Rapid Transmembrane Movement of Newly Synthesized Phosphatidylethanolamine across the Inner Membrane of Escherichia coli

Richard P. H. Huijbregts‡, Anton I. P. M. de Kroon, and Ben de Kruijff

From the Department of Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, NL-3584-CH Utrecht, The Netherlands

For the first time the transmembrane movement of an endogenously synthesized phospholipid across the inner membrane of E. coli is reported. [14C]phosphatidylethanolamine (PE) was biosynthetically introduced into inner membrane vesicles from the PE-deficient strain AD93, by reconstitution with the enzyme phosphatidylserine (PS) synthetase. Upon addition of wild type cell lysate containing PS synthetase, and the metabolic substrates CTP and [14C]serine to inside-out vesicles from AD93, [14C]PS was synthesized, which was for the most part converted into [14C]PE. [14C]PE was introduced in right-side out vesicles by enclosing PS synthetase and CTP in the vesicle lumen and adding [14C]serine. The newly synthesized [14C]PE immediately equilibrated over both membrane leaflets (t1/2 less than one min), as determined by its accessibility toward the amino-reactive chemical fluorescamine. In both inside-out and right-side out vesicles, a 35–65% distribution was found of the newly synthesized PE over the cytoplasmic and periplasmic leaflet, respectively. The transport process of PE was not influenced by the presence of ATP or the proton motive force in inside out vesicles. Pretreatment of both types of vesicles with sulfhydryl reagents, or of right-side out vesicles with proteinase K, did not affect the rate and extent of the transmembrane distribution of the newly synthesized PE.

Phospholipids are the major lipid component of the cell envelope of the Gram-negative bacterium Escherichia coli. Phospholipids are the only lipid constituent in the inner membrane and they build the inner leaflet of the outer membrane. The lipid matrix of the extracellular leaflet of the outer membrane consists of lipopolysaccharide (1, 2). The phospholipid content of the E. coli envelope comprises 75% phosphatidylethanolamine (PE),1 which is a zwitterionic phospholipid, 20% phosphatidylglycerol, and 5% cardiolipin, both anionic phospholipids (3). Phospholipids are synthesized at the cytosolic side of the inner membrane (4). Therefore, in growing cells trans- and intermembrane transport of phospholipids must occur from the endofacial leaflet of the inner membrane to the other destinations in the expanding envelope.

In this study the transmembrane movement of PE across the inner membrane of E. coli was investigated. Besides being the most abundant phospholipid in E. coli, PE has several specific functions in the membrane, recently reviewed by Dowhan (5). PE as a non-bilayer-prefering lipid is essential for the polymorphic regulation of the membrane lipid organization in E. coli (6). It also stimulates several membrane transport systems (7, 8) and is involved as a chaperone in the correct folding of the lac permease in the inner membrane of E. coli (9, 10).

A study done by Donohue-Rolfe and Schaechter (11) demonstrated transport of phospholipids from the inner to the outer membrane of E. coli in vivo, using pulse labeling of phospholipids followed by subfractionation of the cell envelope. PE appeared in the outer envelope with a t1/2 of 2.8 min at 37 °C, whereas phosphatidylglycerol and cardiolipin were transported with half-times shorter than 30 s. Transport of the phospholipids was severely impaired when the proton motive force (pmf) was absent, whereas inhibitors of ATP, protein, or lipid synthesis did not have an effect on the process. Similar pulse labeling studies were performed in the Gram-positive bacterium Bacillus megaterium (12, 13). Rapid transmembrane movement (t1/2 of 3 min at 37 °C) of newly synthesized PE across the plasma membrane was demonstrated that was completely independent of the synthesis of lipid or protein and also independent of sources of metabolic energy.

To investigate the mechanisms of phospholipid transport in more detail, in vitro systems have been used. Recently, transmembrane movement of short chain NBD-labeled phospholipid analogs was characterized in vitro in inner membrane vesicles from the above two prokaryotes. In right-side out vesicles from B. megaterium an inward movement of the analogs was observed after they had been incorporated in the outer leaflet of the membrane (14). In inside-out inner membrane vesicles isolated from E. coli, transmembrane movement of the fluorescent phospholipids was detected after their incorporation in the cytoplasmic leaflet of this membrane (15). Both prokaryotic in vitro systems have in common that the process of transmembrane movement is not or only slightly phospholipid head-group specific, and in both systems transmembrane equilibration is fast with half-times of about 30 s and 7 min at 37 °C in the inner membrane vesicles of B. megaterium and E. coli, respectively. The mechanism of the phospholipid transmembrane movement as well as the suggested involvement of a flipase (14, 15) remain to be elucidated.

