fusion is very slow, and it is not noticeably altered in the presence of any of the anions under investigation (data not shown). It is also significant that the rate of fusion of vesicles is drastically increased at 3 mM calcium, but it remains essentially unchanged in the presence of anions like sulfate, chloride, and thiocyanate. These observations show that anions do not promote fusion, and that anions have little or no effect on the kinetics of calcium-induced fusion of DMPMe vesicles. The effect of anions on the desorption of the bound enzyme is not due to the chaotropic effect because the effects of anions are observed at low concentrations (less than 0.2 M) and also because sulfate and thiocyanate exhibit

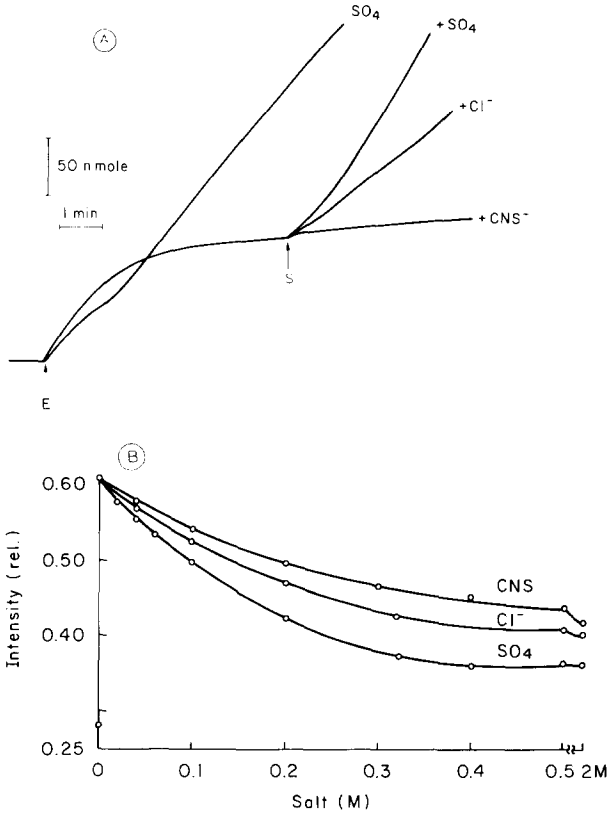


Fig. 1. (A) Reaction progress curves for the hydrolysis of DMPMe (ester) (0.33 mM) with pig pancreatic phospholipase A_2 (0.4 μg added at E) in the presence of 4 ml of 0.3 mM $CaCl_2$ at pH 8.0 and 30°C. Salts (0.1 M) were either present initially or added at S and indicated by + at the end of the first-order phase of hydrolysis of DMPMe. (B) Effect of anions on binding of phospholipase A_2 to DTPMe vesicles. Effect of thiocyanate (top), chloride (middle) and sulfate (bottom) was measured on the fluorescence emission at 333 nm at 30°C, pH 8.0 in 0.6 mM $CaCl_2$, 10 mM Hepes, 8 μM phospholipase A_2 and 20 μM DTPMe.

qualitatively similar effects. We do not believe that the primary effect of anions in the aqueous phase is on the defects in the bilayer interface because fusion and phase transition properties of bilayers [21] are not modified appreciably by anions at these concentrations.

Anion-induced intervesicle transfer of the bound enzyme

As implied in Scheme I, intervesicle transfer of the bound enzyme (E*) could occur by desorption, and the desorbed enzyme could bind to another vesicle. As shown in Fig. 2A, the exchange of the enzyme bound to vesicles of DTPMe is promoted by anions in the sequence sulfate > chloride > thiocyanate. On the other hand, as shown in Fig. 2B, binding of the enzyme to DTPC vesicles is rather weak, both in the presence and in the absence of salts, and the DMPMe vesicles added afterwards are readily hydrolyzed.

The intravesicle scooting and anion-mediated intervesicle transfer of the bound enzyme provide a reasonable basis for interpreting the complexities of the kinetics of phospholipase A_2 on the various forms of the substrate. As shown in Fig. 1A, in the presence of excess DMPMe vesicles only a small fraction of the total accessible substrate is hydrolyzed at low anion concentrations. This suggests that the bound enzyme does not readily exchange with excess vesicles in the absence of anions. Intervesicle exchange promoted by anions gives rise to a steady-state phase of hydrolysis. It may, however, be noted that the initial rate measured under these conditions do not meet the strict criteria for pseudo-zero-order kinetics because the excess substrate is available to the enzyme only for intervesicle exchange (a relatively slow step) but not for the formation of E*S complex. It is particularly important to note that apparently opposite effects of anions can be rationalized on the basis of such considerations. When the rate of hydrolysis in the intravesicle scooting mode ceases due to inaccessibility of the excess substrate vesicles, the anion induced intervesicle exchange increases the rate of hydrolysis (cf. Fig. 1). Similarly, when the rate of hydrolysis in the intravesicle mode is rapid in the early phase of the first-order reaction progress curve, the anion-induced intervesicle exchange decreases the apparent rate of hydrolysis. As shown by a lack of any effect of anions on monomer kinetics, these effects of anions are not due to any direct or specific effects on the catalytic efficacy of the enzyme, but due to a change in the residence time of the enzyme in the interface. Only the hydrolysis in the scooting mode is inhibited by ^2H_2O. However, as shown in Fig. 2B, the rate of hydrolysis of DMPMe vesicles by phospholipase A_2 is inhibited more than 90% in the presence of ^2H_2O. This effect is not due to inactivation of the enzyme in ^2H_2O because if the enzyme in ^2H_2O is added to

