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SPIN-LABELING OF *ESCHERICHIA COLI* MEMBRANE BY ENZYMATIC SYNTHESIS OF PHOSPHATIDYLGLYCEROL AND DIVALENT CATION-INDUCED INTERACTION OF PHOSPHATIDYLGLYCEROL WITH MEMBRANE PROTEINS

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Summary

Escherichia coli membrane particulate fraction has been spin-labeled by incubating with *sn*-glycerol-3-phosphate, CTP, palmitoyl CoA and 12-nitroxide stearoyl CoA. Incorporation of the spin-labeled acyl chain into phosphatidylglycerol was confirmed. ESR spectrum of the spin-labeled phosphatidylglycerol in *E. coli* membrane consisted at least of two components; one due to the labels undergoing rapid anisotropic motions and the other due to strongly immobilized labels (the overall splitting value, approx. 58 G). The relative intensity of the two components was dependent on the concentration of divalent cations. The immobilized component decreased on treatment of the membrane with EDTA and increased on addition of Mg^{2+} or Ca^{2+}. The spectrum at 1 mM Mg^{2+} or Ca^{2+} consisted almost only of the immobilized component. Spin-labeled phosphatidylglycerol in total lipid membrane showed ESR spectrum due to mobile labels and the spectrum was not affected appreciably by the divalent cations. The results suggest the divalent cation-mediated interaction of phosphatidylglycerol with proteins in *E. coli* membrane. Phosphoenolpyruvate-dependent uptake of methyl-α-D-glucoside was markedly accelerated by Mg^{2+}. Ca^{2+} was not effective for the enhancement. The divalent cation-induced interaction of phosphatidylglycerol with proteins was discussed in relation to the sugar transport.

Introduction

The spin-labeling technique has been successfully used to elucidate dynamic structure of lipids in model membranes as well as biological membranes [1,2]. In most of these studies, biological membranes were incubated with sonicated

vesicles of spin-labeled phospholipids. The lipid molecules may be incorporated into membranes by fusion and/or exchange between vesicles and membranes. The incorporated lipids can be rapidly equilibrated in each half of membrane due to rapid lateral diffusion [3,4]. However, the motions in transverse direction of membrane are very much limited and, therefore, distribution of spin-labeled lipids may be different from that in intact biological membranes.

Biosynthetic incorporation of spin-labeled lipids would be ideal for intact spin-labeling of biological membranes. Several attempts have been made successfully for incorporation of 12-nitroxide stearic acid or its methyl ester into membrane phospholipids of *Neurospora crassa* [5—7], *Acholeoplasma laidlawii* [8], and yeasts [9,10]. Enzymatic incorporation of spin-labeled stearate into *sn*-glycero-3-phosphate using isolated liver microsomes has also been carried out successfully [11—13].

In the present investigation we used an *Escherichia coli* membrane particulate fraction for biosynthetic spin-labeling. Phospholipid biosynthesis in the membrane system has been extensively studied and well characterized [14]. The membrane has a particylarly simple lipid composition; phosphatidylethanolamine (80%), phosphatidylglycerol (15%), and cardiolipin (5%). It is expected that the major phospholipid may serve as a two-dimensional matrix, while phosphatidylglycerol and cardiolipin may be more functionally involved. We were able to label the membrane by enzymatic incorporation of spin-labeled stearate into phosphatidylglycerol using a spin-labeled acyl CoA as substrate. We found a marked mobility change in phosphatidylglycerol acyl chains induced by Mg^{2+} and also by Ca^{2+}. The results suggest a divalent cation-mediated interaction between phosphatidylglycerol and membrane proteins.

Materials and Methods

Chemicals

sn-Glycerol-3-phosphate as dicyclohexylammonium salt and CTP trisodium salt were the products of Boeringer Mannheim. Phosphoenolpyruvate and palmitoyl CoA were obtained from Sigma Chemical Co. and CoA from Tanabe Pharmaceutical Co. Ltd. [^{14}C]Methyl-α-D-glucoside and *sn*-[U-^{14}C]glycerol-3-phosphate disodium salt were purchased from New England Nuclear. Silica gel thin-layer plate (Silica gel G, Art. 5721) was the product of Merck and cellulose thin-layer plate (Avicell) was obtained from Funakoshi Co. All other reagents were of analytical grade.

