Volume 336, number 2, 221–224 FEBS 13412 December 1993

Active increase in cardiolipin synthesis in the stationary growth phase and its physiological significance in *Escherichia coli*

Shuichi Hiraoka**, Hiroshi Matsuzaki, Isao Shibuya*

Department of Biochemistry, Saitama University, Urawa 338, Japan

Received 20 October 1993

Activity of the *Escherichia coli* cardiolipin synthase, encoded by *cls*, increased about 10-fold in the stationary growth phase, while other committed-step enzymes in phospholipid biosynthesis rather decreased. A null *cls* mutant lost viability to 10^{-4} of the wild-type cells during the prolonged incubation for 5 days. Cardiolipin was most stable among membrane phospholipids during the incubation. Accordingly, cardiolipin should play a role in survival of the cell and *E. coli* employs a sophisticated way to form cardiolipin according to need even under non-growing conditions.

Cardiolipin; Cardiolipin synthase; Cell survival; *Escherichia coli*; Stationary growth phase

1. INTRODUCTION

Cardiolipin (also known as diphosphatidylglycerol) is unique among membrane phospholipids in its structure, having four acyl chains and two phosphate groups, and is known to be important for the activities of electron transport enzymes in eucaryotic mitochondria [1,2]. In *Escherichia coli*, cardiolipin is one of the major phospholipids in membranes. However, its essentiality is uncertain [3] and its specific functions, if any, have not been understood; the previously reported requirements for this lipid were almost equally satisfied by another major acidic phospholipid, phosphatidylglycerol (for a recent review, see [4]). In addition to its unclear functions, cardiolipin of *E. coli* has several curious features in its biosynthesis, the biological significance of which has not been explained: (i) it is mainly synthesized by cardiolipin synthase, encoded by *cls* [3], via condensation of two phosphatidylglycerol molecules [5,6], in contrast to the eucaryotic counterparts that more economically utilize one molecule each of CDP-diacylglycerol and phosphatidylglycerol [7,8]; (ii) its contents in membranes increase significantly in the stationary growth phase and under conditions where the cellular energy level is lowered [6,9–11]; and (iii) its synthesis is enhanced when phosphatidylethanolamine synthesis is impaired by defects in phosphatidylserine synthase [12,13].

The recent development of a useful assay method for *E. coli* cardiolipin synthase [14,15] has enabled us to examine the synthase activity accurately for many strains under various growth conditions. Here we describe observations that explain many of the unique features in cardiolipin biosynthesis and suggest an important role for this particular lipid in survival of the cell.

*Corresponding author. Fax: (81) (48) 855 6295.

***Present address:* Department of Immunology, Mitsubishi Kasei Institute of Life Sciences, Machida 194, Japan.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmid, and culture conditions*

E. coli K-12 strains W3110 (wild type) [16], JE5513 (*lpp*-2) [17], YA5513 (JE5513 *pgsA3*) [18], JP5513 (JE5513 *pssA1*, formerly *pss*-1) [19], and S101 (W3110 *cls*::*kan*) [20] were described previously. In the last strain, the promoterless *kan* gene of pUC-4K [21] was inserted into the chromosomal *cls* gene to form an operon fusion [3], so that the kanamycin phosphotransferase activity corresponded to the transcriptional expression of the *cls* gene. Plasmid pCL11 was a derivative of pBR322 carrying the intact *cls* locus [22]. Cells were grown in LB medium [23] at 37°C, except for strain JP5513 which was grown at 42°C for the maximum phenotype. Ampicillin was added to the medium at 50 μg per ml when plasmid-harboring cells were cultured. Growth was monitored in a Klett–Summerson photometer; 1 Klett unit corresponded to approximately 5×10^6 cells per ml. Unless otherwise specified, cells were harvested for enzyme assay at Klett unit 100 (exponential phase) or at 2 h after the cessation of turbidity increase (Klett unit 300, stationary phase). Viable cells were counted by plating diluted cultures on LB plates containing 1.5% agar.

2.2. *Assay conditions for enzymes and phospholipids*

Cardiolipin synthase was assayed by the standard method described previously [14,15], except that 200 μM phosphatidyl[2-^{3}H]glycerol and 0.03% Triton X-100 were used, instead of the standard 20 μM substrate and 0.015% Triton X-100, to minimize the possible diluting effect of endogenous phosphatidylglycerol. Activities of *sn*-glycerol-3-phosphate acyltransferase [24], phosphatidylserine synthase [25], and phosphatidylglycerophosphate synthase [26] were measured as previously described. Kanamycin phosphotransferase was assayed using [γ-^{32}P]ATP (ICN Biomedicals, Inc., Irvine, CA) as described [27]. One unit of enzyme activity was defined as 1 nmol of reaction product formed in 1 min. Phospholipids were extracted from the cells uniformly labeled with ^{32}P, separated by two-dimensional thin-layer chromatography, and quantified from their radioactivity as previously described [28].