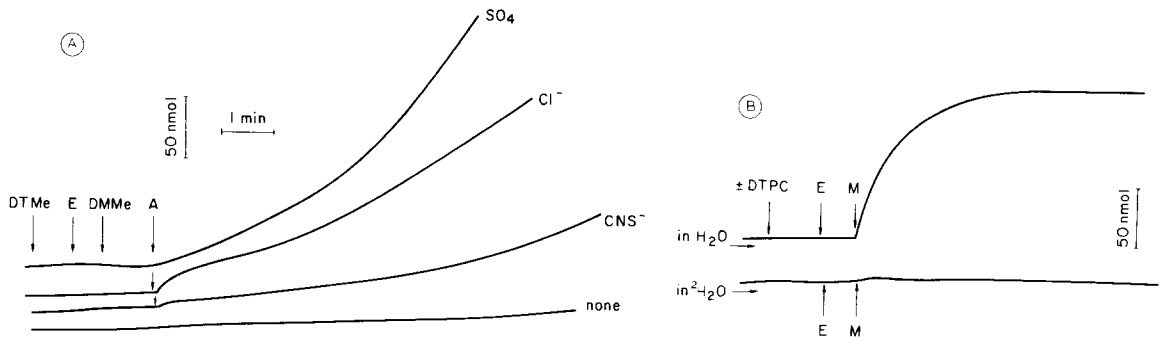


Fig. 2. (A) Reaction progress curves for the hydrolysis of DMPMe (DMMe) vesicles (330 μM) added to a mixture to a mixture of DTPMe (DTMe) (80 μM) and PLA (0.4 μg) in 0.3 mM $CaCl_2$; salts were then added at A. Conditions 30°C, pH 8, 0.3 mM $CaCl_2$, and 0.1 M salts as indicated. (B) Reaction progress curves for the hydrolysis of DMMe vesicles (80 μg added at E) at pH 8, 30°C, 0.3 mM $CaCl_2$. The top curve was obtained in H_2O in the presence or in the absence of DTPC (160 μM) vesicles; bottom curve exhibiting no hydrolysis was obtained in the presence of DMMe (80 μM) and no salt or in the presence of 0.1 M sodium salts of chloride, sulfate or cyanate.

DMPMe dispersions in water, no inhibition is observed. Similarly, hydrolysis of monomer substrate is not inhibited at all in ^2H_2O. Action of ^2H_2O on the reaction progress curve of DMPC with freshly added lysoPC, and DMPC + lysoPC + palmitic acid ternary codispersions was also examined. In all these cases the rate of hydrolysis of the enzyme was 60–85% inhibited in the presence of ^2H_2O. According to the criteria of fluorescence enhancement, binding of phospholipase A_2 to vesicles of DMPMe and other anionic lipids is not altered appreciably as shown in Table I (discussed below). Since the catalytic site of phospholipase A_2 remains functional in ^2H_2O, inhibition of interfacial catalysis in ^2H_2O implies that the coupling between the interfacial recognition region (IRR) and the catalytic site of the bound enzyme is somehow lost in ^2H_2O. Such a large deuterium isotope effect of ^2H_2O vs. H_2O implies that some sort of specific interaction is involved.

TABLE I

THE BINDING PARAMETERS FOR PIG PHOSPHOLIPASE A_2 + LIPID COMPLEX

Conditions described in Materials and Methods. RQY, relative quantum yield.

Phospholipid	Medium	K_d (μM)	n	RQY
Hexadecyl-phosphocholine	H_2O	2.06	37	0.88
	^2H_2O	2.07	30	1.04
cis 18:1 PC	H_2O	4.2	22	0.9
	^2H_2O	3.6	20	1.20
Octadecyl-phosphoglycerol	H_2O	< 0.3 *	37	1.04
	^2H_2O	< 0.28 *	35	1.08
DMPMe	H_2O	< 0.4 *	46	0.96
	^2H_2O	> 0.7 *	50	1.0

* High covariance (> 0.95) between n and K_d.

Action of semisynthetic phospholipase A_2 on DMPMe vesicles

Reaction progress curves for the hydrolysis of DMPMe vesicles by semisynthetic phospholipase A_2 were recorded at high vesicle to enzyme ratios (about 6) in the absence of salts. As shown in the preceding paper [1] and in Fig. 1A, in the presence of 0.3 mM $CaCl_2$ for all the semisynthetic enzymes the reaction progress curve is first order, which suggests that not only the transbilayer movement is slow but also the intervesicle exchange of the bound enzyme is relatively insignificant. Such curves result from binding of one enzyme molecule to one vesicle and the concomitant hydrolysis in the intravesicle scooting mode of the substrate molecules available in the outer monolayer of the vesicle. With each of the semisynthetic phospholipase A_2 the hydrolysis begins immediately suggesting that the binding of the enzyme to DMPMe vesicles is fast. The extent of hydrolysis and k_i values for hydrolysis of DMPMe vesicles by the various semisynthetic phospholipase A_2 are summarized in Fig. 3. As expected,

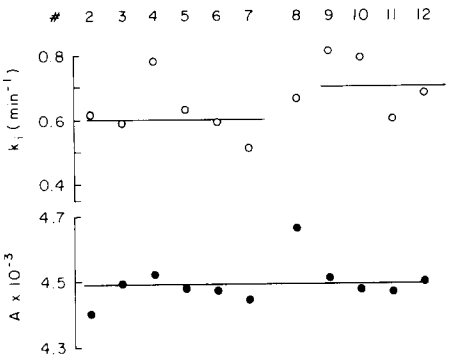


Fig. 3. Amplitude (A) and the first order rate constant (k_i) for the hydrolysis of DMPMe (ester) vesicles (0.32 mM) by several semisynthetic enzymes Nos. 1–12. Reaction conditions as in Fig. 1.

