Biochimica et Biophysica Acta, 646 (1981) 119–125
Elsevier/North-Holland Biomedical Press

BBA 79346

DYNAMIC STATES OF PHOSPHOLIPIDS IN *ESCHERICHIA COLI* B MEMBRANE

ELECTRON SPIN RESONANCE STUDIES WITH BIOSYNTHETICALLY GENERATED PHOSPHOLIPID SPIN LABELS

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(Received February 17th, 1981)

Key words: Phospholipid membrane; Dynamic state; Spin label; ESR; (E. coli B)

Mobility of phospholipid hydrocarbons in the *Escherichia coli* B membrane fractions was studied by labeling phosphatidylethanolamine or phosphatidylglycerol in situ by biosynthetic incorporation of the spin label. For this purpose, CDP-diacylglycerol spin label was synthesized from phosphatidic acid spin label and cytidine 5′-phosphoromorpholidate and purified by thin-layer chromatography. CDP-diacylglycerol spin label was then incorporated into phospholipids biosynthetically. ESR spectra of these *E. coli* B membrane fractions showed that phosphatidylglycerol tended to interact with membrane proteins through the mediation of Mg^{2+}, whereas phosphatidylethanolamine had less of this tendency and was more involved in the formation of the bulk of the bilayer continuum of the membrane. These conclusions were also supported by labeling membranes with exogenous spin-labeled phospholipids, although there was some indication that exogenous phospholipids were incorporated into sites different from the sites of incorporation of phospholipids newly synthesized in situ.

Introduction

The spin-labeling method has significantly contributed to the understanding of the dynamic states of biological and model membranes [1,2]. One of the common methods of spin-labeling the membranes is to incubate membranes with sonicated vesicles of spin-labeled phospholipid. Some groups of researchers, on the other hand, spin-labeled biological membranes biosynthetically [3–6]. The latter method may have an advantage for studying biological membranes in their native state [6].

Escherichia coli has only two major classes of phospholipids in its membrane, phosphatidylethanolamine and phosphatidylglycerol. The pathway of phospholipid biosynthesis in the membrane has been studied extensively [7,8]. The acyl portion of acyl-CoA or acyl-carrier protein is incorporated into *sn*-glycerol 3-phosphate, forming phosphatidic acid. Phosphatidic acid reacts with cytidine triphosphate and the formation of CDP-diacylglycerol results. In the presence of L-serine, CDP-diacylglycerol is converted to phosphatidylethanolamine through phosphatidylserine by a system of membrane enzymes. In the presence of *sn*-glycerol-3-phosphate, CDP-diacylglycerol is converted to phosphatidylglycerol through phosphatidylglycerol phosphate [7,8].

Previously, we reported the enzymatic conversion of 12-nitroxide stearoyl-CoA to phosphatidylglycerol by *E. coli* B membrane fractions, and the data suggested strong interaction of phosphatidylglycerol with proteins in *E. coli* membrane, mediated by

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Trivial name: CUTSCUM, diisobutylphenoxypolyethoxyethanol.

0005-2736/81/0000–0000/$02.50

divalent cations, Mg^{2+} or Ca^{2+} [6]. In the present paper, we report the enzymatic conversion of spin-labeled CDP-diacylglycerol to phosphatidylethanolamine and phosphatidylglycerol by *E. coli* B membrane fractions, and describe the dynamic states of these two major phospholipids. These results will be compared with the results of exogenous spin-labeling of *E. coli* B membrane.

Materials and Methods

Chemicals. sn-Glycerol 3-phosphate dicyclohexylammonium salt was the product of Boehringer Mannheim. Cytidine 5′-monophosphate and morpholine were purchased from Kohjin Biochemicals and Nakarai Chemicals, respectively. L-[3-^{14}C]Serine and *sn*-[U-^{14}C]glycerol 3-phosphate were obtained from New England Nuclear. Silica gel G thin-layer plate (Art. 5721) was the product of Merck. Nonionic detergent CUTSCUM was obtained from Fisher Scientific Company (New Jersey).