3. RESULTS

3.1. *Growth-phase dependent cardiolipin synthase activity*

Cardiolipin synthase activities were measured for the wild-type and mutant cells harvested both in the exponential and stationary growth phases (Table I). The specific enzyme activity in the stationary phase was 13-times higher than that in the exponential phase in strain JE5513. A phase-dependent change in cardiolipin synthase was clearly seen in a time-course experiment with another wild-type strain W3110 (Fig. 1). The specific activity in the cells of overnight culture decreased rapidly upon inoculation into a new medium and turned to increase gradually in the mid-exponential phase, reaching the maximum at about 5 h after the cessation of turbidity increase. Under these conditions, the cellular phosphatidylglycerol level changed from 19% of the total phospholipid phosphorus (exponential phase) to 9% (stationary phase). Since this change corresponded to no more than 5% of the amount of radioactive substrate added in the assay mixture, possible errors, if any, due to the variable dilution of the substrate specific radioactivity were not significant.

Table I also shows the results with mutant strains defective in the committed steps in the biosyntheses of three major phospholipids. Glycerol release was not detected with the membrane fraction from a null *cls* mutant, indicating that the assay method employed was specific to cardiolipin synthase, not measuring the secondary minor reaction of cardiolipin formation which takes place even in null *cls* mutants [3,28]. The phase-dependent change of cardiolipin synthase activity was also seen in *pssA1* and *pgsA3* mutants which had a temperature-sensitive phosphatidylserine synthase [28] and a defective phosphatidylglycerophosphate synthase [29], respectively, suggesting that the observed change in cardiolipin synthase activity did not depend on the phospholipid composition of the membrane. The synthase activity in the exponential phase was significantly higher in these mutants than the wild-type strain and this may explain the higher contents of cardiolipin, relative to those of phosphatidylglycerol, in *pssA1* and *pgsA3* mutants [12,29]. Hirschberg and Kennedy [5] reported an activation of cardiolipin synthesis in crude envelope preparations by CDP-diacylglycerol, which actually accumulates in these mutants [29,30], although CDP-diacylglycerol rather inhibits the activity of highly purified cardiolipin synthase preparations under our assay conditions [31].

Table I

Cardiolipin synthase activities in the exponential and stationary growth phases

Strain	Phospholipid gene	Specific activity (units/mg protein)		
		Exponential (E)	Stationary (S)	S/E
JE5513	Wild	1.6	20.9	13
S101	*cls::kan*	< 0.3*	< 0.3*	–
JP5513	*pssA1*	9.4	29.2	3.1
YA5513	*pgsA3*	5.3	21.1	4.0

*Below the detection limit.

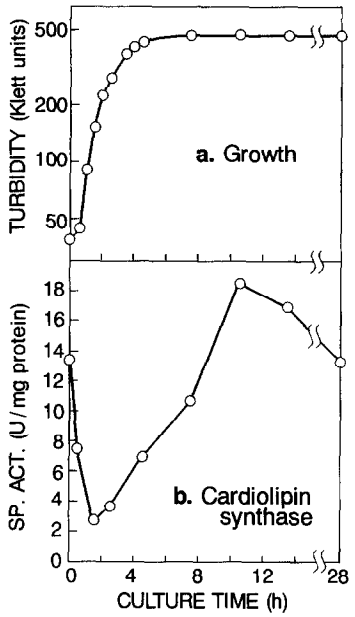

Fig. 1. Growth-phase-dependent cardiolipin synthase activity in wild-type strain, W3110.

In contrast to cardiolipin synthase, activities of three other committed-step enzymes in the phospholipid biosynthetic pathway did not increase but rather decreased in the stationary growth phase (Table II), indicating that cardiolipin synthase is unique in the phospholipid biosynthesis.

To understand the mechanism of phase-dependent change of cardiolipin synthase activity, kanamycin phosphotransferase activities in different growth phases were assayed in a *cls-kan* operon fusion strain. The specific activities (units per mg protein) in strain S101 harvested at Klett 120 (exponential phase), Klett 280 (early stationary phase), and at 6 h after the cessation of turbidity increase (late stationary phase) were 110, 143, and 101, respectively (averages of duplicate measurements). This suggested that the change in the transcriptional expression of the *cls* gene, if not at all, was not large enough to explain the observed changes in cardiolipin synthase activity.