In the present work, transmembrane transport of PE in E. coli inner membrane vesicles is investigated under conditions more closely mimicking the in vivo situation. For this purpose an in vitro system was devised in which the transmembrane movement of endogenously synthesized, radiolabeled PE could be monitored using the amino-reactive probe fluorescamine. This chemical reacts very rapidly with amino groups at slightly

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 31-30-253-2465; Fax: 31-30-252-2478; E-mail: rhuijbregts@chem.uu.nl.

1 The abbreviations used are: PE, phosphatidylethanolamine; PS, phosphatidylserine; pmf, proton motive force; ISO, inside out; RSO, right-side out; Me2SO, dimethyl sulfoxide; HPTLC, high performance thin layer chromatography; FL-PE, fluorescamine product of PE; NBD, 7-nitrobenz-2-oxa-1,3-diazol.
alkaline pH (16) and has been successfully used in determining the transmembrane distribution of PE in several organelles and plasma membranes (17–20). Due to the high amount of PE present in the inner membrane of wild type E. coli, steric interference of the fluorescamine derivative of PE is likely to prevent the reaction of the newly synthesized radiolabeled PE with fluorescamine from going to completion (21). This problem was circumvented by biosynthetically introducing a small amount of PE into inner membrane vesicles of the E. coli strain AD93, which are devoid of PE because of a defect in the enzyme phosphatidylserine synthetase (22). Addition of a cytosolic lysate from wild type cells containing phosphatidylserine synthetase (23), together with the appropriate substrates restores the synthesis of phosphatidylserine (PS) in inner membrane vesicles from AD93. Most of the PS synthesized is subsequently converted into PE by the membrane-bound enzyme PS decarboxylase. An additional advantage of using strain AD93 is that its inner membrane contains a substantial amount of phosphatidic acid (22), which conveniently serves as a precursor for the synthesis of PS in the reconstituted in vitro system.

The synthesis and subsequent transmembrane movement were measured in both inside-out and right-side-out vesicles. The outer leaflet of the inside out vesicles corresponds to the cytosolic side of the inner membrane, which is the site of PE synthesis. The transmembrane movement was measured as the sequestration of the newly synthesized PE molecules from the reaction with fluorescamine. In right-side out vesicles, synthesis of PE was generated in the lumen of the vesicle, and transmembrane movement was monitored by detecting the appearance of PE in the outer leaflet. Both approaches showed that the newly synthesized PE equilibrates across the membrane with a half-time of less than 1 min. In both inside-out and right-side-out vesicles, the newly synthesized PE distributes in a 35 to 65 ratio over the cytoplasmic and periplasmic leaflet, respectively.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**l-(3-3H)Serine was obtained from Amersham Pharmacia Biotech. Fluorescamine, dithiothreitol, N-ethylmaleimide and octyl glucoside were purchased from Sigma. Iodoacetamide was obtained from Fluka. Proteinase K was purchased from Boehringer Mannheim. All other chemicals used were analytical grade.

**Isolation of Inner Membrane Vesicles—**The E. coli mutant strain AD93 (pss93::kan recA srl1) (22) was used for the isolation of inner membrane vesicles. This strain contains no PE and needs divergent cations for growth. Therefore, cells were grown in LB medium supplemented with 50 mM MgCl2.

Cells were harvested in the late log phase, and inside out (ISO) vesicles were isolated as described by Huijbregts et al. (15) with the exception that the cells were lysed in buffer containing in addition 50 mM MgCl2. ISO vesicles were stored in buffer containing 50 mM triethanolamine-HAc, pH 7.5, 250 mM sucrose. Small aliquots of the vesicle suspension were frozen in liquid nitrogen and stored at −80 °C. The protein-phospholipid ratio of the ISO vesicles was between 3 and 6 μg of protein per nmol of phospholipid.

