Role of Phosphatidylinositol in Attachment of Alkaline Phosphatase to Membranes†

Martin G. Low* and Donald B. Zilversmit‡

ABSTRACT: The mechanism of release of alkaline phosphatase from membranes by phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* was studied. Alkaline phosphatase was readily released from pig kidney microsomes by phospholipase C but not by a variety of other treatments, e.g., high ionic strength, extremes of pH, divalent cations, chelating agents, or analogues of the polar head group of phosphatidylinositol. Alkaline phosphatase released from microsomes by phospholipase C did not bind to phospholipid vesicles containing phosphatidylinositol. Alkaline phosphatase solubilized from microsomes by butanol extraction, however, was able to bind to phospholipid vesicles even when they contained no phosphatidylinositol. The ability of butanol-extracted alkaline phosphatase to bind to phospholipid vesicles was destroyed by added phosphatidylinositol-specific phospholipase C. Hydrolysis of added phosphatidylinositol by endogenous phospholipase activity in butanol extracts was also accompanied by loss of binding ability. Loss of binding ability was paralleled by a decrease in the apparent molecular weight of alkaline phosphatase. These results indicate that alkaline phosphatase is attached to membranes by a strong interaction with phosphatidylinositol.

Alkaline phosphatase, 5′-nucleotidase, and acetylcholinesterase are released from a wide variety of membranes by bacterial phosphatidylinositol-specific phospholipases C (Slein & Logan, 1965; Ikezawa et al., 1976; Taguchi & Ikezawa, 1978; Ohyabu et al., 1978; Low & Finean, 1977b,c, 1978). Although these observations have suggested that phosphatidylinositol may be responsible for the attachment of these enzymes to membranes, the molecular details are not understood. In particular, the nature (i.e., covalent or noncovalent) of the presumed interaction between the enzymes and phosphatidylinositol has not been identified. In this paper, we have studied the release of alkaline phosphatase from pig kidney microsomes and its recombination with phospholipid vesicles.

Materials and Methods

Egg yolk phosphatidylcholine and wheat germ phosphatidylinositol were obtained from Lipid Products, Redhill, Surrey, England. [^{14}C]Triolein (60 mCi/mmol) was obtained from New England Nuclear, Boston, MA. [^{32}P]Phosphatidylcholine (~1.5 μCi/μmol) was prepared and purified from rat liver and [^{3}H]phosphatidylinositol (~10 μCi/μmol) from rat liver microsomes as described in a previous paper (Low & Zilversmit, 1980). Myoinositol, *p*-nitrophenyl phosphate, and phospholipase A_2 (*Naja naja*; 1000 units/mg) were obtained from Sigma Chemical Co., St. Louis, MO. Glycerophosphorylinositol was prepared from wheat germ phosphatidylinositol by mild alkaline methanolysis (Kates, 1972). Phospholipase C was purified from *Staphylococcus aureus* culture supernatants with the modified procedure of Low & Zilversmit (1980).

Pig kidneys were homogenized with 2 volumes of 0.25 M sucrose (at 4 °C) in a Waring blender and centrifuged at 10000*g* for 30 min. The supernatants were centrifuged at 100000*g* for 60 min, and the resulting microsomal pellet was resuspended in 0.25 M sucrose to give a final protein concentration of ~50 mg/mL. The microsomal suspension was stored at −20 °C until required. The alkaline phosphatase specific activity was ~0.6 unit/mg of protein; total phospholipid and phosphatidylinositol contents were 430 and 48 nmol/mg of protein, respectively.

Alkaline phosphatase was released from pig kidney microsomes by treatment with phospholipase C from *S. aureus*; 0.32 mL of microsomal suspension, 0.4 mL of 100 mM Hepes[1]–NaOH buffer, pH 7.4, and 1 μg of phospholipase C in a final volume of 4 mL were incubated for 30 min at 37 °C. The mixture was cooled on ice and centrifuged at 100000*g* for 60 min at 4 °C. One milliliter of the supernatant was applied to a column (1.5 × 48 cm) of Sephadex G-75 Superfine equilibrated in 100 mM NaCl, 0.1 mM $MgCl_2$, 0.01 mM zinc acetate, 10 mM Tris-HCl, pH 8.2 (4 °C), and 0.02% w/v sodium azide (buffer A). The column was eluted at a flow rate of 2.5 mL/h, and 2-mL fractions were collected. The alkaline phosphatase was eluted in the void volume and the pooled fractions (4 mL) were used for binding experiments. In some experiments (see Table II) the Sephadex G-75 step was omitted. This reduced the interval between release of alkaline phosphatase and the start of the binding experiment from 24 to 2 h.

