Effect of Phospholipase C Hydrolysis of Membrane Phospholipids on Membranous Enzymes*

RICHARD D. MAVIS,‡ ROBERT M. BELL,§ AND P. ROY VAGELOS

From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

SUMMARY

The response of several Escherichia coli membranous enzymes to hydrolysis of up to 95% of membrane phospholipid has been investigated. Purified phospholipase C of Bacillus cereus was utilized in these studies. The rate and extent of digestion of E. coli phospholipids was independent of whether the lipid was associated with membrane protein or extracted from membranes and sonically dispersed. Phosphatidylethanolamine and phosphatidicglycerol were completely hydrolyzed, while cardiolipin was partially resistant to hydrolysis by phospholipase C. Acyl-CoA:glycerol 3-phosphate acyltransferase and NADH oxidase were inactivated at a rate very similar to the rate of hydrolysis of total lipids. Acyl-CoA:1-alkylglycerol 3-phosphate acyltransferase was inactivated to an extent of 50% during hydrolysis of 50% of membrane phospholipid. The remaining activity was stable to continued hydrolysis of phospholipid. Glycerol 3-phosphate dehydrogenase and succinic dehydrogenase remained completely active after hydrolysis of 95% of membrane phospholipids. These results show the heterogeneity of membranous enzymes with respect to their dependence upon the presence of intact membrane phospholipids. The lack of effect of membranous lipid-protein interactions on the accessibility of phospholipids to phospholipase C hydrolysis was shown in general by the similarity in the rates of hydrolysis by phospholipase C of membranous and isolated phospholipids. More specifically, the similarity in the rate of hydrolysis of phospholipids and the rates of inactivation of certain membranous enzymes suggests that these enzymes are dependent on phospholipids whose susceptibility to phospholipase C hydrolysis is similar to the bulk of membrane phospholipids.

The apparent independence of the bulk of membrane protein and the bulk of membrane lipid as indicated by the work of Glaser et al. with spectroscopic probes (1) reveals the need for studying membranous lipid-protein interactions at a more specific level. That such interactions exist is obvious from the phospholipid requirements of several purified membranous enzymes (2-8) as well as from the dependence of temperature characteristics of various membranous enzyme and transport activities on the phase properties of membrane lipid (9-13). These interactions are not detectable spectroscopically in intact membrane, presumably because they do not represent the bulk of membrane protein or possibly because they are associated with small amounts of phospholipid not detectable by the methods used in such experiments. Therefore a more specific probe, such as enzymic activity, would appear to be useful in studying the role of lipid-protein interaction in membrane function. We have recently reported differences in the response of various membranous enzymes of Escherichia coli to changes in membrane fatty acid composition (9). The heterogeneity of lipid-protein interaction revealed by that study suggested that further investigation of the phospholipid requirements of these enzymes would be of interest. We have recently shown that these enzymes have normal specific activities in membranes of an E. coli mutant with altered phospholipid composition.1

The phospholipid requirements of several membranous enzymes have been shown by various methods, the most conclusive of which is purification of the enzyme to the extent that activation by added phospholipid can be shown. The extreme difficulty encountered in the purification of some membranous enzymes has led to the utilization of more indirect methods. Phospholipase digestions have been utilized with several systems (14-19), but interpretation of these results requires caution. Phospholipase A produces fatty acids and lysophosphatides, compounds whose surfactant properties are known to inhibit several enzymes nonspecifically. Thus, enzyme inhibition caused by phospholipase A can be due to the surfactant properties of the hydrolysis products rather than to the removal of required phospholipids. The products of phospholipase C hydrolysis, diglyceride and water-soluble phosphoryl compounds, do not have surfactant properties. Thus inactivation of a membranous enzyme by phospholipase C can be more safely interpreted as suggesting the requirement of intact phospholipid. The purity of phospholipase preparations is critical in such a demonstration. Contaminating proteases or other

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activities may inactivate membranous enzymes independently of phospholipid hydrolysis. In the present work, a highly purified preparation of phospholipase C from Bacillus cereus (20) was used to investigate the effect of phospholipid hydrolysis on several membranous enzymes of E. coli. The use of membrane preparations from 32P-labeled cells provided a convenient method of following lipid hydrolysis concurrently with the spectrophotometric determination of several enzymatic activities.