For the isolation of right-side out (RSO) vesicles, cells were harvested in the late log phase and washed with 10 mM Tris-HCl, pH 8.0 at room temperature. Cells were resuspended in a buffer containing 30 mM Tris-HCl, pH 8.0, 20% (w/v) sucrose (75 ml per gram of wet cells), prewarmed to 37 °C. EDTA was added to the cell suspension up to a concentration of 5 mM. After 3.5 min, lysosome was added to a final concentration of 80 μg/ml, and 1.5 min later the cells were stabilized by adding 10 mM MgCl2. When the formation of spheroplasts as monitored under the microscope was complete (about 30 min after the addition of lysosome), the spheroplasts were pelleted by centrifugation at 10,500 × g for 15 min at room temperature. From the spheroplasts, RSO vesicles were prepared as described by Huijbregts et al. (24). RSO vesicles were stored in buffer containing 50 mM triethanolamine-HAc, pH 7.0, 50 mM KCl. Small aliquots of the vesicle suspension were frozen in liquid nitrogen and stored at −80 °C. The protein-phospholipid ratio of the RSO vesicles was in the range of 4.5–7 μg of protein per nmol of phospholipid.

**Transmembrane Movement of PE across E. coli Inner Membrane**

**In Vitro Synthesis of [3H]PE in AD93 ISO Inner Membrane Vesi-

icles—**Synthesis of PE in ISO vesicles of the pss strain AD93 was accomplished by reconstitution with S-135 lysate (25) from wild type strain W3899 (22). ISO vesicles (final concentration 0.5 mM phospholipid) were added to PS synthetase buffer (50 mM triethanolamine-HAc, pH 7.5, 100 mM KC1, 15 mM MgCl2), which contained 1 mM CTP, l-[3-3H]serine, and wild type S-135 lysate, at final concentrations of 1 mM, 0.1 μM, and 0.57 mg/ml protein, respectively, and which had been prewarmed to 37 °C. After the indicated periods of incubation at 37 °C, samples corresponding to 10 nmol of phospholipid were drawn from the suspension, and immediately subjected to phospholipid extraction for determination of synthesized radiolabeled lipids. In addition, samples of 10 nmol phospholipid were drawn, diluted 2.5-fold in ice-cold PS synthetase buffer, and put on ice to determine the accessibility of [3H]PE to fluorescamine (see below).

Where indicated, ISO vesicles (0.75 mM phospholipid) were preincubated in PS synthetase buffer with 2 mM N-ethylmaleimide on ice for 30 min before the in vitro synthesis. Alternatively, vesicles were incubated with 1 mM dithiothreitol on ice for 15 min, and subsequently with 10 mM iodoacetamide on ice for 15 min. After the pretreatments the vesicle suspensions were added to the premix containing the components necessary for the synthesis of [3H]PE described above. Where indicated, 10 mM ATP was present during the synthesis reaction.

**In Vitro Synthesis of [3H]PE in AD93 RSO Inner Membrane Vesi-

icles—**Synthesis of PE in RSO vesicles of AD93 was achieved by enclosing CTP and the enzyme PS synthetase in the lumen of the vesicles using repetitive freeze-thawing. Suspensions of RSO vesicles (0.2 mM phospholipid) in PS synthetase buffer containing 10 mM CTP and lysate (2.31 mg/ml protein) were subjected to 6 cycles of freezing in liquid nitrogen and thawing on ice. After the final thawing step, the suspensions were diluted in RSO buffer (50 mM triethanolamine-HAc, pH 7.0, 100 mM KC1), to a vesicle concentration of 0.05 mM phospholipid, and centrifuged at 135,000 × gmax in a TL100 ultracentrifuge (Beckman) at 4 °C for 10 min. The vesicles were washed three times in RSO buffer and then resuspended in this buffer at a concentration of 0.4 mM phospholipid. To assess the contribution to the synthesis of [3H]labeled amino-phospholipids by any remaining non-enclosed PS synthetase, CTP and lysate were added after, instead of before, the 6 freeze-thaw cycles.

To start amino-phospholipid synthesis, the vesicle suspension was added to an equal volume of RSO buffer containing MgCl2 (final concentration 50 mM) and l-[3-3H]serine (final concentration 0.222 mM), which had been prewarmed to 37 °C. After different times of incubation, samples containing 10 nmol phospholipid were drawn and either subjected to phospholipid extraction or diluted 2-fold in ice-cold buffer (50 mM triethanolamine-HAc, pH 8.0, 100 mM KC1), and put on ice for the reaction with fluorescamine.