Alkaline phosphatase was also solubilized from pig kidney microsomes with the butanol extraction procedure described by Morton (1954). Microsomes were diluted with 5 volumes of ice-cold H_2O and then mixed with 3 volumes of ice-cold butanol. The mixture was kept on ice for 10 min with vigorous shaking at intervals and centrifuged at 40000*g* for 15 min at 4 °C, and the lower phase was collected. Butanol was removed from the lower phase by dialysis at 4 °C against 50 volumes of buffer A for 1, 5, and 15 h, consecutively. This material was used directly for binding experiments and chromatography. In some experiments (see Figure 2 and Table III) the lower phase was incubated (for 1 h at 25 °C or 24 h at 4 °C) before removing the butanol by dialysis. These incubations

† From the Division of Nutritional Sciences and Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. *Received February 5, 1980.* This research was supported by U.S. Public Health Service Research Grant HL 10940 from National Heart, Lung and Blood Institute, U.S. Public Health Service.

* Address correspondence to this author. Present address: Department of Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298.

‡ Career Investigator of the American Heart Association.

[1] Abbreviations used: PC, phosphatidylcholine; PI, phosphatidylinositol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

0006-2960/80/0419-3913$01.00/0

did not affect the total alkaline phosphatase activity. Specific activity of the butanol-extracted alkaline phosphatase was ~0.6 unit/mg with a recovery of ~70%. Total phospholipid was 10–20 nmol/mg of protein.

The ability of alkaline phosphatase to bind to phospholipid membranes was generally determined by cholate dialysis. A solution of 0.25 mL of labeled phospholipids (see below) in sodium cholate was mixed with 0.25 mL of alkaline phosphatase (phospholipase C released or butanol extracted), incubated on ice for 1 h, and then dialyzed at 4 °C against 250 volumes of buffer A first for 4–6 h and then for 15 h. A 0.5-mL amount of the dialysate was mixed with 0.7 mL of 70% (w/v) sucrose in buffer A, and 1 mL of this mixture was layered under 3 mL of 30% w/v sucrose in buffer A in a centrifuge tube. After centrifugation for 24 h at 100000*g* (4 °C) the upper 2 mL was collected by aspiration. In experiments with butanol-extracted alkaline phosphatase a small pellet was also formed; this was resuspended and included in the lower 2-mL fraction. In general 80–100% of the applied alkaline phosphatase and 90–100% of the applied radioactivity were recovered after centrifugation. In experiments with phospholipase C released alkaline phosphatase, 0.5 mL of the dialysate was also loaded directly onto a column (1 × 45 cm) of Sepharose 6B equilibrated in buffer A. The column was eluted at 4 °C with buffer A at a flow rate of 2.5 mL/h, and 1-mL fractions were collected. Recoveries of alkaline phosphatase and radioactivity from the fractionation were in the range of 80–100%. Three different mixtures of phospholipids were used in these experiments: (a) phosphatidylcholine (20 μmol), [^{32}P]phosphatidylcholine (~0.15 μCi; 0.1–1 μmol), [^{14}C]triolein (~0.15 μCi), and butylated hydroxytoluene (70 nmol); (b) phosphatidylcholine (18 μmol), [^{32}P]phosphatidylcholine (~0.15 μCi; 0.1–1 μmol), phosphatidylinositol (2 μmol), [^{3}H]phosphatidylinositol (~0.5 μCi; 50 nmol), [^{14}C]triolein (~0.15 μCi), and butylated hydroxytoluene (70 nmol); (c) as in (b) but containing in addition microsomal lipids (2.4 μmol of lipid phosphorus). The lipids were dissolved in chloroform, dried in vacuo, and then dissolved in 1 mL of 10% (w/v) sodium cholate in buffer A. In most binding experiments this solution was mixed directly with alkaline phosphatase; however, in some experiments the cholate was removed by dialysis and the dialysate was used for determining the binding of alkaline phosphatase to preformed phospholipid vesicles.

Alkaline phosphatase was assayed as described previously with 2 mM *p*-nitrophenyl phosphate as substrate (Low & Finean, 1977b). One unit of activity hydrolyzes 1 μmol of substrate per min at 37 °C.

Phospholipids were extracted from microsomes (Low & Finean, 1977a) and analyzed by thin-layer chromatography on $(NH_4)_2SO_4$-impregnated silica gel H plates as described by Kaulen (1972). Phospholipids were eluted from the silica gel with three portions of methanol/concentrated HCl (98:2) at 65 °C. Lipid phosphorus was determined according to Bartlett (1959). The hydrolysis of [^{3}H]phosphatidylinositol by butanol-extracted microsomes was determined by extracting the lipids from incubation mixtures (Low & Finean, 1977a) and analyzing the lower chloroform phase for residual radioactivity. Protein was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin (Pentex, Miles Laboratories, Inc.) as standard.

