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Purification and characterization of cardiolipin synthase from *Escherichia coli*

Ragolia, Louis, Ph.D.

City University of New York, 1994

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PURIFICATION AND CHARACTERIZATION

OF

CARDIOLIPIN SYNTHASE

FROM

ESCHERICHIA COLI

by

LOUIS RAGOLIA

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1994

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

PURIFICATION AND CHARACTERIZATION

OF

CARDIOLIPIN SYNTHASE

FROM

Escherichia coli

by

Louis Ragolia

Advisor: Professor Burton E. Tropp

Escherichia coli cardiolipin synthase catalyzes the conversion of two phosphatidylglycerol molecules to cardiolipin and glycerol. This enzyme was amplified in strain BL21(DE3) bearing recombinant plasmid pLR3, which was itself constructed by inserting the *cls* gene downstream from a T7 RNA promoter. Membranes from BL21(DE3)/pLR3 have over 1200 times more cardiolipin synthase activity than do comparable membranes from wild type cells. The enzyme was purified to homogeneity by extraction with Triton X-114 and chromatography on DEAE-cellulose. The purified enzyme

migrated as a single band (46 kD) on SDS-PAGE. This, along with SDS-PAGE analysis of induced protein, supports the notion that *cls* is the structural gene for cardiolipin synthase. Cardiolipin synthase activity was determined in a mixed micelle assay in which phosphatidyl[2-^{3}H]glycerol was the substrate. The enzyme is inhibited by the product of the reaction, cardiolipin, and by phosphatidate. However, it is not inhibited by two other anionic phosphoglycerides, phosphatidylinositol and *bis*-phosphatidate. Phosphatidylethanolamine partially offsets inhibition by cardiolipin but not by phosphatidate. Magnesium chloride has the opposite effect. Cardiolipin inhibition of cardiolipin synthase probably plays an important role in regulating cardiolipin synthesis in *E. coli.*

To Michele, Mom and Dad

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ABBREVIATIONS

(amp)	Ampicillin
(CL)	Cardiolipin
(CDP)	Cytidine-5'-diphosphate
(DAG)	Diacylglycerol
(DBP)	3,4-Dihydroxybutyl-1-phosphonate
(DEAE)	Diethylaminoethyl
(DNA)	Deoxyribonucleic acid
(*E. coli*)	*Escherichia coli*
(EDTA)	Ethylenediaminetetraacetate
(H_{II})	Inverted hexagonal phase
(IPTG)	Isopropyl β-D-thiogalactopyranoside
(kb)	Kilobase
(kD)	Kilodalton
(KOAc)	Potassium acetate
(MES)	2-[N-Morpholino]ethane sulfonic acid
(PAGE)	Polyacrylamide gel electrophoresis
(PE)	Phosphatidylethanolamine
(PG)	Phosphatidylglycerol
(PGP)	Phosphatidylglycerolphosphate
(PS)	Phosphatidylserine
(RNA)	Ribonucleic acid
(SDS)	Sodium dodecyl sulfate
(TCA)	Trichloroacetic acid
(TEMED)	N,N,N',N'-Tetramethylethylenediamine
(TX-100)	Triton X-100
(TX-114)	Triton X-114

TABLE OF CONTENTS

List of Tables

List of Figures

CHAPTER 1

INTRODUCTION

Escherichia coli (*E. coli*), like other gram negative bacteria, are enclosed by two membranes which together comprise the cell envelope (Figure 1). While both layers of the inner (cytoplasmic) membrane consist primarily of phosphoglycerides, the outer membrane has phosphoglycerides on the interior layer and lipopolysaccharide on the exterior. There are approximately 2×10^7 phosphoglyceride molecules per *E. coli* cell, of which about 75% are phosphatidylethanolamine (PE), 20% are phosphatidylglycerol (PG), 5% are cardiolipin (CL), and 0.6% are other minor lipids.[1] The structures for the three major phosphoglycerides are given in Figure 2. Application of molecular genetic techniques and colony autoradiography have provided phosphoglyceride biosynthetic mutants which have in turn facilitated the elucidation of the phosphoglyceride biosynthetic pathway (Figure 3).[2] Since there are no internal organelles, and all of the phosphoglycerides are confined to the cell envelope, *E. coli* represents a relatively simple system and an attractive organism for exploring phosphoglyceride metabolism.