Before the in vitro phospholipid synthesis, RSO vesicles with PS synthetase and CTP enclosed were treated in several ways as indicated. RSO vesicles (0.35 mM phospholipid) were preincubated with N-ethylmaleimide or dithiothreitol/iodoacetamide in RSO buffer as described for the ISO vesicles except that all incubation times were doubled. Alternatively, vesicles (0.2 mM phospholipid) were incubated with 0.5 mg/ml proteinase K in RSO buffer for 15 min either on ice or at 37 °C. Proteinase K-treated RSO vesicles were washed twice in RSO buffer before starting the in vitro phospholipid synthesis.

Furthermore, the in vitro phospholipid synthesis in RSO vesicles was performed in RSO buffer in the absence of divalent cations or with either 50 mM CaCl2, 50 mM BaCl2, or a combination of 40 mM MgCl2 and 10 mM BaCl2, replacing the 50 mM MgCl2 present in the standard experiment.

**Transmembrane Distribution of Newly Synthesized [3H]PE in AD93 Inner Membrane Vesicles—**To determine the pool of PE that is accessible to fluorescamine in both ISO and RSO vesicles, samples (corresponding to 10 nmol phospholipid) drawn from the synthesis reactions, were diluted in ice-cold buffer as already mentioned (final pH 7.5), and incubated on ice with 2 mM fluorescamine, added from a 100 mM stock solution in Me2SO. 15 s after the addition of fluorescamine, the reaction was terminated by adding ethanolamine to a final concentration of 250 mM. To determine the maximal pool of PE accessible to fluorescamine, samples were diluted in buffer containing octylglucoside at a final concentration of 40 mM. The lipids of the samples were extracted and analyzed.

**General Procedures—**Phospholipids of isolated membrane fractions, and from samples of in vitro phospholipid synthesis reactions and fluorescamine accessibility experiments were extracted according to Bligh and Dyer (26).

The amount of [3H]labeled amino-phospholipids synthesized was de-
RESULTS

A reconstituted system containing inner membrane vesicles isolated from cells of pss⁺ strain AD93 and a wild type lysate containing the enzyme PS synthetase was used to study the transmembrane movement of in vitro synthesized PE in the inner membrane of E. coli. Fig. 1A shows the synthesis of the amino-phospholipids PE and PS in ISO vesicles from AD93 incubated with CTP, lysate, and [¹⁴C]serine in PS synthetase buffer (lane 1). CTP is required for the synthesis of the intermediate CDP-diglyceride from the phosphatidic acid present in the vesicles. In the presence of serine, CDP-diglyceride is converted into PS by the enzyme PS synthetase. Omitting one of the components resulted in less or no synthesis of amino-phospholipids. When CTP was omitted from the incubation, some amino-phospholipids were synthesized (Fig. 1A, lane 2). This is probably due to the presence of a small amount of CDP-diglyceride in the membrane of the vesicles. The observation that an incubation of ISO vesicles of AD93 with [¹⁴C]labeled glycerol-3-phosphate at 37 °C yielded [¹⁴C]labeled phosphatidyglycerol (data not shown) supports this notion. When the lysate isolated from wild type E. coli, which is the source of the enzyme PS synthetase, was left out of the reaction mixture (Fig. 1A, lane 3) no amino-phospholipid was synthesized. Mg²⁺ was not essential for the synthesis of PS in AD93 inner membrane vesicles, but had a stimulatory effect (compare lanes 1 and 4). Amino-phospholipid synthesis increased in the concentration range of 0–20 mM Mg²⁺, whereas PS decarboxylation was maximal at 15 mM Mg²⁺ (data not shown). Therefore, this concentration of Mg²⁺ was used in all further experiments. In the absence of ISO vesicles no lipids were synthesized (lane 5), as expected, because the vesicles are suppliers of both the precursor phosphatidic acid and the enzymes involved in phospholipid metabolism, except for PS synthetase. This result implies that the lysate was essentially membrane free. Apart from the amino-phospholipids, sometimes minor components running faster than PE appeared on the silica plate (Fig. 1A, lane 1). The presence of these spots depended on the batch of vesicles used in the in vitro synthesis reaction. The nature of the extra spots is unknown, although their rate of flow values are similar to those of phosphatidyglycerol and cardiolipin.