EXPERIMENTAL PROCEDURE

Materials—Glycerol-3-P, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide, phenazine methosulfate, 5,5'-dithio-dibutyryl-2-nitrobenzoic acid, deoxyribonuclease, phospholipase D, NADH, NAD+, glucose-6-P, and glucose-6-P dehydrogenase were purchased from the Sigma Chemical Co., St. Louis, Mo. Palmityl-CoA and oleyl-CoA were from P-L Biochemicals, Milwaukee, Wis. 1-Acylglycerol 3-phosphate was prepared by treatment of 1-acylglycerol 3-phosphorylcholine with phospholipase D (21). 1-Acylglycerol 3-phosphorylcholine (prepared by treatment of egg lecithin with phospholipase A) was purchased from Cyclo Chemical, Los Angeles, Calif. [32P]Phosphoric acid was purchased from New England Nuclear.

Growth of E. coli and Preparation of 32P-Labeled Membrane Fraction—E. coli strain H139 was grown at 37° in 1% Difco Bacto Tryptone, 0.5% sodium chloride, 0.2% glycerol, 1.7 mM thiamine, 2.0 mM pantothenate, 0.2% sodium succinate, and 2 to 5 mCi of [32P]phosphate per liter. Cells were harvested in late exponential phase, washed once with cold 0.02 M potassium phosphate buffer, pH 7.0, resuspended in one-fiftieth the original culture volume of the same buffer and frozen. The suspension was thawed and the cells were broken in a French pressure cell previously cooled in ice. The cell extract was made 2.5 mM in MgCl2 and a few crystals of deoxyribonuclease were added. The extract was incubated on ice for 30 min and then centrifuged at 6,000 × g for 10 min to remove unbroken cells. The membrane fraction was then collected by centrifugation for 45 min at 50,000 × g. After washing twice by resuspension and homogenization with a Teflon-glass homogenizer in cold 0.02 M potassium phosphate buffer, pH 7.0, the membrane fraction was resuspended in the same buffer at a protein concentration of 0.078 pmole of NADH in a final volume of 0.2 ml. The reaction was initiated by addition of membrane fraction. Succinic dehydrogenase activity was measured as the phenazine methosulfate-mediated reduction of the tetrazolium dye 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide to its formazan which absorbs at 550 nm. A millimolar extinction coefficient of 8.75 was used for the formazan. NADH oxidase activity was measured spectrophotometrically by following a decrease in absorbance at 340 nm. Incubation mixtures contained 2.2 pmole of histidine buffer, pH 6.5, 0.4 pmole of MgSO4 and 0.078 pmole of NADH in a final volume of 0.2 ml. The reaction was initiated by addition of membrane fraction. Sucrose dehydrogenase activity was measured as the phenazine methosulfate-mediated reduction by succinate of the tetrazolium dye used in the glycerol-3-P dehydrogenase assay. Incubation mixtures contained 1 pmole of disodium succinate, 22.5 pmole of KCN, 6 μg of 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide, 20 μg of phenazine methosulfate, 0.4 pmole of MgSO4, 2.2 pmole of histidine buffer, pH 6.5, and a final volume of 0.2 ml. Reduction of the tetrazolium dye was followed as in the glycerol-3-P dehydrogenase assay. Glucose-6-P dehydrogenase was assayed in a final volume of 0.6 ml containing 2 μmol of glucose-6-P, 15 μmol of NADP, 1.75 μmol of MgCl2, and 30 μmol of Tris-HCl buffer, pH 7.8. Reduction of NADP was followed at 340 nm. All spectrophotometric assays were performed on a Gilford model 2400 recording spectrophotometer at 25°.

RESULTS

The assay described here, with radioactively labeled phospholipids extracted from E. coli grown in the presence of [32P] phosphate, provides a convenient and reliable method of meas-
uring phospholipase C activity. The use of the two phases, ether and water, allowed ready separation of unhydrolyzed lipid from phosphoryl moieties released by phospholipase activity. As shown in Fig. 1, the rate of phospholipid degradation was linear up to 25 min and proportional to the amount of enzyme added. Linearity with time was observed during degradation of up to 80% of the lipid in the assay. No activation by Ca++ or inhibition by EDTA was observed in this assay.