Results

Release of Alkaline Phosphatase from Microsomes. Incubation of microsomes from pig kidney with phosphatidylinositol-specific phospholipase C (0.25 μg/mL) for 30 min at 37 °C released approximately 80% of the alkaline phosphatase into the supernatant (Table I). Larger amounts of phospholipase C have been shown to release 95–100% of the alkaline phosphatase. The effects of high ionic strength, high and low pH, and divalent cations were also tested but none of these released significant amounts of alkaline phosphatase (Table I). The absence of activity in supernatants was not due to inactivation of the alkaline phosphatase, as in most cases the total activity was not substantially inhibited by these modifications of the incubation mixture (Table I). The effect of 10 mM EDTA or pH 4.0 buffer could not be tested as these treatments inactivated the alkaline phosphatase (data not shown).

Table I: Release of Alkaline Phosphatase from Pig Kidney Microsomes[a]

additions to incubn mixture	alkaline phosphatase act. (%) total	supernatant
no addition	95.4	0.25
NaCl (1 M)	117.5	0.35
KCl (1 M)	104.0	0.21
$MgCl_2$ (100 mM)	82.7	0.32
$CaCl_2$ (100 mM)	94.1	0.50
Tris/acetate, pH 5.0 (75 mM)	87.9	0.70
Tris/acetate, pH 9.0 (75 mM)	104.3	0.49
$Na_2CO_3/NaHCO_3$, pH 10.1 (37.5 mM)	52.6	0.29
EDTA (1 mM)	87.5	0.26
inositol (250 mM)	112.2	0.11
glycerophosphorylinositol (5 mM)	107.3	0.32
phospholipase C (0.25 μg/mL)	95.0	80.20
phospholipase A_2 (2.5 units/mL)	96.6	0.36
phospholipase A_2 (2.5 units/mL) + bovine serum albumin (2.5 mg/mL)	95.6	0.36

[a] Incubations contained pig kidney microsomes (0.125 mg of protein/mL), 10 mM Hepes/NaOH buffer, pH 7.4 (this buffer was omitted when pH 5, 9, or 10.1 buffers were added), and a variety of agents as described in the table (the final concentration in incubation is given). Mixtures were incubated for 30 min at 37 °C, a sample was kept on ice for assay of total activity, and the remainder was centrifuged at 100000*g* for 1 h and the supernatant sampled. In the experiments with NaCl, KCl, inositol, glycerophosphorylinositol, and phospholipases A_2 or C the samples were assayed for alkaline phosphatase directly. In the other experiments the samples were dialyzed, at 4 °C, against 2 × 100 volumes of buffer A before assay of alkaline phosphatase. Values are expressed as a percentage of the activity determined in an unincubated microsomal sample, and each is the mean of two separate experiments.

Phospholipase A_2 (*N. naja*) also did not release alkaline phosphatase from the microsomes in the presence or absence of defatted bovine serum albumin. The results were the same in the presence or absence of 1 mM $CaCl_2$. Qualitative thin-layer chromatography of the pellets showed extensive hydrolysis of the major phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, and indicated that the phospholipase was active under these conditions. The extent of phosphatidylinositol hydrolysis was not determined because one cannot interpret the failure to release alkaline phosphatase; even with extensive hydrolysis of the total phosphatidylinositol, that involved in the binding of alkaline phosphatase may not have been degraded. These results are in agreement with those obtained previously where it was shown that the nonspecific phospholipases C, although ex-

Table II: Binding of Phospholipase C Released Alkaline Phosphatase to Phospholipid Vesicles[a]

incubn mixture	% of recovered act. alkaline phosphatase upper fraction	alkaline phosphatase lower fraction	[^{32}P]PC upper fraction	[^{32}P]PC lower fraction
alkaline phosphatase I				
+cholate	0.1	99.9		
+PC/PI/cholate	0.4	99.6	88.5	11.5
+PC/PI/ML/cholate	0.5	99.5	86.6	13.6
alkaline phosphatase II				
+cholate	0.2	99.8		
+PC/PI/cholate	0.4	99.6	92.4	7.6
+PC/PI/ML/cholate	0.7	99.3	94.4	5.6

[a] Alkaline phosphatase released from microsomes by phospholipase C was incubated with phospholipids dissolved in cholate or with cholate alone, dialyzed, and centrifuged (see Materials and Methods). Values refer to the percentage of the total recovered alkaline phosphatase or [^{32}P]PC found in each fraction and are the means of two experiments done with different alkaline phosphatase preparations. Alkaline phosphatase I (~0.2 unit) was used without purification; alkaline phosphatase II (~0.1 unit) had been chromatographed on Sephadex G-75 as described under Materials and Methods (ML = microsomal lipids).

tensively hydrolyzing microsomal phospholipids, were unable to release alkaline phosphatase (Ikezawa et al., 1976; Low & Finean, 1977b).

