Function of Phospholipids in Escherichia coli

INFLUENCE OF CHANGES IN POLAR HEAD GROUP COMPOSITION ON THE LIPID PHASE TRANSITION AND CHARACTERIZATION OF A MUTANT CONTAINING ONLY SATURATED PHOSPHOLIPID ACYL CHAINS

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Gerd Pluschke and Peter Overath
From the Max Planck-Institut für Biologie, Corrensstrasse 38, 74 Tübingen, Federal Republic of Germany

The clst mutation conferring a defect in cardiolipin synthesis (Pluschke, G., Hirota, Y., and Overath, P. (1978) J. Biol. Chem. 253, 5048-5055) has been introduced into an Escherichia coli strain defective in unsaturated fatty acid synthesis in order to study the effect of changes in polar head group composition on the ordered ↔ fluid phase transition of the membrane phospholipids. The defect in cardiolipin formation is compensated by an increase in phosphatidylglycerol content, resulting in a decrease of the midpoint of the phase transition by 6°C. Starvation of the clst mutant strain for the unsaturated fatty acid supplement leads to the same polar head group composition as wild type Escherichia coli. The results demonstrate that, under defined conditions, saturated acyl chains of reduced average length into the phospholipids, and growth is inhibited although the membrane remains in a fluid state. A revertant of this strain is described which retains the parental fabB", "fadE", and clst markers and grows in the absence of an unsaturated fatty acid supplement. A clst" derivative of the revertant can multiply in a restricted temperature range (35-43°C). It contains only saturated phospholipid acyl chains of an anomalously short average length of 14 carbon atoms but has the same polar head group composition as wild type E. coli. The results demonstrate that, under defined conditions, saturated acyl chains of reduced length are functionally equivalent to unsaturated chains.

The membrane lipids of Escherichia coli consist of glycerophospholipids, predominantly phosphatidylethanolamine (80%) and negatively charged phosphatidylglycerol and cardiolipin. Exponentially growing cells contain about 15% phosphatidylglycerol and 5% cardiolipin. In the stationary phase and a variety of suboptimal growth conditions, the cardiolipin content tends to increase at the expense of phosphatidylglycerol (1). The fatty acyl chains of these phospholipids have an average length of more than 16 carbon atoms, about half of which carry one cis double bond. When E. coli wild type grows at 37°C, essentially all of its membrane lipids are in the fluid state. The transition temperature of the broad ordered ↔ fluid transition is centered at Tc ≈ 10°C; the upper end of the transition is at about 25°C (2).

Studies with E. coli, as well as other microorganisms, notably Acholeplasma laidlawii, have contributed to the understanding of the relationship between lipid structure and membrane function because the lipid composition in these cells can be manipulated by genetic or nutritional means (3-6). In particular, the influence of changes in the hydrocarbon chain composition of phospholipids on the ordered ↔ fluid phase transition as well as a variety of parameters such as cell growth, transport processes, etc., has been investigated. Much less is known about the structural and functional effects of changes in the polar head group composition. The isolation of a mutant defective in the synthesis of cardiolipin (7) has made a comparison of the phase transition in cells of normal and cardiolipin-deficient phospholipid composition possible. Such a comparison is presented in this paper. In the second part, the isolation and characterization of a mutant which grows continuously with only saturated acyl chains in the membrane phospholipids will be described.

MATERIALS AND METHODS

Organisms—The strains of E. coli K12 used in the present study (see Table I) are derivatives of the well characterized fatty acid-requiring strain K1062 (9). This strain is defective in unsaturated fatty acid synthesis (fabB) and in fatty acid degradation (fadB), and carries an undefined mutation allowing supplementation with trans-unsaturated fatty acids. In addition, some strains contain a defect in cardiolipin synthesis (clst; see Ref. 7).

Media—Medium 1: Vogel-Bonner mineral salts medium (10) supplemented with 0.3% casamino acids (Difco, vitamin-free), 0.5% glycerol, 0.5% Brij 35 (polyoxyethylene dodecylether), and 0.01% palmito-elicheic acid (trans-Δ^5,16:1) acid. Medium 2: Prepared according to Ref. 11, containing 0.5% glycerol and 1-α-manois in the following concentrations: 0.1% arginine and serine, 0.01% glutamic acid, glutamine, leucine, lysine, and valine; and 0.006% in all the others. Medium 3: 10 g of tryptone, 5 g of NaCl, 1 mm MgSO_4, 0.5% Brij 35, and 0.01% olate (cis-18:1). Media were solidified by the addition of 15 g/liter of agar. Growth was followed by the determination of the absorbance at 420 nm in a Gilford 300 spectrophotometer.