Synthesis of spin-labeled acyl CoA

This was done by the reaction of CoA and anhydride of 12-nitroxide stearic acid. The method was basically the same as that of Devaux et al. [15] published independently. The spin-labeled stearate was synthesized by the method of Waggoner et al. [16] with a slight modification [17] and its anhydride by the reaction with dicyclohexylcarbodiimide [18]. A tetrahydrofuran solution of the acid anhydride (0.63 mmol in 18 ml) was mixed with aqueous solution of CoA (0.11 mmol in 18 ml, pH 8 adjusted with NaOH). The mixture was then added with tetrahydrofuran to make it homogeneous and allowed to react at room temperature. The proceeding of esterification was checked by assaying

free thiol group [19]. At the end of reaction, the pH was lowered to 6, tetrahydrofuran was evaporated off, and 1.8 ml of 7% perchloric acid [20] was added in an ice bath. The precipitate was washed three times with ether, dissolved in 1.5 ml of chloroform/methanol (2 : 1, v/v), and applied to preparative cellulose thin-layer chromatography [21]. The plate was developed with butanol/glacial acetic acid/water (5 : 2 : 3, by vol). The ultraviolet-absorbing band was scraped off and extracted three times with 10-ml portions of water. After adjusting the pH to 5.5, 11 ml of 7% perchloric acid was added and the precipitate was washed three times with ether. The product still contained a trace amount of 12-nitroxide stearic acid as judged from the ultraviolet absorption but used without further purification. The concentration of spin-labeled CoA was determined by absorption at 260 nm corrected for that of 2,2,6,6-tetramethylpiperidine-1-oxyl.

Preparation of membrane particulate fraction

Growth of *E. coli* B and preparation of the membrane particulate fraction were done in a phosphate buffer (10 mM phosphate/10 mM $MgCl_2$/5 mM dithiothreitol, pH 7.2) according to the procedure described previously [22]. The suspending medium was then switched to a Tris · HCl buffer (33 mM Tris · HCl/10 mM $MgCl_2$/2 mM NaCN, pH 7.2, hereafter called the Tris buffer) for the present spin-label study. Dithiothreitol was omitted because of its reducing activity of nitroxide radicals and NaCN was added since it protected nitroxides from reduction by the membrane fraction.

Spin-labeling of membrane particulate fraction by enzymatic synthesis of phosphatidylglycerol

The reaction mixture contained 270 μM of *sn*-glycerol-3-phosphate, 25 μM of spin-labeled acyl CoA, 25 μM of palmitoyl CoA, 1 mM of CTP, and the membrane particulate fraction (2.3 mg of protein) in a final volume of 2 ml of the Tris buffer. The mixture was incubated at 17°C for 2 h under gentle shaking. For a control, addition of CTP and *sn*-glycerol-3-phosphate was omitted. After the incubation, the mixture was centrifuged at 20000 × *g* for 25 min at 2°C and the pellet was washed three times with 8 ml of the Tris buffer containing 0.5% bovine serum albumin additionally. The resulting pellet was finally washed with 8 ml of the Tris buffer without bovine serum albumin. The washed pellet was resuspended in 10—30 μl of the same buffer and applied to ESR measurement at 23°C with a commercial X-band spectrometer (JEOLCO Model ME-2X). Protein was determined according to Lowry et al. [23].

For investigation of effect of divalent cations on the membrane, the washed spin-labeled membrane was treated with 8 ml of 33 mM EDTA/Tris buffer (pH 7.3) containing 2 mM NaCN and centrifuged. The pellet was resuspended in the Tris buffer. After ESR measurement, the suspension was added with 8 ml of the Tris buffer containing indicated concentrations of $MgCl_2$ or $CaCl_2$ and centrifuged. The pellet was resuspended in the same buffer and applied to ESR measurement.