With this assay, phospholipase C was purified from the culture medium of B. cereus essentially according to the procedure of Zwaal et al. (20). The resulting preparation, which had a specific activity of 860 μmoles of lipid phosphorus released per min per unit of absorbance at 280 nm, migrated as a single major band on disc gel electrophoresis in sodium dodecyl sulfate. In order to test for possible proteolytic activity, an incubation of 1 mg of commercial, soluble glucose-6-P dehydrogenase with 1.2 enzyme units of the purified phospholipase C was performed for 1 hour at room temperature. No loss of dehydrogenase activity resulted.

The susceptibility to hydrolysis of E. coli membranous phospholipids by phospholipase C was compared to that of phospholipids extracted from the E. coli membrane by following their rates of hydrolysis under identical conditions. The effect of sonication of the membrane on susceptibility of phospholipids to hydrolysis was similarly studied. As shown in Fig. 2, the rate of hydrolysis of E. coli membrane phospholipids by purified phospholipase C was very similar in intact membrane fraction, sonicated membrane fraction, and a suspension of sonically dispersed lipids isolated from the membrane fraction, under the conditions used. This similarity was confirmed in an experiment (not shown) with one-third the concentration of 32P-lipid utilized in the experiment of Fig. 2.

The specificity of the purified phospholipase C toward individual phospholipids was investigated by analyzing the [32P]phospholipids extracted at various times during the phospholipase digestions described in Fig. 2. As shown in Fig. 3A, the rate of hydrolysis varied for the three major classes of phospholipids in the E. coli membrane fraction. Phosphatidylethanolamine, the major phospholipid of E. coli, comprising 75 to 80% of the total membrane phospholipids, was hydrolyzed most rapidly. After 80 min of digestion, only 20% of the phosphatidylethanolamine remained. Phosphatidylglycerol, comprising about 13% of E. coli phospholipids, was hydrolyzed at a very similar rate. Cardiolipin, a 5 to 10% component of E. coli phospholipids, was hydrolyzed slower than phosphatidylethanolamine or phosphatidylglycerol. During the first 5 min of digestion, cardiolipin appeared to increase. After 10 min, cardiolipin began to decrease and hydrolysis of cardiolipin continued until 60% of the initial cardiolipin remained at 60 min. Very little further decrease in cardiolipin occurred in the next hour of incubation during which phosphatidylethanolamine and phosphatidylglycerol decreased at a constant rate.

Similar specificity with respect to rates of hydrolysis of individual phospholipids was observed in sonicated membrane fraction (Fig. 3B) or in phospholipids extracted from membrane fraction and dispersed in buffer by sonic oscillation (Fig. 3C). In both preparations, phosphatidylethanolamine and phosphatidylglycerol were hydrolyzed at similar rates, phosphatidylethanolamine decreasing to 11% in 80 min of digestion during which phosphatidylglycerol decreased to about 20%. Cardiolipin was hydrolyzed more slowly than phosphatidylglycerol or phosphatidylethanolamine. An initial hydrolysis of about 20 to 30% of the cardiolipin in the first 10 to 20 min of digestion was followed by a virtual cessation of further hydrolysis of cardiolipin. Incubation for an additional 100 min produced very little loss of cardiolipin while a further decrease in both phosphatidylethanolamine and phosphatidylglycerol of 30 to 40% occurred. No initial increase in cardiolipin, as seen in intact membrane fraction, was observed in sonicated membrane fraction or in isolated, sonically dispersed phospholipid from the membrane fraction.

The effect of hydrolysis of membrane phospholipids on several membranous enzyme activities of E. coli was investigated. As shown in Fig. 4, membranous palmitoyl-CoA:glycerol-3-P acyltransferase activity was inactivated by treatment with purified phospholipase C at a gradually decreasing rate very similar to the rate of hydrolysis of membrane phospholipids.

