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Amplification and Substantial Purification of Cardiolipin Synthase of *Escherichia coli*

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A simple, specific, and sensitive assay procedure for cardiolipin synthase of *Escherichia coli* has been developed. This measures the radioactivity of glycerol formed from phosphatidyl[2-^{3}H]glycerol and is mainly based on the findings that 400 mM phosphate and 0.015% Triton X-100 markedly activate the enzyme. Cardiolipin synthase was amplified 760-fold upon induction with isopropyl β-D-thiogalactoside in cells harboring a pBR322 derivative in which the *cls* gene encoding this enzyme was preceded by the *tac* promoter. Under these conditions, cardiolipin content increased, membrane potential decreased, spheroplasts became fragile, cells lost viability, and inducer-resistant mutants appeared at a high frequency. The amplification enabled the isolation of an enzyme preparation with a specific activity approximately 10,000-times higher than that of wild-type whole cell lysate. This purification was simply achieved by extraction of the crude membrane fraction with Triton X-100 and a single phosphocellulose column chromatography. This preparation, together with the crude envelope fraction, was used to characterize the basic properties of *E. coli* cardiolipin synthase, some of which were utilized in setting up the assay conditions.

Cardiolipin is one of the major membrane phospholipids in *Escherichia coli* and is unique in its structure among lipids, suggesting that it has specific roles in membrane functions. *E. coli* cardiolipin also has several interesting features in its biosynthesis: (i) it is synthesized from two molecules of phosphatidylglycerol (*1, 2*), in contrast to its eucaryotic counterparts that more economically utilize CDP-diacylglycerol and phosphatidylglycerol (*3, 4*); (ii) its content in membranes changes significantly with growth phase and some cellular conditions (*2, 5*); (iii) despite the ample presence of the substrate phosphatidylglycerol, its synthesis is limited and probably regulated; (iv) its formation in the envelope fraction is strongly stimulated by CDP-diacylglycerol (*1, 6*); and (v) its synthesis is greatly enhanced when phosphatidylethanolamine synthesis is impaired by defects in phosphatidylserine synthase (*7-9*). The biological significance of these phenomena, however, is not understood.

Enzymes responsible for cardiolipin synthesis have not been extensively purified from any organisms, probably because of their low cellular content and hydrophobic nature. This enzymatic activity has been one of the most recalcitrant to study of all phospholipid biosynthetic activities in all species. Therefore, conclusions concerning the reaction mechanisms and properties of the enzymes involved are based on observations made *in vivo* or with crude membrane preparations (*1, 2, 10-12*). To understand the biological implications of various features of cardiolipin biosynthesis at the molecular level, it seemed essential to purify and characterize the responsible enzyme, cardiolipin synthase. To do this, both the establishment of a reliable assay procedure and the amplification of the enzyme by molecular genetic means seemed desirable.

Assay methods for *E. coli* cardiolipin synthase activity have been described (*2, 6, 11*) but none of them seem to be satisfactory for routine use. They yielded specific activities which varied widely for comparable membrane preparations [from 1 (*2*) to 100 (*11*) pmol of cardiolipin formation per min per mg of protein] by laborious procedures requiring TLC (*2*). The lack of a convenient assay has prevented many investigators from pursuing studies on this activity.

An *E. coli* gene, *cls*, was shown to be responsible for cardiolipin synthesis (*13*) and was cloned by complementation of the lethal phenotype of the *cls-1* (formerly *cls*) allele on the *pssA1* (formerly *pss-1*) background under certain culture conditions (*11*). Although rigorous proof is still needed, several lines of circumstantial evidence point to its structural, rather than regulatory, nature (*10, 11*). A 10-fold amplification of cardiolipin synthase was achieved with a pBR322 derivative bearing this gene (*11*) but it was not enough to facilitate purification.

We describe here a useful assay method for *E. coli* cardiolipin synthase which is based on newly found properties of the enzyme. The use of this method and the amplification of the *cls* gene expression by molecular genetic means have enabled us to obtain a highly purified preparation of cardiolipin synthase. We also describe the characteristic nature of the cells overproducing this enzyme.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*E. coli* K-12 derivatives used were JM109 [*recA1 endA1 gyrA96 thi-1 hsdR17*

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Abbreviation: IPTG, isopropyl β-D-thiogalactoside.