Lipid analysis of membrane particulate fraction

Total lipid was extracted from the membrane fraction by the method of

Bligh and Dyer [24]. The extract was developed on a Silica gel G plate with chloroform/methanol/ammonia (7 M) (60 : 35 : 5, by vol). The authentic phosphaditylglycerol, phosphatidic acid, phosphatidylethanolamine, and spin-labeled CoA were also developed for references. The spot for each lipid was scraped off and extracted with chloroform/methanol (2 : 1, v/v). The extract was redissolved in 50 μl of chloroform/methanol (2 : 1, v/v) and ESR spectrum was measured. Relative distribution of the spin label in various lipid fractions was determined from the signal intensity. In some experiments, *sn*-[U-^{14}C]-glycerol-3-phosphate was used as a substrate for the enzymatic synthesis. After extraction of phospholipids and separation by thin-layer chromatography, radioactivity of each fraction was measured as described previously [22].

Chemical synthesis of spin-labeled phosphatidylglycerol and its incorporation into total lipid bilayer membrane

Spin-labeled phosphatidylglycerol was chemically synthesized according to T. Maeda by exchange of the polar head group of spin-labeled phosphatidylcholine with glycerol under catalysis of phospholipase D. The method was similar to that used for non-labeled phosphatidylcholine [25]. Spin-labeled phosphatidylcholine was prepared from egg lecithin and 12-nitroxide stearic acid [18]. Phospholipase D was prepared as acetone powder from savoy cabbage [26]. The reaction mixture for the synthesis of spin-labeled phosphatidylglycerol contained 500 mg of phospholipase D dissolved in 16.5 ml of 0.5 M sodium acetate buffer (pH 5.6), 11 ml of glycerol, 50 mg of spin-labeled phosphatidylcholine dissolved in 7.5 ml of ether, and 0.5 ml of 1 M $CaCl_2$. The mixture was allowed to react for 2 h at 37°C under constant stirring in a stoppered flask and then extracted three times with water and applied to thin-layer chromatography on Silica gel G plate. After development with chloroform/methanol/ammonia (60 : 35 : 5, by vol), the plate was cut between the bands of phosphatidylcholine and phosphatidylglycerol. The plate containing phosphatidylglycerol was then developed with chloroform/methanol/acetic acid (65 : 25 : 8, by vol) and the band of phosphatidylglycerol was scraped off. Spin-labeled phosphatidylglycerol was extracted with chloroform/methanol (1 : 1, v/v), giving almost only one spot on thin layer chromatogram.

The spin-labeled phosphatidylglycerol and total lipid extract were dissolved in chloroform at a weight ratio of 1 : 22 and chloroform was evaporated to dryness under reduced pressure. The lipid was added with 33 mM Tris · HCl buffer and sonicated for 2 min in an ice bath.

Assay of phosphoenolpyruvate-dependent sugar uptake by memrbane particulate fraction

Phosphoenolpyruvate-dependent uptake of methyl-α-D-glucoside was assayed according to Kaback [27] with some modifications. Membrane particulate fraction was washed with 33 mM EDTA/Tris buffer (pH 7.3) containing 2 mM NaCN and suspended in the Tris buffer. Aliquots of the membrane suspension containing 0.44 mg of protein were diluted to a final volume of 100 μl including 36.3 mM Tris · HCl (pH 7.2), 2.2 mM NaCN, 0.3 M LiCl, 0.1 M phosphoenolpyruvate, and the indicated concentration of $MgCl_2$ or $CaCl_2$. After preincubation at 23°C for 15 min, 10 μl of 364 μM [^{14}C]methyl-α-D-glucoside was

added and the incubation was continued for 3 min. The sample was diluted with 4 ml of 0.5 M LiCl to terminate the reaction and filtered through Millipore filter HAWP 02500. The filter was dried and the radioactivity was counted according to Long and Dittmer [28].

Results

Incorporation of phosphatidylglycerol spin label into membrane by enzymatic synthesis

In order to confirm that spin-labeled acyl CoA can be a substrate for the enzymatic synthesis of phospholipids, the membrane particulate fraction was incubated with spin-labeled CoA, [^{14}C]glycerol-3-phosphate, and CTP at 37°C for 20 min. Spin-labeled CoA was pretreated with bovine serum albumin. After the incubation, phospholipids were extracted and developed on Silica gel plate as described in Materials and Methods. Radioactivity measurement showed that about 5% of the spin-labeled CoA was converted into phosphatidylglycerol. The spot for phosphatidic acid also showed some, but negligible, radioactivity.

