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Analysis of the Kinetics of Phospholipid Activation of Cobra Venom Phospholipase A_2*

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A kinetic analysis of the "dual phospholipid model" for cobra venom phospholipase A_2 (Hendrickson, H. S., and Dennis, E. A. (1984) *J. Biol. Chem.* 259, 5734–5739) was applied to the activation of phospholipase A_2-catalyzed hydrolysis of a thiol ester analog of phosphatidylethanolamine (thio-PE) in Triton X-100/phospholipid mixed micelles by various phosphorylcholine-containing activators. Activation of thio-PE hydrolysis by didecanoylphosphatidylcholine (PC) was found to be a function of the surface concentration of activator rather than bulk concentration. Its presence did not affect the initial binding of enzyme to phospholipid in the micelle surface as determined kinetically. After initial binding of enzyme to the surface, the activation appears to be due to enzyme-lipid binding in the surface. Activation does not appear to affect the affinity of the enzyme for phospholipid substrate, but rather affects the catalytic efficiency of the enzyme as characterized by the value of V_{max}. The monomeric phospholipid dibutyryl-PC, when used as an activator at 57 mM (bulk concentration), also showed effects of surface dilution with Triton X-100, which would not be expected unless the lipid is incorporated into the micelles to some extent at these high concentrations. A thiol ester analog of phosphatidylcholine, thio-PC, was less effective than didecanoyl-PC as an activator, but appeared to be more effective than decylphosphorylcholine. A conformational change of the enzyme upon binding of the activator, after enzyme is bound to substrate at the interface, is discussed as a possible mechanism for this activation.

An unusual aspect of the action of cobra venom phospholipase A_2 on phospholipids in Triton X-100/phospholipid mixed micelles is the substrate specificity of the enzyme. PC[1] is a much better substrate than PE in Triton X-100 micelles with only one phospholipid present (1). However, in the presence of PC or phosphorylcholine-containing lipids, PE is at least as good and in many cases better than PC as a substrate (2–4). This specificity reversal is not seen with phospholipases A_2 from other sources.[2] Activation of the hydrolysis of PE in Triton X-100/PE mixed micelles by dibutyryl-PC, which is monomeric and not significantly incorporated into the micelle at moderate concentrations, indicates a mechanism of direct activation of the enzyme rather than activation as a result of a change in the properties of the interface (2).

There are three obvious ways that the activation can take place: (i) by increasing the binding of enzyme to the bulk substrate, (ii) by increasing the binding of the enzyme to substrate in the surface, or (iii) by increasing the V_{max} of the enzyme. In order to better understand the activation process and differentiate between these possibilities, a detailed kinetic analysis of cobra venom phospholipase A_2 using thiol ester lipid analog substrates (5) was applied to the activation of thio-PE hydrolysis by various phosphorylcholine-containing activators. The activating effects of didecanoyl-PC, thio-PC, dibutyryl-PC (which are also substrates) and decylphosphorylcholine on the hydrolysis of thio-PE by cobra venom phospholipase A_2 are presented here.

EXPERIMENTAL PROCEDURES

Phospholipase A_2 was purified to homogeneity from *Naja naja naja* (cobra) venom as described by Deems and Dennis (6) and modified by Darke *et al.* (7). Thio-PC and thio-PE were prepared as described in the accompanying manuscript (5). Decylphosphorylcholine was synthesized from decanol by the choline phosphorylation procedure of Brockerhof and Ayengar (8). Didecanoyl-PC and dibutyryl-PC were obtained from Calbiochem-Behring, and the latter was purified by silicic acid chromatography. Preparation of Triton X-100/phospholipid mixed micelles and the enzyme assay are described in the accompanying manuscript (5). The kinetic rates for the enzyme reaction with thio-PE in the presence or absence of activators were initially linear and eventually decreased as the percentage of hydrolysis increased. Lag phases were not observed in any experiment. Data were fitted to the kinetic equations derived previously (5) by nonlinear regression analysis using the programs in KINFIT, a kinetics program written by Knack and Röhm (9).