Binding to Phospholipid Vesicles of Alkaline Phosphatase Released by Phospholipase C. Alkaline phosphatase was released from pig kidney microsomes with phospholipase C, and its ability to bind to phospholipid vesicles was determined. The alkaline phosphatase was mixed with phospholipids dissolved in cholate, the cholate was removed by dialysis, and the mixture was centrifuged in a discontinuous sucrose density gradient to separate free and vesicle-bound alkaline phosphatase (Table II). It is clear that under these conditions less than 1% of the alkaline phosphatase is associated with the phospholipid vesicles. Essentially all of the alkaline phosphatase remained in the lower fraction while the majority of the phospholipid had moved into the upper fraction. Only the data for [^{32}P]phosphatidylcholine are shown, but [^{3}H]phosphatidylinositol and [^{14}C]triolein gave a similar distribution.

An effort was made to initiate the interaction of the alkaline phosphatase with phospholipid vesicles as soon as possible after its release from the membrane (alkaline phosphatase I; Table II). This was done to minimize the possible "denaturation" of the phosphatidylinositol binding site subsequent to release. However, this meant that phospholipase C was present during the incubation of alkaline phosphatase and phospholipid vesicles and that further phosphatidylinositol hydrolysis could not be excluded. Small amounts of phospholipase C were used for the release of alkaline phosphatase to minimize this hydrolysis. Phosphatidylinositol hydrolysis during the subsequent incubation with phospholipid/cholate solution (alkaline phosphatase I; Table II) was only 5–10%, but no binding was observed. It is still possible that a small amount of residual phospholipase C might release any alkaline phosphatase that had reassociated with phospholipid vesicles. This possibility was eliminated by separating the released alkaline phosphatase from phospholipase C on a Sephadex G-75 column before the addition of the phospholipid/cholate mixture. In these experiments phosphatidylinositol hydrolysis could not be detected (less than 3%) but significant binding to the phospholipid vesicles was not observed (alkaline phosphatase II; Table II).

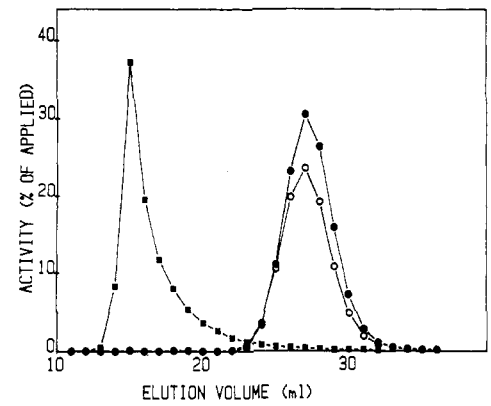

FIGURE 1: Chromatography of phospholipase C released alkaline phosphatase on Sepharose 6B. Alkaline phosphatase (~0.2 unit) released from microsomes by phospholipase C was incubated with [^{32}P]PC/[^{3}H]PI/microsomal lipids/cholate (● and ■) or cholate alone (○), dialyzed, and loaded onto a column of Sepharose 6B (see Materials and Methods). Values shown are the percentage of applied alkaline phosphatase activity (○ and ●) or [^{32}P]PC (■) in each fraction. [^{14}C]Triolein and [^{3}H]PI have elution profiles similar to those of [^{32}P]PC.

In some of these experiments the phospholipid vesicles used for the binding contained pig kidney microsomal lipids as well as phosphatidylcholine and phosphatidylinositol. This was done to rule out the possibility that other microsomal lipids besides phosphatidylinositol were involved in the binding of alkaline phosphatase to membranes. However, vesicles containing the microsomal lipids did not bind significant amounts of alkaline phosphatase (Table II).

In most of these experiments alkaline phosphatase was incubated with phospholipids dissolved in cholate and the binding determined after the removal of cholate. Some experiments, however, were done with preformed phospholipid vesicles (PC/PI or PC/PI + microsomal lipids) from which the cholate had already been removed, and similar results were obtained (data not shown).

In several experiments gel filtration on a Sepharose 6B column was substituted for centrifugation to separate free and vesicle-bound alkaline phosphatase after the removal of cholate. A typical elution profile is shown in Figure 1. Apparently none of the released alkaline phosphatase elutes in the fractions containing the major peak of phospholipid. Less than 3% of the phosphatidylinositol had been hydrolyzed. The alkaline phosphatase eluted in a position ($V_e/V_o = 2.0$) similar to that of controls, in which released alkaline phosphatase was treated with cholate without phospholipids (Figure 1). The same result was obtained in a total of five experiments with PC/PI or PC/PI + microsomal lipids.