In Fig. 1B both the lipid synthesis and the proportion of [¹⁴C]PE in ISO vesicles from AD93 are quantified. In the time window shown, none of the components necessary for PS synthesis became limiting, since the curve is almost linear with a small lag time at the start of the synthesis. The amount of amino-phospholipids synthesized during a 15 min incubation at 37 °C corresponded to 0.35 mol % of the endogenous phospholipid present in the vesicles. The appearance of PE is expressed as a percentage of the total amount of amino-phospholipid (PS + PE) synthesized at each time point. The amount of radioactive lipid and the proportion of PE of the newly formed lipids were determined as described under "Experimental Procedures." The error bars depict the standard deviation (n = 3).

determined by liquid scintillation counting of the phospholipid extracts. The amount of PE as a proportion of the amount of radiolabeled amino-phospholipids, and the proportions of PE and the fluorescamine product of amino-phospholipids synthesized during a 15 min incubation at 37 °C corresponded to 0.35 mol % of the endogenous phospholipid present in the vesicles. The appearance of PE is expressed as a percentage of the total amount of amino-phospholipid (PS + PE) synthesized at each time point. The amount of radioactive lipid and the proportion of PE of the newly formed lipids were determined as described under "Experimental Procedures."
The accessibility of newly synthesized PE to fluorescamine after the indicated periods of in vitro lipid synthesis in AD93 ISO, expressed as a percentage of the amount of PE labeled by fluorescamine in the presence of 40 mM octylglucoside. The accessibility of the newly synthesized PE in ISO vesicles was determined as described under "Experimental Procedures." The error bars depict the standard deviation (n = 3).

As protease treatment of ISO vesicles degrades phospholipid biosynthetic enzymes, sulfhydryl reagents were used to investigate if proteins play a role in the transmembrane movement of PE. A pretreatment of ISO vesicles with N-ethylmaleimide or with a combination of dithiothreitol and iodoacetamide did not affect the transmembrane movement of PE (data not shown). Neither the synthesis of amino-phospholipids nor the relative amount of PE were influenced by these pretreatments (data not shown). To investigate the energy requirements of the transmembrane movement of PE, the in vitro synthesis was carried out in the presence of 10 mM ATP. No effect of ATP could be detected on the rate and extent of the transmembrane distribution of newly synthesized PE (data not shown). The ATP added was able to generate a pmf as was verified by 9-amino-6-chloro-2-methoxyacridine fluorescence measurements (25) (data not shown). The presence of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone during in vitro PE synthesis in ISO vesicles of AD93 did not affect the accessibility of PE to fluorescamine either, indicating that the pmf is not involved in the process of transmembrane movement of newly synthesized PE.

The transmembrane movement of PE in ISO vesicles of AD93 was monitored by determining the PE pool, which is protected from the reaction with fluorescamine, i.e., the pool that has disappeared from the outer (cytoplasmic) leaflet of the ISO vesicles. To confirm the data obtained from ISO vesicles, it was investigated whether the complementary situation, the appearance of the marker molecule at the periplasmic leaflet, is detectable. For this purpose, CEO vesicles isolated from cells of AD93, were used. All of the enzymes involved in PS synthesis, except for PS synthetase, are located in the lumen of the ISO vesicle. Synthesis of PE requires the presence of the enzyme PS synthetase, CTP, and serine in the lumen of the RSO vesicle. Serine can be transported across the inner membrane of E. coli (29), whereas CTP and PS synthetase cannot. Therefore, these components were enclosed in the lumen of the vesicles by repetitive freeze-thawing of a suspension of RSO vesicles in PS synthetase buffer containing lysate and CTP.

Fig. 4 shows that when RSO vesicles, reisolated after the enclosure procedure were incubated with [14C]serine at 37 °C, amino-phospholipids were synthesized (lane 2). There was hardly any synthesis in the control experiment in which CTP and lysate were not enclosed but added immediately after freeze-thawing (Fig. 4, lane 1). In vivo, serine is transported...
Figure 4. In vitro synthesis of [14C]PE and its accessibility to fluorescamine in AD93 RSO vesicles. A phosphor imaging picture shows a HPTLC analysis of RSO vesicles without (lane 1), and with CTP and lysate enclosed in the lumen before (lane 2) and after incubation with 2 mM fluorescamine for 15 s in the absence (lane 3) and presence of 40 mM octylglucoside (lane 4). In lane 1 CTP and lysate were added to the vesicles after the freeze-thaw procedure. For further details see “Experimental Procedures.”