Isolation of Phospholipids—Phospholipids were extracted by the method of Bligh and Dyer, as modified by Ames (12), and precipitated by acetone. Phospholipids were separated by CM-cellulose column chromatography. Columns (2.5 × 45 cm) were packed as described (13) with preswollen CM-cellulose (CM52, sodium form, Whatman) in methanol. Prior to applying the lipids, methanol was removed from the column by eluting with 10 bed volumes of chloroform. Then the lipid (up to 500 mg) in chloroform was applied to the column and first eluted with 5 bed volumes of 5% methanol in chloroform to remove residual nonpolar lipids. Stepwise elution was accomplished with 10 to 20 bed volumes of chloroform with increasing methanol content. The methanol concentrations in chloroform required to elute the phospholipids are: 10% for phosphatidylethanolamine, 18% for phosphatidylglycerol, and 22% for cardiolipin. Fission of the phospholipids was followed by thin layer chromatography. Thin layer chromatography, identification, and quantification of ^32P-labeled phospholipids

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has been described before (7). The fatty acid composition of the phospholipids was determined as described previously (14).

**Measurement of Lipid Phase Transition—**For fluorescence measurements, cells were diluted in Vogel-Bonner mineral salts buffer containing 0.1 M NaCl, 10 mM morpholinopropane sulfonate, 0.1 mM EDTA (pH 7.3) prepared by sonication with a Branson Sonifier for 3 min at 42°C under a stream of nitrogen. After a 5-fold dilution, these samples were used directly for fluorescence measurements with PhNap as indicator.

**RESULTS**

**Lipid Phase Transition in Cardiolipin-deficient E. coli—**The parental strain K1062 used in this study is auxotrophic for unsaturated fatty acids (fabB), defective in the degradation of fatty acids (fadE), and carries an undefined mutation allowing supplementation with trans-unsaturated fatty acids. Its growth requirement is met by a variety of cis- or trans-unsaturated but not by saturated fatty acids. Strain K1062 was grown in the presence of palmitelaidate as supplement and the three main phospholipids were isolated. The hydrocarbon chain composition of the separated components is essentially the same (cf. the legend to Table II). Comparison of the midpoint of the lipid phase transition, Tc, of the individual components, of artificial lipid mixtures and cells shows that the phase transition temperature is mainly determined by phosphatidyl ethanolamine (Table II; compare also Ref. 15). Phosphatidylglycerol decreases the transition temperature while cardiolipin has only a minor effect because the transition of this lipid is close to that of phosphatidylethanolamine.

Recently, a mutant of E. coli was described (genotype cls) which has a defect in the synthesis of cardiolipin from phosphatidylglycerol (7). This defect does not affect the growth properties of the cell. The decrease in cardiolipin content is compensated by an increase in phosphatidylglycerol content. The change in the polar head group composition in this mutant should lead to a decrease of the transition temperature, since the Tc value of phosphatidylglycerol is more than 20°C lower than that of cardiolipin (Table II). Therefore, two isogenic derivatives of strain K1062 were constructed, one containing the wild type (T2GP: fabB fadE cls*) and the other containing the mutant cls allele (T2GP: fabB fadE cls). Both strains were grown at 40°C in the presence of palmitelaidate as supplement. As can be seen from samples No. 1 in Table III which correspond to time 0 in Fig. 1, the fatty acid composition of both strains is essentially the same. Regarding the polar head groups, strain T2GP contains 75%2 phosphatidylethanolamine, 25% phosphatidylglycerol, and traces of cardiolipin; strain T2GP contains 81% phosphatidylethanolamine, 9% phosphatidylglycerol, and 10% cardiolipin. The midpoint of the broad phase transition, Tc, is 6°C lower for the cardiolipin-deficient cls strain than for the cls* strain (see samples No. 1 in Fig. 2). Thus, the absence of cardiolipin and the corresponding increase in phosphatidylglycerol content decreases the phase transition in vivo in a direction and to an extent expected from the properties of the isolated lipids.

The growth behavior of both strains upon deprivation of the unsaturated fatty acid supplement was investigated. The cultures were washed free of trans-Δ1-16.1 and grown further at 40°C. At various times, samples were taken for determination of the number of surviving cells (Fig. 4A), the increase in cell mass (Fig. 4B), the fatty acid and polar head group composition (Table III), and the phase transition (Table III and Fig. 2).

