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Immobilized Phospholipase A_2 from Cobra Venom

PREVENTION OF SUBSTRATE INTERFACIAL AND ACTIVATOR EFFECTS*

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The activation of cobra venom phospholipase A_2 by activators (containing phosphorylcholine moieties) appears to depend upon the aggregation state of the enzyme, and the presence of a lipid-water interface. The characteristics of this activation were studied by comparing the behavior of the enzyme immobilized on an agarose gel to that of the soluble enzyme. The immobilized enzyme displays only a few per cent of the soluble enzyme activity toward micellar dipalmitoylphosphatidylcholine (PC). However, the relative loss of activity is much less with micellar dipalmitoylphosphatidylethanolamine or soluble diheptanoyl-PC. The affinity for Ca^{2+} is increased about 10-fold by immobilization while the apparent pK_a of the enzyme is decreased by 0.5–0.8 pH units. Activation energies are similar for the two enzyme forms and are independent of the physical state of the substrate used. Catalytic constants of the enzyme toward monomeric PC are not changed by immobilization. Yet, activators of the soluble enzyme have negligible effect on the immobilized enzyme, either in the presence or absence of an interface. Monomeric activators promote the binding of the soluble enzyme to the immobilized form. Apparently, immobilization mainly produces monomerically constrained enzyme which cannot be activated under any condition, whereas normally, activators in the presence of lipid-water interfaces induce the formation of enzyme dimers or possibly higher order aggregates.

Phospholipase A_2 (EC 3.1.1.4) is a small water-soluble enzyme that hydrolyzes the fatty acid ester bond at the *sn*-2 position of 1,2-diacyl-*sn*-phosphoglycerides. Unlike other esterases, phospholipase A_2 acts preferentially on substrates that are part of a lipid-water interface. Previous studies have suggested that the interface plays an important role in the enzymatic activity (for a review, see Ref. 1). Several explanations have been suggested for this specific requirement for interfacial phospholipid. Among them, the dual phospholipid model was suggested by our laboratory (2, 3) for the cobra venom enzyme. Support for this model has come principally from the activation of PE[1] hydrolysis by phosphorylcholine-containing lipids (4-6). This model requires the enzyme to undergo at least two binding steps before catalysis occurs; thus it must bind more than one phospholipid to work effectively. The studies using phospholipids in mixed micelles with Triton X-100 showing "surface dilution kinetics" (7) also fit this model. Activation does not appear to affect the affinity of the enzyme for phospholipid substrate, but rather it affects the catalytic efficiency of the enzyme (8, 9), Recently several mechanisms were proposed which could explain these observations (1). A conformational change of the enzyme upon binding of the phosphorylcholine-containing lipid (activator) appears to be the most likely mechanism for the activation, and some experiments also suggest a functional role for a dimeric enzyme (2).

We (10) have previously shown that the enzyme can be covalently immobilized to porous glass beads by diazo coupling. We have now utilized an improved coupling system to immobilize the enzyme and have compared the behavior of the cobra venom phospholipase A_2 in soluble form and immobilized form toward monomeric and micellar substrates in order to better understand the interfacial activation processes. This has also enabled us to study the effect of activators on both enzyme forms. A preliminary report of these results has been presented (11).

EXPERIMENTAL PROCEDURES

Materials—Phospholipase A_2 was purified from cobra venom (*Naja naja naja*) obtained from the Miami Serpentarium as described elsewhere (12) and recently modified (37). Palmitoyl lyso-PC, dipalmitoyl-PC, and *N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14) were obtained from Calbiochem-Behring. Dodecylphosphorylcholine was a gift of Dr. H. S. Hendrikson (St. Olaf College, Northfield, MN). Sphingomyelin and agarose CL-6B 200 were from Sigma. Diheptanoyl-PC was from Avanti Polar Lipids and dipalmitoyl-PE was from Mann Research Laboratories. Triton X-100 was provided by Rohm and Haas.

Dihexanoyl-PC and dihexanoyl-PE were synthesized and kindly provided by Dr. A. Pluckthun and F. F. Davidson in this laboratory (13). Diheptanoyl-PE was synthesized by similar methods (13) starting from purified egg yolk PE (14). Briefly, *N*-tritylbromide was used to protect the amino group. *N*-Tritylphosphatidylethanolamine was deacylated with tetrabutylammonium hydroxide (25% in methanol) and reacylated with heptanoic anhydride in the presence of *p*-*N,N*-dimethylaminopyridine. Detritylation of *N*-trityldiheptanoyl-PE was accomplished by anhydrous trifluoroacetic acid.

1,2-Dibutylcarbamoyl-*sn*-glycero-3-phosphorylcholine was synthesized according to Gupta and Bali (15). Products gave a single spot by one-dimensional thin layer chromatography on silica gel plates (Analtech) using chloroform/methanol/water (65:25:4, v/v) and chloroform/methanol/acetic acid/water (65:15:10:4, v/v) as developing solvents. Iodine vapor and molybdate spray (16) were employed for detection. Both lipids gave one peak by $^{31}P\{^1H\}$ NMR (broad band decoupling, sweep width ±8000 Hz). ^1H NMR spectral features for diheptanoyl-PE in $CDCl_3$ are as follows (peaks ppm downfield from internal tetramethylsilane): 0.88 (t, terminal CH_3); 1.28 (broad s, acyl-CH_2); 1.59 (m, β-CH_2); 2.27–2.33 (m, sn_1- and sn_2-0(CO)CH_2); 2.44 (s, NH_2); 3.16 (broad multiplet, CH_2-N); 4.1 (m), 4.2 (m), and

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; lyso-PC, 1-palmitoyl-*sn*-glycero-3-phosphorylcholine; CMC, critical micellar concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

4.35–4.4 (m) (glycerol CH_2OP, choline CH_2OP, glycerol $CH_2O(CO)$); 5.22 (m, glycerol CH). The phospholipid concentrations were determined by phosphorus analysis (17). All other chemicals used in these studies were of the best available grade.

Covalent Coupling of Phospholipase A_2 to Agarose Gel—Activation of the agarose gel with tresyl choride (2,2,2-trifluoroethanesulfonate chloride, Fluka) was performed at room temperature in dry acetone as described by Nilsson and Mosbach (18). The coupling reaction was run in a 0.2 M borate (pH 7.8) buffer containing 0.5 M NaCl and 10 mM $CaCl_2$. In a typical experiment 1 g of wet activated gel was incubated with 1 mg of pure phospholipase A_2 for 2 h at 0–4 °C under mild stirring. The gel was then washed with the same buffer. The per cent of coupled protein was determined from the protein content of washings. The reaction was then quenched with 100 mM ethanolamine (30 min, 4 °C). The gel was collected on a glass filter funnel and washed successively with 50–100 ml of 0.2 M acetate buffer (pH 3.5) containing 0.5 M NaCl, 50–100 ml of 0.5 M NaCl, and with glass distilled water. The wet gel was always resuspended in water to a final "gel concentration" of 90–120 mg ml^{-1}. A control gel was treated in the same conditions but without phospholipase A_2 coupling (unreacted gel).