Synthesis and purification of spin-labeled CDP-diacylglycerol. Spin-labeled phosphatidylcholine was prepared by the method of Hubbell and McConnell [9]. Spin-labeled phosphatidic acid was derived from spin-labeled phosphatidylcholine by the action of phospholipase D [10]. Cytidine 5′-phosphoromorpholidate was prepared from cytidine 5′-monophosphate and morpholine [11]. Synthesis of CDP-diacylglycerol spin label from spin-labeled phosphatidic acid and cytidine 5′-phosphoromorpholidate and its partial purification was by the method of Agranoff and Suomi [11]. Partially purified CDP-diacylglycerol spin-label was dissolved in chloroform/methanol (1 : 1, v/v) and applied to sequential preparative thin-layer chromatography. The sample was first developed with chloroform/methanol/7 M ammonium hydroxide (60 : 35 : 5, v/v). The ultraviolet-absorbing band was scraped off and extracted five times with 60 ml chloroform/methanol (1 : 1, v/v) for 15 min at 45°C. The combined extract was concentrated under reduced pressure and applied to the second thin-layer plate, which was developed with diisobutylketone/acetic acid/water (40 : 30 : 7, v/v). The ultraviolet-absorbing band was extracted in a similar way. CDP-diacylglycerol thus obtained showed a single spot on thin-layer chromatography. The yield, based on phosphorus analysis [12], was 11% of the amount of spin-labeled phosphatidylcholine. The micellar solution of 1.6 mM CDP-diacylglycerol spin label was adjusted to pH 7.5 with 2 M ammonium hydroxide and stored at −20°C.

Preparations of membrane fractions. Growth of *E. coli* B and the preparation of its membrane fractions were carried out as described previously [6,13].

Partial purification of phosphatidylserine synthetase. Soluble phosphatidylserine synthetase was purified up to the stage of the treatment with high concentration of $MgCl_2$ by the method reported previously [14]. The supernatant, after centrifugation, was used for spin-labeling experiments.

Labeling of E. coli B membrane fractions by endogenous synthesis of spin-labeled phospholipids. For the synthesis of spin-labeled phosphatidylethanolamine, membrane fractions containing 2 mg protein were washed with 4 ml 33 mM EDTA/33 mM potassium phosphate buffer (pH 7.0). After centrifugation at 20 000 × *g* for 25 min at 2°C, the pellet was suspended in 0.2 ml 33 mM potassium phosphate buffer (pH 7.1) containing 2 mM NaCN. NaCN was previously reported by us to protect spin-labeled compounds from the reducing activity of membrane fractions [6]. 20 nanomol spin-labeled CDP-diacylglycerol were added to the suspension. The complex between membrane fractions and CDP-diacylglycerol was formed within 5 min at room temperature, because, after this time, all of the spin-labels were recovered in the membrane pellet on centrifugation of the mixture. The reaction mixture contained 200 μM L-serine, 2 ml phosphatidylserine synthetase solution, 33 mM potassium phosphate buffer, 2 mM NaCN and the membrane · CDP-diacylglycerol complex in a final volume of 6 ml.

For the synthesis of spin-labeled phosphatidylglycerol, 33 mM Tris-HCl buffer (pH 7.3) was used in place of potassium phosphate buffer in order to obtain some better conversion (Ishinaga, M., Nishihara, M. and Kito, M., unpublished data). The reaction mixture contained 120 μM *sn*-glycerol 3-phosphate, 0.5 ml phosphatidylserine synthetase solution, which had some activity of phosphatidylglycerol phosphate synthetase, 15 mM $MgCl_2$, buffer and the membrane · CDP-diacylglycerol complex in a final volume of 6 ml. As a control, membrane · CDP-diacylglycerol complex was incubated in phosphate

buffer containing only 15 mM $MgCl_2$ and 2 mM NaCN.

After incubation for 2 h at 17°C with gentle shaking, each sample was centrifuged. The pellet was suspended in 30 μl of a buffer (33 mM potassium phosphate/10 mM $MgCl_2$/2 mM NaCN, pH 7.1, hereafter called buffer A). The suspension was treated with 20 mg charcoal in 8 ml buffer A for 15 min at room temperature to remove unreacted CDP-diacylglycerol. Charcoal was removed by a low-speed centrifugation. After the recovery of the membrane fractions by high speed centrifugation, charcoal treatment of membrane suspension was repeated twice more. The suspension of the pellet in 30 μl buffer A was used for ESR measurements.