3.2. *Cardiolipin-dependent survival of the cell*

To examine the physiological implication of cardiolipin in the stationary phase, viable cells were counted for prolonged cultures of strain W3110, its isogenic null *cls* mutant S101, and S101 harboring pCL11 (Fig. 2). Day 1 in the figure was 2 h after the cessation of turbid-

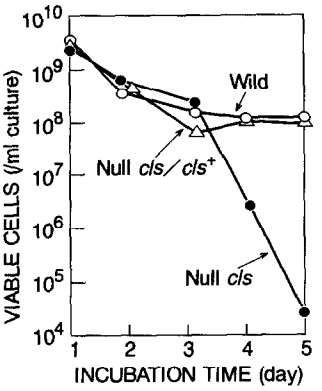

Fig. 2. Cardiolipin-dependent survival of *E. coli* cells. Strains W3110 (○) and S101 (*cls::kan*) (●) were incubated with shaking in LB medium in Monod (L-shaped) tubes at 37°C. S101 harboring pCL11 (△) was incubated under the same conditions, except for the addition of ampicillin in the medium. Starting at 2 h after the cessation of turbidity increase (day 1), aliquotes were withdrawn at intervals for viable cell count.

ity increase. Viable cells in 1 ml of the culture of S101 at day 5 was 10^{-4} of that of W3110. Under these conditions, the viability loss of S101/pCL11 was no more than that of W3110, indicating that the faster death of S101 was due to the lack of cardiolipin synthase. Three repeated experiments gave essentially the same results (data not shown). In W3110 at day 5 under these incubation conditions, the cellular contents of phosphatidylethanolamine and phosphatidylglycerol decreased to 16% and 13% of those at day 1, respectively, whereas 63% of cardiolipin of day 1 remained at day 5, resulting in a phospholipid headgroup composition of 31% phosphatidylethanolamine, 3% phosphatidylglycerol, and 57% cardiolipin. At day 5, the total phospholipid content in S101 was 50% of that in W3110. The results indicated that cardiolipin was more stable than other major phospholipids and this might be related to the lower surviving ability of strain S101.

4. DISCUSSION

The activity of cardiolipin synthase, but not of other phospholipid enzymes, has been found to increase significantly in the stationary growth phase in *E. coli* cells. A special care has been taken to minimize the possible assay errors due to a phase-dependent change in the amount of endogenous phosphatidylglycerol, despite the previous suggestions that the endogenous and exogenous phosphatidylglycerol fractions do not mix [6,14]. The assay method employed here is highly specific for the *E. coli* cardiolipin synthase and much more sensitive than those described previously [14,15]. Although Tunaitis and Cronan previously reported that the cardiolipin synthase activity in the late stationary phase was about half that observed in the exponential phase [6], it does not necessarily conflict with the present result in which we showed a decrease in the synthase activity upon prolonged culture in the stationary phase (Fig. 1).

Table II

Activities of phospholipid enzymes in strain W3110 in the exponential and stationary growth phases

Enzyme	Specific activity (units/mg protein)		
	Exponential (E)	Stationary (S)	S/E
G3P* acyltransferase	0.23	0.10	0.4
Phosphatidylserine synthase	20.6	14.6	0.7
PGP* synthase	12.1	6.8	0.6

*G3P, *sn*-glycerol-3-phosphate; PGP, phosphatidylglycerophosphate.

The active increase in the synthase activity explains, at least partly, the well-known increase in cardiolipin content in the stationary phase. It is possible, however, that the cardiolipin-specific phosphodiesterase, the activity of which has been reported to depend on ATP or the substrate in energetized membranes [32,33], is also involved in the stationary-phase increase of cardiolipin. The actual contribution of this putative catabolic enzyme in the regulation of cardiolipin content remains to be quantitatively examined.

The molecular mechanism underlying the observed change in cardiolipin synthase activity is at present unknown. The transcriptional expression of the *cls* gene, as assessed by the kanamycin phosphotransferase activity in a *cls-kan* operon fusion strain, did not seem to change much during the course of culture. Cardiolipin synthase activities were similar in *pssA1* and *pgsA3* mutants, in which phospholipid compositions are markedly different from that of the wild-type strain, suggesting that the cardiolipin synthase activity was not regulated by the phospholipid environment. Whatever the mechanism is, the active increase in cardiolipin synthase activity appears to be physiologically significant; the *cls* null mutation, without appreciable growth phenotypes [3], caused a remarkably faster loss of viability under the present experimental conditions. Since the lag times necessary before the growth resumption of stationary-phase cells were essentially the same for strains W3110 and S101 (unpublished data), cardiolipin must play a role in survival of the cell during the non-growth period, possibly ensuring the minimum phospholipid content in the membranes and also stabilizing the membrane structures.

With regard to the importance of cardiolipin in the stationary phase, we point out the unique nature of the *E. coli* cardiolipin synthase; its substrate is solely phosphatidylglycerol that amply exists in the membrane. In contrast to this and other procaryotic cardiolipin synthases [4], eucaryotic counterparts catalyze the condensation of phosphatidylglycerol and CDP-diacylglycerol, the reaction apparently more economical to

form cardiolipin. However, the mechanism to utilize only phosphatidylglycerol should be advantageous for procaryotic cells that have to survive in variable and severe environments; CDP-diacylglycerol is maintained at a low and definite level and its synthesis requires a new energy supply [4]. Accordingly, the active increase in cardiolipin synthesis under adverse conditions must be a physiologically significant regulation and *E. coli* appears to have adopted the type of enzyme that allows such a regulation.