Individual lipid function, transbilayer movement, intermembrane translocation, and biosynthetic regulation of phosphoglycerides still remain unclear. Cell growth ceases when the total phosphoglyceride level is decreased by about 30%, suggesting that phosphoglycerides are essential and are not present in a large excess.[3] Wild type *E. coli* will maintain a constant phosphoglyceride composition in response to most changes in environment. Two exceptions include the decreased amount of unsaturated fatty acids present in cells cultured at an elevated temperature and the increased CL level present in cell envelopes at stationary phase.[1,4] Mutants exhibit changes in their phosphoglyceride composition when specific phosphoglyceride biosynthetic enzymes are deleted.[5,6] Overproduction of biosynthetic enzymes does not usually result in a proportional increase of the corresponding phosphoglyceride.[7,8]

The structural roles phosphoglycerides play, maintaining cell membrane integrity and functioning as a permeability barrier, are well known and accepted.[9] Why, however, are the three major phosphoglycerides of *E. coli* present in specific proportions? For that matter, why is there more than one type of phosphoglyceride at all? What is the explanation for such an enormous chemical diversity among the minor phosphoglycerides and why

are most biosynthetic mutants conditionally lethal? All of these questions suggest that there must be other important biological functions, besides structural, for these phosphoglycerides. Recently, some additional biological functions for phosphoglycerides have been demonstrated. In *E. coli*, PG is involved in the translocation of proteins across membranes[10] and both PG and CL are involved in binding the dnaA protein.[11,12] In eukaryotes, phosphatidylinositol linked *sn*-1,2-diacylglycerol[13] is involved in signal transduction with protein kinase C, platelet activating factor is a phosphatidylcholine variant,[14] and the reversal by PG and CL of the inhibition of transcription and replication by histones has been observed *in vitro*.[15]

The cell envelope requires a proper balance of fluid and non-fluid hydrocarbon chains within the phosphoglycerides of the bilayer.[16] Almost all membranes contain phosphoglycerides which can form non-lamellar structures such as inverted hexagonal phase (H_{II}). Temporary sites of non-lamellar structure have been linked to membrane fusion, cell division, and transbilayer movement of lipids as well as proteins.[17] It is known that divalent cations induce H_{II} formation in CL model systems.[17]

Cardiolipin (diphosphatidylglycerol), a major anionic phosphoglyceride in the *E. coli* cell envelope, has a unique structure (Figure 2) and its chemistry and biomembrane function have been reviewed.[18] The presence of four hydrocarbon chains makes CL extremely hydrophobic. The distance between phosphorus atoms is approximately equal to the distance between phosphorus atoms within a DNA molecule.[18] In eukaryotes almost all of the CL in the cell is within the inner mitochondrial membrane.[18] Many proteins involved in oxidative phosphorylation have high-affinity binding sites for CL.[19,20] It is believed that CL may surround these proteins and act as a buffer for protons (Thomas Haines, personal communication).

Mutants affecting biosynthetic enzymes have been isolated along the phosphoglyceride biosynthetic pathway (Figure 3). Mutations in CL synthase, phosphatidylserine (PS) synthase, and phosphatidylglycero-phosphate (PGP) synthase, all perturb the CL level.[21]

The first *E. coli* mutant defective in CL synthesis was isolated by comparing the lipid distributions of temperature sensitive mutants.[22] Further analysis revealed that the temperature sensitive phenotype and the defect in CL synthesis were independent mutations. Resistance to 3,4-dihydroxybutyl-1-phosphonate (DBP), a glycerol-3-phosphate analog, was used to isolate

another CL deficient mutant.[23] Although both mutants had more than a 5-fold decrease in CL level, CL was still being synthesized. This could only be true if the mutations were leaky or if CL was being synthesized by an alternate pathway.