Binding of Butanol-Solubilized Alkaline Phosphatase to Phospholipid Vesicles. Alkaline phosphatase was solubilized from pig kidney microsomes by brief extraction by butanol at 4 °C followed by immediate dialysis. Its ability to bind to phospholipid vesicles containing phosphatidylinositol was determined. The extracted alkaline phosphatase was mixed with a solution of phospholipids dissolved with cholate. The cholate was removed by dialysis, and the mixture was centrifuged in a discontinuous sucrose density gradient to separate free and vesicle-bound alkaline phosphatase (Table III). After centrifugation, most of the alkaline phosphatase had moved into the upper fraction, along with most of the phospholipid (alkaline phosphatase I; Table III), suggesting substantial binding of the alkaline phosphatase to phospholipid vesicles. However, if the butanol extract was incubated at 25 °C, before removing the butanol by dialysis, different results were obtained. Most

Table III: Binding of Butanol-Extracted Alkaline Phosphatase to Phospholipid Vesicles[a]

incubn mixture	% of recovered act. alkaline phosphatase upper fraction	alkaline phosphatase lower fraction	[^{32}P]PC upper fraction	[^{32}P]PC lower fraction
alkaline phosphatase I				
+cholate	1.7	98.3		
+PC/cholate	73.2	26.8	94.3	5.7
+PC/cholate + PLC	14.6	85.4	95.7	4.3
+PC/PI/cholate	71.0	29.0	94.7	5.3
alkaline phosphatase II				
+cholate	0.3	99.7		
+PC/cholate	25.2	74.8	91.9	8.1
+PC/PI/cholate	22.8	77.2	93.6	6.4
alkaline phosphatase III				
+cholate	2.4	97.6		
+PC/cholate	51.2	48.8	96.7	3.3
+PC/cholate + PLC	13.2	86.8	94.9	5.1
+PC/PI/cholate	51.9	48.1	94.9	5.1

[a] Alkaline phosphatase (~1 unit) extracted from microsomes with butanol was incubated with phospholipids dissolved in cholate or in cholate alone, dialyzed, and centrifuged (see Materials and Methods). The values refer to the percentage of total recovered alkaline phosphatase and [^{32}P]PC (similar distributions were found for [^{14}C] triolein and [^{3}H]PI) found in each fraction and are the means of two experiments done with different butanol extracts. Alkaline phosphatases I, II, and III indicate no incubation of butanol extract, incubation at 25 °C for 1 h, and incubation at 4 °C for 24 h, respectively. In some experiments 0.5 μg of phospholipase C (PLC) was included in the incubation mixture.

of the alkaline phosphatase now remained unbound in the lower fraction (alkaline phosphatase II; Table III). This loss of binding ability was prevented by the inclusion of EDTA (1 mM) during the incubation at 25 °C (~35% of the alkaline phosphatase activity was inhibited by the EDTA). Seventy-three percent of the alkaline phosphatase activity in this extract was able to bind to phospholipid vesicles (data not shown). Decreased binding was also observed if the extract was kept at 4 °C for 24 h before removal of butanol (alkaline phosphatase III; Table III). Incubation of the extracts at 25 or 4 °C did not, however, appear to change the total alkaline phosphatase activity. Binding does not appear to require added phosphatidylinositol, as a similar proportion of the alkaline phosphatase was found in the upper fraction when only phosphatidylcholine was used to form the phospholipid vesicles (Table III). However, the binding was substantially inhibited by the inclusion of phospholipase C in the incubation mixture (Table III).

Some binding experiments were also done with preformed phospholipid vesicles from which cholate had already been removed. However, substantially less alkaline phosphatase appeared to bind under these conditions compared with that observed with phospholipid/cholate mixtures. This may in part have been due to inefficient separation by the centrifugation technique; a considerable proportion of the phospholipid remained in the lower fraction.

Chromatography of Butanol-Solubilized Alkaline Phosphatase on Sepharose 6B. The molecular weight of the butanol-solubilized alkaline phosphatase was greatly affected by the conditions during the exposure to butanol. The enzyme prepared by exposure to butanol for 1 h at 25 °C gave a major peak of alkaline phosphatase (~80% of applied activity) on Sepharose 6B with an elution volume (Figure 2; $V_e/V_0 = 2.0$)

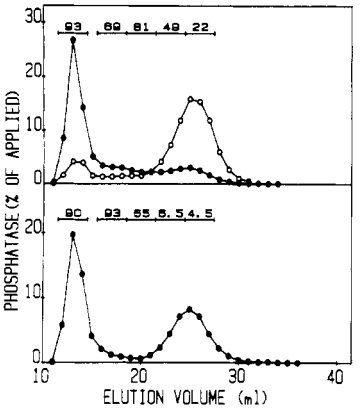

FIGURE 2: Chromatography of butanol-extracted alkaline phosphatase on Sepharose 6B. 0.5 mL of butanol-extracted alkaline phosphatase (~2 units; see Materials and Methods) was loaded onto a column (1 × 45 cm) of Sepharose 6B and eluted (2.5 mL/h) in buffer A, and 1-mL fractions were collected. The ordinate shows the percentage of applied alkaline phosphatase recovered in each fraction (total recovered, 80–95% of applied activity). These values are representative of two similar experiments done with different butanol extracts. Upper figure: (●) no incubation of extract; (○) extract incubated for 1 h at 25 °C. Lower figure: (●) extract incubated at 4 °C for 24 h (see Materials and Methods). Values above the curves are the percentages of alkaline phosphatase in pooled fractions which bind to PC vesicles determined as in Table III (these values refer to the curves with solid symbols).