Spacer Coupling—The tresyl chloride-activated gel was suspended in 0.2 M borate/NaOH (pH 9.5) buffer containing 1 M 1-amino-5-ol pentane (Fluka), and kept under gentle agitation for 30 min at 20 °C. The gel was extensively washed with water and transferred stepwise to dry acetone. The spacer was then activated with tresyl chloride and coupled with phospholipase as described above.

Enzyme Activity—The enzymatic activity of the soluble and immobilized enzyme was titrimetrically measured toward long chain PE (5 mM) and PC (5 mM) in mixed micelles with Triton X-100 (19). When short chain substrates were employed, Triton X-100 was omitted. Assays were conducted at pH 8.0 and 40 °C. Except where noted, Triton X-100 was 20 mM and $CaCl_2$, 10 mM. All the values given are averages of at least duplicate assays.

Sampling Procedure—The gel-bound enzyme was vortexed gently to homogeneity, then an aliquot was taken with a calibrated automatic pipette. The tip was carefully wiped with soft paper towels before introduction in the assay vessel. In experiments using diheptanoyl-PC (1 mM) as substrate, we found a good linear correlation between the enzyme quantity and its activity, at least in the range of gel concentrations generally used in these studies (0.25–3.5 μg of protein). The error between each assay (using different tips and pipettes) was less than ±15%.

Protein Determination—Protein concentrations were determined by the method of Lowry *et al.* (20) using the appropriate correction factor (12).

Amino Acid Analysis—Amino acid analyses were carried out on a Beckman 117 or 141 analyzer, after 22 h hydrolysis in constant boiling HCl (containing 1 mg/ml phenol) at 105 °C in vacuum sealed tube. Before loading on the column, hydrolysates were filtered on a 0.45-μm pore size hydrophilic Nylon-66 filter (Rainin Instrument Co.).

Binding of the Soluble Enzyme to the Immobilized Enzyme—Preparation IV (see below) and unreacted control gel were used in these experiments. 100 μl of gel (7.6 μg of immobilized phospholipase A_2, 9.2 mg of gel) or of the unreacted gel (9.8 mg of gel) were incubated under mild agitation for 1 h at 25 °C in 40 mM borate buffer (1 ml) containing 1 mM $CaCl_2$ and various quantities of soluble phospholipase A_2 (0–20 μg). Then the medium was centrifuged 2 min using a bench centrifuge and the optical density of the supernatant measured at 220 nm. Under these conditions, the specific binding represents the difference between the total amount of enzyme bound to the preparation IV gel and the amount of enzyme bound to the unreacted control gel. Equation 1 was used

$$E_{bound} = E_{total}[(\Delta A_{IV} - \Delta A_c)/A_t] \quad (1)$$

where E_{bound} is the amount of specifically bound protein (μg), E_{total} is the total amount of added protein (μg), and ΔA is the difference in optical density at 220 nm between the total optical density of the supernatant not exposed to gel (A_t) and the optical density of the supernatant which was exposed to gel followed by centrifugation. Subscripts IV and c refer to preparation IV and control gel, respectively.

RESULTS

Coupling—Coupling of cobra venom phospholipase A_2 was performed using four different conditions as summarized in Table I. In preparation I, the protein concentration (125 μg of protein ml^{-1} of coupling buffer) was sufficient to ensure the presence of aggregated enzyme; in preparation II, the same enzyme concentration was used but in the presence of 0.5 mM lyso-PC (CMC = 7 μM (21)) as a protective agent for the active site; in preparation III, the enzyme concentration was the same but a spacer arm was present on the gel; and in preparation IV, the enzyme concentration was 30 μg ml^{-1} so that more of the protein would be monomeric (22). The better yield obtained in preparation IV as compared to preparation I may be explained by the higher concentration of protein monomers in preparation IV than in preparation I. In coupling to the aggregated form, if only one of the subunits is covalently bound to the gel, then subsequent washings would dissociate the aggregates and remove all noncovalently bound subunits from the gel. The protein concentration calculated from the amino acid analysis of preparations I and III was 4.3 and 5.9 μM, respectively, corresponding to 58 μg ml^{-1} and 80 μg ml^{-1} of gel suspension. The agreement of these values with those determined by the protein content of the washings (in Table I) proves that the latter method provides an accurate means of determining the amount of bound enzyme. Preparation I was used for the studies reported herein, unless otherwise specified.

TABLE I

Preparation of immobilized phospholipase A_2

Preparation	Gel	Enzyme concentration	Lyso-PC (0.5 mM)	Yield	Enzyme bound
		μg ml^{-1}		*%*	*μg ml^{-1} of gel*
I	Agarose	125	–	60	55
II	Agarose	125	+	69	93
III	Agarose + spacer	125	–	80	77
IV	Agarose	30	–	87	76

Stability of the Enzyme-Agarose Bond—To determine if the enzyme was covalently bound to the agarose beads, the gel was vigorously stirred, then centrifuged, and the supernatant assayed for the activity using a 3.2 mM solution of diheptanoyl-PC (most sensitive assay). Under these conditions, only 0.23% of the activity of the immobilized enzyme was freed in the supernatant. In another experiment, immobilized phospholipase A_2 was treated with 1 or 20 mM Triton X-100, and the aqueous phase removed by centrifugation or filtration with a 0.22-μm pore diameter filter (Millipore GSWP). The activity was assayed using the dipalmitoyl-PC/Triton X-100 assay on the aqueous fraction and the gel fraction. No activity was detected in the aqueous fraction; more than 99% of the activity was associated with the gel. Using soluble phospholipase A_2 with the same conditions shows that less than 5% of the activity remains associated with the filter. Even after 5 months of storage at 4 °C, the protein released in the aqueous phase was less than 1%, as determined by the activity of both the gel and aqueous fraction on diheptanoyl-PC. All of the data indicate that the enzyme was covalently bound to the agarose beads.