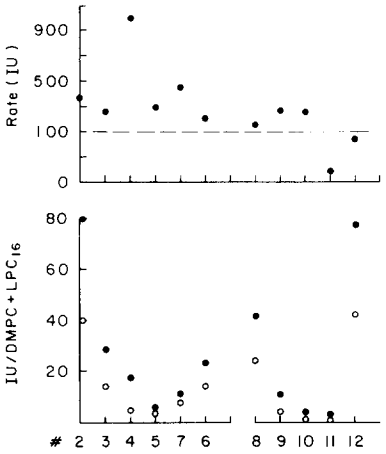


Fig. 4. Specific activities of the various phospholipase A_2s on DMPMe (ester) (0.32 mM) vesicles (top); these rates were initiated by 0.1 M sulfate as in Fig. 1. (Bottom) Specific activities for the hydrolysis of DMPC vesicles (160 μM) activated with freshly added lysoPC (final mole fraction 0.07 in DMPC) in the absence (●) and in the presence (○) of 0.1 M NaCl. Conditions 25°C, pH 8.0, 10 mM $CaCl_2$.

the maximum extent of hydrolysis $A = 4300$ substrate molecules hydrolyzed by each bound enzyme, is constant for all the enzymes. The values of k_i for the semisynthetic phospholipase A_2 derived from the pig phospholipase A_2 are in the 0.6 ± 0.2 per min range. The variability in k_i for the enzymes derived from bovine phospholipase A_2 is somewhat larger than that for pig phospholipase A_2. No change in k_i would be expected if the factors governing catalytic turnover in the interface by the bound enzyme were not altered by the amino acid substitutions. On the basis of intrinsic uncertainties involved in the measurement of k_i we believe that the k_i values for the various substituted pig and the bovine phospholipase A_2 enzymes are not significantly different, thus implying that the effect of amino acid substitutions on the interfacial catalytic turnover rate in the scooting mode is relatively minor. Since the extent of hydrolysis per enzyme molecule remains the same for all the semisynthetic enzymes, based on the arguments developed in the preceding papers it would imply that the monomeric enzyme in the interface is catalytically active.

If anions are added to the reaction mixture when the hydrolysis by the first order process has stopped, as shown in Fig. 1A, hydrolysis of the excess substrate begins immediately with a pseudo-first-order process, and all the accessible substrate (63% of the total substrate present) can ultimately be hydrolyzed by the enzyme which was added initially. The steady-state rates of hydrolysis are summarized in Fig. 4. Specific activities for the hydrolysis of excess DMPMe vesicles, in the presence of sulfate, differ by a factor of about 20 for the various enzymes. As also shown in the bottom panel of Fig. 4, the specific activities for the hydrolysis of DMPC vesicles activated by freshly added lysoPC differ by a factor of over 100. A comparison of the two substrate systems shows that there is no apparent correlation between the activities of the enzyme in these two substrate interfaces. Since the DMPC + lysoPC substrate system initially does not have any net surface charge, it is reasonable to assume that the change in the relative activities is due to a change in the interfacial binding and/or exchange of the enzyme. Thus, the interfacial residence times of the various enzyme in the two substrate interfaces could be significantly different.

Quantitative evaluation of the interfacial residence time of phospholipase A_2 on the substrate bilayer is not possible yet. However, some indication of long residence times can be obtained from experiments of the type shown in Fig. 1A. In the presence of anions at the start of the reaction, the reaction progress curves have an inflexion before the steady-state phase of hydrolysis occurs. This

ultimately leads to the hydrolysis of all the accessible substrate. It is very difficult to measure the time to inflexion accurately and we are still investigating the variables that control such a feature of reaction progress curves. However, when the time for inflexion is more than 1 min, it can be determined with reasonable reproducibility. For example in the presence of 0.1 M sulfate, the inflexion times are: Ala-7 (No. 6, 5 min) > Glu-6-Ala7 (No. 7, 1.5 min) = AMPA (No. 3, 1.7 min) > pig phospholipase A_2 (No. 2, 0.9 min). Similarly, the inflexion times for the bovine phospholipase A_2 are: 4-Asn (No. 11, 2.5 min) > Glu-4 (No. 10, 1.8 min) > bovine AMPA (No. 9, 1.5 min) > bovine phospholipase A_2 (No. 8) = Arg-6 (No. 12, 0.4 min). It may be noted that the enzymes with high specific activity based on a high steady-state rate in the presence of salts exhibit lower time for reaching the inflexion point. The significance of the inflexion time can be evaluated in the light of our earlier observation. In the first paper [1] it was shown that the intervesicle exchange time of the enzyme from DTPMe vesicles in the presence of anions is significantly larger than it is from the product-containing vesicles. This suggests that the enzyme added initially to DMPMe vesicles remains bound to these vesicles, and the rate of intervesicle exchange increases somewhat with the mole fraction of the products, that is, with the extent of hydrolysis. Since k_1 for the various semisynthetic enzymes are approximately the same, it would imply that a difference in the effect of sulfate on the rate of hydrolysis is on a step other than those in the catalytic turnover. Since one of the products of hydrolysis is an anion, it is also tempting to surmise that anions in the aqueous phase dislodge the enzyme from the interface more readily while competing with fatty acids for the anion binding site.