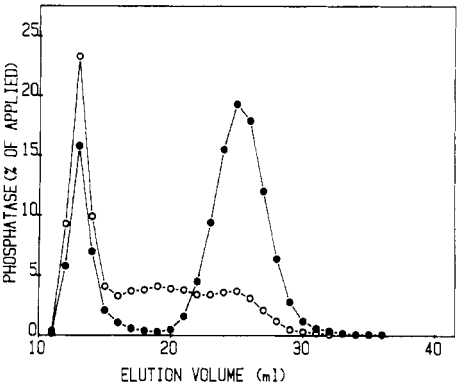

FIGURE 3: Effect of phospholipase C on molecular weight of butanol-extracted alkaline phosphatase. 0.4 mL of butanol-extracted alkaline phosphatase, 0.1 mL of phospholipase C (50 μg/mL), and 0.5 mL of 10% w/v sodium cholate in buffer A were incubated on ice for 1 h and then dialyzed against buffer A. 0.5 mL of the dialysate was chromatographed on a column of Sepharose 6B as described in Figure 2. Values shown are the percentages of applied alkaline phosphatase recovered in each fraction and are representative of two similar experiments done with different butanol extracts. (○) Control (no added phospholipase C); (●) phospholipase C treated.

similar to that obtained for phospholipase C released alkaline phosphatase as seen in Figure 1. Approximately 15% of the activity was eluted in the void volume. However, with alkaline phosphatase prepared by brief exposure to butanol at 4 °C (Figure 2) more of the activity (~50–60%) was eluted in the void volume and only about 20% in the low molecular weight peak ($V_e/V_0 = 2.0$). With alkaline phosphatase prepared by exposure to butanol for 24 h at 4 °C the two peaks contained approximately equal amounts of activity (Figure 2). If EDTA (1 mM) was included in the butanol extract during the incubation at 25 °C, only about 30% of the alkaline phosphatase was found in the low molecular weight peak when the extract was chromatographed on Sepharose 6B (data not shown). Treatment of the butanol-solubilized alkaline phosphatase with phospholipase C, in the presence of cholate prior to fractionation, markedly increased the proportion of the activity eluted in the low molecular weight peak (Figure 3).

The properties of the excluded and low molecular weight forms of alkaline phosphatase were compared by determining their ability to bind to phospholipid vesicles. Figure 2 shows that the high molecular weight form has a much greater ability to bind than does the low molecular weight form and that this difference appears to be independent of the relative amounts of the two forms of the enzyme.

Hydrolysis of Phosphatidylinositol during Butanol Extraction of Alkaline Phosphatase. [^{32}P]Phosphatidylcholine (0.9 μmol) and [^{3}H]phosphatidylinositol (0.1 μmol) were added to 1 mL (~6 mg of protein) of the lower phase of the butanol-extracted microsomes. After incubation at 25 °C for 1 h or at 4 °C for 24 h, less than 3% of the original [^{3}H]-phosphatidylinositol remained. However, during these incubations less than 5% of added [^{32}P]phosphatidylcholine was hydrolyzed. When EDTA (1 mM) was added to the extract at the same time as the phospholipids, the hydrolysis of [^{3}H]phosphatidylinositol was substantially inhibited and approximately 95% remained after incubation at 25 °C for 1 h. These results suggest the presence of a divalent cation dependent phospholipase in the lower phase of the butanol-extracted microsomes.

The phosphatidylinositol content of the butanol-extracted microsomes was determined by thin-layer chromatography, and values in the range of 1.5–3.8 nmol/mg of protein were obtained (compared to 48 nmol/mg of protein in microsomes). The phosphatidylinositol content of butanol-extracted microsomes incubated for 1 h at 25 °C decreased by 50% in one experiment but showed no substantial change in two subsequent experiments. Apparently the bulk of chloroform/methanol-extractable phosphatidylinositol is not accessible to this phospholipase. The observed decrease in molecular weight and loss of binding ability of the alkaline phosphatase to phospholipid vesicles (see preceding two sections) could be due to this phospholipase activity. In that instance the phosphatidylinositol involved in the binding reaction would have to be more accessible to this phospholipase than the bulk of the phosphatidylinositol in the butanol-extracted microsomes.

Discussion

Previous work by several investigators has shown that alkaline phosphatase, 5′-nucleotidase, and acetylcholinesterase are released from membranes by phosphatidylinositol-specific phospholipases C but not by other phospholipases with different specificities (Slein & Logan, 1965; Ikezawa et al., 1976; Taguchi & Ikezawa, 1978; Low & Finean, 1977b,c). The released enzymes have a molecular weight in the region 100 000–200 000 and do not appear to be associated with membrane fragments (Ohyabu et al., 1978; Low & Finean, 1977c, 1978). These observations have suggested that phosphatidylinositol is involved in the attachment of these enzymes to membranes. Such an attachment could be mediated through a phosphatidylinositol bridge between the enzyme molecule and the membrane; e.g., the polar head group could be attached to the enzyme with the diglyceride portion in a hydrophobic portion of the membrane.