RESULTS

Kinetic Analysis—A reaction scheme, proposed previously for the dual phospholipid model for phospholipase A_2 action (5), is shown in Equation 1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ESS \xrightarrow{k_3} ES + \text{Prod} \qquad (1)$$

This involves binding of the water-soluble enzyme to a phospholipid in the micelle surface, followed by binding to additional phospholipid in the interface and then catalytic hy-

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; thio-PC, 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycerol-3-phosphorylcholine; thio-PE, 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycerol-3-phosphorylethanolamine; didecanoyl-PC, 1,2-bis(decanoyl)-*sn*-glycerol-3-phosphorylcholine; dibutyryl-PC, 1,2-bis(butyryl)-*sn*-glycerol-3-phosphorylcholine.

[2] A. Pluckthun and E. A. Dennis, manuscript in preparation.

drolysis. The rate expression derived previously (5) is shown in Equation 2.

$$v = \frac{V_{max} X_s S_0}{K_m K_s + K_m S_0 + X_s S_0} \quad (2)$$

The following symbols are used: v ((mole/volume)time^{-1}), bulk velocity; V_{max} ((mole/volume)time^{-1}), maximal velocity; S_0 (mole/volume), total phospholipid substrate (bulk concentration); X_s (mole fraction, unitless), total phospholipid substrate (surface concentration); X_a (mole fraction, unitless), total phospholipid activator (surface concentration); A_0 (mole/volume), total bulk concentration of activator; $K_s = k_{-1}/k_1$ (mole/volume); and $K_m = (k_{-2} + k_3)/k_2$ (mole fraction, unitless).

In the reaction scheme shown in Equation 1, one of the two phospholipids binds to an activator site causing acceleration of the hydrolysis of the other phospholipid bound at the catalytic site. Experiments have not yet differentiated between these two sites, so it is not known which site is involved in the initial binding of enzyme to the micelle surface. Activation of PE hydrolysis by PC involves binding of two different phospholipids to the enzyme. Each phospholipid can bind to both sites, perhaps with similar affinities; however, the relative affinity of a given phospholipid for each site may be quite different. This greatly complicates the kinetic analysis.

Fig. 1 shows the possible equilibria which may occur with two different phospholipids, denoted *A* and *B*. If *A* represents didecanoyl-PC and *B* represents thio-PE, then only the equilibria to the right of the *dashed line* in Fig. 1 are significant. This is because the affinity of thio-PE for initial binding to the enzyme is about an order of magnitude tighter than that of didecanoyl-PC (5), and the concentrations of didecanoyl-PC used in these experiments are generally an order of magnitude less than those of thio-PE. Thus, the first phospholipid bound will be B, and this may be either at the activator or catalytic site, and the second phospholipid bound, which can be either A or B, would be at the other site.

As discussed previously (5), two types of kinetic experiments can be performed in order to determine K_s, K_m, and V_{max}. In Case I, varying the bulk substrate concentration, S_0, at constant surface concentration of substrate, X_s, allows the determination of the initial binding, K_s. In Case II, varying the surface concentration of substrate, X_s, at constant bulk concentration of substrate, S_0, allows the determination of K_m and V_{max}. In this way, the kinetic parameters can be determined with activators present at constant bulk concentration, A_0 (mole/volume), or constant surface concentration, X_a (mole fraction).

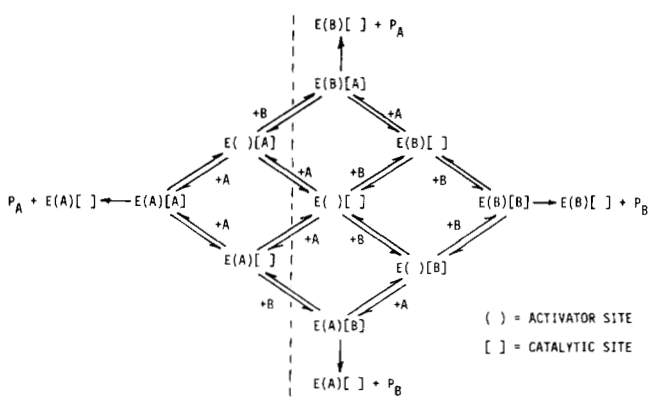

FIG. 1. **Possible equilibria for two different phospholipids interacting with phospholipase A_2.** If *A* represents didecanoyl-PC and *B* represents thio-PE, then only the equilibria to the *right* of the *dashed line* are significant.