Figure 5. Time course of the synthesis of [14C]labeled amino-phospholipids (●) and of the appearance of PE (○) in AD93 RSO vesicles with CTP and lysate enclosed in the lumen. Also shown is the time course of the synthesis of [14C]-labeled amino-phospholipids in RSO vesicles to which lysate and CTP were added after the freeze-thaw procedure (□). The amount of amino-phospholipids synthesized is expressed as mol% of the amount of endogenous phospholipid present in the vesicles. Appearance of PE is expressed as a percentage of the amount of amino-phospholipid synthesized at each time point. Procedures for the enclosure of components in RSO vesicles, for the determination of the amount of amino-phospholipids synthesized, and of the proportion of PE are described under “Experimental Procedures.” The error bars depict the standard deviation (n = 3).

Synthesis of radioactive lipid is quantified as mol% of endogenous phospholipid. The amounts of PE are depicted as a percentage of total radioactive amino-phospholipid. The amounts of FL-PE are depicted as a percentage of maximally accessible FL-PE after solubilization of the inner membrane vesicles with 40 mM octylglucoside. Percentages are expressed as mean values ± S.D. (n ≥ 3). In the control experiments with isoleucine suspensions were incubated for 15 min at the temperature indicated before reisolation and in vitro synthesis.

Table I

| Synthesis PE FL-PE |
|-------------------|
| Control 0 °C      | 0.12 ± 0.035 | 86 ± 1.0 | 69 ± 2.0 |
| Proteinase K 0 °C | 0.06 ± 0.01  | 83 ± 0.4 | 65 ± 2.3 |
| Control 37 °C     | 0.08 ± 0.01  | 84 ± 1.2 | 72 ± 4.2 |
| Proteinase K 37 °C| 0.01 ± 0.00  | 84 ± 3.1 | 63 ± 5.7 |

Across the inner membrane by several transporters, which depend on the pmf (30, 31). However, generation of a pmf by the artificial electron donor system ascorbate-phenazine methosulfate (32) did not affect the amount of amino-phospholipids synthesized in RSO vesicles. Therefore, in all further experiments RSO vesicles were incubated with serine without generating a pmf.

Quantification of the synthesis of amino-phospholipids and the proportion of [14C]PE is shown in Fig. 5. After 5 min of incubation at 37 °C, the amount of amino-phospholipids synthesized in RSO vesicles with PS synthetase and CTP enclosed corresponded to 0.15 mol% of the endogenous phospholipids and hardly increased after this time point. The amount of lipid synthesized in the vesicles freeze-thawed in the presence of CTP and lysate was about 7 times larger than in the control experiment in which these components were added to the vesicle suspension after the freeze-thaw procedure (Fig. 5). This demonstrates that CTP and PS synthetase were enclosed in the lumen of the vesicles by the freeze-thaw procedure and that the synthesis observed was not generated by residual CTP and PS synthetase, which were not removed by the washing procedure. Fig. 5 also shows that after 5 min already about 80–90% of the PS synthesized in RSO vesicles with CTP and lysate enclosed were incubated with sulfhydryl reagents or proteinase K. Neither treatment with sulfhydryl reagents (data not shown) nor proteinase K (Table I) resulted in a significant change in the fluorescamine accessible pool of PE.
Proteinase K treatment did decrease the amount of amino-phospholipids synthesized (Table I). The effect was stronger when the incubation with proteinase K was performed at 37 °C than at 0 °C. Proteinase K probably inactivates the enzyme CDP-diglyceride synthetase, which is postulated to be an integral membrane protein with 7 transmembrane helices.  

Proteinase K did not affect the decarboxylation of the newly synthesized PS, in agreement with localization of PS decarboxylase at the cytosolic side of the inner membrane (34).

It was shown by DeChavigny et al. (22) that AD93 needs divalent cations in the periplasm for growth. It was found that Mg\(^{2+}\) and Ca\(^{2+}\) support growth although Ba\(^{2+}\) does not (28). The effect of the presence of several divalent cations during the in vitro synthesis of PE in RSO vesicles was investigated. No differences could be detected in the synthesis, the amount of PS converted into PE, or the accessibility of PE to fluorescamine when either no divalent cations or a variety of divalent cations (absence of divalent cations, 50 mM CaCl\(_2\), 50 mM BaCl\(_2\), or a combination of 40 mM MgCl\(_2\) and 10 mM BaCl\(_2\)) replaced the 50 mM MgCl\(_2\) present in the standard experiment (data not shown).