In the enzymatic spin-labeling, palmitoyl CoA was also added as substrate and the reaction mixture was incubated at 17°C for 2 h. According to radioactivity measurement, phosphatidylglycerol produced during the incubation amounted to approx. 8% of total phosphatidylglycerol in the membrane. ESR measurement of lipid fractions extracted from the membrane showed incorporation of the nitroxide stearate into phosphatidylglycerol. The fraction due to spin-labeled acyl CoA also showed a weak signal but its intensity was several times smaller than that for phosphatidylglycerol. No signal was detected from phosphatidylethanolamine and phosphatidic acid fractions.

ESR spectrum of phosphatidylglycerol spin label in membrane

ESR spectrum of the membrane particulate fraction labeled by enzymatic synthesis of spin-labeled phosphatidylglycerol is shown in Fig. 1. The spectrum apparently consists of at least two components; one corresponding to labels largely immobilized (the overall splitting value, approx. 58 G) and the other due to those undergoing rapid anisotropic motions. As a control, the membrane was incubated in the absence of glycerol-3-phosphate and CTP. This membrane also gave an ESR spectrum as shown in Fig. 1, although the signal intensity was much weaker than that for the membrane containing spin-labeled phosphatidylglycerol. The lipid analysis of the control membrane indicated that the signal was mainly due to spin-labeled acyl CoA remaining after the washing. Further washing with bovine serum albumin did not decrease the signal appreciably.

In order to compare the results of enzymatic spin-labeling with those of exogeneous spin-labeling, the membrane particulate fraction was incubated with sonicated vesicles of chemically-synthesized phosphatidylglycerol spin label. However, the lipid analysis showed that most of the phospholipid spin labels were hydrolyzed to 12-nitroxide stearate. The hydrolysis may be due to phospholipase A present in *E. coli* membranes [29]. The ESR spectrum was therefore quite similar to that for the membrane labeled directly with the spin-labeled stearate.

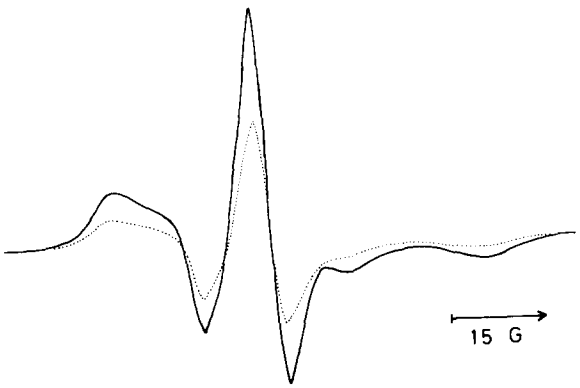


Fig. 1. ESR spectrum of *E. coli* membrane labeled by enzymatic synthesis of phosphatidylglycerol spin label. *E. coli* B membrane particulate fraction was incubated with *sn*-glycerol-3-phosphate, 12-nitroxide stearoyl CoA, palmitoyl CoA, and CTP as described in Materials and Methods. The membrane was washed three times with bovine serum albumin and once with Tris buffer. ESR spectrum was measured at 23°C. The dotted spectrum is that for a control membrane incubated in the absence of *sn*-glycerol-3-phosphate and CTP.

Effect of divalent cations on membrane

The enzymatically spin-labeled membrane was first treated with EDTA since the membrane preparation, the enzymatic synthesis, and the washing were always done in media containing $MgCl_2$. The EDTA treatment markedly affected the ESR spectrum of the membrane (see Fig. 2). The strongly immobilized component decreased while the mobile component increased. The spectral change was reversible; addition of Mg^{2+} restored the spectrum (compare the dotted spectrum in Fig. 2 with the solid line spectrum in Fig. 1). The spectral change was dependent on the Mg^{2+} concentration. To illustrate the dependence, ratio of the peak height at field positions a and b (see Fig. 2) was plotted against concentration in Fig. 3. The effect saturated at about 1 mM $MgCl_2$ and a half maximal effect was obtained at about 0.5 mM. Ca^{2+} gave essentially