Experiments summarized in Fig. 4 illustrate the effect of the quality of interface on the kinetics of hydrolysis by phospholipase A_2. These results raise several interesting questions. The one of immediate concern to us regards the nature of the interaction between the enzyme and anions. The results shown in Figs. 1 and 4 demonstrate that, indeed, anions in the aqueous phase compete with anions in the interface. In the presence of a zwitterionic interface, the effect of anions in the aqueous phase is to inhibit the rate of hydrolysis by 40 to 70% presumably by promoting desorption of the bound enzyme. On the other hand, under the conditions used for specific activity measurements with DMPMe vesicles as substrate, the increased intervesicle exchange by anions leads to an increased hydrolysis of the excess substrate vesicles. The concept of intravesicle scooting and intervesicle exchange, therefore, account for the apparently conflicting kinetic effects. An optimal residence time of phospholipase A_2 on the interface would lead to a maximal steady-state rate of hydrolysis of all the available substrate; otherwise in the intravesicle scooting mode only a fraction of the substrate vesicles would by hydrolyzed with a rapid initial rate which would be limited by the rate constant for intravesicle scooting.

The microenvironment of Trp-3 in phospholipase A_2

Both bovine and pig phospholipase A_2 contain only one tryptophan residue in position 3, which can be used as an intrinsic fluorescence probe for the microenvironment of the N-terminus region. The major features of the fluorescence emission spectra of these proteins are summarized in Fig. 5. The emission peaks (top panel) were only slightly

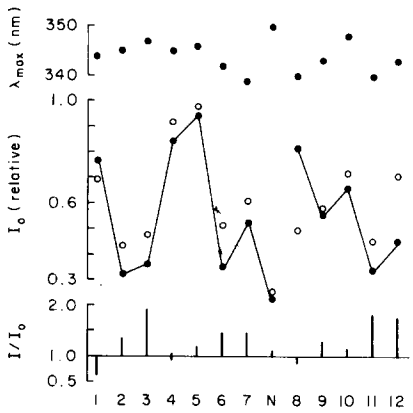


Fig. 5. Fluorescence properties of phospholipase A_2s (top panel) emission maximum in aqueous phase, pH 8.0, 30 °C, 0.1 M KCl. (Middle) Relative quantum yields (= 1 for *N*-acetyltryptophanamide (NATA) under these conditions) in water (pH 8.0, ●) and in ^2H_2O (pH 7.6, ○) at 30 °C in 0.1 M KCl. (Bottom) Relative fluorescence quantum yield in 0.1 M sulfate vs. that in 10 mM Hepes and 1 mM chloride. Conditions 30 °C, 10 mM Hepes and 1 mM $CaCl_2$. The connecting line is drawn only for clarity and has no theoretical significance.

influenced by amino acid substitutions, and compared to the emission peak for N7-peptide of pig phospholipase A_2, the peaks for all the enzymes were shifted to lower wavelengths which suggests that Trp-3 in these proteins is somewhat shielded [12–14]. However, the emission maxima are above 340 nm for the substituted enzymes, which suggests that Trp-3 is not buried. Unlike the small difference in emission maxima the quantum yields of these proteins varied substantially. Lowest quantum yields obtained, for example for pig phospholipase A_2 (No. 2) in aqueous phase, were about 30% of the values for *N*-acetyltryptophanamide under identical conditions. Some of the substitutions resulted in a decreased quantum yield, while others increased it. In the case of pig phospholipase A_2, substitution of Ala-1 with either Gly (No. 4) or D-Ala (No. 5), as well as that of Arg-6 with Glu-6 (No. 7) resulted in significantly higher fluorescence quantum yields. In contrast, introduction of Arg-6 (No. 12) in bovine phospholipase A_2 drastically reduced the quantum yield, which was also the case for insertion of Asp-4 (No. 11) for Gln. The high quantum yield resulting from the removal of Arg-6 and by introduction of an anionic group suggests that cationic groups in the vicinity of Trp-3 cause quenching. If the N-terminus region was in a helical conformation, the charged residues in position 1, 4, 6 and 10 would be one face of the helix, and hydrophobic residues like Leu-2, Trp-3, Phe-5, Met-8 could be on the other face. If this helix is localized in a particular conformation, the chirality of Ala-1 would also be a crucial factor. A probable role of H-bonding network in interactions of Ala-1 has been discussed elsewhere [11,14].

The relative quantum yields of the various proteins also change in ^2H_2O (middle panel, Fig. 5). Most of the semisynthetic phospholipase A_2 show an increase in the quantum yield in ^2H_2O compared to H_2O although pig prophospholipase A_2 (No. 1) and bovine phospholipase A_2 (No. 8) show a decrease. We observed a similar change in the fluorescence intensity of phospholipase A_2 in the presence vs. absence of sulfate (bottom panel, Fig. 5). Thus for all the phospholipase A_2 the magnitude of the ^2H_2O effect appears to be related to the change in the fluorescence intensity induced by anions. In both cases, an increase in the fluorescence intensity is observed without a significant change in the position of the emission peak. The effect of salt concentration on the fluorescence intensity of pig phospholipase A_2 is shown in Fig. 6. The fluorescence intensity of pig phospholipase A_2 increases with the salt concentration; the apparent decrease with thiocyanate is probably due to competition with the small amount of chloride present in the buffer solution.