In order to study the effect of Mg^{2+}, membrane fractions were treated with 8 ml 33 mM EDTA/33 mM potassium phosphate buffer (pH 7.0) containing 2 mM NaCN, hereafter called buffer B, for 5 min at room temperature and centrifuged. The pellet was suspended in 30 μl of the same buffer. After ESR measurements, each suspension was retreated with 8 ml buffer A and centrifuged. Each pellet was resuspended in 20 μl buffer A and was used for ESR measurement.

Chemical synthesis of spin-labeled phospholipids. Spin-labeled phosphatidylethanolamine and phosphatidylglycerol were prepared from phosphatidylcholine spin label by the action of phospholipase D in the presence of ethanolamine and glycerol, respectively [6,15].

Labeling of E. coli B membrane fractions with exogenous phospholipid spin-labels. A 1 : 1 mixture of phosphatidylethanolamine spin label and total lipid (total weight, 30 μg) was sonicated for 2 min at 0°C in 0.2 ml buffer B. Membrane fractions of *E. coli* B containing 2 mg proteins, which were treated beforehand with buffer B, were sonicated for 30 s at 0°C with the mixture of phosphatidylethanolamine spin label and total lipids in 0.3 ml of the same buffer. EDTA treatment was reported to inhibit phospholipase A_2 activity of the outer membrane [16]. The addition of 1.5 ml buffer B was followed by a centrifugation. The pellet was washed with buffer A and applied to ESR measurement. For studying the effect of Mg^{2+}, membrane fractions were treated with buffer B for 5 min at room temperature and retreated with buffer A as described above.

In the case of spin-labeling of membrane fractions with phosphatidylglycerol label, EDTA-pretreated membrane fractions containing 4 mg proteins were sonicated with 10 μg of 1 : 1 mixture of the label and total lipids.

Labeling of total lipids with phospholipid spin labels. Total lipids were extracted from *E. coli* B membrane fractions by the method of Bligh and Dyer [17].

About 300 μg total lipids and 5 μg phosphatidylethanolamine or phosphatidylglycerol spin label were mixed in buffer A or buffer B. After the sonication for 15 s at 0°C, samples were centrifuged at $1\,000 \times g$ for 5 min. Each pellet was suspended in 40 μl supernatant and ESR studies were carried out.

ESR measurements and other methods. All ESR measurements were done at 23°C with a commercial X-band spectrophotometer (JEOLCO Model ME-2X). Protein determination was by the method of Lowry et al. [18]. Phospholipid was determined by the method of Bartlett [12].

Results

Substrate activity of spin-labeled CDP-diacylglycerol for enzymatic synthesis of spin-labeled phosphatidylethanolamine and phosphatidylglycerol. To confirm the conversion of spin-labeled CDP-diacylglycerol to phosphatidylethanolamine and phosphatidylglycerol, membrane fractions containing 740 μg proteins were incubated with 0.1 μmol CDP-diacylglycerol label in the presence of 15 mM $MgCl_2$, 0.1% CUTSCUM and 0.2 mM L-[3-^{14}C]serine or 0.11 mM *sn*-[U-^{14}C]glycerol 3-phosphate in a final volume of 0.3 ml 33 mM potassium phosphate buffer (pH 7.2). After 10 min at 37°C, 10 ml chloroform/methanol (2 : 1, v/v) and 100 μg of phosphatidylethanolamine or phosphatidylglycerol were added. Chloroform layers, obtained after washing with 20 ml 2 M KCl and water, were dried with gaseous nitrogen. Phospholipids were developed on silica gel G plate with chloroform/methanol/acetic acid/water (65 : 25 : 5 : 1, v/v) and loacted with iodine vapor. Radioactivities of each phospholipid fraction showed the synthesis of phosphatidylethanolamine, 0.94 and 0.054 nmol in the presence and absence of CUTSCUM, respectively. Synthesis of phosphatidylglycerol was also calculated as 1.02 and 0.104 nmol

in the same manner. The stimulated conversion of spin-labeled CDP-diacylglycerol to phospholipids in the presence of the detergent CUTSCUM, as with phospholipid synthesis in *Bacillus* sp. [19], might be explained by the solubilization or disruption of the intact membrane structure and the modification by the detergent of the physical state of the substrate, supporting the close interaction between the substrate molecule and the corresponding enzyme(s).