Let us first consider strain T2GP. As shown in Fig. 4B, the fatty acid and polar head group composition (Table III) and the phase transition (Table III and Fig. 2).
Cells of strain T2GP (cls) and T20GP (cls') were grown at 40°C in Medium 1 with palmitylaldehyde (trans-Δ^16:1) acid as supplement. Under these conditions, the generation time of both strains was essentially the same. Exponentially growing cells were rapidly centrifuged, washed twice with Vogel-Bonner buffer at temperatures of 40°C, further incubated at 40°C, and calculated changes in the transition temperature, AT, was estimated from the difference of published values for pure phospholipids (20, 21): 18:0 phosphatidylethanolamine, T_c = 74°C; trans-Δ^16:1 phosphatidylethanolamine, T_c = 35°C (ΔT_c = 39°C); 12:0 phosphatidylethanolamine, T_c = 29°C; 14:0 phosphatidylethanolamine, T_c = 47.5°C (ΔT_c = 18.5°C); 14:0 phosphatidylethanolamine, T_c = 47.5°C (ΔT_c = 25.5°C). T_c values for cardiolipin and phosphatidylethanolamine containing the same fatty acid composition are nearly the same (cf. Table II).

### Table III

<table>
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<tr>
<th>Strain</th>
<th>Sample number*</th>
<th>Fatty acyl chain composition</th>
<th>Average chain length of fatty acids</th>
<th>Polar head group composition</th>
<th>Change in phase transition temperature</th>
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<td>12:0</td>
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</table>

* Numbers refer to samples taken as indicated in Fig. 1 by arrows.

growth continues for about one generation. After 2 h, the absorbance decreases. The number of viable cells (Fig. 1A) first increases for a short time, then viability decreases exponentially. This behavior is accompanied by changes in the hydrocarbon chain and polar head group composition (Table III). After removal of the supplement, only saturated acyl chains are incorporated into phospholipids. The average chain length decreases, initially caused by the incorporation of 12:0 and 14:0, and at a later stage, remains constant due to a relatively faster rate of formation of 16:0. During starvation, phosphatidylethanolamine is gradually converted to cardiolipin which finally reaches the abnormally high content of 25%.

Three different laboratories have previously reported (2, 22, 23) that about half the lipid must be in the fluid state to allow growth of E. coli. This conclusion appears also applicable to strain T2GP, since the upper end of the transition, T_c = 33°C, for cells containing exclusively saturated acyl chains of an average chain length of 13.4 carbon atoms (compare sample No. 6 in Table III) is well below the growth temperature, T = 40°C.

The behavior of the cardiolipin-deficient strain T2GP is distinctly different in several respects. The absorbance shows a 3-fold increase in the first 2 h of starvation and then remains constant (Fig. 2B). Only relatively small changes in the number of colony formers are observed (Fig. 2A). The cells accumulate large amounts of 12:0 and 14:0 in their phospholipids, which causes a continuous decrease in the average chain length. The polar head group composition remains essentially constant (Table III). The change in the hydrocarbon chain composition causes an increase in the phase transition temperature from 22 to 31°C (ΔT_c = 9°C). This increase is also in agreement with the change expected from the behavior of pure lipid components (ΔT_c = 8.5°C). However, in contrast to strain T2GP, all the lipids are fluid after 5 h of starvation for palmitylaldehyde, since the upper end of the transition, T_c = 33°C, is about 39°C. The extrapolated transition temperature, T_c = 33°C, for cells containing exclusively saturated acyl chains of an average chain length of 13.4 carbon atoms (compare sample No. 6 in Table III) is well below the growth temperature, T = 40°C.

### Cell Types

**Cells Containing Only Saturated Acyl Chains in Phospholipids**—The behavior of strain T2GP suggested that E. coli cells containing only saturated acyl chains in membrane phospholipids should be viable. Attempts to grow strain T2GP on a variety of media without unsaturated fatty acid supplement failed. Also, supplementation of this strain by different mixtures of saturated fatty acids did not support growth. An alternative possibility was that variants of strain T2GP could be selected which would be able to grow in the absence of unsaturated fatty acids. Cells of strain T2GP were starved for unsaturated fatty acids and plated on a synthetic medium.
Polar Head Group Composition and Lipid Phase Transition

33°C. As shown in the lower part of Fig. 3, phospholipids from this strain contain only saturated fatty acids. Equal amounts of 12:0, 14:0, and 16:0 imply an average chain length of 14 carbon atoms. Unsaturated acyl chains are undetectable (<1%). The identity of the fatty acids was confirmed by mass spectroscopy.

The nature of the mutational change(s) in strain T114GP that allow(s) growth without unsaturated fatty acid supplement is unknown. The property could not be transduced from strain T114GP to T2GP using phage P1. This observation, together with the low frequency of occurrence of this derivative, may indicate the requirement of more than one muta-

lacking fatty acids (cf. Medium 2 under “Materials and Methods”) at 40°C. Revertant colonies appeared at a low frequency (<10⁻²). Under the same condition, strain T20GP did not give rise to revertants. Most of the revertants obtained from strain T2GP had regained the ability to synthesize unsaturated fatty acids and were discarded. One mutant, designated T114GP, retained the parental genotype (fadB, fadE, cls). The mutant is cold-sensitive and can grow only at temperatures above

Fig. 1. Viable cell count (A) and change in absorbance (B) of cultures of strains T2GP and T20GP starved for unsaturated fatty acids. At time 0, the cells were resuspended in Medium 1 without fatty acid supplement as described in the legend to Table III. Samples were taken at different times, diluted and plated at 40°C on Medium 3 plates. Arrows indicate when samples were taken for fluorescence measurements and determination of fatty acid and polar head group composition (cf. Table III and Fig. 2).