![Fig. 1. Assay of phospholipase C. Time and enzyme dependence. A, time course of assay. O, no enzyme; A, 5 μl of enzyme; □, 15 μl of enzyme. B, enzyme dependence of assay. Time of assay was 10 min.](image1)

![Fig. 2. Hydrolysis of membrane phospholipids by purified phospholipase C. O, intact membrane fraction; ●, sonicated membrane fraction; ▲, isolated lipid sonically dispersed in buffer. All incubations were performed at room temperature in 0.24 ml of 0.02 M potassium phosphate buffer, pH 7.0 containing 0.2 μl of purified phospholipase C. Each incubation contained an amount of [32P]lipid corresponding to 2 mg of intact membrane protein. Samples of 20 μl were withdrawn for phospholipid analysis (described under “Experimental Procedure”) at the times indicated.](image2)
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**Fig. 3.** Hydrolysis of individual phospholipids by purified phospholipase C. Phospholipid analysis was performed on the samples from the incubations in Fig. 2. A, intact membrane fraction; B, sonicated membrane fraction; C, isolated lipid sonically dispersed in buffer. ○, phosphatidylethanolamine; □, phosphatidylglycerol; Δ, cardiolipin.

**Fig. 4.** Effect of phospholipase C hydrolysis of membrane phospholipids on membranous palmityl-CoA:glycerol-3-P acyltransferase activity. Membrane fraction (10 mg of protein) was incubated at room temperature in 0.9 ml of 0.02 M potassium phosphate buffer with (closed symbols) and without (open symbols) 0.6 unit of purified phospholipase C. Samples of 10 μl were withdrawn at times indicated for enzyme assay or phospholipid analysis. Δ, percentage of [3P]lipid remaining; ○, percentage of palmityl-CoA:glycerol-3-P acyltransferase activity remaining. Specific activity at zero time was 1.2 nmoles per min per mg of protein.

After 100 min of incubation, 75% of the phospholipids were hydrolyzed and 90% of the glycerol-3-P acyltransferase activity was lost. Similarly, membranous NADH oxidase was inactivated by phospholipase C treatment at a rate comparable to that of phospholipid hydrolysis (Fig. 5). Inactivation of membranous oleyl-CoA:1-acylglycerol-3-P acyltransferase (Fig. 6) proceeded at a rate identical with phospholipid hydrolysis until 45% of the activity remained, at which point inactivation ceased. The residual activity remained constant during an additional hour of treatment resulting in continued hydrolysis of phospholipids. Glycerol-3-P dehydrogenase and succinic dehydrogenase activities in membrane fractions were unaffected by hydrolysis of over 80% of membrane phospholipids as shown in Figs. 7 and 8.
Fig. 6. Effect of phospholipase C hydrolysis of membrane phospholipids on membranous oleyl-CoA:1-acylglycerol-3-P acyltransferase activity. An incubation was performed as in Fig. 4. Oleyl-CoA:1-acylglycerol-3-P acyltransferase activity was assayed at times indicated. Specific activity at zero time was 2.3 nmols per min per mg of protein.

Fig. 7. Effect of phospholipase C hydrolysis of membrane phospholipids on membranous glycerol-3-P dehydrogenase. An incubation was performed as in Fig. 4. Glycerol-3-P dehydrogenase was assayed at times indicated. Specific activity at zero time was 11.6 nmols per min per mg of protein.

Fig. 8. Effect of phospholipase C hydrolysis of membrane phospholipids on membranous succinic dehydrogenase. An incubation was performed as in Fig. 4. Succinic dehydrogenase activity was assayed at times indicated. Specific activity at zero time was 29.4 nmols per min per mg of protein.

Attempts to reactivate palmityl-CoA:glycerol-3-P acyltransferase or NADH oxidase activities of phospholipase C-treated membranes by addition of suspensions of phosphatidylglycerol or phosphatidylethanolamine purified from lipid extracts of E. coli were unsuccessful. The glycerol-3-P acyltransferase and NADH oxidase activities of partially inactivated membrane fraction washed free of phospholipase were stable to incubation at room temperature for at least 15 min or freezing at -15°C.

Since the stability of the glycerol-3-P dehydrogenase and succinic dehydrogenase during digestion of 80% of total membrane phospholipid could be attributed to preferential association of these enzymes with the 20% of phospholipid which remained intact, an attempt was made to completely hydrolyze the membrane phospholipids. Incubation of membrane fraction with 10 times the concentration of purified phospholipase C used in Figs. 2 to 7 resulted in hydrolysis of 96% of the phospholipids in 155 min. Analysis of the unhydrolyzed phospholipid revealed that 2% of the original phosphatidylethanolamine, 4.5% of the original phosphatidylglycerol, and 32% of the original cardiolipin remained. No loss of glycerol-3-P dehydrogenase or succinic dehydrogenase activities over that observed in control incubations without phospholipase C was observed in these membranes.