Activity of the Immobilized Phospholipase A_2—The specific activity of the agarose-immobilized enzyme toward 5 mM dipalmitoyl-PC was between 20 and 30 μmol min^{-1} mg^{-1} of protein which was only 1.7–2.5% of the specific activity of the soluble phospholipase A_2 (Table II). Using 5 mM dipalmitoyl-PE alone as substrate leads to less loss in enzyme activity. Such results show that a limitation on diffusion-controlled migration of micellar substrates cannot account for the observed loss of activity with immobilized enzyme since micelles of dipalmitoyl-PE are at least as large as dipalmitoyl-PC micelles. Moreover, chromatography of various micelles on agarose gel clearly indicates that they can penetrate into the

TABLE II
Activity of soluble and immobilized phospholipase A_2 *toward various substrates*

Substrate	Triton X-100 (20 mM)	Specific activities (% of soluble)		
		Soluble	Preparation I	Preparation II
		μmol min⁻¹ mg⁻¹		
Dipalmitoyl-PC (5 mM)	+	1120	19 (1.7)	28 (2.5)
Dipalmitoyl-PE (5 mM)	+	13	5.3 (41)	4.3 (33)
Dipalmitoyl-PE (5 mM) + sphingomyelin (1 mM)	+	380	16 (4.2)	8.0 (2.1)
Diheptanoyl-PC (0.8 mM)	−	86	77 (90)	64 (75)
Diheptanoyl-PC (3.2 mM)	−	3400	280 (8.3)	210 (6.1)

gel matrix (23, 24). When sphingomyelin (1 mM) was added to the assay, the specific activity of the immobilized enzyme on dipalmitoyl-PE was similar to dipalmitoyl-PC. While PE hydrolysis of the immobilized enzyme was increased 2–3-fold by sphingomyelin, this was not nearly as dramatic as the 30-fold activation observed with the soluble enzyme. Overall, these results show that the loss of enzyme activity is less on nonactivated substrates (PE) than on activated substrate (PE plus sphingomyelin or PC). Apparently, the immobilized enzyme has lost most of its ability to be activated by phosphorylcholine-containing compounds.

It is well known that lipolytic enzymes such as phospholipase A_2 are activated by interfaces. Using diheptanoyl-PC as substrate at a submicellar concentration (0.8 mM) or at concentration above (3.2 mM) the CMC of 1 mM shows that the soluble enzyme is 40-fold more active on micelles than on monomers. In contrast, the immobilized enzyme was only 3–4-fold more active. Moreover, the immobilized enzyme appeared to have almost full activity on monomeric substate, but to be only 6–8% as active on micellar substrate. Note that no hydrolytic activity was associated with the control gel. Similar results were obtained using preparations III (4%) and IV (12%) (data not shown). Within the experimental error of the quantitation of the bound protein and the kinetic assays, all four preparations showed the same general lack of activation in the presence of activators and interfaces. In summary, these data show that the interfacial activation of the immobilized enzyme is some 10-fold less than the activation of the soluble enzyme. The fact that the sphingomyelin activation of PE hydrolysis was also about 10-fold more for the soluble form than for the immobilized form might indicate that about 10% of the bound enzyme is in a state that can still be activated either by the lipid interface or by activators.

Dependence of Enzymatic Activity on pH—Fig. 1 shows the pH dependence of the soluble and immobilized phospholipase A_2 toward diheptanoyl-PC. The experimental data obtained with this substrate fits the theoretical curve assuming a pK_a 6.1 for the soluble enzyme. The apparent pK_a of the immobilized enzyme was shifted down to 5.3 (<1 mg of gel/assay). That this change was not due to the presence of the gel was verified by the fact that the pK_a of the soluble enzyme was not significantly modified by the addition of up to 9.8 mg of unreacted gel to the assay. The same fit with the theoretical curve assuming a pK_a of 6.1 was obtained for the soluble enzyme using diheptanoyl-PE (Fig. 1). The experimental data obtained for the immobilized enzyme toward diheptanoyl-PE leads to an apparent $pK_a = 5.6$. However, the data at highest pH values do not match the theoretical curve very well, but the experimental deviation is greatest for these points. The data can be fit to one ionizable group with pK_a 6.1 for the soluble enzyme activity on PE and PC. This pK_a could be that of the histidine (pK_a 6.5) found to be essential to the enzyme activity (5), although it does not have to be. The pK_a is shifted down by 0.5–0.8 pH unit after immobilization of the enzyme on the Sepharose matrix. The curves found for the soluble enzyme were superimposable and the maximum activity was reached at about pH 7.5. The maximum activity of the immobilized enzyme was also reached at this value using diheptanoyl-PE but with diheptanoyl-PC as substrate, the maximum activity was reached at about pH 6.5. In all cases maximal activity was reached by pH 8.0 and, therefore, all assays were conducted at this pH.

Dependence of Enzymatic Activity on Temperature—The influence of temperature was investigated in order to determine if the enzyme activity is diffusion controlled. Fig. 2 shows Arrhenius plots for the phospholipase A_2, soluble and immobilized forms, acting on micellar and monomeric diheptanoyl-PC. All these plots are linear with correlation coefficients between 0.87 and 0.99; thus the hydrolysis rate obeys the Arrhenius equation to a good approximation. Activation energies (E_{act}) calculated from these data were 6.9 and 7.1 kcal mol^{-1} for the soluble enzyme hydrolyzing monomers and micelles of diheptanoyl-PC; E_{act} was 7.6 and 7.5 kcal mol^{-1} for the same substrate forms using the immobilized enzyme. The similarity of these values between both enzyme forms for both physical states of the substrate, and the fact that there is no change in slope for the immobilized enzyme at high temperature suggests that catalysis is not diffusion controlled (25, 26).