The relative fluorescence quantum yield for the various phospholipase A_2 in the presence of sulfate against that in ^2H_2O is plotted in Fig. 7. Even

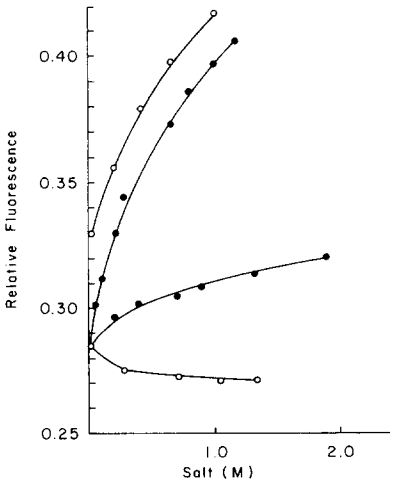


Fig. 6. Increase in fluorescence intensity of pig phospholipase A_2 as a function of salt concentration: (from top) sulfate in ^2H_2O, sulfate, chloride, and thiocyanate in water.

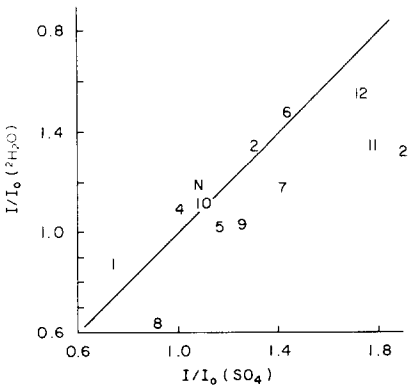


Fig. 7. Relative fluorescence increase (ratios of quantum yields) by 1 M sulfate (abscissa) vs. the relative quantum yield in ^2H_2O (ordinate) for the phospholipase A_2s.

though there is some scatter (correlation coefficient 0.77, slope 10.5 ± 0.2, intercept 0.05 ± 0.2), the trend is unmistakable clear, that is both ^2H_2O and sulfate ions lead to a similar change in the fluorescence quantum yield of the various phospholipase A_2s. It may be pertinent to note that those enzymes which fall farthest away from the line are the ones derived from bovine phospholipase A_2 (Nos. 8, 9, 11, 12). We do not yet understand the reasons underlying the scatter in the data, however, there are several factors leading to uncertainties in this correlation. Anion binding affinities for different proteins could be somewhat different, and therefore the degree of saturation of the binding site could be different at 1 M sulfate used for these measurements. Also specific interactions of tryptophan in the ground and the excited state with its neighbors can not be ruled out.

The origins of enhancement in tryptophan fluorescence in ^2H_2O is probably complex. Such changes have been reported for water-soluble indole derivatives [22–24]. However, the changes in the fluorescence quantum yield in ^2H_2O reported here are considerably more pronounced and are specific for the microenvironment of Trp-3. Thus the efficacy of anions for increasing the fluorescence intensity of free phospholipase A_2 is in the order sulfate > chloride > thiocyanate. The change induced by sulfate is also observed in the presence of ^2H_2O, suggesting that their effects on the increase in the fluorescence intensity are complementary.

The reduced quantum yield of Trp-3 in some proteins compared to that for *N*-acetyltryptophanamide is believed to arise from internal quenching in the static and dynamic modes involving various polar groups in the vicinity of the fluorophore [24–27]. It is possible to distinguish dynamic from static quenching by comparing the change in the intensity with that in the fluorescence lifetime [25]. In the case of dynamic quenching, the extent of decrease in the quantum yield and the lifetime is the same. In statically quenched systems the decrease in lifetime is negligible compared to the change in the intensity. The fluorescence intensity decay of phospholipase A_2 is complex, and a reasonable fit could be obtained with a minimum of three lifetimes, $\tau_1 = 1$ to 1.5 ns, $\tau_2 =$ about 3 ns, and $\tau_3 = 7$ to 8 ns. We observed some differences

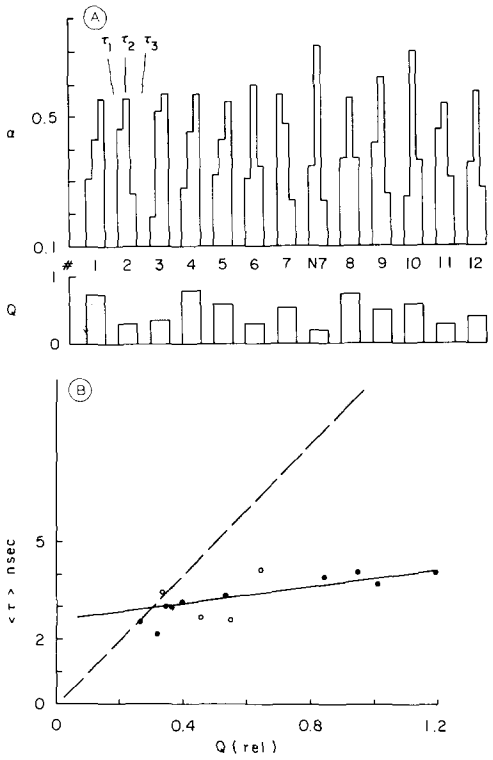


Fig. 8. (A) (Top panel) The relative amplitudes of the three fluorescence life-times: τ_1 (1 ns), τ_2 (3 ns) and τ_3 (7 ns) for the semisynthetic phospholipase A_2s. (Bottom) Relative quantum yields (experimental and theoretical, left and right). Conditions as in Fig. 4. (B) Plot of the relative quantum yield (abscissa) and calculated average lifetimes of semisynthetic phospholipase A_2 at pH 8.0, 30°C in 0.1 M KCl, 10 mM Hepes and 10 mM $CaCl_2$.