DISCUSSION

The purity of the phospholipase C used here is comparable to that obtained by Zwaal et al. (20). Since the assay used here was performed at room temperature, the specific activity reported here (560 units per absorbance unit at 280 nm) compares favorably with that reported by Zwaal et al. (20), who assayed the enzyme at 37°C. The stability of the soluble enzyme, glucose-6-P dehydrogenase, in an incubation with 1.2 units of phospholipase per mg of dehydrogenase argued against the presence of any significant amounts of proteolytic activity in our preparation of phospholipase C. This preparation therefore appeared suitable for a study of the specific effects of hydrolysis of membrane phospholipids to diglycerides.

The similarities of rates and specificity of phospholipase C hydrolysis of intact E. coli membrane, sonicated membrane, and isolated E. coli phospholipid suggests that the physical state of membranous phospholipids is similar to the physical state of sonically dispersed phospholipids, and that no protection from hydrolysis is afforded the bulk of membrane phospho-
The limited hydrolysis of cardiolipin showed here is in concurrence with the observation of Zwaal et al. (20) that this enzyme is sensitive to the chemical nature of the lipid substrate. They reported that red blood cell sphingomyelin was not hydrolyzed by phospholipase C, thereby explaining the previously observed maximum hydrolysis of 70% of red blood cell membrane phospholipids (1). The present work showed that up to 50% of E. coli cardiolipin was hydrolyzed by phospholipase C at a rate comparable to the rates of hydrolysis of phosphatidylethanolamine and phosphatidylglycerol, while the remaining cardiolipin was stable to further incubation with the phospholipase.

The resistance of a major fraction of cardiolipin to hydrolysis by phospholipase C may be explained in a number of ways. One possibility is that two classes of cardiolipin exist, differing in physical state or chemical composition. A heterogeneity of bacterial cardiolipin has been previously suggested (30, 31). The occurrence of partial hydrolysis of cardiolipin in isolated lipid as well as in the intact membrane fraction would seem to eliminate a role of the membrane protein in causing heterogeneity of the physical state of E. coli cardiolipin, or at least require a separate explanation for partial hydrolysis of cardiolipin in isolated lipid. Thus a chemical heterogeneity, most probably due to differences in the nature of the fatty acyl residues is indicated. A decided preference of phospholipase C for phospholipid substrate containing unsaturated acyl residues has been shown (32). Another explanation for partial hydrolysis might be an association of the less polar cardiolipin with diglyceride produced by phospholipase C hydrolysis. Bangham and Dawson have attributed a cessation of phospholipase C hydrolysis to the accumulation of diglyceride which was shown to affect the charge properties of lipid micelles (33). The small amount of cardiolipin present in E. coli relative to the other major phospholipids might also be the reason for its limited hydrolysis. It should be noted, however, that treatment of intact membranes with 10 times the concentration of phospholipase C used in the experiment of Fig. 2 resulted in the hydrolysis of over 95% of both phosphatidylethanolamine and phosphatidylglycerol, while 32% of the original cardiolipin remained unhidrolyzed; i.e. the final concentration of cardiolipin was twice that of unhydrolyzed phosphatidylglycerol or phosphatidylethanolamine. Thus a lesser affinity of the enzyme for cardiolipin must be postulated if concentration effects are to be invoked. It is of interest that cardiolipin was resistant to phospholipase C hydrolysis in membrane preparations derived from mitochondria (18, 19). In contrast, synthetic cardiolipin, or cardiolipin isolated from beef heart, was degraded by phospholipase C in a two-phase system of water and diethyl ether (34).

The apparent initial increase in cardiolipin observed during incubation of intact membranes with phospholipase C may be due to conversion of phosphatidylglycerol to cardiolipin by enzymes present in the membrane. This conversion has been shown previously in vivo and in vitro (24, 35, 36). Sonication apparently destroyed this activity in the present work, since no increase in cardiolipin occurred when sonicated membrane fractions were studied.