In Fig. 2, a break in the plot is evident for the soluble enzyme acting on monomeric diheptanoyl-PC and is equivalent to a 40% decrease in the activity of the soluble enzyme. This break at about 307 K (34 °C) could be explained by a

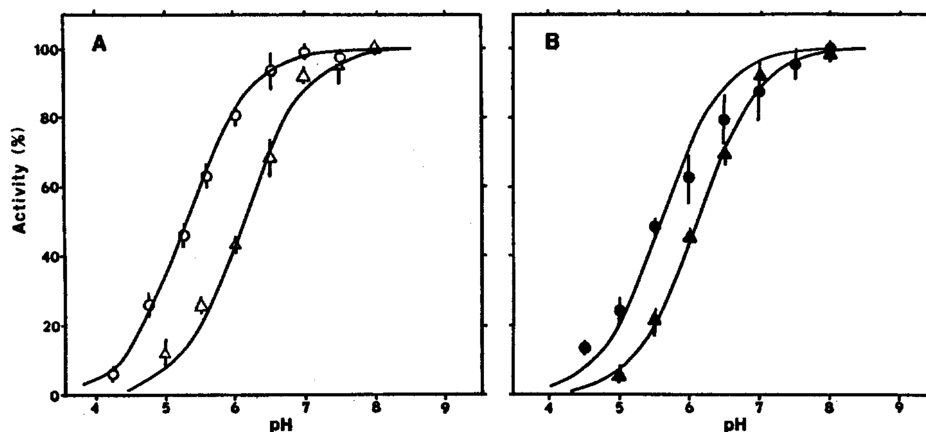

FIG. 1. **Effect of pH on enzyme activity.** Activity is shown (*A*) toward diheptanoyl-PC (5 mM) in the presence of 10 mM $CaCl_2$ for (△) soluble enzyme and (○) immobilized enzyme (preparation I) and (*B*) toward diheptanoyl-PE (1 mM) in the presence of 10 mM $CaCl_2$ for (▲) soluble enzyme and (●) immobilized enzyme (preparation I). Average of duplicate determinations are shown along with experimental deviations. Theoretical curves for pK_a values discussed in text are shown by *solid lines*.

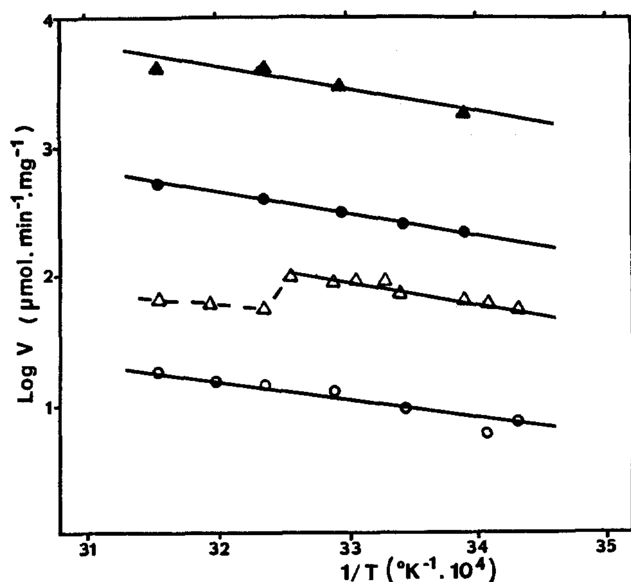

FIG. 2. **Arrhenius plots of the enzyme activity.** The effect of temperature on activity was determined toward micellar diheptanoyl-PC (5 mM) for (▲) soluble and (●) immobilized enzyme (preparation IV), and toward monomeric diheptanoyl-PC (0.5 mM) for (△) soluble and (○) immobilized enzyme (preparation IV).

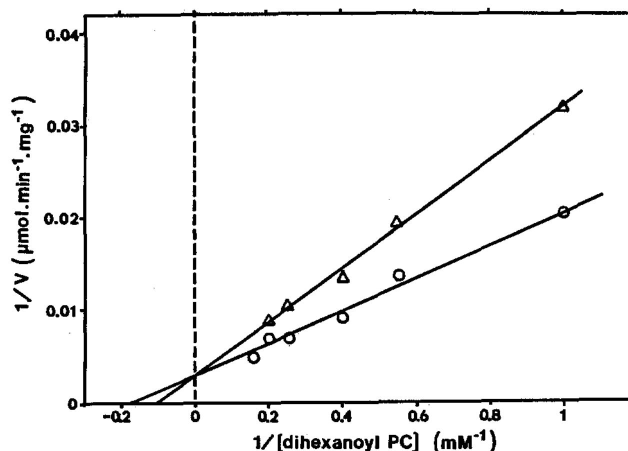

FIG. 3. **Lineweaver-Burk plot of the enzyme activity toward monomeric dihexanoyl-PC by (△) soluble enzyme and (○) immobilized enzyme (preparation I).**

conformational change of the soluble enzyme. This change does not appear when the phospholipase A_2 acts on micellar substrate or for the immobilized enzyme. Aggregated substrate and immobilization of the phospholipase A_2 may, in fact, stabilize the enzyme conformation.

Dependence of Enzymatic Activity on Ca^{2+}—Before we attempted to determine the effect of Ca^{2+} on activity, both the soluble and the immobilized enzymes were treated to remove all traces of Ca^{2+}. The soluble enzyme, previously dialyzed against water, was incubated with EGTA (2 mM), then dialyzed 4 h against 2 mM EGTA, and then exhaustively dialyzed against glass distilled water. The immobilized enzyme (preparation II) was incubated 0.5 h with 10 mM EGTA under agitation, washed with 25 volumes of EGTA (1 mM) and 100 volumes of glass distilled water. The final volume of the gel was adjusted to the starting volume. Dipalmitoyl-PC/Triton X-100 mixed micelles as substrate were prepared in the absence of Ca^{2+} using glass distilled water.

The apparent dissociation constants from double reciprocal plots of activity *versus* Ca^{2+} concentration were 0.1 and 1.1 mM for the immobilized and soluble enzyme, respectively. Thus, the affinity of the immobilized enzyme for Ca^{2+} is about 10-fold greater than that of the soluble enzyme. The soluble enzyme is totally inactive at a EGTA concentration as low as 0.3 mM, while the immobilized enzyme still displays 5–10% of the maximal activity in the presence of 5 mM EGTA.

Activity on Monomeric Substrate—Kinetic constants for the soluble and immobilized phospholipase A_2 were determined using dihexanoyl-PC at concentrations well below its CMC (CMC 10–15 mM (27–29)) to ensure the presence of only monomeric substrate. The double reciprocal plots presented in Fig. 3 are linear for both enzyme forms. Both enzymes had V_{max} values of 330 µmol min^{-1} mg^{-1} while the apparent K_m values ($K_{m(app)}$) were 9.5 and 5.9 mM, respectively, for the soluble and the immobilized enzyme. The lower $K_{m(app)}$ found for the immobilized enzyme could be the result of a different partitioning of the substrate between the bulk phase and the agarose phase. One effect of this would be to increase the concentration of the substrate in the immediate environment of the bound enzyme. Another effect could be formation of a lipid monolayer around the beads to give rise to an interface similar to those in micelles, although the addition of unreacted gel shows no activation toward the soluble enzyme.