relA1 supE44 Δ(lac-proAB)/F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)] (*14*), CB64 (*trp-75 cysB93 tfr-8; E. coli* Genetic Stock Center), and CB64-CLR (*Δcls*::*kan cysB93 tfr-8*) (*10*). Plasmid pPD324 was derived from pCL11, a 9.2-kb derivative of pBR322 carrying the *cls* gene (*11*), by digesting with *Bam*HI and *Bal*31 and ligating with a *Bam*HI linker. By doing this, the genomic region upstream of the *cls* coding sequence was shortened to 0.1 kb, thus eliminating most of another putative coding region present in pCL11 (Asami, Y., Ohta, A., and Shibuya, I., unpublished observation). The 0.27-kb *Bam*HI fragment containing the *tac* promoter (*15*) that had been excised from plasmid pKK223-3 (Pharmacia) was inserted into the unique *Bam*HI site of pPD324. Plasmid pNT6 (5.9 kb) carried the *tac* promoter in front of the *cls* gene in the same orientation.

Culture Conditions—Broth media LB (*16*) and NBY (*12*) and a synthetic M63 medium (*16*) were described previously. They were supplemented with 50 μg per ml of ampicillin and, where indicated, isopropyl β-D-thiogalactoside (IPTG) of up to 0.5 mM. Growth at 37°C was monitored by measuring the turbidity with a Klett-Summerson photometer (green filter). One Klett unit corresponded to approximately 5×10^6 cells per ml. Viable cells were counted by plating diluted cultures on NBY plates containing ampicillin and 1.5% agar.

Cardiolipin Synthase Assay—In an early phase of the study, cardiolipin synthase was assayed essentially by the method of Tunaitis and Cronan (*2*) as modified previously (*11*). This utilized phosphatidyl[^{14}C]glycerol (2,500 cpm/nmol) as substrate and the radioactivity of cardiolipin formed was determined by extraction and separation by TLC. Later, reaction conditions were optimized and a simpler method was developed that determines the radioactivity of released glycerol. In this method, the 200-μl reaction mixture contained 400 mM potassium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 0.015% Triton X-100, 1 mg/ml of bovine serum albumin, 20 μM phosphatidyl[2-^{3}H]glycerol (26,000 dpm/nmol), and an enzyme preparation corresponding to approximately 40 μg of protein in the case of crude membrane fractions or 20 ng of protein in the case of purified preparations. The reaction was terminated after 10 min of incubation at 37°C by adding 1.6 ml of methanol, followed by the addition of 3.2 ml of chloroform and 1 ml of 0.88% KCl containing 0.1% glycerol. After vigorous mixing, two layers were separated by a brief centrifugation. Radioactivity of the aqueous layer was determined in a liquid scintillation spectrometer. One unit of enzyme activity was defined as 1 nmol of glycerol formed in 1 min.

Membrane Potential and Spheroplast Stability—Membrane potential was assessed by measuring the fluorescence quenching by intact cells essentially as described (*17*) by using the dye 3,3′-dipropylthiacarbocyanine iodide (*18*). Strain JM109 harboring either pBR322 or pNT6 was grown to Klett 50 in M63 medium with ampicillin in the presence or absence of IPTG (500 μM) for 2 h. Cells were washed with and suspended in M63 lacking carbon sources to give Klett 50 at 4°C. Fluorescence was measured in a spectrofluorometer (Hitachi model 850) with excitation and emission at 520 nm and 581 nm, respectively. A 2 μl amount of 1 mM fluorescence dye was added to 3 ml of cell suspension in a cuvette with continuous stirring at 37°C followed by an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, at 10 μM. Relative membrane potential was defined as: $100\,[1-(b-a)/(c-a)]$, where a was the fluorescence intensity with cells; b, that with cells and the dye; and c, that with cells, the dye, and the uncoupler.

Spheroplast stability was measured as follows. JM109/pBR322 and JM109/pNT6 were cultured in LB supplemented with ampicillin and IPTG and suspended in 30 mM Tris-HCl (pH 8.0). They were converted to spheroplasts by adding 1.3 mM EDTA, 0.005% lysozyme, and sucrose of various concentrations, and the turbidity at 660 nm was taken as a rough measure of membrane stability.

Other Assays—Phospholipid composition was determined by radioactivity measurements of the lipid spots on two-dimensional thin-layer chromatograms for cells labeled uniformly with ^{32}P, as described (*12*). Phospholipid-phosphorus was assayed by the method of Morrison (*19*), and protein was assayed by the method of Lowry *et al.* (*20*) as modified by Peterson (*21*) with crystalline bovine serum albumin as a standard. Molecular weight assay and quantitation of individual proteins were carried out by SDS-PAGE as described by Laemmli (*22*), followed by staining with Coomassie Brilliant Blue and scanning in a densitometer (model ACD-18, Gelman Sciences, Ann Arbor, MI).