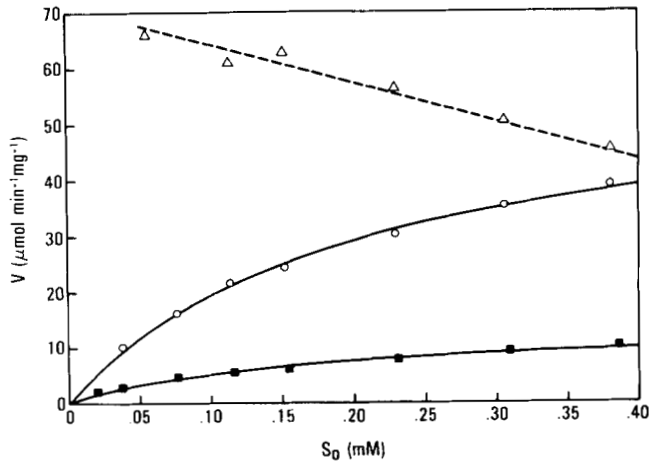

FIG. 2. **Velocity of phospholipase A_2-catalyzed hydrolysis of thio-PE as a function of total substrate (thio-PE), S_0, at constant $X_s = 0.0196$.** ■, no activator; ○, didecanoyl-PC at constant $X_a = 0.00244$; △, didecanoyl-PC at constant $A_0 = 0.0476$ mM. *Solid lines*, computer fits of data to Equation 3.

Activation of Thio-PE Hydrolysis—For Case I, the initial binding of enzyme to thio-PE substrate on the interface was determined at constant surface concentration of substrate, X_s. At constant bulk concentration of didecanoyl-PC activator, the rates decreased with increasing substrate, S_0, whereas at constant surface concentration of activator, the rates increased with increasing substrate in a hyperbolic (saturation) manner (Fig. 2). A constant bulk concentration of activator, the surface concentration of activator necessarily decreases with increasing substrate, since the ratio of thio-PE to Triton X-100 is fixed. This decrease in rate, then, is a function of decreasing surface concentration of activator, and activation must be a function of surface concentration of activator rather than bulk concentration. The values of K_s determined in the absence and presence of activator are quite similar (Table I). Activation appears to occur as a result of the second binding step of the enzyme and does not appear to affect the apparent K_s and only slightly decreases the apparent K_m for substrate binding.

For Case II, the secondary binding of enzyme to thio-PE in the interface was determined at constant bulk concentration of activator so as to maintain a constant ratio of activator to substrate in the surface (Fig. 3). For thio-PE without activator, the curve for v *versus* X_s appeared hyperbolic (5). With didecanoyl-PC present as an activator, the curve appeared more sigmoidal. This sigmoidal-like behavior was also seen with thio-PC alone as substrate. From a computer fit to the Hill equation

$$v = V_{max} X_s^n / (K'^n + X_s^n); \; K' = K_m(K_s/S_0 + 1)$$

values of V_{max} and K_m were calculated. With decylphosphorylcholine as an activator of thio-PE hydrolysis, the curve appeared hyperbolic rather than sigmoidal. Dibutyryl-PC as an activator at 57 mM bulk concentration was fitted to a hyperbolic saturation curve. A slightly better fit was obtained when the hyperbola was allowed to begin at $v = 170$ μmol min^{-1} mg^{-1}. The kinetic parameters, K_m and V_{max}, determined in the presence of these activators are given in Table I.

Activation Factor—In order to compare and evaluate various activators, we assumed that the activator binds to a specific site which shows saturation behavior. The data given

TABLE I

Kinetic parameters for phospholipase A_2-catalyzed hydrolysis of thio-PE in the presence of various activators

Activator	Substrate		Activator		K_s	K_m	V_{max}
	S_0	X_s	A_0	X_a			
	mM		*mM*		*mM*	*mol fraction*	*μmol min⁻¹ mg⁻¹*
None		0.0196			0.18 ± 0.03		
None	0.476					0.10 ± 0.01	89 ± 8
Didecanoyl-PC		0.0196		0.00244	0.23 ± 0.02		
Didecanoyl-PC	0.476		0.0595			0.037 ± 0.004[a]	1100 ± 100[a]
Dibutyryl-PC	0.476		57.1			0.038 ± 0.004[b]	1300 ± 60[b]
						0.081 ± 0.009[c]	1500 ± 100[c]
Decylphosphoryl-choline	0.476		1.90			0.071 ± 0.007	920 ± 50

[a] The curve, v *versus* X_s, was sigmoidal with a Hill coefficient of 1.55 ± 0.05.
[b] Based on a hyperbola beginning at $v = 0$.
[c] Based on a hyperbola beginning at $v = 170$ μmol min^{-1} mg^{-1}.