The activity of the enzyme was also assayed toward 1 mM dihexanoyl-PE in the presence of 1 mM $CaCl_2$. This Ca^{2+} concentration was employed because at higher Ca^{2+} concentrations the PE precipitates. The specific activity found for the soluble enzyme and the immobilized enzyme were 13.7 and 48.3 µmol min^{-1} mg^{-1}, respectively. Taking into account the low activity of the soluble enzyme at low Ca^{2+} concentration (lower Ca^{2+} affinity than the immobilized enzyme) and its thermal modification, both enzymes have virtually the same specific activity.

Effect of Substrate Micellization on the Enzyme Activity—Cobra venom phospholipase A_2, like most other lipolytic enzymes, acts more efficiently on emulsified or micellar substrate than on soluble substrate. Thus a dramatic increase in the enzyme activity appears at substrate concentrations close to their CMC. Fig. 4 shows the variation of the soluble enzyme activity using diheptanoyl-PC as substrate. At concentrations below 1.3–1.4 mM, the apparent CMC (CMC = 1.47–1.60 mM (28, 30)), the enzyme displays little activity, but when the apparent CMC is reached, the activity increases dramatically. The immobilized enzyme (preparations I and III) showed only a small increase in activity above the CMC. The concentration of micellar substrate was calculated according to a treatment developed elsewhere (29). It assumes that for the substrate in micelles, $[S_{mic}] = [S_t] - [S_{mon}]$, where $[S_t]$ is the total substrate concentration and $[S_{mon}]$ the monomeric substrate concentration. We assume that $[S_{mon}]$ is the apparent CMC. A plot of v *versus* $[S_{mic}]$ should be a hyperbolic saturation curve with a linear double reciprocal plot. This linear plot is presented in Fig. 4*B*. Linear regression analysis show that the apparent K_m for both the soluble and immobilized enzyme (preparation I) are similar, about 0.5–0.7 mM in $[S_{mic}]$ units corresponding to a total substrate concentration of about 1.8–2 mM at V_{max}/2. Nevertheless, V_{max} for the soluble enzyme is about 10-fold higher than that observed with the immobilized enzyme. V_{max} values are, respectively, 4450 and 510 µmol min^{-1} mg^{-1}. Based on the ratios $V_{max}/K_{m(app)}$ for each enzyme form, the immobilized enzyme displays about 11.5% of the soluble enzyme activity which agrees well with the values given in Table II.

Similar studies were carried out using diheptanoyl-PE as substrate. Short chain PE exhibits a limited solubility in

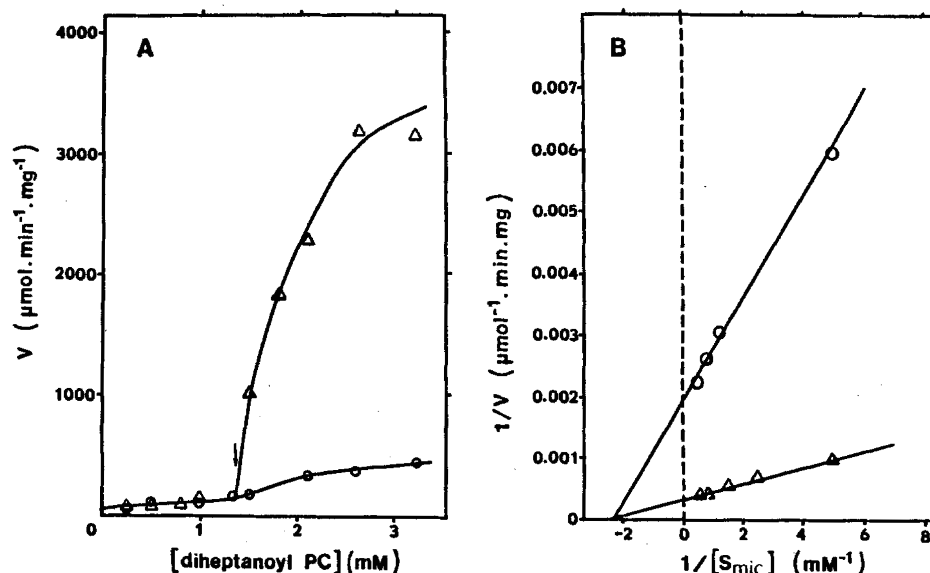

FIG. 4. **Effect of the micellization of the substrate on enzymatic activity.** *A*, hydrolysis of deheptanoyl-PC by (△) soluble enzyme and (○) immobilized enzyme (preparation I). The *arrow* indicates the approximate CMC of diheptanoyl-PC. *B*, Lineweaver-Burk plot of the data as a function of micellar substrate, $[S_{mic}]$, where ($[S_{mic}] = [S_t] - \text{CMC}$).

water above which the PE precipitates. The solubility of diheptanoyl-PE is about 1 mM (13). The activity of the soluble enzyme increases until a plateau is reached at a substrate concentration close to the solubility limit (Fig. 5). The immobilized enzyme also reaches its maximum activity, representing about 20–25% of the soluble enzyme activity, at the solubility limit of the substrate. A double reciprocal plot of these data (Fig. 5*B*) again shows a similar $K_{m(app)}$ (0.51 mM) between the two forms of phospholipase A_2 while V_{max} is 820 μmol min^{-1} mg^{-1} for the soluble enzyme and 120 μmol min^{-1} mg^{-1} for the immobilized enzyme.

Effect of Activators—Phosphorylcholine-containing compounds are activators of the cobra venom phospholipase A_2 acting on PE (4–6), sphingomyelin being among the best (5). To delineate the effect of these compounds on the immobilized enzyme compared to the soluble enzyme, further experiments were carried out with monomeric diheptanoyl-PE (Table III). The addition of Triton X-100 does not modify the rate of hydrolysis for either form of the enzyme. Dodecylphosphorylcholine (CMC = 1 mM (31)) increases the soluble enzyme activity but has no effect on the immobilized enzyme. Soluble dibutylcarbamoyl-PC has no effect on either the soluble or the immobilized enzyme; however, the addition of Triton X-100 generates the formation of an interface and leads to the activation of the soluble enzyme (13) but not the immobilized enzyme. Sphingomyelin in the presence of detergent has an important activation effect on the soluble enzyme and about a 10-fold less effect on the immobilized enzyme. These data show that monomeric phosphorylcholine-containing compounds are activators of the soluble enzyme acting on PE, but only when an interface is provided. The immobilized enzyme was activated neither by the interface nor by the activators even if an interface was available.