Amplification and Purification of Cardiolipin Synthase—For the routine assay for cardiolipin synthase, the envelope fractions were prepared from 50-ml cultures in 300-ml Erlenmeyer flasks. Strain JM109/pNT6 was grown with vigorous shaking in LB medium supplemented with ampicillin. IPTG was added at Klett 50 to give the concentration of 300 μM and culture was continued for 2 h. Cells were collected by centrifugation, washed with 50 mM Tris-HCl (pH 7.5), and resuspended in 2 ml of 50 mM Tris-HCl (pH 7.5) containing 10 mM β-mercaptoethanol. They were disrupted by sonication, unbroken cells were removed by centrifugation at $3,000\times g$ for 10 min, and the envelope fraction was collected by centrifugation at $39,000\times g$ for 1 h, resuspended in 0.3 ml of the same buffer, and stored frozen. For purification of the enzyme, cells were grown in a jar fermentor (model KRU-2, Kanto Rikaki Mfg., Tokyo) in 4 liters of LB medium supplemented with 0.2% glucose and ampicillin. Cultivation was continued for 4 h after the addition of IPTG. Cells were collected, washed, and suspended in 40 ml of the buffer that additionally contained 20 mM $MgCl_2$ and a few micrograms of DNase I. They were disrupted twice in a French pressure cell operated at 20,000 psi.

For purification, the thawed membrane fraction, 262 mg protein in 9.1 ml, was collected by centrifugation at $39,000\times g$ for 1 h, suspended with a Teflon homogenizer in 26 ml of buffer A, which contained 10 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 1% Triton X-100, and 20% sucrose, and stirred for 1 h on ice. Insoluble material was removed by centrifugation at $145,000\times g$ for 1 h and the supernatant was divided and, if appropriate, stored frozen at −70°C without significant loss of enzymatic activity. Phosphocellulose (Whatman P11) packed in a column (26 mm wide × 135 mm high) was equilibrated with buffer A and charged with 25.5 ml of the supernatant fraction. The column was washed with 1.5 liters of 100 mM potassium phosphate (pH 7.5) containing 10 mM β-mercaptoethanol and 0.2% Triton X-100, then eluted with 600

mM potassium phosphate (pH 7.5) containing 500 mM KCl, 10 mM β-mercaptoethanol, and 0.2% Triton X-100. Eluate was collected in 5-ml fractions and subjected to assays for protein and enzymatic activity. Active fractions, typically fractions 9 through 22 (total 70 ml), were combined, mixed with the same volume of 40% sucrose, and stored frozen at −70°C.

Materials—Restriction enzymes, T4 DNA ligase, *Bal*31 nuclease, and a *Bam*HI linker were purchased from Takara Shuzo, Kyoto and plasmid pKK223-3 was from Pharmacia, Uppsala, Sweden. They were used according to the recommendation of the manufacturers. Ampicillin, IPTG, lysozyme, DNase I, 3,3′-dipropylthiacarbocyanine iodide, and carbonyl cyanide *m*-chlorophenylhydrazone were obtained from Sigma Chemical, St. Louis, MO, U.S.A., precoated silica gel TLC plates were from E. Merck AG, Darmstadt, Germany, and phosphocellulose P11 was from Whatman International, Maidstone, U.K. Polyethylene glycol mono-*p*-isooctylphenyl ether, exactly equivalent to Triton X-100, was purchased from Nacalai Tesque, Kyoto and is described simply as Triton X-100 in this paper. ^{3}H- and ^{14}C-labeled precursors were from the Radiochemical Centre (Amersham, U.K.), and [^{32}P]P_i was from the Japan Radioisotope Association, Tokyo. All other chemicals were reagent grade or better.

Phosphatidyl[2-^{3}H]glycerol and phosphatidyl[^{14}C]glycerol were synthesized enzymatically using *sn*-[2-^{3}H]glycerol 3-phosphate and *sn*-[^{14}C]glycerol 3-phosphate, respectively, and an *E. coli* envelope fraction amplified with phosphatidylglycerophosphate synthase and purified as described previously (*11*). [Acyl-^{14}C]phospholipids were prepared from a *pssA1* mutant SD10 (*12*), grown at 42°C in Pennassay broth containing 20 mM $MgCl_2$, 0.2% glycerol, and 3.5 μmol (6.7 μCi per ml) of sodium [1-^{14}C]-acetate, and purified by one-dimensional TLC with chloroform-methanol-acetic acid (100 : 41.7 : 16.7, v/v). Analysis of these lipids by chemical deacylation and phospholipase A digestion showed that radioactivity was localized exclusively and evenly in both acyl chains.