Acknowledgements: We acknowledge the participation of Koichi Tanaka in the experiments of Table II and Fig. 2. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

[1] Ioannou, P.V. and Golding, B.T. (1979) Prog. Lipid Res. 17, 279–318.
[2] Hoch, F.L. (1992) Biochim. Biophys. Acta 113, 71–133.
[3] Nishijima, S., Asami, Y., Uetake, N., Yamagoe, S., Ohta, A. and Shibuya, I. (1988) J. Bacteriol. 170, 775–780.
[4] Shibuya, I. (1992) Prog. Lipid Res. 31, 245–299.
[5] Hirschberg, C.B. and Kennedy, E.P. (1972) Proc. Natl. Acad. Sci. USA 69, 648–651.
[6] Tunaitis, E. and Cronan Jr, J.E. (1973) Arch. Biochem. Biophys. 155, 420–427.
[7] Hostetler, K.Y., Galesloot, J.M., Boer, P. and van den Bosch, H. (1975) Biochim. Biophys. Acta 380, 382–389.
[8] Tamai, K.T. and Greenberg, M.L. (1990) Biochim. Biophys. Acta 1046, 214–222.
[9] Chamberlain, B.K. and Webster, R.E. (1976) J. Biol. Chem. 251, 7739–7745.
[10] Cronan Jr., J.E. (1968) J. Bacteriol. 95, 2054–2061.
[11] Cronan, J.E., Jr. and Vagelos, P.R. (1972) Biochim. Biophys. Acta 265, 25–60.
[12] Ohta, A. and Shibuya, I. (1977) J. Bacteriol. 132, 434–443.
[13] DeChavigny, A., Heacock, P.N. and Dowhan, W. (1991) J. Biol. Chem. 266, 5323–5332.
[14] Hiraoka, S., Nukui, K., Uetake, N., Ohta, A. and Shibuya, I. (1991) J. Biochem. (Tokyo) 110, 443–449.
[15] Shibuya, I. and Hiraoka, S. (1992) Methods Enzymol. 209, 321–330.
[16] Bachmann, B.J. (1987) in: *Escherichia coli* and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umbarger, H.E., Eds.) pp. 1190–1219, American Society for Microbiology, Washington.
[17] Hirota, Y., Suzuki, H., Nishimura, Y. and Yasuda, S. (1977) Proc. Natl. Acad. Sci. USA 74, 1417–1420.
[18] Asai, Y., Katayose, Y., Hikita, C., Ohta, A. and Shibuya, I. (1989) J. Bacteriol. 171, 6867–6869.
[19] Nishijima, S. (1992) Ph.D. Thesis, Saitama University, Urawa, Japan.
[20] Nishino, T., Kitamura, E., Matsuzaki, H., Nishijima, S., Matsumoto, K. and Shibuya, I. (1993) Biosci. Biotech. Biochem. 57, 1805–1808.
[21] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
[22] Ohta, A., Obara, T., Asami, Y. and Shibuya, I. (1985) J. Bacteriol. 163, 506–514.
[23] Miller, J.H. Experiments in Molecular Genetics, (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor.
[24] Scheideler, M.A. and Bell, R.M. (1992) Methods Enzymol. 209, 55–63.
[25] Ohta, A., Shibuya, I., Maruo, B., Ishinaga, M. and Kito, M. (1974) Biochim. Biophys. Acta 348, 449–454.
[26] Ohta, A., Waggoner, K., Radominska-Pyrek, A. and Dowhan, W. (1981) J. Bacteriol. 147, 552–562.
[27] Haas, M.J. and Dowding, J.E. (1975) Methods Enzymol. 43, 611–628.
[28] Shibuya, I., Miyazaki, C. and Ohta, A. (1985) J. Bacteriol. 161, 1086–1092.
[29] Miyazaki, C., Kuroda, M., Ohta, A. and Shibuya, I. (1985) Proc. Natl. Acad. Sci. USA 82, 7530–7534.
[30] Kobayashi, T., Ohta, A. and Shibuya, I. (1986) J. Biochem. (Tokyo) 99, 1393–1400.
[31] Hiraoka, S. Ph.D. Thesis, (1992) Saitama University, Urawa, Japan.
[32] Cole, R., Benns, G. and Proulx, P. (1974) Biochim. Biophys. Acta 337, 325–332.
[33] Cole, R. and Proulx, P. (1977) Can. J. Biochem. 55, 1228–1232.