The inactivation of acyl-CoA:glycerol-3-P acyltransferase at a rate very similar to that of total phospholipid hydrolysis suggests that either this enzyme is dependent on phospholipid whose accessibility to phospholipase C is similar to that of the bulk of membrane lipids or that intact membrane structure is necessary for the activity of this enzyme. Previous work from this laboratory (9) showed that the presence of trans-unsaturated fatty acids in membrane phospholipids perturbed the temperature characteristics of this enzyme, implying an association with phospholipid. However, increasing the degree of unsaturation of membrane lipids had no effect on the Arrhenius plot of this activity, suggesting its independence of phase changes in membrane lipids.

The 50% inactivation of acyl-CoA:1-acylglycerol-3-P acyltransferase observed during phospholipid hydrolysis is similar to the partial inactivation of this enzyme previously observed upon treatment of the E. coli membrane fraction with maleic anhydride (26). Thus it appears that either two enzymes, differing in their phospholipid dependence and response to maleylation, catalyze this reaction, or, if a single enzyme is responsible, it is converted to a half-active form by either maleylation or hydrolysis of membrane phospholipids. Since inactivation of the labile portion of this activity is complete after the initial hydrolysis of 50% of the phospholipid, it would appear that this enzyme is dependent upon phospholipid which is part of a more accessible fraction of membrane phospholipids. Alternatively, destruction of membrane integrity sufficient to maximally inactivate this enzyme could result from hydrolysis of 50% of the membrane lipid. This is in contrast to the inactivation of acyl-CoA:glycerol-3-P acyltransferase and NADH oxidase which appears to require total hydrolysis of membrane phospholipid.

NADH oxidase, like acyl-CoA:glycerol-3-P acyltransferase, was inactivated by phospholipase C at a rate very similar to that of phospholipid hydrolysis. Thus the complex process of electron transport appears to require phospholipid with an accessibility to phospholipase digestion equal to that of the bulk of membrane lipid or at least require intact membrane. The role of phospholipid in mitochondrial electron transport has been indicated in a number of ways among which was inactivation by phospholipase C (18). In contrast, membraneous NADH oxidase of Bacillus megaterium is reportedly stable to phospholipase C treatment (37).

The two dehydrogenases studied here, which are not dependent on electron transport systems but simply catalyze the direct reduction of phenazine methosulfate by substrate, remain active after hydrolysis of over 95% of membraneous phospholipid. This observation together with our previous demonstration that Arrhenius plots of membraneous glycerol-3-P dehydrogenase are independent of membrane fatty acid composition (9), strongly suggests the autonomy of this enzyme with respect to membrane phospholipids.

Mitochondrial succinic dehydrogenase is reportedly stable to phospholipase C hydrolysis (19) although an earlier report is in conflict with this observation (38). Succinic dehydrogenase has been solubilized and purified from mitochondria, and while lipids appeared to stabilize the enzyme under certain conditions (39), an absolute requirement for phospholipid was not evident. Arrhenius plots of mitochondrial succinic dehydrogenase from rat liver and sweet potato have been shown to be discontinuous at temperatures coinciding with phase changes in membrane lipids detected by spin label probes (40, 41). Disruption of the
mitochondria with a nonionic detergent eliminated the break in the Arrhenius plots but apparently did not inactivate the enzyme. Thus an enzyme-lipid interaction resulting in a reflection of the phase properties of the lipid in the activity of the enzyme does not necessarily imply an absolute dependence upon phospholipid for activity. Conversely, the enzyme acyl-CoA:glycerol-3-P acyltransferase, has an apparent dependence on intact phospholipid but does not reflect the phase properties of membrane phospholipids in its temperature characteristics.

All of the enzymes studied here have been shown to be a part of the cytoplasmic membrane (42-44). Thus the heterogeneity of these enzymes with respect to phospholipid dependence is characteristically of a single membrane structure. Currently popular models of membrane structure show various degrees of intercalation of proteins by lipid within a single membrane (45). The bulk of the lipid, however, is thought to exist in a bilayer structure independent of protein. Thus only a small fraction of the cytoplasmic membrane (42-44). Thus the heterogeneity of these enzymes with respect to phospholipid dependence is consistent with such a model. Membranous enzymic activities showed varying degrees of dependence on imported here are consistent with such a model.

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