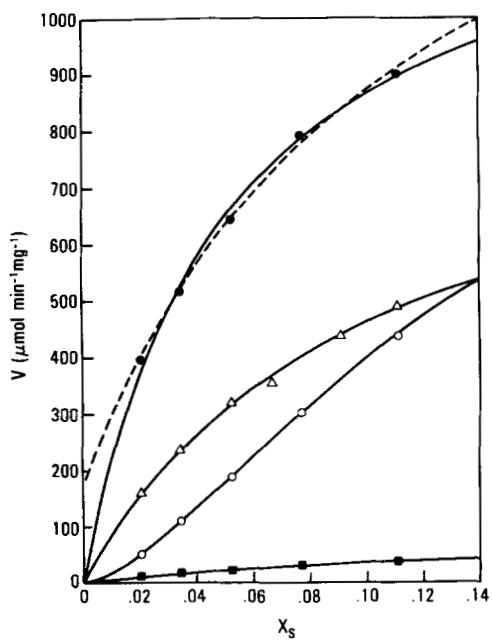

FIG. 3. **Velocity of phospholipase A_2-catalyzed hydrolysis of thio-PE as a function of surface concentration of substrate (thio-PE), X_s, at constant S_0 = 0.476 mM.** ■, no activator; ○, didecanoyl-PC as activator, A_0 = 0.0595 mM; △, decylphosphorylcholine as activator, A_0 = 1.9 mM; ●, dibutryl-PC as activator, A_0 = 57.1 mM. *Solid lines*, computer fits of data to Equation 3 or Hill equation (with didecanoyl-PC); *dashed line*, computer fit to hyerbola beginning at $v = 170$ μmol min^{-1} mg^{-1}.

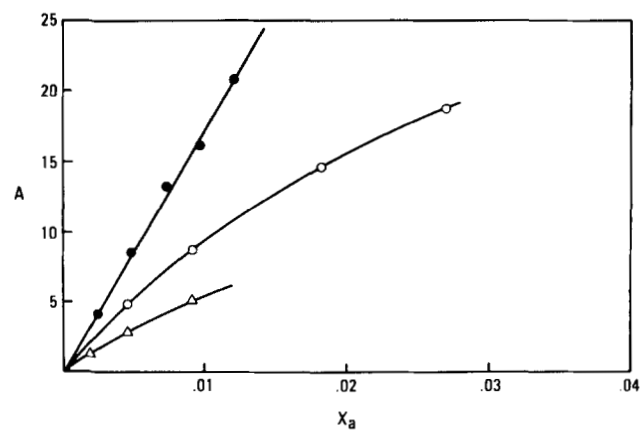

FIG. 4. **Activation factor, A, for thio-PE hydrolysis as a function of surface concentration of activator, X_a.** ●, didecanoyl-PC, X_s = 0.0196, S_0 = 0.160 mM; ○, didecanoyl-PC, X_s = 0.073, S_0 = 0.8 mM; △, thio-PC, X_s = 0.073, S_0 = 0.8 mM. Lines for ○ and △ data points are computer fits to hyperbola.

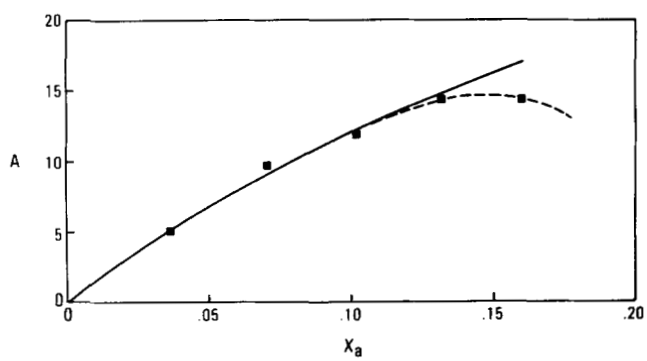

FIG. 5. **Activation factor, A, for thio-PE hydrolysis as a function of surface concentration of decylphosphorylcholine, X_a.** X_s = 0.0476, S_0 = 0.476 mM. *Solid line*, computer fit to hyperbola; *dashed line*, inhibition.