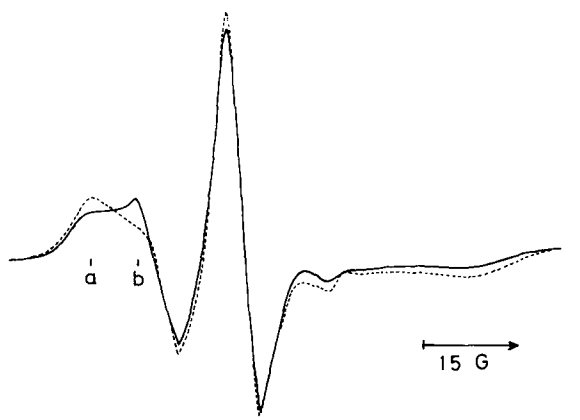


Fig. 2. Effect of EDTA-treatment and Mg^{2+}-retreatment on the ESR spectrum of *E. coli* membrane labeled by enzymatic synthesis of phosphatidylglycerol spin label. ———, The spin-labeled membrane treated with 33 mM EDTA/Tris buffer (pH 7.3); - - - - -, the EDTA-treated membrane added with 10 mM $MgCl_2$ in Tris buffer.

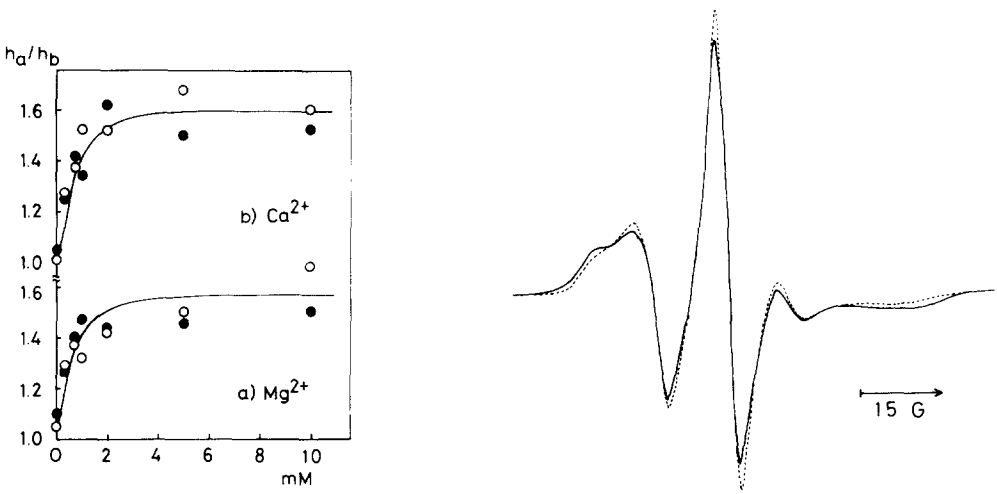


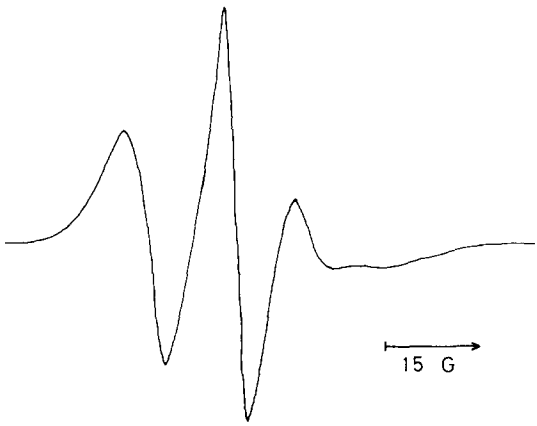
Fig. 3. Concentration dependence of the effect of Mg^{2+} (a) and Ca^{2+} (b) on the ESR spectrum of *E. coli* membrane labeled by enzymatic synthesis of phosphatidylglycerol. The spin-labeled membrane was first treated with 33 mM EDTA, washed, and then added with indicated concentration of the divalent cations. The peak height ratio at the field positions a and b as designated in Fig. 2 was plotted against the cation concentration. ●, Data obtained from spectra recorded at a higher magnification.