Binding Experiments—These experiments constitute an attempt to determine under which conditions the soluble cobra venom phospholipase A_2 can bind to the immobilized one. As shown in Fig. 6, in the absence of any activator (phosphorylcholine-containing compounds) or detergent, there is no specific binding of the soluble form to the immobilized form. When the monomeric activator, dodecylphosphorylcholine (0.9 mM), was present during incubations, a specific binding, following a saturation curve was observed. In these conditions, 2.1 μg of the soluble protein were bound to 7.6 μg of immobilized protein, leading to a stoichiometry of about 0.3. The leveling off, occurred for a total amount of added soluble enzyme of about 8 μg, most probably indicating the formation of well defined aggregates without any denaturation of the protein.

Since dodecylphosphorylcholine is an activator for the cobra venom phospholipase A_2 and is also a detergent, this

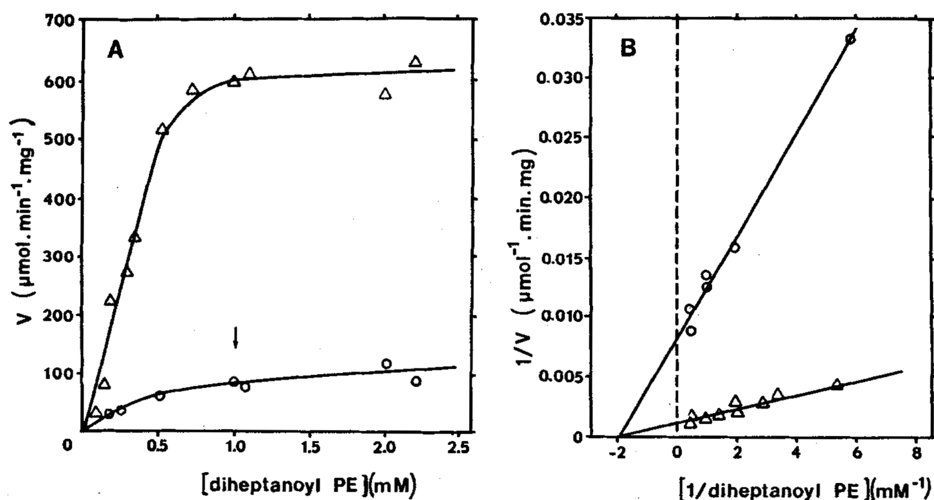

FIG. 5. **Activity of the soluble and immobilized phospholipase A_2 toward diheptanoyl-PE.** *A*, hydrolysis by (△) soluble enzyme and (○) immobilized enzyme (preparation I). The *arrow* indicates the limit of solubility of the substrate. *B*, Lineweaver-Burk plot of the data. The two lowest concentrations with the soluble enzyme were omitted for calculation of the line by linear regression analysis.

TABLE III

Effect of ligands on soluble and immobilized phospholipase A_2 *activity toward diheptanoyl-PE*

Immobilized enzyme, preparation I; 0.2 mM diheptanoyl-PE.

Ligand	Triton X-100 (2 mM)	Soluble activity	Immobilized (preparation I) activity	
			μmol min^{-1} mg^{-1}	% of soluble
		μmol min^{-1} mg^{-1}		
None	−	81	25	31
None	+	71	25	35
Dodecylphosphorylcholine (1 mM)	−	241	22	9
Dibutyrylcarbamoyl-PC (5 mM)	−	85	32	38
Dibutyrylcarbamoyl-PC (5 mM)	+	377	43	11
Sphingomyelin	+	1740	71	4

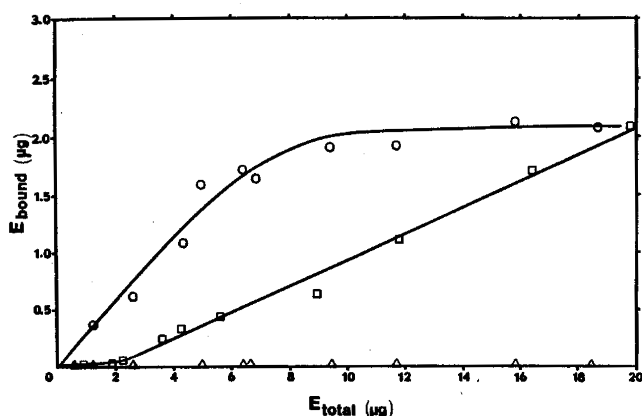

FIG. 6. **Binding of soluble phospholipase A_2 to immobilized phospholipase A_2.** The amount of specifically bound phospholipase A_2 (E_{bound}) to the gel preparation IV, was determined as described in the text and plotted *versus* the total amount of added protein (E_{total}). Data are given for experiments carried out in the presence of (△) buffer alone, (□) buffer plus 0.6 mM Zwittergent 3-14, and (○) buffer plus 0.9 mM dodecylphosphorylcholine.

effect could be due to a specific action of the activator or to a less specific action of the detergent. To distinguish between these two effects, a detergent (Zwittergent 3-14) known to not be an activator was used in the same kind of experiment. In the presence of 0.6 mM Zwittergent 3-14 (CMC = 0.32 mM), binding increased almost linearly with the addition of soluble enzyme apparently without any saturation limit. This result indicates either the formation of various sized aggregates or some denaturation. Thus the activator appears to induce the formation of well defined aggregates.

DISCUSSION

Immobilization

A number of immobilization techniques leading to covalent attachment of biomolecules are known and have been widely applied. Recently a new method of activating hydroxyl groups using *p*-toluenesulfonyl chloride or tresyl chloride allowing subsequent immobilization of ligands was described (18, 32). This method was shown to have advantages over previous ones because the ligands are bound directly to the carbon atoms of the support and side reactions such as matrix cross-linking do not occur during activation and coupling (33). The bond is formed via a nucleophilic attack by sulfhydryl or amino groups and leads to a thio-ether or secondary amine bond between the ligand and gel (18, 32, 33). There are no free sulfhydryl groups in the cobra phospholipase A_2, but there are 6 or 7 lysines (34). Thus, linkage to the phospholipase is presumably via lysines. This is consistent with the observation that an amino acid analysis of preparations I and III showed a decrease of 0.6–0.9 lysine residues when compared with the soluble enzyme.

Many possible factors may be responsible for the behavior of immobilized enzymes and must be taken into account in analyzing the kinetic studies reported here. These considerations are (i) that the conformation of the enzyme can be modified by the immobilization, (ii) the substrate partitioning may not be equal between the bulk phase and the gel phase and absorption of the substrate on gel bead can increase the local concentration of the substrate, (iii) the environment of the immobilized form is not the same as that of the free enzyme, and (iv) diffusion may control the reaction rate (26). Each is discussed below.