Biosynthetic spin-labeling of phospholipids in E. coli B membrane fractions from spin-labeled CDP-diacylglycerol. ESR spectra of membrane fractions after spin-labeled phospholipid syntheses in the condition without CUTSCUM (see Materials and Methods) are shown in Fig. 1. All these spectra appeared to have at least two components, a strongly immobilized (peak a) and a mobile component (peak b). The spectrum of membrane fractions which had synthesized phosphatidylglycerol spin label (Fig. 1, solid line) was richer in the immobilized component (peak a) than the spectra of the membrane containing primarily phosphatidylethanolamine spin-label. This result suggests that phosphatidylglycerol has a stronger tendency to become immobilized, presumably through its interaction with membrane proteins.

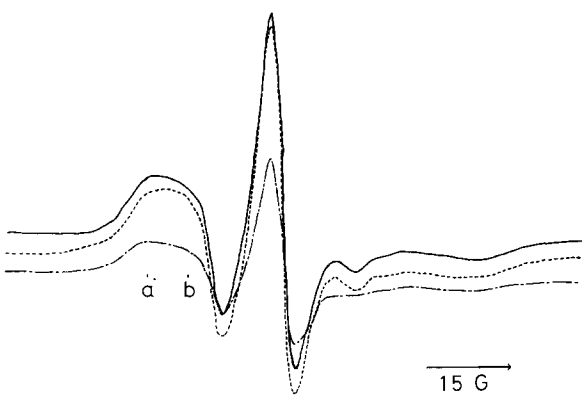

Fig. 1. ESR spectra of membrane fractions that synthesized phospholipids from CDP-diacylglycerol spin label. *E. coli* B membrane fractions were incubated with CDP-diacylglycerol spin label for 2 h at 17°C in the presence of L-serine or *sn*-glycerol-3-phosphate. After charcoal treatment, ESR spectra of membrane fractions were measured in the presence of 10 mM $MgCl_2$. As a control, membrane fractions which had been incubated with CDP-diacylglycerol only were also applied to ESR measurement. ———, membrane fractions that had synthesized phosphatidylglycerol; - - - - -, membrane fractions that had synthesized phosphatidylethanolamine; · — · — ·, control membrane fractions. The ratio of spectral intensities in these three preparations was about 2 : 2 : 1. Peaks a and b are those of immobilized component and mobile component of the spectrum, respectively. The positions of these peaks were at magnetic fields lower from the top of central peak by 21 and 14 G, respectively.

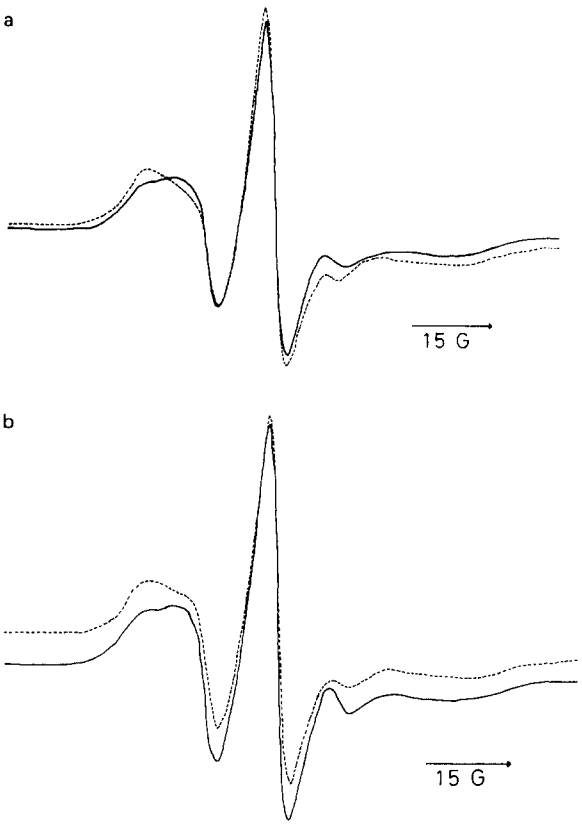

Fig. 2. EDTA treatment and Mg^{2+} retreatment of membrane fractions containing endogenously synthesized phospholipid spin-label. Membrane fractions that had synthesized phosphatidylglycerol (a) and membrane fractions that had synthesized phosphatidylethanolamine (b) in Fig. 1 were treated with EDTA and retreated with Mg^{2+}. ESR spectra were measured after each step. ———, EDTA treatment; - - - - - -, Mg^{2+} retreatment.