above suggest that activation depends on surface concentration rather than bulk concentration of activator. Activation at a given concentration of activator can be measured experimentally by an activation factor, $A = (v_a - v_0)/v_0$, where v_a and v_0 are velocities in the presence and absence of activator, respectively. Saturation of activator binding can be expressed by the equation, $A = A_{max}X_a/(K_a + X_a)$.

The effectiveness of various activators is shown in Figs. 4 and 5, where the activation factor, A, is plotted as a function of surface concentration of activator, X_a. The activating effect of didecanoyl-PC at two different fixed concentrations of thio-PE was greater at the lower thio-PE concentration. This suggests a competition between thio-PE and didecanoyl-PC for the site of activation. Thio-PC appears to be less effective than didecanoyl-PC as an activator. This is difficult to interpret, however, since hydrolysis of thio-PC contributes to the observed rate. The monoalkyl lipid decylphosphorylcholine appears to be less effective as an activator than diacyl-PCs. However, this may be due to its tighter binding at the active site, bringing the enzyme closer to saturation and giving rise to a hyperbolic-type curve. At high decylphosphorylcholine concentrations, inhibition is seen, and this may also be due to tight binding of decylphosphorylcholine to the active site of the enzyme. The effectiveness of these activators is summarized in Table II. With dibutyryl-PC, A_{max} was not determined. However at 57 mM, the enzyme is probably close to

TABLE II

Effectiveness of various activators of phospholipase A_2-catalyzed hydrolysis of thio-PE

Activator	S_0[a]	K_a	A_{max}
	mM	*mol fraction*	
Didecanoyl-PC	0.774	0.038 ± 0.0004	45 ± 0.3
Thio-PC	0.774	0.032 ± 0.008	23 ± 5
Decylphosphoryl-choline	0.476	0.21 ± 0.06	37 ± 7

[a] Triton X-100 concentration = 9.68 mM.

saturation (2), and $A = 25$ (at $X_s = 0.073$), which is probably close to A_{max}.

DISCUSSION

Activation of thio-PE hydrolysis by didecanoyl-PC does not significantly affect the initial binding of enzyme to thio-PE in the micelle surface as characterized by the value of K_s. Activation is a function of the surface concentration of activator rather than bulk concentration and thus appears to be the result of enzyme-lipid binding in the surface which occurs after the initial binding of the enzyme to the surface.

Activation of thio-PE hydrolysis by didecanoyl-PC in the surface results in a sigmoidal relationship between the reaction rate and surface concentration of substrate (or activator, since the ratio of substrate to activator is fixed). This sigmoidal effect was seen with thio-PC as a substrate alone, but was not obvious with thio-PE alone as a substrate in the absence of an activator. Possible causes for this are discussed in the accompanying manuscript (5). These results may indicate a basic difference in the way thio-PC and thio-PE interact with the enzyme in the secondary binding within the interface. For example, micellar PC may induce dimer (or higher aggregate) formation and PE not. Another possible cause for this sigmoidal behavior may be the necessity of more than one phospholipid molecule to interact with the enzyme for activation to occur. This could represent additional phospholipid molecule(s) adjacent to the substrate molecule which would be in contact with the enzyme when the substrate is bound. If this were the case, then the PC head group would be effective in this activation, whereas the PE head group would not be. This could involve a zwitterionic charge interaction of the enzyme with the PC head group which is not possible with the PE head group due to the greater hydration about the primary amino group. This could keep the enzyme from close contact with the interface or prevent a conformational change resulting in a more efficient active site. Triton X-100 molecules adjacent to the substrate molecule could also have the same effect due to the large size of the polyether head group. The decrease in activity observed upon the addition of Triton X-100 may result from the breakup of patches or aggregates of phospholipid molecules surrounding the substrate molecule which the enzyme must bind to be active. The nature of the head groups of these surrounding lipid molecules may be a critical factor affecting the activity of the enzyme, according to this explanation.