(i) The higher affinity of the immobilized cobra venom phospholipase A_2 for Ca^{2+} can be explained by a conformational change. This change seems to affect the Ca^{2+} binding site, but not the catalytic site since in the presence of a saturating concentration of Ca^{2+}, V_{max} for both enzyme forms were similar with a monomeric substrate.

(ii) The substrate may be partitioned differently between the support and the bulk solution. In this case, the catalytic constants, determined on monomeric dihexanoyl-PC, for the immobilized enzyme should be different from those of the soluble enzyme. It is well known that lipolytic enzymes have an increased activity on a soluble substrate when this substrate is adsorbed on an "artificial" interface such as glass beads (35). This effect is not implicated in the enzyme activity difference observed between the soluble and the immobilized form reported here because the soluble phospholipase A_2 activity is not altered by the addition of gel beads.

(iii) The interaction between the immobilized enzyme and its substrate may take place in a different microenvironment from that existing in free solution. The pH around the gel particles can differ from the pH of the bulk solution due to charge repulsion-attraction (25). The quenching of the coupling reaction by ethanolamine generates a secondary amino group and a primary hydroxyl group (matrix-NH-CH_2-CH_2-OH); both have high pK_a values and are not charged at least at pH 8.0, where most of the assays were performed. The observed shift of the apparent pK can be accounted for in part by a change in the microenvironment but this can also be the result of a subtle modification of the enzyme.

It is possible that the gel matrix causes cage effects. However, it was shown that the addition of control gel up to 10 times the gel concentration used in immobilized enzyme assays does not modify the pK_a of the essential residue or the activity of the soluble enzyme. This argues against a modification of the mechanism of activation of the enzyme after immobilization.

(iv) Diffusion may control completely or partially the reaction rate. This point is particularly important in the studies using micellar substrates. The agarose gel used has an exclusion limit of 4×10^6 daltons corresponding to the exclusion of an "equivalent sphere" with a Stokes radius between 400 and 500 Å. This pore diameter is sufficiently large to allow diffusion of the 4:1 Triton X-100/phospholipid mixed micelles which have Stokes radii of about 76 Å and a micellar molecular mass of about 100,000 daltons (23). We observed a good specific activity using PE/Triton X-100 (Stokes radius = 79 Å (4)) mixed micelles. This is in agreement with free diffusion of these micelles inside the gel matrix. In the case of the

soluble enzyme, the diffusion of the enzyme molecule is rapid when compared to that of the substrate micelle. This situation is reversed in the case of the immobilized enzyme. Activation energy values found are similar regardless of the substrate physical state (monomers or micelles) and the enzyme physical state (soluble or immobilized). This shows that for all combinations, only the catalytic steps are rate-limiting while internal and external diffusion of the substrate and of the immobilized enzyme are not. The pure diheptanoyl-PC micelles have a broad size distribution with micellar molecular masses between 30,000 and 60,000 daltons in the concentration range used (36). Thus, the decreased activity with micellar PC cannot be attributed to limitation of the substrate diffusion.

Activation

Pancreatic and venom phospholipases exhibit dramatic rate enhancements when their substrates pass from a monomeric state to a micellar state (1). At least four hypotheses have been suggested for this interfacial activation (reviewed in Ref. 1).

(i) *The Substrate Theory*—This theory assumes that the substrate becomes more susceptible to enzyme action when it is incorporated in a lipid-water interface. Such an orientation effect should cause a fall in activation energy and a subsequent increase in V_{max}.

(ii) *The Enzyme Theory*—This theory suggests that a conformational change might occur in the enzyme molecule upon interaction with certain lipid-water interfaces or with activators. Such a conformational change could be a dimerization of the enzyme.

(iii) *The Product Theory*—This theory postulates that the rate-limiting step is the product release and it becomes faster in the interface than in the bulk.

(iv) *The Concentration Effect*—This effect implies that the effective substrate concentration that the enzyme sees is much higher at the lipid-water interface than in the bulk phase, thus decreasing the apparent K_m value.

Using monomeric diheptanoyl-PC and dihexanoyl-PE as substrate, the immobilized enzyme had virtually the same specific activity as the soluble form. The catalytic constants determined on monomeric dihexanoyl-PC are close for both forms of the enzyme. The substrate concentration range used for this study was far enough below the CMC to avoid "premicelle" formation but not low enough to hinder the accurate determination of V_{max} and K_m. The immobilized enzyme is active on monomeric substrates and on nonactivated micellar substrates. These results demonstrate that immobilization does not affect the catalytic site of the enzyme and its binding to micelles. Thus the loss of the immobilized enzyme activity on activated micellar substrate cannot be attributed to the immobilization procedure.

As shown in Fig. 4, soluble enzyme displays interfacial activation whereas the immobilized shows only a small activation. This activation can be attributed to a small fraction of immobilized enzyme (perhaps 10%) which can be activated by the lipid-water interface or activators. The lack of interfacial activation of the immobilized enzyme is probably due to the inability of this enzyme form to be activated by the lipid interface. The apparent K_m is the same for the two forms of the enzyme, but V_{max} of the soluble form is about 10-fold higher than V_{max} for the immobilized one. The substrate binding appears to be unchanged by the interface while the catalytic step seems to be enhanced. All of these data seem to rule out theories which consider that the origin of the interfacial activation is mainly located at the substrate level. The origin of the interfacial activation, at least for the cobra venom phospholipase A_2, appears to be located at the enzyme level.

These studies show that soluble activators such as dibutylcarbamoyl-PC do not increase the soluble enzyme activity of monomeric substrate unless an interface is present. In previous studies (4), it was shown that soluble PC (such as dibutyryl-PC) activates the enzyme on micellar PE even if PC is not incorporated into the lipid-water interface. This is consistent with a direct interaction between the activator and the enzyme, suggesting the presence of an activator site and a catalytic site on the cobra venom phospholipase A_2 (6). Immobilization apparently fixes the enzyme conformation in such a manner that it is no longer activatable by phosphorylcholine-containing compounds or by interfaces. Immobilization presumably physically prevents the aggregation of most of the enzyme, although some of the protein molecules may be immobilized in a close enough proximity to aggregate. The activation of the cobra venom phospholipase A_2 toward PE by PC-containing compounds requires an interface (13). Taken together these results suggest that normally the enzyme interacts with the activator interfacial phospholipid leading to an aggregated protein. These results show that immobilization prevents both activation by lipid interfaces and activation by activators. The remaining question is: are both due to aggregation of the enzyme which is prevented by immobilization?