RESULTS

Characteristics of Cardiolipin Synthase and Its Assay Conditions—To establish a dependable and reasonably simple assay method for cardiolipin synthase, as well as to understand its basic properties, we examined the various factors that might affect the enzymatic activity, by using both the wild-type envelope fraction and an enzyme preparation highly purified as described later. The activity was critically dependent on the Triton concentration with the maximum activation at 0.015% for both enzyme preparations (Fig. 1). At 0.03% of the detergent, the concentration used in the previous assay methods (*2*, *11*), the synthase activity was about one-third of the maximum with the crude envelope fraction and almost completely inhibited with the purified preparation. Cardiolipin synthase activity in purified preparations was significantly enhanced by potassium phosphate with the maximum at 400 mM (Fig. 2A) and by 1 mg/ml of bovine serum albumin (Fig. 2B). The positive effects of these materials had not previously been recognized. The activating effect of phosphate could not be replaced with KCl, suggesting a specific interaction of phosphate with the enzyme; in the presence of 50 mM potassium phosphate, the enzyme activity only gradually increased up to 3-fold by increasing the KCl concentration to 800 mM. The activity was maximum at neutral pH (Fig. 2C), as previously reported (*2*). Also in good agreement with the previous observation for the crude envelope fraction (*2*) was the independence of the activity on metal ions: Mg^{2+} of up to 80 mM had essentially no effect on the activity and EDTA had only a weak inhibiting effect (80 mM EDTA inhibited the activity about 30%). These observations were used to set up the optimum reaction conditions for this enzyme, as described in "MATERIALS AND METHODS."

The previously described assay methods for cardiolipin synthase determined the radioactivity of cardiolipin formed upon separation by TLC (*1*, *2*, *6*). However, ^{32}P-labeled substrates are undesirable because of short half-lives and ^{14}C-labeled substrates were not reliably quantitated in the

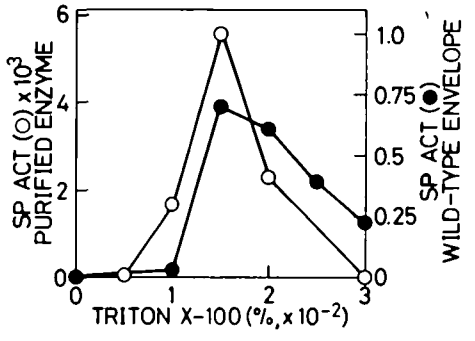

Fig. 1. **Critical dependence of *E. coli* cardiolipin synthase activity on Triton X-100.** The phosphocelluose-purified enzyme preparation (○) and the wild-type envelope fraction (●) were assayed for cardiolipin synthase activity under the optimum assay conditions as described in "MATERIALS AND METHODS," except for Triton concentration. Specific activity (sp act) is the units of enzyme per mg of protein.

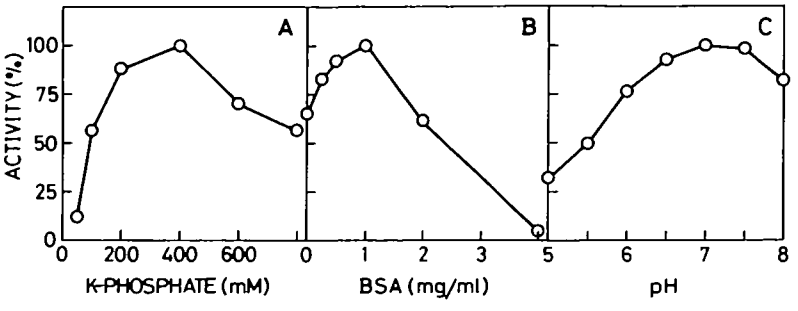

Fig. 2. **Characteristics of the *E. coli* cardiolipin synthase reaction.** Activities were assayed for an enzyme preparation purified by phosphocellulose column chromatography as described in "MATERIALS AND METHODS," except that one component of the reaction mixture was altered as indicated in the *x*-axis of each panel, and expressed as percentages of the maximum values. Panels are: A, activation by potassium (K-) phosphate; B, activation by bovine serum albumin (BSA); and C, pH dependency.