Fig. 2. Phase transition temperatures of the membrane lipids in cells starved for unsaturated fatty acids as a function of the molar percentage of saturated fatty acids. The arrows in Fig. 1 indicate when samples were taken for fluorescence measurements and fatty acid analysis. Transition temperatures of cells were taken from scans with increasing temperature using PhNap as fluorescence indicator (22). The width of the transition indicates the high (Tₜ) and low temperature end (Tᵢ) of the phase transition. The fatty acid composition is given in Table III.

Fig. 3. Gas-liquid chromatograms of fatty acid methyl esters derived from phospholipid ethanolamine of strains K12Ymel and T114GP. Fatty methyl esters from phospholipid extracts of cells growth in Medium 2 (Apiezon L column at 220°C). Fatty acid composition: K12Ymel: 12:0, 2%; 14:0, 5%; 16:0, 30%; 16.1, 33%; 17c, 9%; 18:1, 21%; T114GP: 12:0, 31%; 14:0, 35%; 16:0, 34%.
were measured by \( \text{fadE} \) T11SGP. Temperature dependence values for polar head group composition similar to wild type and the change in absorbance was measured for several generations. Initially in Medium 2 at 40°C were shifted to the desired temperature when the cells were grown at 40°C. The fatty acid composition of the strain transductants had obtained the wild type parental strains T114GP and T116GP. The other 12 strains T117GP has a lipid phase transition temperature (22). In contrast, the behavior of strain T2GP (see Fig. 2 and Table III). The increase in the transition temperature observed for both strains upon starvation for unsaturated fatty acids can be accounted for in terms of changes in polar head group and hydrocarbon chain composition. Furthermore, the contribution of these structural changes to the rise in the transition temperature can be calculated from the transition temperature of synthetic lipids (Table III). Thus, phospholipids in E. coli membranes behave in a way predicted from the isolated components regarding both the hydrocarbon chains (4) and the polar head groups.

In the second part, a mutant of E. coli is described which contains only saturated fatty acids of abnormally low average chain length but a normal polar head group composition. Therefore, saturated acyl chains with an average length of 14 carbon atoms fulfill the requirement of a stable and fluid bilayer at the physiological temperature of this intestinal bacterium. A further reduction in chain length is likely preceded by a decrease in bilayer stability. An increase would shift the ordered ↔ fluid transition above the upper limiting growth temperature. The lower limiting growth temperature of strains T117GP and T118GP is about 25°C higher than in wild type E. coli and coincides with the midpoint of the phase transition, i.e. growth is inhibited when more than 50% of the phospholipids are in the ordered state. A reduction in average bilayer thickness by about 5 Å expected from the reduction in average chain length is compatible with adequate function of all membrane-associated proteins required for growth (compare also conclusions in Refs. 24 and 25).

It has previously been shown that branched-chain fatty acids bearing bulky methyl substituents (24) or bromo-substituted fatty acids (26) are suitable supplements for the growth of fatty acid auxotrophs. Therefore, E. coli phospholipids are functional in the absence of acyl chains bearing \( \text{cis-} \) or \( \text{trans-} \) double bonds, provided the supplement ensures that at least half of the lipids are in a fluid state at the growth temperature. Similarly, the growth inhibition observed in starvation experiments for \( \text{cis-} \)unsaturated fatty acids have been related to the increase in phase transition temperature close to the growth temperature (22). In contrast, the behavior of strain T2GP (Figs. 1 and 2) indicates that inhibition of growth can occur under conditions where essentially all of the phospholipids are still in a fluid state. At least one additional mutation affecting most likely a regulatory mechanism involving, for instance, cyclic AMP (27) is required in order to overcome this growth inhibition. The reconstruction experiment (Fig. 4) suggests that strains growing continuously without unsatu-
rated fatty acid supplement should in principle also be ob-
tained from a cls* strain such as T20GP.
Why do fabB fadE mutants synthesize shorter chain fatty
acids upon removal of the supplement? The preferential in-
corporation of these acids into the phospholipids may reflect
a competition between rapid incorporation and further elon-
gation of the acyl chains. The existence of such a regulatory
mechanism has been proposed by Cronan et al. (28), who
found that E. coli cells produce abnormally long chain length
fatty acids when uncoupled from phospholipid synthesis. The
preferential synthesis of fatty acids of reduced length is not
observed to the same extent in a strain with a temperature-
sensitive fabA mutation during growth at semipermissive
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