in the relative amplitudes of the three lifetimes of the various semisynthetic phospholipase A_2s. The results plotted in Fig. 8A show that generally speaking the longer lifetime component increases in those cases where the steady-state emission quantum yields are also higher. However, in all the cases, the increase in the average lifetime were significantly smaller than those in the relative quantum yields (Fig. 8B). In this figure, the slope of 1 in the semilog plot would have been expected if the fluorescence of Trp-3 in semisynthetic phospholipase A_2 was only dynamically quenched, and slope of zero for the static quenching. A small positive slope of about 0.15 suggests that the dynamic quenching component may be about 15%. Thus only a small part of the internal quenching is dynamic, and that the static quenching plays a

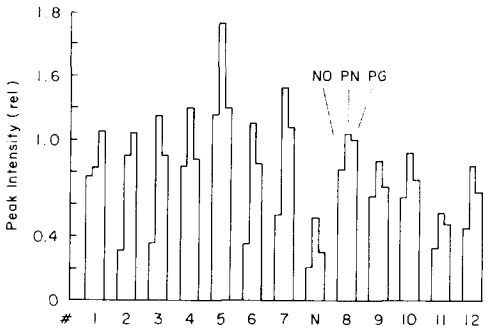


Fig. 9. Relative fluorescence quantum yields of the phospholipase A_2s in 5 mM octadecenylphosphocholine (PN) and in 1.5 mM octadecylphosphorylglycerol (PG) and no additive, NO.

major role in the fluorescence intensity changes associated with the amino acid substitutions in phospholipase A_2.

Fluorescence properties of phospholipase A_2 in a lipid environment

Binding of phospholipase A_2 to the lipid-water interface gives rise to an emission maximum that is blue-shifted to 333 nm from 342 nm, and has higher emission intensity. The relative peak intensities of phospholipase A_2 alone (NO) in anionic (PG) and zwitterionic (PN) interfaces are shown in Fig. 9. In most of these cases the relative quantum yield approaches the value of about 1, that is probably coincidentally observed for *N*-acetyltryptophanamide in water. Lifetime measurements were also done in some of these cases. Here, also we observed an increased contribution of longer lifetime components, and the increases in the average lifetime were considerably smaller ($< 20\%$) compared to the increase in the steady-state fluorescence quantum yield (results not shown). This suggests that binding of the enzyme to the lipid interface eliminates the internal static quenching component.

Discussion

Evidence for the presence of an anion binding site on phospholipase A_2 can be inferred from a variety of observations. Reaction progress curves for monolayers [28] and bilayers [1,5,19] of anionic phospholipids do not exhibit a latency phase; the presence of cationic additives inhibit the activity of phospholipase A_2 [29]; Trp-3 is not accessible to cationic quenchers from the aqueous phase [6]; At concentrations well below critical micelle concentration anionic amphipaths form microaggregates with phospholipase A_2 [30,43]. The significance of the putative anion binding site for anchoring the enzyme to the substrate interface has been elaborated in this paper.

Anions in the aqueous phase promote intervesicle transfer of phospholipase A_2. The order of effectiveness is sulfate > chloride > thiocyanate. One of the simplest interpretation of intervesicle exchange promoted by anions is that phospholipase A_2 containing a bound anion like sulfate can not bind the interfacial anion. This competition for the anion binding site effectively increases the aqueous phase concentration of the enzyme, and therefore the probability of intervesicle exchange also increases. Phospholipase A_2 anchored to an anion in the interface by such an 'ion-lock' would increase the interfacial residence time, and therefore increase the probability of formation of E*S complex. Anchoring of phospholipase A_2 through an anion binding site also accounts for the ability of anions in the aqueous phase to promote 'solubilization' of intracellular phospholipases by KCl [31], NaCl [32], and H_2SO_4 [33,34].

The kinetic implications of the effect of anions on the reaction progress curve are far reaching. Anions in the aqueous phase or cations in the bilayer phase increase intervesicle exchange by reducing the residence time of phospholipase A_2 in the interface, which effectively reduces the catalytic turnover in the scooting mode. This accounts for a poor binding of phospholipase A_2 to zwitterionic DMPC vesicles unless anionic additives are present. The latency period observed in PC vesicles is a cumulative effect of the products formed by a small fraction of the enzyme bound to the unmodified DMPC vesicles [19]. The latency period is further increased in the presence of anions in the aqueous phase by effectively decreasing the residence period of the bound enzyme in the presteady-state phase. In the steady-state phase of hydrolysis of DMPC vesicles, the residence period increases as the enzyme is anchored to the fatty acid anion in the interface. Since a fatty acid alone in DMPC vesicles does not promote hydrolysis unless lysophospholipids are also

present [5], it would imply that the negative charge in the interface is only a necessary but not a sufficient condition for interfacial catalysis in the scooting mode. This is probably because phospholipase A_2 anchored to the anion in the interface can not bind to the substrate unless the substrate can be destabilized enough to bind to the catalytic site of phospholipase A_2 ($E^* + S \rightarrow E^*S$). Destabilization of the substrate molecule in the bilayer interface is probably promoted by organizational defects or instabilities [5]. In terms of Scheme I, $E \rightarrow E^*$ step can be viewed as the anchoring step in which the anion binding site binds to the anionic group in the interface. On the other hand the $E^* + S \rightarrow E^*S$ step requires destabilization of the substrate molecule in the interface.