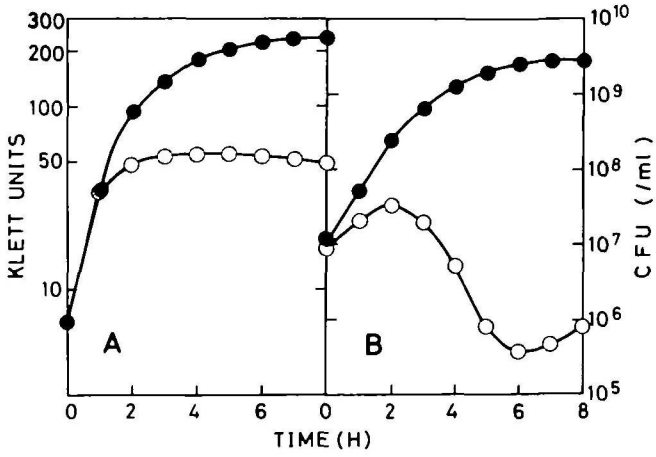

Fig. 3. **Lethal effect of the overproduction of cardiolipin synthase.** Strain JM109 harboring pNT6 was grown overnight in NBY medium supplemented with ampicillin and inoculated at time zero into the same medium in the presence (○) and absence (●) of 500 μM IPTG. Turbidity (panel A) and colony forming units (CFU, panel B) were measured at intervals as described in "MATERIALS AND METHODS."

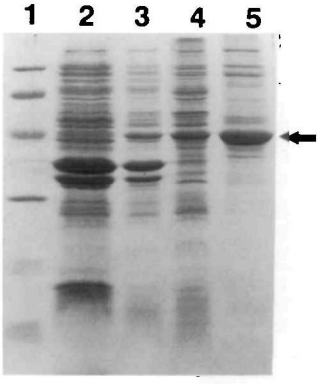

Fig. 4. **Coomassie-stained gels after SDS-PAGE of cell fractions made from strain JM109 harboring pNT6 and the vector pKK223-3.** Cells were grown in Erlenmeyer flasks and treated with 300 μM IPTG as described in "MATERIALS AND METHODS." Lane 1 corresponds to markers of 97.4, 66, 45, 30, and 14.4 kDa. The plasmids, growth conditions, and fractions are: lane 2, whole cell extract with pKK223-3; lanes 3-5, with pNT6; lane 3, the envelope fraction; lane 4, the Triton extract; and lane 5, the phosphocellulose fraction. Each sample contained 30 μg of protein, except for lane 2 which contained 50 μg. The arrow indicates the *cls* gene product of 45 kDa.

TABLE I. **Various levels of cardiolipin synthase activity depending on the expression of the *cls* gene.** The envelope fractions prepared from the cells grown in Erlenmeyer flasks were assayed for cardiolipin synthase as described in "MATERIALS AND METHODS."

Strain	Plasmid	Specific activity[a]	Amplification
JM109	pBR322	0.99	1
	pPD324	21.5	22
	pNT6	30.8	31
	pNT6+IPTG	755	762
CB64	pBR322	1.35	—
CB64-CLR	pBR322	<0.03[b]	—

[a]Units per mg of protein. [b]Below the detection limit.

TABLE II. **Phospholipid composition of strain JM109 harboring various plasmids.** Cells were labeled uniformly with [^{32}P]P_i (90 μCi, 18 μCi/ml) in NBY medium supplemented with ampicillin. At Klett 60, 500 μM IPTG was added to the strains indicated as IPTG+, and culture was continued for 2 h. Lipids were extracted and quantitated as described in "MATERIALS AND METHODS."

Plasmid	IPTG	% of total[a]			
		PE	PG	CL	PA
pBR322	+	78.0	18.6	3.2	0.2
pPD324	+	80.0	12.1	7.5	0.4
pNT6	−	77.8	11.5	9.4	1.3
pNT6	+	80.4	5.4	12.7	1.5

[a]Molar percent of lipid phosphorus calculated from the radioactivity of each spot. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid.