**DISCUSSION**

In the present study, for the first time the transmembrane movement of newly synthesized PE across the inner membrane of *E. coli* is reported. Amino-phospholipids were synthesized in a newly developed in vitro system consisting of isolated inner membrane vesicles derived from strain AD93. This strain lacks the enzyme PS synthetase and thus cannot make PE. A wild type lysate was added to the vesicles as source of the enzyme PS synthetase. A rapid redistribution of the newly synthesized PE over the two membrane leaflets was determined in both ISO and RSO vesicles using the amino-reactive chemical fluorescamine.

The in vitro phospholipid synthesis system takes advantage of the fact that PS synthetase, unlike all other enzymes involved in phospholipid metabolism, is found in the cytosol upon isolation (23). Furthermore, acidic phospholipids are the trigger for PS synthetase to bind to the membrane and to become active (35). Since they are the sole kind of phospholipids in AD93 (22), activation of PS synthetase is ensured. Moreover, there is a considerable pool of phosphatidic acid (22) in this strain which conveniently serves as a substrate for the synthesis of PS in the in vitro system. The decarboxylation of PS to PE is still intact in AD93, and therefore it is expected that the same applies for the subsequent transport processes.

The amount of amino-phospholipids synthesized in ISO vesicles of AD93 increased with the concentration of Mg\(^{2+}\) up to 20 mM, the highest concentration tested. This can be attributed to the enzyme CDP-diglyceride synthetase, which has an absolute requirement for Mg\(^{2+}\) (36). PS synthetase activity is not influenced by divalent cations (37, 38). In wild type *E. coli* membranes PS is not detectable because it is immediately converted into PE by PS decarboxylase (39). Also, in the inner membrane vesicles from strain AD93, rapid decarboxylation of the PS synthesized occurred but the reaction was never complete. PS decarboxylase does not require divalent cations for activity (40), yet a concentration of 15 mM Mg\(^{2+}\) at the cytosolic side of the inner membrane vesicles was found to be optimal for the conversion of PS into PE in AD93 vesicles. This suggests that anionic phospholipids have an inhibitory action on the PS decarboxylase that can in part be overcome by Mg\(^{2+}\), possibly by shielding the charge of these lipids.

The reason for choosing fluorescamine for the determination of the distribution of the newly synthesized PE is its fast rate of reaction (1/2 of 200–500 ms) with amino-groups (16). Furthermore, fluorescamine is an uncharged molecule, whereas most other amino-reactive chemicals are anionic. Pilot experiments (data not shown) with one of these reagents, trinitrobenzenesulfonic acid, showed that its reaction with PE in AD93 ISO vesicles was very slow on ice, probably due to repulsion by the negatively charged membrane surface. In the topology experiments vesicles were incubated with 2 mM fluorescamine for 15 s. These parameters were chosen for two reasons. First, the amount of PE that reacted with fluorescamine did not increase at concentrations above 2 mM, suggesting that 2 mM fluorescamine was able to label all the accessible amino groups. Second, to minimize membrane penetration the shortest technically possible time of 15 s was used. Under these conditions 2 mM fluorescamine labeled at least 85% of the PE when the vesicles were opened by octylglucoside (Fig. 2B). The complementary results obtained with ISO and RSO vesicles (Fig. 6) validate the assay conditions chosen.

From *in vivo* studies (11) performed in *E. coli*, it is known that the transport of phospholipids from the cytoplasmic leaflet of the inner membrane to the outer membrane is pmf dependent. In contrast, the pmf did not affect the transmembrane movement of newly synthesized PE in ISO vesicles (this study), nor the translocation of NBD-labeled phospholipids across the inner membrane of wild type cells (15). This implies that the pmf is involved in the transport of phospholipids from the periplasmic leaflet of the inner membrane to that of the outer membrane. Interestingly, a number of protein transport processes across the outer membrane (for examples see Refs. 41 and 42) also require the pmf across the inner membrane.

Rietveld et al. (43) showed that the polymorphic properties of phospholipids induced by divalent cations in strain AD93 are necessary for the growth of *E. coli*. It was observed that protein translocation was facilitated when divalent cations were enclosed in the lumen of ISO vesicles isolated from strain AD93 (44), demonstrating that these properties are required for efficient protein translocation. In this study no differences were detected in the synthesis, the PE/PS ratio or the transmembrane movement of PE in RSO vesicles from AD93 in the absence or presence of 50 mM of several divalent cations. These results indicate that the structural requirement of divalent cations at the periplasmic side of the inner membrane (22) is not a prerequisite for the transport of phospholipids across the inner membrane in strain AD93.