In a previous study (2), high concentrations (60 mM) of dibutyryl-PC appeared to eliminate the effect of surface dilution with Triton X-100, at least up to a Triton X-100 to PE ratio of 16:1. It was assumed that dibutyryl-PC was entirely monomeric and that activation of the enzyme occurred by a bimolecular reaction between soluble dibutyryl-PC and either soluble enzyme or enzyme bound to PE in the interface. The present study, which utilizes a spectrophotometric assay system, allowed the range of the earlier study to be extended to 50:1 Triton to phospholipid and does show a measurable decrease in relative rate at high Triton to phospholipid ratios, although not as much as for substrate alone or micellized activators. When the relative rate of thio-PE hydrolysis in the presence of 57 mM dibutyryl-PC determined spectrophotometrically (Fig. 3) is plotted as a function of the Triton to thio-PE ratio, these data are consistent (agree within ±10%) with that obtained previously (2) by pH-stat with oxy-PE in which the Triton to phospholipid ratio ranged from 4:1 to 16:1. At such a high concentration of dibutyryl-PC in the presence of an increased concentration of Triton micelles, there may well be some incorporation of dibutyryl-PC into the micelles. Previous experiments which showed only low incorporation of dibutyryl-PC into Triton X-100 micelles only used concentrations of dibutyryl-PC up to 7.5 mM (10). Even incorporation of 2–5% of the dibutyryl-PC into the micelles would give a surface concentration of dibutyryl-PC comparable with that of other good activators. Surface dilution observed here may thus be due to some dibutyryl-PC which is incorporated into the micelles at high bulk concentration of this lipid and of Triton X-100. A slightly better fit of the data for dibutyryl-PC activation was achieved by beginning the hyperbola at 170 μmol min^{-1} mg^{-1} (Fig. 3). This would represent at most only about 10% of the activation not being subject to surface dilution.

The differences in activating efficiencies of didecanoyl-PC, thio-PC, and decylphosphorylcholine may be related to head group area in the interface. The sulfur atoms present in the head group of thio-PC confer more hydrophobic character to the head group. This is reflected in the lower critical micelle concentration of thio-PC as compared to the didecanoyl-PC which has the same hydrocarbon chain length (11) and the lower solubilities of thio-PC and thio-PE in Triton X-100 mixed micelles. Thio-PC may thus have a somewhat smaller head group area in the interface than didecanoyl-PC. Decylphosphorylcholine, with only one hydrocarbon chain/head group, would have an even smaller head group area. It is also possible that this compound has a different conformation of the choline phosphate group.

An alternate explanation for the apparent low activating efficiency of decylphosphorylcholine as characterized by its high K_a value is the possibility that not all of this compound is incorporated into the micelle. Decylphosphorylcholine has a critical micelle concentration of 10 mM.[3] At a concentration of 1.9 mM, it would be monomeric; but in the presence of Triton X-100 micelles, one would expect most of this compound to be partitioned into the micelle. If, however, only a small portion is partitioned into the micelle, its surface concentration would be much less, and this would account for the high apparent K_a value. Dibutyryl-PC has an A_{max} value at least comparable to those of the other activators. Since it is not known to what extent this lipid partitions into the micelle, a value of K_a cannot be determined for either a bound or soluble form.

Activation of thio-PE hydrolysis by didecanoyl-PC appears from these experiments to result from binding of the activator to the enzyme in the interface after initial binding of enzyme to the substrate thio-PE in the surface. Activation does not appear to affect the affinity of the enzyme for the phospholipid substrate, but rather affects the catalytic efficiency of the enzyme. The simplest interpretation of these results would involve a conformational change of the enzyme upon binding of the activator, and this could be effected after the enzyme is bound to substrate at the interface via its catalytic site or an additional site(s). Just how the binding of a phosphoryl-

[3] H. S. Hendrickson, unpublished observations.

choline-containing lipid to the activator site causes a conformational change in the enzyme is unclear at this time. Among the many possible explanations for the precise role of the activator phospholipid are the possibilities that the conformational change accelerates product release, accelerates attack of the catalytic amino acid residue(s), or causes enzyme dimerization and/or aggregation. Such a change must be less important for the phospholipases A_2 from other sources.[2]

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