Association between phosphatidylinositol and alkaline phosphatase could occur in two ways. It could be due to a specific, noncovalent binding of the PI molecule at a binding site on the enzyme molecule. Alternatively, there might be a covalent linkage between alkaline phosphatase and the phosphatidylinositol molecule. In this paper we have attempted to distinguish between these possibilities.

If noncovalent interactions are responsible for attachment of alkaline phosphatase to phosphatidylinositol, disruption of these interactions should lead to the release of alkaline phosphatase. However, exposure of microsomes to high ionic strength, divalent cations, or extremes of pH did not release significant amounts of alkaline phosphatase into the supernatant. This suggested that simple polar interactions are not responsible for the attachment of alkaline phosphatase to the membrane. The possibility that the interactions involved were more specific was explored by using analogues of the polar head group (inositol and glycerophosphorylinositol), but they did not release alkaline phosphatase either. Apparently, these agents are unable to compete with the polar head group of phosphatidylinositol in the bridge between membrane and alkaline phosphatase. The failure of these procedures to release alkaline phosphatase from the membranes could not be due to nonspecific hydrophobic interactions of single phosphatase molecules with the membrane, because this model would not account for the release of the alkaline phosphatase by phospholipase C.

Noncovalent binding of alkaline phosphatase to phosphatidylinositol is also inconsistent with our observation that phospholipase C released alkaline phosphatase does not reassociate with phosphatidylcholine/phosphatidylinositol vesicles. The failure of alkaline phosphatase to reassociate with vesicles might have been due to the continued presence of one of the products of hydrolysis at the binding site or to an irreversible change in the structure of the binding site subsequent to release of the phosphatase by the phospholipase C. Although these possibilities cannot be excluded by the present data, we think that the evidence presented here argues against a noncovalent binding of the phosphatase to phosphatidylinositol present in the membrane.

Extraction of membrane lipids with butanol has been widely used as a method for solubilizing, and subsequently purifying, membrane-bound alkaline phosphatase from a wide range of tissues. In most cases the majority of the activity appears to be extracted as water-soluble dimers with a relatively low molecular weight (100 000–200 000). These remain in a nonaggregated state after removal of the butanol and do not require detergents to maintain their solubility. We have found that butanol treatment partially delipidates the enzyme and removes 90–95% of the phosphatidylinositol. However, a large proportion of the butanol-solubilized enzyme was able to bind to phosphatidylcholine vesicles, and the binding was substantially inhibited by phospholipase C. It therefore appears that some phosphatidylinositol remains with the phosphatase after butanol treatment, which suggests that it is strongly bound to this enzyme.

The ability of the alkaline phosphatase to bind to phosphatidylcholine vesicles was substantially decreased when the extract was incubated at 25 °C for 1 h or at 4 °C for 24 h prior to the removal of butanol. During this treatment hydrolysis of added [^{3}H]phosphatidylinositol was observed. This supports the hypothesis that phosphatidylinositol is involved in the linkage of alkaline phosphatase to membranes or membranelike structures.

Chromatography of the alkaline phosphatase indicated that after butanol treatment at 25 °C the phosphatase structure was modified. Most of the alkaline phosphatase in an extract prepared by brief exposure to butanol was excluded from a Sepharose 6B column. However, in extracts which were incubated at 25 °C prior to removal of the butanol, only a small proportion of the activity was excluded from the column. Furthermore, the high molecular weight form of the alkaline phosphatase had a much higher ability to bind to phosphatidylcholine vesicles than the lower molecular weight form. It appears that prolonged exposure of alkaline phosphatase

to butanol at 25 °C reduces its molecular weight as well as its ability to bind to phospholipid vesicles. The additional observation that treatment of butanol-extracted alkaline phosphatase with added phospholipase C also decreases its molecular weight and binding ability emphasizes the importance of phosphatidylinositol in maintaining the alkaline phosphatase in a form which has high molecular weight and ability to bind to phospholipid membranes.

The occurrence of both high and low molecular weight alkaline phosphatase has also been observed by other investigators (Ghosh & Fishman, 1968; Nose, 1976; Doellgast et al., 1977), and it has been suggested that the low molecular weight form results from the proteolytic degradation of the high molecular weight form of the enzyme (Chang & Moog, 1972). We cannot preclude the occurrence of proteolysis in our extraction experiments; however, the observation that EDTA inhibits both phosphatidylinositol hydrolysis and the decrease in molecular weight of alkaline phosphatase during incubation of the butanol extract seems to indicate that one or more endogenous phospholipases are involved in decreasing the molecular weight of the phosphatase.