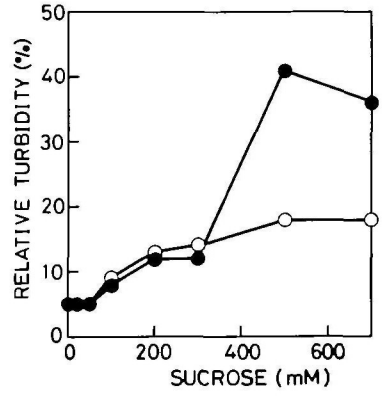

Fig. 5. **Fragility of spheroplasts from the cells amplified with cardiolipin synthase.** Absorbancies at 660 nm of the spheroplast suspensions in various concentrations of sucrose were measured as described in "MATERIALS AND METHODS" and expressed as percentages of those of intact cells. Symbols are; ○, JM109/pNT6 induced with IPTG; and ●, JM109/pBR322.

presence of silica gel (data not shown). To overcome these difficulties, a method to determine the radioactivity of glycerol formed from phosphatidyl[2-^{3}H]glycerol was developed as described in "MATERIALS AND METHODS." This eliminated both the time-consuming process of TLC and the problem of self-adsorption of radioactivity. When [2-^{3}H]glycerol was added in the reaction mixture in place of the substrate, more than 96% of the radioactivity was recovered in the aqueous phase, in accordance with a previous analysis (*23*).

By combining the above two revisions, a new assay method for cardiolipin synthase was obtained ("MATERIALS AND METHODS"). To verify the specificity of the method, the reaction products from [acyl-^{14}C]phosphatidylglycerol and [acyl-^{14}C]cardiolipin in both the lipid and aqueous fractions were examined using the envelope fraction from JM109/pKK-223-3. From 70,000 dpm of [acyl-^{14}C]phosphatidylglycerol, only two spots, 13,000 dpm of cardiolipin and 55,900 dpm of the unaltered substrate, were detected on a two-dimensional autoradiogram. Other areas of the plate showed only 0.4% of the total radioactivity, indicating that other reactions such as the formation of acyl phosphatidylglycerol (*24*) (formerly considered as bis-phosphatidic acid; *2, 25*) were practically absent. With 70,000 dpm of [acyl-^{14}C]cardiolipin, no other spots of more than the detection limit (about 100 dpm) were observed, indicating that our method did not allow phospholipase reactions. The solvent system used is able to separate cardiolipin from other minor acidic phospholipids, including phosphatidic acid and acyl phosphatidylglycerol (*26*). Therefore, the method developed here is useful for the specific assay of *E. coli* cardiolipin synthase.

With a purified enzyme preparation and 70,000 dpm of

TABLE III. **Solubilization and purification of cardiolipin synthase from the amplified cells.** From the induced cells of strain JM109 harboring pNT6 cultured in a jar fermentor, the envelope fraction was prepared, extracted with Triton X-100, and fractionated by phosphocellulose column chromatography as described in "MATERIALS AND METHODS."

Fraction	Total enzyme (units)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Envelope fraction	65,800	262	251	100	1
Triton extract	36,300	54.6	665	55	2.6
Phosphocellulose	26,200	5.46	4,800	40	19

[acyl-^{14}C]cardiolipin, 1,020 dpm of phosphatidylglycerol was formed when 300 mM glycerol was present in the reaction mixture, whereas two spots of 200-300 dpm with relative mobilities of phosphatidylglycerol and phosphatidylmannitol were observed when 300 mM D-mannitol was present. The results suggested that cardiolipin synthase also catalyzes, though weakly under the present assay conditions, the reverse reaction of cardiolipin synthesis with a rather loose substrate specificity, as proposed from *in vivo* observations (*9*).

Amplification of Cardiolipin Synthase—The addition of 200 μM IPTG into a culture of strain JM109 harboring plasmid pNT6 resulted in cessation of increase in the turbidity after 2 h (Fig. 3A). Membrane proteins were analyzed after growth arrest by SDS-PAGE for strain JM109 harboring various plasmids. Accumulation of a protein band corresponding to molecular weight of approximately 45,000 was seen in IPTG-induced JM109/pNT6 (Fig. 4, lane 3). Since this was the only overproduced protein with a molecular weight corresponding to the size of the putative coding region of the *cls* gene (Asami, Y., Ohta, A., and Shibuya, I., unpublished observation), this protein must be the *cls* gene product, CLS. Examination by SDS-PAGE of the culture fluid after the removal of the cells, as well as of the cytoplasmic soluble fraction and cell debris, showed that the CLS amplified by IPTG was present exclusively in the membrane fraction (data not shown). Cardiolipin synthase activity coincided well with the genotypes of the cells (Table I). Synthase was amplified up to 760-fold in the induced cells and was not detected in a *cls* null mutant CB64-CLR by the present sensitive assay, indicating also that the assay method did not detect the putative secondary pathway for cardiolipin formation (*10, 12*). A higher amplification with plasmid pNT6 than with plasmid pPD324 in the absence of IPTG must be a reflection of the basal expression from the *tac* promoter. Amplification of cardiolipin synthase was also attempted with the cells harboring another inducible plasmid in which the lambda P_R promoter was placed in front of the *cls* gene. Upon heat inactivation of the *cI857* product, cardiolipin synthase was amplified to a level similar to that of pNT6 in the presence of IPTG (data not shown).