Since we have previously noted the Mg^{2+}-dependent interaction of phosphatidylglycerol with proteins in *E. coli* B membrane [6], we now compare the effect of Mg^{2+} on phosphatidylethanolamine and phosphatidylglycerol. Fig. 2a shows the spectra of membrane fractions which had synthesized phosphatidylglycerol after EDTA treatment (solid line)

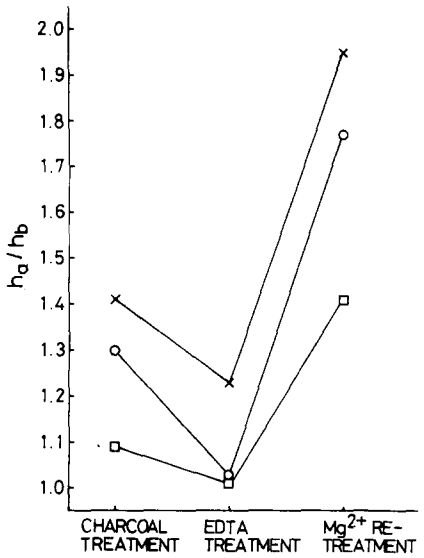

Fig. 3. Effect of Mg^{2+} on peak-height ratio h_a/h_b. From heights of peaks a and b (see Fig. 1) of each spectrum (Figs. 1 and 2), ratios of h_a/h_b after each treatment were plotted. The h_a/h_b values from the control experiment were calculated similarly from ESR spectra, which are not shown. ○, membrane fractions that had synthesized phosphatidylglycerol; □, membrane fractions that had synthesized phosphatidylethanolamine; X, membrane fractions for control experiments.

and following Mg^{2+} retreatment (dotted line). These spectra show that the removal of Mg^{2+} from membranes reduces the immobilized component. In Fig. 2b, ESR spectra obtained after the same treatment of membrane fractions, which had synthesized phosphatidylethanolamine, are shown. From these spectra, the effect of Mg^{2+} on membrane phosphatidylethanolamine appears to be qualitatively similar to that on phosphatidylglycerol.

As a quantitative measure of the spectral change, we took the peak-height ratio h_a/h_b for each spectrum (h_a and h_b: heights of peak a and peak b, respectively, measured from the baseline as noted in Fig. 1). As shown in Fig. 3, the membrane fractions containing endogenously synthesized phosphatidylglycerol spin label not only possessed greater h_a/h_b values, but also were affected more by the EDTA treatment and the Mg^{2+} retreatment than membrane fractions containing phosphatidylethanolamine spin-label. Since the ratio h_a/h_b reflects the relative amount of immobilized component, these results suggest that phosphatidylglycerol interacts more strongly with proteins by the mediation of Mg^{2+} than does phosphatidylethanolamine.

In Fig. 3, we note that the membranes from the control incubation of Fig. 1 also responded strongly to treatments with EDTA and Mg^{2+}. This observation probably reflects the interaction of negatively charged CDP-diacylglycerol, which should be the major spin-labeled compound in the membranes, with membrane proteins. Since the membrane fractions, which had synthesized phosphatidylethanolamine spin label, still contained substantial amounts of CDP-diacylglycerol, their response to EDTA and Mg^{2+} (Fig. 3) most likely reflects the responses of remaining CDP-diacylglycerol molecules. A part of the spectral change of membrane fractions, which had synthesized phosphatidylglycerol spin label, would be also explained in the same manner.

Mg^{2+} retreatment on each sample produced higher values of h_a/h_b than before EDTA treatment (Fig. 3). The reason for this phenomenon is not clear, but there is a possibility of redistribution of membrane components induced by EDTA treatment and/or Mg^{2+} retreatment.