Fig. 4. ESR spectrum of *E. coli* membrane labeled with 12-nitroxide stearoyl CoA in the presence (———) and absence (- - - - - -) of Mg^{2+}. The membrane was incubated with 2.5 μM each of spin-labeled acyl CoA and palmitoyl CoA in the Tris buffer. The spin-labeled membrane was treated with 33 mM EDTA/Tris buffer (- - - - - -) and retreated with 10 mM $MgCl_2$ (———).

the same effect on the membrane. ESR spectrum of the spin-labeled membrane in the presence of Ca^{2+} was indistinguishable from that in the presence of Mg^{2+}. The concentration dependence of the peak height ratio is reproduced in Fig. 3, which demonstrates the close similarity between effects of Ca^{2+} and Mg^{2+}.

Effect of Mg^{2+} on *E. coli* membrane was investigated using spin-labeled acyl CoA as another probe. The membrane particulate fraction was incubated with 2.5 μM each of spin-labeled CoA and palmitoyl CoA at 17°C for 5 min. The spin-labeled membrane gave a two-component spectrum as shown in Fig. 4. The immobilized component had the overall splitting value of approx. 56 G; slightly but definitely smaller than that for phosphatidylglycerol spin label. Treatment of the membrane with EDTA increased the mobile component at the expense of the immobilized component (see Fig. 4). Addition of 10 mM $MgCl_2$ restored the spectrum. ESR spectrum of 12-nitroxide stearate incorporated into the membrane also consisted of two components. The overall splitting of the more immobilized component was approx. 51 G, much smaller than that for phosphatidylglycerol spin label in the membrane. Removal of Mg^{2+} by EDTA increased the mobile component at the expense of the immobilized component.

Finally, effect of the divalent cations on total lipid bilayer membrane was studied. ESR spectrum of chemically-synthesized phosphatidylglycerol spin label in the lipid membrane consisted almost of one component (see Fig. 5). This spectrum due to labels undergoing rapid anisotropic motions is similar to the mobile component observed for the *E. coli* membrane containing enzymatically-synthesized phosphatidylglycerol spin label. Mg^{2+} and Ca^{2+} did not give



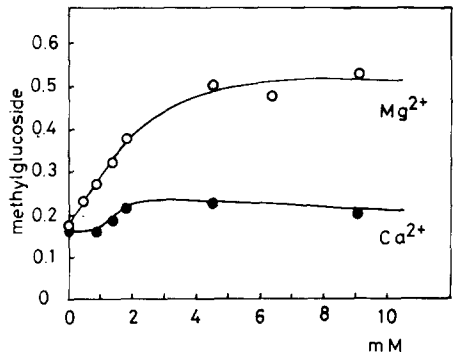


Fig. 5. ESR spectrum of chemically-synthesized phosphatidylglycerol spin label in total lipid bilayer membrane. The lipid mixture was sonicated in 33 mM Tris · HCl buffer.

Fig. 6. Effect of Mg^{2+} and Ca^{2+} on phosphoenolpyruvate-dependent uptake of methyl-α-D-glucoside in *E. coli* membrane. The uptake was assayed using radioactive substrate as described in Materials and Methods and plotted in units of nmol of methylglucoside per mg of protein against final concentration of divalent cations.

any appreciable effect on the lipid membrane. ESR spectrum of spin-labeled acyl CoA in total lipid bilayer membrane also consisted of one component, although the signal due to labels in aqueous phase was considerably large. Addition of Mg^{2+} did not affect appreciably the spectrum of the lipid phase labels.

Divalent cations and sugar uptake in membrane particulate fraction

Phosphoenolpyruvate-dependent uptake of methyl-α-D-glucoside was investigated as a function of concentration of divalent cations. The uptake was markedly enhanced by Mg^{2+} (see Fig. 6). The enhancement saturated at approx. 6 mM and a half maximal effect was obtained at approx. 1.4 mM. This effect was observably only when both phosphoenolpyruvate and Mg^{2+} were present. On the other hand, Ca^{2+} was not effective for the sugar uptake (see Fig. 6).

Discussion

E. coli membrane has been successfully labeled by enzymatic synthesis of a phospholipid spin label, phosphatidylglycerol spin label. Exogeneous spin-labeling was not possible for the membrane system because of degradation by inherent phospholipase A. In the enzymatic synthesis, we used both spin-labeled acyl CoA and palmitoyl CoA as substrate in the hope of producing spin-labeled phosphatidylglycerol, the major fraction of which contains one paramagnetic and one non-paramagnetic acyl chains in a molecule. When a mixture of *cis* vaccenoyl CoA and plamitoyl CoA was used as substrate, for example, 69% of phosphatidylglycerol produced had palmitoyl chain at C_1 and *cis* vaccenoyl chain at C_2 position [22]. The present spin-labeling method can be easily extended to label the membrane with a specific phospholipid spin label. The membrane can be labeled with phosphatidic acid by incubation with *sn*-glycerol-3-phosphate and spin-labeled acyl CoA in the absence of CTP.