It is perhaps relevant to note that many of the published butanol extraction procedures used in the initial stages of alkaline phosphatase purification involve either standing overnight at 4 or 20 °C or a brief warming to 37 °C prior to removal of butanol (Ghosh & Fishman, 1968; Wachsmuth & Hiwada, 1974; Ramasamy & Butterworth, 1974; Cathala et al., 1975; Latner & Hodson, 1976; Malik & Butterworth, 1976; Doellgast et al., 1977), conditions which we have found to favor the hydrolysis of added [^{3}H]phosphatidylinositol by endogenous phospholipase. It seems to us that the low molecular weight alkaline phosphatase finally isolated by these investigators may be a degraded form of the enzyme produced by the action of endogenous phospholipases. Perhaps the phosphatidylinositol-specific phospholipases C which have been identified in many mammalian tissues [for references, see Michell (1975)] are responsible for the production of low molecular weight alkaline phosphatase during butanol extraction.

The observation that alkaline phosphatase can be prepared in a high and a low molecular weight form suggests that this enzyme may be attached to biological membranes in either one of these forms. Possibly single molecules of alkaline phosphatase could be attached to the membranes by a bridge involving a single phosphatidylinositol molecule. On the other hand, it is possible that aggregates, held together by phosphatidylinositol, are associated with the membrane. In that instance, the release of phosphatase from the membrane by phospholipase C would be due to the disruption of the aggregate.

It has recently been suggested (Shukla et al., 1980) that 5′-nucleotidase in rat liver is not covalently linked to phosphatidylinositol. This conclusion was based on the absence of radioactivity associated with 5′-nucleotidase released from [^{3}H]inositol-labeled hepatocytes by phospholipase C. Although these observations cannot completely exclude the possibility of covalent linkage between phosphatidylinositol and 5′-nucleotidase, they suggest that it is unlikely. In contrast, our results with pig kidney alkaline phosphatase give no support to the involvement of noncovalent interactions. To resolve this question, it will be necessary to use different techniques for determination of the nature of the interaction between phosphatidylinositol and membrane enzymes. Thus, the demonstration of covalently bound inositol in purified alkaline phosphatase released from membranes by phospholipase C would provide very strong evidence for covalent linkage between phosphatidylinositol and the enzyme. However, in view of the low amounts of alkaline phosphatase present in mammalian tissues, this experiment would require a technique for the analysis of inositol considerably more sensitive than those currently available.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Cathala, G., Brunel, C., Chappelet-Tordo, D., & Lazdunski, M. (1975) *J. Biol. Chem. 250*, 6040–6045.

Chang, C. H., & Moog, F. (1972) *Biochim. Biophys. Acta 258*, 166–177.

Doellgast, G. J., Spiegel, J., Guenther, R. A., & Fishman, W. H. (1977) *Biochim. Biophys. Acta 484*, 59–78.

Ghosh, N. K., & Fishman, W. H. (1968) *Biochem. J. 108*, 779–792.

Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T., & Ohyabu, T. (1976) *Biochim. Biophys. Acta 450*, 154–164.

Kates, M. (1972) in *Techniques of Lipidology*, North-Holland Publishing Co., Amsterdam.

Kaulen, H. D. (1972) *Anal. Biochem. 45*, 664–667.

Latner, A. L., & Hodson, A. W. (1976) *Biochem. J. 159*, 697–705.

Low, M. G., & Finean, J. B. (1977a) *Biochem. J. 162*, 235–240.

Low, M. G., & Finean, J. B. (1977b) *Biochem. J. 167*, 281–284.

Low, M. G., & Finean, J. B. (1977c) *FEBS Lett. 82*, 143–146.

Low, M. G., & Finean, J. B. (1978) *Biochim. Biophys. Acta 508*, 565–570.

Low, M. G., & Zilversmit, D. B. (1980) *Biochim. Biophys. Acta 596*, 223–234.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Malik, N., & Butterworth, P. J. (1976) *Biochim. Biophys. Acta 446*, 105–114.

Michell, R. H. (1975) *Biochim. Biophys. Acta 415*, 81–147.

Morton, R. K. (1954) *Biochem. J. 57*, 595–603.

Nose, K. (1976) *J. Biochem. (Tokyo) 79*, 283–288.

Ohyabu, R., Taguchi, R., & Ikezawa, H. (1978) *Arch. Biochem. Biophys. 190*, 1–7.

Ramasamy, I., & Butterworth, P. J. (1974) *Biochim. Biophys. Acta 370*, 477–486.

Shukla, S. D., Coleman, R., Finean, J. B., & Michell, R. H. (1980) *Biochem. J. 187*, 277–280.

Slein, M. W., & Logan, G. F. (1965) *J. Bacteriol. 90*, 69–81.

Taguchi, R., & Ikezawa, H. (1978) *Arch. Biochem. Biophys. 186*, 196–201.

Wachsmuth, E. D., & Hiwada, K. (1974) *Biochem. J. 141*, 273–282.