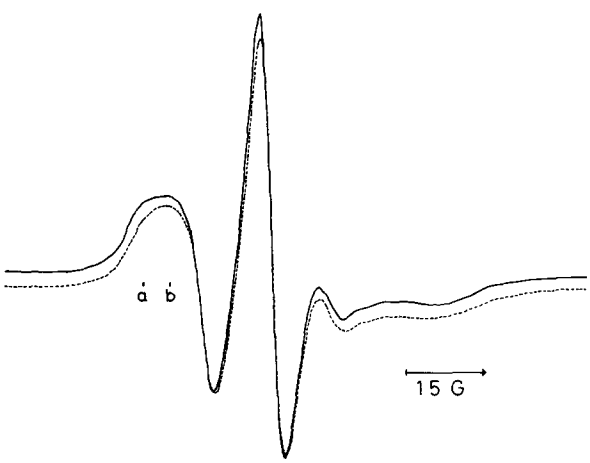

Fig. 4. Labeling of membrane fractions with exogenous phospholipid spin labels. *E. coli* B membrane fractions were sonicated with a 1 : 1 mixture of total lipids and phospholipid spin label in buffer B. After membrane fractions were washed with buffer A, their ESR spectra were measured. ———, phosphatidylglycerol spin-labeling, ------, phosphatidylethanolamine spin-labeling. The positions of peaks a and b should be noted as the immobilized and the mobile component, respectively.

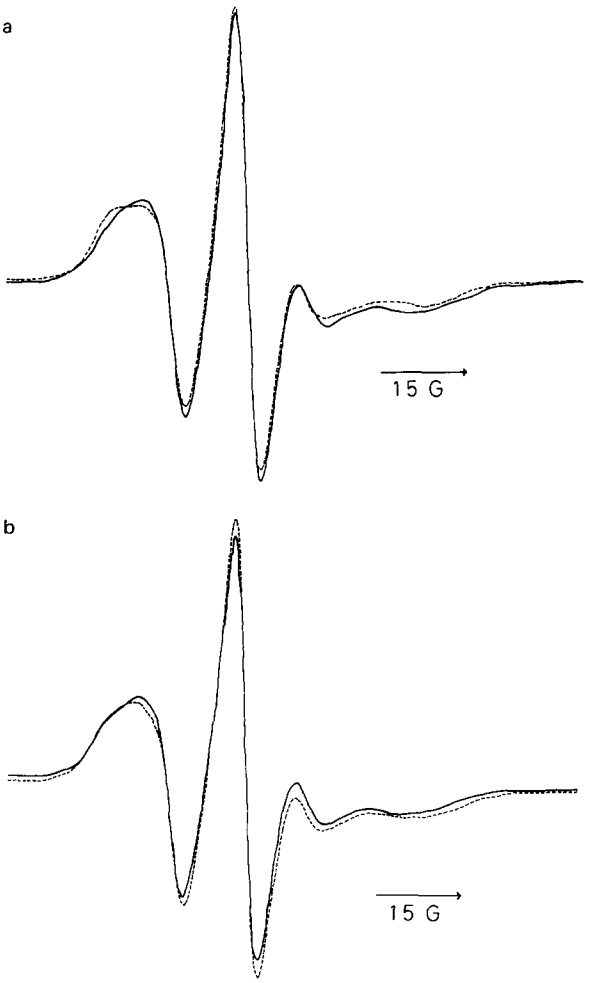

Fig. 5. EDTA treatment and Mg^{2+} retreatment of membrane fractions exogenously spin-labeled with phospholipid labels. *E. coli* B membrane fractions exogenously spin-labeled with phosphatidylglycerol (a) and phosphatidylethanolamine (b) labels in Fig. 4 were treated with EDTA and retreated with Mg^{2+}. ESR spectra were measured in each step. ———, EDTA treatment; - - - - - -, Mg^{2+} retreatment.

Labeling of membrane fractions with exogenous phospholipid spin labels. For comparison with the results of biosynthetic spin-labeling, *E. coli* B membrane fractions were spin-labeled exogenously with chemically synthesized phospholipid labels as described in Materials and Methods. Fig. 4 shows the ESR spectra of membrane fractions labeled with phosphatidylglycerol (solid line) and phosphatidylethanolamine (dotted line) spin labels. Both spectra are composed of more than two components, but generally show higher mobilities of labeled compounds than in the case of biosynthetically incorporated spin labels.