Binding

These studies show that there is no specific binding of the soluble enzyme to the immobilized enzyme, at least in the range of soluble enzyme concentration used. If a micellar detergent, which is not an activator but still possesses some of the properties of an activator is present, some binding occurs. However, the aggregates formed increase in size with the addition of soluble enzyme. Since the phospholipase A_2 from cobra venom does not bind appreciably to pure detergent micelles (2), it is possible that these aggregates are formed nonspecifically or by phospholipase A_2 molecules which are denatured. Nevertheless, in the presence of a monomeric activator, the soluble enzyme binds to the immobilized enzyme. The size of these aggregates appears to be well determined. The stoichiometry of less than unity agrees with the formation of dimers or possibly higher order aggregates. This may also reflect an equilibrium state in which the formation of soluble aggregates would be favored over the formation of mixed aggregates. Kinetic studies using a mixed system (*i.e.* immobilized plus soluble enzyme) should give information about the relationship between this aggregation and activation. Nevertheless, assuming that the equilibrium constant for the formation of soluble aggregates and mixed aggregates (no totally immobilized aggregates can be formed) is the same, we should observe a 50% increase in activity in assays made with a 1.1 stoichiometry of soluble to immobilized enzyme. The fact that only 30% of the immobilized enzyme can bind soluble enzyme would reduce this to a 15–20% increase in activity in the best case. These values are too close to the error range of the pH stat assay to be accurately determined. In fact, in such experiments we did not find any significant increase in activity in the mixed system. Thus it appears likely that activators promote the dimerization and possibly higher order aggregation of the phospholipase A_2.

The studies presented herein show that interaction of the cobra venom phospholipase A_2 with phosphorylcholine-containing activators probably leads to a dimeric or aggregated form of this enzyme. This activation originates from an increase in the catalytic efficiency of the enzyme rather than

from an increase of its affinity for the substrate. Thus the phospholipase A_2 activity is modulated by a lipid-protein interaction via the postulated activator site probably leading to a conformational change which also results in a protein-protein interaction with formation of an enzyme dimer. This conformational change and probably the aggregate is much more active on substrates in lipid-water interface and is prevented by immobilization.

REFERENCES

1. Dennis, E. A. (1983) *The Enzymes* **XVI**, 307–353
2. Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 1950–1954
3. Dennis, E. A., Darke, P. L., Deems, R. A., Kensil, C. R., and Plückthun, A. (1981) *Mol. Cell. Biochem.* **36**, 37–45
4. Roberts, M. F., Adamich, M., Robson, R. J., and Dennis, E. A. (1979) *Biochemistry* **18**, 3301–3308
5. Adamich, M., Roberts, M. F., and Dennis, E. A. (1979) *Biochemistry* **18**, 3308–3314
6. Plückthun, A., and Dennis, E. A. (1982) *Biochemistry* **21**, 1750–1756
7. Deems, R. A., Eaton, B. R., and Dennis, E. A. (1975) *J. Biol. Chem.* **250**, 9013–9020
8. Hendrickson, H. S., and Dennis, E. A. (1984) *J. Biol. Chem.* **259**, 5734–5739
9. Hendrickson, M. S., and Dennis, E. A. (1984) *J. Biol. Chem.* **259**, 5740–5744
10. Adamich, M., Voss, F. H., and Dennis, E. A. (1978) *Arch. Biochem. Biophys.* **189**, 417–423
11. Lombardo, D., and Dennis, E. A. (1985) *Fed. Proc.* **44**, 1204
12. Deems, R. A., and Dennis, E. A. (1981) *Methods Enzymol.* **71**, 703–710
13. Plückthun, A., Rohlfs, R., Davidson, F. F., and Dennis, E. A. (1985) *Biochemistry* **24**, 4201–4208
14. Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965) *J. Am. Oil. Chem. Soc.* **42**, 53–56
15. Gupta, C. M., and Bail, A. (1981) *Biochim. Biophys. Acta* **663**, 506–515
16. Dittmer, J. C., and Lester, R. L. (1964) *J. Lipid Res.* **5**, 126–127
17. Turner, J. D., and Rouser, G. (1970) *Anal. Biochem.* **38**, 423–436
18. Nilsson, K., and Mosbach, K. (1981) *Biochem. Biophys. Res. Commun.* **102**, 449–457
19. Dennis, E. A. (1973) *J. Lipid Res.* **14**, 152–159
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
21. Haberland, M. E., and Reynolds, J. A. (1975) *J. Biol. Chem.* **250**, 6636–6639
22. Deems, R. A., and Dennis, E. A. (1975) *J. Biol. Chem.* **250**, 9008–9012
23. Robson, R. J., and Dennis, E. A. (1978) *Biochim. Biophys. Acta* **508**, 513–524
24. Robson, R. J., and Dennis, E. A. (1983) *Accts. Chem. Res.* **16**, 251–258
25. Laidler, K. J., and Bunting, P. S. (1980) *Methods Enzymol.* **64**, 227–248
26. Goldstein, L. (1976) *Methods Enzymol.* **54**, 397–443
27. Hershberg, R. D., Reed, G. H., Slottom, A. J., and de Haas, G. H. (1976) *Biochim. Biophys. Acta* **424**, 73–81
28. Tausk, R. J. M., Karmiggelt, J., Oudshoorn, C., and Overbeek, J. T. G. (1974) *Biophys. Chem.* **1**, 175–183
29. Plückthun, A., and Dennis, E. A. (1981) *J. Phys. Chem.* **85**, 678–683
30. Van Eijk, J. H., Verheij, H. M., Dijkman, R., and de Haas, G. H. (1983) *Eur. J. Biochem.* **132**, 183–188
31. Plückthun, A. (1982) Ph.D. dissertation, University of California, San Diego
32. Nilsson, K., and Mosbach, K. (1980) *Eur. J. Biochem.* **112**, 397–402
33. Nilsson, K., Norrlöw, O., and Mosbach, K. (1981) *Acta Chem. Scand. Ser. B Org. Chem. Biochem.* **35**, 19–27
34. Darke, P. L., Jarvis, A. A., Deems, R. A., and Dennis, E. A. (1980) *Biochim. Biophys. Acta* **626**, 154–161
35. Brockman, H. L., Law, J. H., and Kézdy, F. J. (1973) *J. Biol. Chem.* **248**, 4965–4970
36. Tausk, R. J. M., Van Esch, J., Karmiggelt, J., Voordouw, G., and Overbeek, J. Th. G. (1974) *Biophys. Chem.* **1**, 184–203
37. Hazlett, T. L., and Dennis, E. A. (1985) *Toxicon* **23**, 457–466