Cardiolipin content was increased in cells harboring plasmid pNT6 and further after induction with IPTG (Table II). Cardiolipin-phosphorus reached this level 1 h after IPTG addition and did not significantly increase thereafter (15.8% after 4 h). This increase in induced cells must be a concerted result of two causes: *i.e.*, amplification of cardiolipin synthase and the growth cessation which in general causes cardiolipin accumulation by an unknown mechanism (*2, 5*).

Lethal Effect of the Amplification—The addition of IPTG to the culture of JM109/pNT6 gradually arrested the increase in turbidity (Fig. 3A) and cells lost viability rather quickly (Fig. 3B). Addition to the culture medium of sucrose, NaCl, and $MgCl_2$ to the levels that suppress the phenotypes of *pssA1* mutants (*8, 12, 27*) did not remedy the defect. Under these lethal conditions, membrane potential, as assessed by fluorescence quenching, was significantly lowered by amplification of cardiolipin synthase: the ratios of the relative membrane potentials, as defined in "MATERIALS AND METHODS," for JM109/pNT6 without induction and JM109/pNT6 with induction to that for JM109/pBR322 were 0.91 and 0.19, respectively. Spheroplasts from the induced cells were more fragile than those from uninduced cells (Fig. 5). Viable cell counts decreased upon induction of JM109/pNT6 but began to increase after prolonged incubation of an arrested culture (Fig. 3B). When the cells grown in the absence of IPTG were plated onto NBY-agar containing 500 μM IPTG, IPTG-resistant mutants appeared with an approximate frequency of 7×10^{-6}.

Purification of Cardiolipin Synthase—The optimal conditions for the solubilization, purification, and storage of cardiolipin synthase with minimum loss of enzymatic activity were sought. Triton X-100 of concentration 1% and 1 mg per 1 mg membrane protein was found most suited to the solubilization of the synthase among various detergents, including *n*-octyl-β-D-glucoside, deoxycholate, and nonanoyl-*N*-methylglucamide. If solubilized in the presence of 20% sucrose, the synthase could be stored frozen without appreciable loss of activity at least for 3 months. Among the various chromatographic conditions tested, only a phosphocellulose column (Whatman P11) with a stepwise elution using phosphate ion was found effective in purification; other chromatographic procedures, including DEAE-cellulose and gel filtration with Sephacryl S-300 (HR), did not further improve the purity. We therefore set up a procedure for solubilization and purification of cardiolipin synthase as described in "MATERIALS AND METHODS."

As summarized in Table III, cardiolipin synthase was purified 19-fold from the amplified cell envelope to yield a preparation of high specific activity, 4,800, which corresponded to approximately a 10,000-fold increase in the purity of the enzyme in comparison with unamplified whole cells. The level of amplification reached in the cells cultured in a jar fermentor (Table III) was always lower than that in 50-ml cultures in Erlenmeyer flasks with reciprocal shaking (Table I), probably because the aeration was not enough in the former cultures. The final phosphocellulose fraction was subjected to SDS-PAGE (Fig. 4, lane 5) and bands were quantitated by staining and scanning in a densitometer. The density of the major band CLS was approximately 80% of the total protein, although the homogeneity of this band has not been rigorously determined. The active phosphocellulose fraction was used for elucidation of the basic properties of *E. coli* cardiolipin synthase (Figs. 1, 2, and 4;

Tables I and III).