The k_i and A are not appreciably different for the semisynthetic pancreatic phospholipase A_2. These results in general support the hypothesis that N-terminus in phospholipase A_2 is part of the interfacial recognition site, where it anchors interfacial anionic groups. The amino acid substitutions that we have tested do not appreciably change the parameters for the scooting kinetics (Fig. 3). However, such substitutions do modify the average residence time of phospholipase A_2 in a zwitterionic interface (Fig. 4). This is reflected in the apparent affinity of phospholipase A_2 (cf. Fig. 1B). Implications of this conclusion on the reaction progress curve are only suggested at this stage. For example, the anion binding characteristics of the various phospholipase A_2 were best reflected in the shapes of the reaction progress curves where intervesicle exchange dominates. The inflexion points in the reaction progress curves (e.g., Fig. 1A) seem to indicate that slower steady-state rates are observed when the time for inflexion is long. Based on the arguments developed in the results section, we believe that the inflexion time is related to the desorption rate constant in the presence of products, k_p.

Ion selectivity remains the same under most of the conditions we have examined: sulfate > chloride > thiocyanate. This selectivity sequence also suggests that the binding site is a cation of small radius, such as an unsubstituted ammonium ion rather than guanidinium of arginine side chain [35]. There are several cationic groups in the N-terminus region: Ala-1, Arg-6 and Lys-10 [10]. Arg-6 does not appear to be absolutely necessary for anchoring, although it quenches Trp-3 fluorescence. We suspect that interaction of Ala-1 with anions modulates the environment of Trp-3, as does the interaction of Ala-1 with ^2H_2O. The increase in the fluorescence intensity of the semisynthetic phospholipase A_2, as well as the increase in the fluorescence intensity on binding of the enzyme to the interface suggest that the intramolecular interactions within the protein are substantially modified, thus leading to dequenching. Intramolecular interactions with polar groups are considered to be the major factors in intermolecular static quenching [24–27,36]. Unfortunately, X-ray crystallographic data on the N-terminus region of phospholipase A_2 shows little detail to assign relative position of these residues [39–60].

Our recent results (to be published) show that some of the snake venom phospholipase A_2 do not exhibit interfacial catalysis in the scooting mode. Such enzymes have fewer basic residues in the cluster of cations on the D-helix (positions 53–62). Since this helix has also been implicated in interfacial recognition [40] and catalytic functions [41,42], a functional anion binding site on phospholipase A_2 could involve several cationic residues including those at positions 53,65,62 as well as the N-terminus. A modification in these residues could be ultimately responsible for a difference in the interfacial specificity of phospholipase A_2 from different sources.

In designing the experiments described here, our working hypothesis has been that phospholipase A_2 can bind anions in the aqueous phase, and it can anchor to anions in the interface. Since anchoring of the enzyme to the interface depends upon the binding to the interfacial anions, competition from anions in the aqueous phase arises. Quantitative implications of this model are far from trivial, and a complete description would require understanding of ion binding equilibrium in the interface, and of the factors regulating on and off rate constants for the enzyme with free and with the interfacial anions. For such reasons the discussion in this series of papers has been essentially qualitative. However, the conclusions derived from these considerations do provide a deeper understanding of the interfacial catalytic

processes. The major points being, binding of anions somehow brings about a perturbation in the microenvironment of Trp-3 which must involve Arg-6, and a similar change is brought about by ^2H_2O. Both of these changes are somehow connected to the interfacial catalytic activity. Anchoring of phospholipase A_2 to anions in the interface promotes catalysis, and the presence of ^2H_2O inhibits interfacial catalysis. X-ray crystallographic and kinetic data on phospholipase A_2 suggests that a system of H-bonded moieties links the amino terminus to the catalytic system [35,37–39]. Transamination of D-Ala-1 substitution on the N-terminus leads to a complete loss of catalytic activity in the interface [39], as is the case with prophospholipase A (No. 1) which has seven extra amino acid residues attached to the N-terminus. Apparently, it can bind to anionic interface (cf. Fig. 9) but not to a zwitterionic interface, and catalytically it is not active in anionic or zwitterionic interface. Based on such observations it has been suggested that H-bonding network between Ala-1, Gln-4 and Asn-71 is necessary for a conformation of the enzyme that promotes interfacial catalysis. In the light of the results reported in this paper, it is tempting to suggest that this arrangement is achieved when the enzyme is anchored to the interface.

Acknowledgements

We wish to thank Karl Koehler for many useful discussions and critical reading of the manuscript. The lifetime measurements were carried out with the instrument purchased by a shared instrument grant No. RR01703 (NIH) to Professor J.R. Lackowicz. The lipids used in this study were kindly provided by Professor Ramirez. This work was supported by PHS (GM29703) and a travel grant from NATO.