Labeling with phosphatidylethanolamine spin label requires more steps. The membrane will be first labeled with phosphatidylserine spin label by incubation with spin-labeled CDP-diglyceride and serine in the presence of phosphatidylserine synthetase [17,30,31]. The membrane-bound spin label will then be converted to phosphatidylethanolamine spin label by catalytic action of phosphatidylserine decarboxylase. Study of *E. coli* membrane using these enzymatically-synthesized phospholipid spin labels is being carried out in this laboratory.

The ESR spectrum of phosphatidylglycerol spin label in *E. coli* membrane consisted at least of two components. The narrower component was similar to the spectrum of spin-labeled phosphatidylglycerol in total lipid vesicles and can be attributed to the labels located in lipid bilayer region of the membrane. The broader component due to immobilized labels can be related to the membrane proteins since no such component was detected in the spectrum of total lipid vesicles. Such component has been observed in various biological membranes labeled with fatty acid spin labels as well as phospholipid spin labels and assigned to lipids surrounding membrane integral proteins [2,32]. The lipid alkyl chains interact with hydrophobic surface of membrane proteins and their flexibility is greatly reduced.

The divalent cation Mg^{2+} and Ca^{2+} increased the immobilized component at the expense of the mobile component in *E. coli* membrane, while these ions gave no appreciable effect on the total lipid membrane. The results strongly suggest an increased interaction between phosphatidylglycerol and membrane proteins mediated by the divalent cations. The ions probably bridge the polar head group of phosphatidylglycerol and some ionizable amino acid residues of proteins and the lipid acyl chains become bound on the apolar surface of proteins. The interaction may not require strict specificity for the head group in view of similar effect obtained for spin-labeled acyl CoA and spin-labeled stearate.

Specific requirement of phosphatidylglycerol for phosphoenolpyruvate-dependent sugar transport in *E. coli* membrane has been indicated in a study by Milner and Kaback [33] using phospholipase D treatment, although Long and Dittmer [28] obtained a contradictory result using the same technique. Kundig and Roseman [34,35] have studied reconstitution of *E. coli* phosphotransferase activity and demonstrated an essential role of phosphatidylglycerol and Mg^{2+} or Ca^{2+} for reconstitution of enzyme II-B, an integral protein. The reconstituted pellet was active for phosphorylation of sugars when II-A and the phospho-HPr generating system was added. The major phospholipid, phosphatidylethanolamine exhibited no activity. Phosphatidylserine and cardiolipin showed only slight activity and phosphatidic acid no activity. The present finding may be pertinent to the interaction of phosphatidylglycerol with II-B mediated by Mg^{2+}. Ca^{2+} was also effective for binding of phosphatidylglycerol to proteins. The difference between Mg^{2+} and Ca^{2+} in the enhancement of sugar uptake may suggest that Ca^{2+} was active for the lipid · protein complex formation, whereas Mg^{2+} had not only the structural role but also some catalytic role. Or, the complex formed by Ca^{2+}-bridging may not be functionally potent.

Milner and Kaback [33] reported that proline uptake in *E. coli* was only slightly affected by phospholipase D treatment, which decreased the amount of

phosphatidylglycerol. Shechter et al. [36] studied temperature dependence of proline uptake and phosphoenolpyruvate-dependent sugar uptake in *E. coli* using an unsaturated fatty acid auxotroph. These authors observed a correlation between structural transition in lipids and proline uptake but no such correlation for the sugar uptake. The results are consistent with an idea that the thermal characteristics of the membrane is governed by the major phospholipid, while phosphatidylglycerol acts as an functional lipid. The present results suggest that phosphatidylglycerol molecules bind to the integral protein(s) bridged by divalent cations and the lipoprotein complex may be the active component for the phosphotransferase system.

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