DISCUSSION

Some basic properties of *E. coli* cardiolipin synthase that were relevant to the activity assay and purification were studied. A significant activation of the enzyme by rather high concentrations of phosphate (Fig. 2A) has not previously been recognized and the optimum concentration of Triton X-100 for the activity was found to be lower than that previously employed (*2*). The activity did not depend on magnesium ions, as already described (*2*), in sharp contrast to the eucaryotic counterparts (*4, 28*). These observations facilitated the development of a new assay procedure that is more sensitive than the previous ones; it gave specific activities approximately 1,000-times, 125-times, and 10-times higher than those obtained with comparable wild-type membrane preparations by Tunaitis and Cronan (*2*, Table IV), Cole and Proulx (*6*), and Ohta *et al.* (*11*), respectively. The revisions of the assay procedure also included the measurement of glycerol formed, instead of cardiolipin as in all the previous methods (*1, 2, 6, 11*). This made possible the use of ^{3}H-labeled substrate, which is more desirable than the ^{32}P-labeled substrate, and eliminated the time-consuming process of TLC. Further, we conclude that the present method is highly specific for *E. coli* cardiolipin synthase. It was not influenced by the putative secondary pathway to form cardiolipin (*10, 12*). Tunaitis and Cronan (*2*) reported the formation of another acidic phospholipid from [^{32}P]phosphatidylglycerol, which was later identified as acyl phosphatidylglycerol (*24*), whereas our reaction conditions did not allow the formation of this compound. Phospholipase reactions were also not detected. However, the values obtained with the present method do not necessarily represent the absolute activities of cardiolipin synthase, since we do not know about the behavior of endogenous non-labeled phosphatidylglycerol. Tunaitis and Cronan suggested that the endogenous and exogenous phosphatidylglycerol fractions do not mix (*2*). Further studies are needed to clarify the relationships between the two fractions of phosphatidylglycerol.

The introduction of a powerful promoter, *tac*, in front of the *cls* gene resulted in high-level overproduction of cardiolipin synthase upon induction with IPTG. Under these conditions, cardiolipin content increased substantially (Table II), in contrast to the amplification of other phospholipid synthesizing enzymes that do not influence appreciably the phospholipid composition (*29, 30*). This could be a reflection of the unique reaction of cardiolipin synthase that does not require *de novo* phosphatidyl synthesis (*2*); the substrate phosphatidylglycerol is present in large amounts, whereas other phospholipid enzymes require their substrates to be synthesized *de novo*, probably by regulated reactions. It is also interesting to note that the degree of conversion of phosphatidylglycerol to cardiolipin was not proportional to the level of cardiolipin synthase (Tables I and II). Detailed analysis with the purified enzyme should facilitate the understanding of the underlying mechanism of regulation of this reaction.

Upon addition of IPTG to the culture of JM109/pNT6, cells lost viability quickly. Since the plasmid harbored only the *cls* gene downstream of the *tac* promoter, this lethal effect must have been a specific result of the overproduction of CLS. It was probably caused by the accumulation of synthase molecules in the cytoplasmic membrane, rather than by the increased cardiolipin content, since *E. coli pssA1* (*9, 27*) and a *pssA* null mutant (*7*) are viable even with more elevated cardiolipin contents (more than 30%) under culture conditions that do not allow the *cls*-amplified cells to survive. The observed depression of the membrane potential and reduced spheroplast stability (Fig. 5) also correlated with the inviable phenotype of the induced cells and could have resulted from deleterious accumulation of the overproduced hydrophobic protein in the cytoplasmic membrane. The IPTG-resistant mutants that emerged under otherwise lethal conditions must have overcome or evaded this defect. Except for those with simple down mutations in the promoter region or in the replication of the plasmid, these mutants should be useful in studies to understand the structure-function and structure-topology relationships of a typical membrane-integrated enzyme, as well as the mechanism for the chromosomal suppression of the amplification lethality.

Although we tried various approaches to purify the amplified cardiolipin synthase while retaining the enzymatic activity, only the phosphocellulose column chromatography proved useful. Therefore, the purity of the final preparation was not more than 80%, despite an apparent 10,000-fold purification relative to wild-type extracts. Homogeneous but inactive protein preparations were obtained by further SDS-PAGE which should be useful for sequence analysis. Further analyses of the purified enzyme, though not homogeneous, should facilitate the understanding on the molecular basis of the various characteristic features observed *in vivo* in cardiolipin synthesis.

The notion that the *cls* gene encodes cardiolipin synthase is further supported by the present observations: (i) the 45 kDa protein was the sole amplified band upon induction of pNT6 (Fig. 4), indicating that it is the *cls* gene product; (ii) the extent of amplification, as well as the concentration during purification, of CLS and cardiolipin synthase activity coincided well (Table I and Fig. 4); and (iii) cardiolipin synthase activity was not detected in a *cls* null mutant (Table I). The decisive conclusion of this issue awaits a complete purification of active enzyme molecules or an activity reconstitution from the SDS-PAGE-purified protein band.

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