The newly synthesized PE was found to equilibrate within a minute across the inner membrane resulting in a distribution of about 1 to 2, cytoplasmic to periplasmic leaflet, irrespective of whether ISO or RSO vesicles were used and irrespective of the amount of PE synthesized. Whether this distribution reflects the so far unknown steady state transverse distribution of PE in the inner membrane of wild type *E. coli* cells remains an open question. PS synthesis, decarboxylation, and subsequent transmembrane transport of PE followed each other closely in time. Whether they are mechanistically coupled is not known.

The fast rate of the transmembrane transport of PE can be understood when considering that *E. coli* is a rapidly growing organism that divides once every half-hour. In this time period the amount of phospholipids in both membranes must have doubled, requiring fast transport processes. Also in *B. megaterium* (12, 13) rapid transmembrane equilibration of newly synthesized PE was detected with a t\(_{1/2}\) of about 3 min, suggesting that fast flip-flop of lipids is a general property of bacterial plasma membranes and thus fundamentally differs from the much slower processes occurring in plasma membranes of eu-
The rapid transmembrane movement of newly synthesized PE is indicative of a facilitated or active rather than a passive transport process, since it was shown that PE equilibrates with a \( t_{1/2} \) of 7 min at 22 °C across the membrane of lysosomes (46). Several processes involving active transmembrane movement of phospholipids have been reported. The amino-phospholipid translocase is an ATPase that maintains the asymmetric distribution of amino-phospholipids across the mammalian plasma membrane (47, 48). Recently, it was published that ABC transporters involved in multidrug resistance in eukaryotic cells also have the ability to transport phospholipids across the membrane (49). The process described here, however, is independent of ATP and can therefore not be linked to these classes of transport proteins. It is of interest to compare lipid transport across the bacterial and endoplasmic reticulum membranes. Both membranes are the main sites of cellular membrane lipid synthesis (3, 50) and both display rapid ATP-independent transmembrane movement of lipids (Ref. 51 and this study). However, translocation across the endoplasmic reticulum membrane is sensitive to sulfhydryl reagents and proteases (51, 52), whereas translocation across the E. coli inner membrane appears to be insensitive. This may indicate that rapid transmembrane movement of PE across the E. coli inner membrane proceeds by a different, novel transport mechanism. Alternatively, the putative flipase in the E. coli inner membrane may be much less sensitive to such treatments than that of the endoplasmic reticulum.

Previously, we demonstrated rapid transmembrane movement of NBD-labeled PE across the E. coli inner membrane (15). The NBD-PE equilibrated much more rapidly across the membrane with a \( t_{1/2} \) of 7 min and reached an equilibrium distribution of 6 to 1, cytoplasmic to periplasmicleaflet. This might reflect the differences in chemical nature of the two types of PE and/or differences in the protocols used. PE, synthesized from lipid precursors present in the membrane, might more efficiently make use of a flip-flop machinery in the membrane than the NBD-labeled PE that was incorporated into ISO vesicles by external addition. Different flip-flop characteristics of NBD-labeled phospholipids on the one hand and endogenous phospholipids and spin-labeled phospholipids on the other have been reported in other systems (53).

Presently, two separate membrane systems are used for the \textit{in vitro} analysis of the transport of cytoplasmically synthesized components to the outer membrane of \textit{E. coli}. The transport of the precursors of outer membrane proteins across the inner membrane is analyzed in ISO vesicles (54, 55). The subsequent insertion of these proteins in the outer membrane is investigated by adding these proteins in the mature form to outer membranes (33). In the present study specific components could be encased in RSO vesicles to generate synthesis of phospholipids. These molecules subsequently appeared at the periplasmic outer side of the vesicles. Therefore, this approach offers the perspective of investigating the transport of a variety of protein and lipid components from the site of synthesis to the periplasmic side and beyond to the outer membrane all in one \textit{in vitro} system.

Acknowledgments—We thank Gerda de Korte-Kool for the isolation of lysate, and Jan Biermann for the kind gift of the \textit{4C}-labeled glycerol-3-phosphate. William Dowhan is acknowledged for his suggestion on the use of inner membrane vesicle of AD93 as source for the \textit{in vitro} synthesis of PE.