The exogenously labeled membranes were treated with EDTA and then Mg^{2+} (Figs. 5a and b). Clearly the detachment of Mg^{2+} from membrane fractions led to the decrease of immobilized fraction (peak a noted in Fig. 4) for both labeled compounds. However, the addition of Mg^{2+} produced a more prominent increase in the immobilized fraction of phosphatidylglycerol (Figs. 4 and 5a) than that of phosphatidylethanolamine (Figs. 4 and 5b). These results again suggest that the immobilization of hydrocarbon chains of phosphatidylglycerol in membrane fractions is dependent on the presence of Mg^{2+}, whereas the effect of Mg^{2+} is less pronounced with phosphatidylethanolamine.

Spin-labeling of total lipids. Finally, bilayers made of total lipids of *E. coli* B were spin-labeled by the addition of exogenous phospholipid spin labels. Both phosphatidylethanolamine and phosphatidylglycerol spin labels were in rapid anisotropic motions in this environment and the mobility of the former lipid was found to be slightly more restricted (overall splitting, 42 G vs. 40 G), contrary to the results from studies on membrane fractions. The addition of EDTA and Mg^{2+} did not produce any noticeable changes in ESR spectra (data not shown).

Discussion

As described in the Introduction, we previously reported the enzymatic synthesis of spin-labeled phosphatidylglycerol from 12-nitroxide stearoyl-CoA by *E. coli* B membrane fractions, and these membranes biosynthetically labeled in situ were analyzed by the ESR approach. The results suggested a Mg^{2+}-mediated interaction of phosphatidylglycerol with membrane proteins [6].

As a next step, we wanted to investigate the dynamic state of the other class of phospholipid, phosphatidylethanolamine, in *E. coli* membrane, again by using the technique of biosynthetic incorporation of spin label. However, the exclusive synthesis of phosphatidylethanolamine from acyl-CoA was difficult, because the addition of *sn*-glycerol 3-phosphate to the reaction mixture,

necessary for phosphatidic acid synthesis, inevitably led to the synthesis of phosphatidylglycerol [7,8]. In order to avoid this difficulty, we prepared CDP-diacylglycerol spin label chemically and used it as a substrate for the biosynthesis of phospholipid spin labels in *E. coli* membrane.

Some difficulty was encountered in our attempt to remove the remaining CDP-diacylglycerol label from the membrane fractions. The use of bovine serum albumin, which could successfully remove 12-nitroxide stearoyl-CoA [6], was not so effective. Charcoal treatment produced better results, but CDP-diacylglycerol was not eliminated completely from membrane fractions, presumably because it had a high affinity to membrane bilayer owing to the presence of two hydrophobic acyl chains.

In spite of this difficulty, it is clear that the endogeneously synthesized phosphatidylglycerol spin label tended to be immobilized more than the phosphatidylethanolamine spin label, and presumably this immobilization occurs because of the interaction of phosphatidylglycerol with intrinsic membrane proteins. These results agree with studies which reported a positive role of phosphatidylglycerol in the functions of membrane proteins in *E. coli* [20–22]. The phosphatidylethanolamine molecules in the membrane thus have more tendency to form the bulk of the bilayer continuum. From the experiments with Mg^{2+} and EDTA, it seems that the interaction between phosphatidylglycerol and proteins is mediated by divalent cations. These conclusions have been substantiated also by experiments using chemically synthesized phospholipid spin labels added exogenously.

In spite of this general similarity in the behavior of endogenously generated and exogenously added phospholipid spin-labels, in some aspects they showed distinctly different behavior. For example, the degree of immobilization was definitely greater for endogenously produced phospholipid labels (compare Figs. 1 and 4). The reason for the difference is not clear, but it is possible that the regions into which labeled compounds are incorporated differ depending on the labeling method. For example, incorporation may occur into only one side or both sides of bilayer or into the region distant from or near to membrane proteins. In any case, this observation indicates that the endogenous labeling method may be useful for probing the details of membrane structure and also that subtle local differences may exist in the membrane fluidity and discussions of 'average' or 'bulk' fluidity do not mean much in real biological membranes.

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