References

1 Jain, M.K., Rogers, J., Jahagirdar, D.V., Marecek, J.F. and Ramirez, F. (1986) Biochim. Biophys. Acta 860, 435–447
2 Van Dam-Mieras, M.C.E., Slotboom, A.J., Pieterson, W.A. and DeHaas, G.H. (1975) Biochemistry 14, 5387–5394
3 Araujo, P.S., Rosseneu, M.Y., Kremer, J.M.H., Van Zoelen, E.J.J. and DeHaas, G.H. (1979) Biochemistry 18, 580–586
4 Hille, J.D.R., Donne-op den Kelder, G.M., Sauve, P., DeHaas, G.H. and Egmond, M.R. (1981) Biochemistry 20, 4068–4073
5 Jain, M.K., Edmond, M.R., Verheij, H.M., Apitz-Castro, R.J., Dijkman, R. a DeHaas, G.H. (1982) Biochim. Biophys. Acta 688, 341–348
6 Jain, M.K. and Maliwal, B.P. (1985) Biochim. Biophys. Acta 814, 135–140
7 Van Dam-Mieras, M.C.E., Slotboom, A.J., Pieterson, W.A. and DeHaas, G.H. (1975) Biochemistry 14, 5387–5394
8 Verheij, H.M., Slotboom, A.J. and DeHaas, G.H. (1981) Rev. Physiol. Biochem. Pharmacol. 91, 91–203
9 Verger, R. and DeHaas, G.H. (1976) Annu. Rev. Biophys. Bioeng. 5, 77–117
10 Slotboom, A.J., verheij, H.M. and DeHaas, G.H. (1982) in Phospholipids, (Hawthorne, J.N. and Ansell, G.B., eds.), pp. 359–434, Elsevier Biomedical Press, Amsterdam
11 Slotboom, A.J. and DeHaas, G.H. (1975) Biochemistry 14, 5394–5399
12 Van Scharrenburg, G.J.M., Puijk, W.C., Egmond, M.R., Van der Schaft, P.H., DeHaas, G.H. and Slotboom, A.J. (1982) Biochemistry 21, 1345–1352
13 Van Scharrenburg, G.J.M., Puijk, W.C., Seeger, P.R., DeHaas, G.H. and Flotboom, A.J. (1984) Biochemistry 23, 1256–1263
14 Van Scharrenburg, G.J.M., Jansen, E.J.M., Edmond, M.R., DeHaas, G.H. and Slotboom, A.J. (1984) Biochemistry 23, 6285–6294
15 Birch, D.J.S. and Imhoff, R.E. (1981) Rev. Sci. Instr. 52, 1206–1212
16 Birch, D.J.S. and Imhoff, R.E. (1981) Chem. Phys. Lett. 32, 56–60
17 Volwerk, J.J., Dedieu, A.G.R., Verheij, H.M., Dijkman, R. and DeHaas, G.H. (1979) Recueil 98, 214–220
18 Jain, M.K. and DeHaas, G.H. (1983) Biochim. Biophys. Acta 736, 157–162
19 Apitz-Castro, R.J., Jain, M.K. and DeHaas, G.H. (1982) Biochim. Biophys. Acta 688, 349–356
20 Jain, M.K., Streb, M., Rogers, J. and DeHaas, G.H. (1984) Biochem. Pharm. 33, 2541–2551
21 Jain, M.K. and Wu, N.M. (1977) J. Membrane Biol. 34, 157–201
22 Stryer, L. (1966) J. Am. Chem. Soc. 88, 5708–5712
23 Szabo, A.G. and Rayner, D.M. (1980) J. Am. Chem. Soc. 102, 554–563
24 Creed, D. (1984) Photochem. Photobiol. 39, 537–562
25 Lackowicz, J.R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York
26 Chong, M.C., Petrich, J.W., McDonald, D.B. and Flemming, G.R. (1983) J. Am. Chem. Soc. 105, 3819–3824
27 Lumry, R. and Hershberger, M. (1978) Photochem. Photobiol. 27, 819–825
28 Verger, R., Mieras, M.C.E. and DeHaas, G.H. (1973) J. Biol. Chem. 248, 4023–4034
29 Hendrickson, H.S., Trygstad, W.M., Loftness, T.L. and Sailer, S.L. (1981) Arch. Biochem. Biophys. 212, 508–514
30 Hille, J.D.R., Egmond, M.R., Dijkman, R., Van Oort, M.G., Jirgensons, B. and DeHaas, G.H. (1983) Biochemistry 22, 5347–5353
31 Kennagi, R., Koizumi, K. and Masuda, T. (1981) J. Biol. Chem. 256, 1177–1184

32 Colbeau, A., Canult, P. and Vignais, P.M. (1974) Biochimie 56, 275–288
33 Apitz-Castro, R.J., Cruz, M.R., Mas, M.A. and Jain, M.K. (1981) Thromb. Res. 23, 347–353
34 Jesse, R.L. and Franson, R.C. (1979) Biochim. Biophys. Acta 575, 467–470
35 Wright, E.M. and Diamond, J.M. (1977) Physiol. Rev. 57, 109–156
36 Anderson, J.S., Bowitch, G.S. and Brewster, R.L. (1983) Biopolymers 22, 2459–2476
37 Wells, M.A. (1974) Biochemistry 13, 2265–68
38 Renetseder, R., Brunie, S., Dijkstra, B.W., Denth, J. and Sigler, P. (1985) J. Biol. Chem. 260, 11627–11634
39 Dijkstra, B.W., Kalk, K.H., Brenth, J., DeHaas, G.H., Egmond, M.R. and Slotboom, A.J. (1984) Biochemistry 23, 2759–2766
40 Dutton, M.J., Eaker, D. and Hider, R.C. (1983) Eur. J. Biochem. 137, 537–544
41 Vensel, L.A. and Kantrowicz, E.R. (1980) J. Biol. Chem. 255, 7306–7310
42 Maraganore, J.M. and Heinrickson, R.L. (1985) Biochem. Biophys. Res. Commun. 131, 129–138
43 Jain, M.K., Rogers, J., Marecek, J.F., Ramirez, F. and Eibl, H. (1986) Biochim. Biophys. Acta 860, 462–474