The gene responsible for the defect in CL synthesis, *cls*, maps at minute 27 of the *E. coli* genetic map.[24] The *cls* gene was cloned by Ohta *et al.*[24] and later by our laboratory. *E. coli cls* null mutants have been isolated after disrupting the *cls* gene by either replacement with or insertion of a kanamycin-resistant gene, followed by a homologous recombination with the chromosome. These *cls* null mutants continue to grow normally and synthesize low levels of CL, even though all CL synthase activity is absent.[25] The alternate route for CL synthesis is attributed to PS synthase,[25] which may synthesize small amounts of CL from CDP-diacylglycerol and PG by a mechanism similar to that used by eukaryotes. CL seems to offer a growth advantage at alkaline pH[26] and cells lacking *cls* have a slightly longer doubling time,[25] and exhibit increased resistance to DBP.[23]

How is the CL level regulated in *E. coli*? It seems likely that CL synthase is under some form of regulation since *E. coli* have a reserve of the substrate, PG. An approximate 2.5-fold increase in *cls* gene expression, as

well as an increase in the CL to PG ratio, is observed as cells progress from early to late log phase under aerobic conditions.[27] Cells bearing the *cls* gene on a high copy number plasmid have about ten times more CL synthase than do wild type cells. However, the presence of this additional CL synthase has only a very slight influence on the CL level.[24] These observations raise questions about the physiological significance of changes in *cls* expression and suggest that CL synthesis is regulated at the enzymatic level.

In *E. coli* the enzyme responsible for CL synthesis, CL synthase, catalyzes phosphatidyl group transfer from one PG molecule to another accompanied by the release of glycerol[28,29] (Figure 4). This mechanism is different from the eukaryotic system in which CL is synthesized from CDP-diacylglycerol and PG.[30]

Synthesis of novel phosphoglycerides has also been attributed to CL synthase.[31] It appears that CL synthase has slight activity in the reverse direction and a relaxed substrate specificity. At high concentrations, CL synthase will recognize certain straight-chain sugar alcohols (ie. mannitol), resulting in the synthesis of phosphatidyl, and diphosphatidyl derivatives of these sugar alcohols.[31] This ability is lost in the absence of a functional *cls* gene.[31]

The *pss* gene, encoding PS synthase, catalyzes the synthesis of PS from CDP-diacylglycerol and *L*-serine.[7,32] A temperature sensitive *pss* mutant, *pss*-1, grows at non-permissive temperatures only when the medium is supplemented with Na^+, K^+, NH_4^+, Mg^{2+} or sucrose.[5] Under these conditions the phosphoglyceride distribution is drastically altered. The PE level decreases by approximately 2-fold and the CL level increases about 3-fold.[5] If a *cls* mutation is introduced into a strain with a *pss* mutation the double mutant will no longer grow at the non-permissive temperature, even when the medium is supplemented with Na^+, K^+, NH_4^+, Mg^{2+} or sucrose, and will synthesize even less CL than do *cls* null mutants.[33]

PGP synthase catalyzes the synthesis of PGP from CDP-diacylglycerol and glycerol-3-phosphate.[34,35] Mutants in the *pgsA* gene, encoding PGP synthase, have been isolated and characterized.[6,34, 37] When the phosphoglycerides are isolated from a *pgsA* mutant, PE constitutes approximately 95% of the total phosphoglyceride while PG and CL make up about 2% of the cellular phosphoglycerides.[35] When the PGP synthase level is approximately 10% of the wild type level, cell growth ceases.

CL synthase, as with many other biosynthetic phosphoglyceride enzymes, is located in the crude membrane fraction. The purification of CL

synthase has been difficult for a number of reasons: (i) the low level of CL synthase in the cell; (ii) the hydrophobic nature of the enzyme; and (iii) the CL synthase substrate is itself a phosphoglyceride.

Hiraoka *et al.* have partially purified CL synthase from *E. coli* and examined some of its enzymatic properties.[36] CL synthase is a 46 kD protein which is stimulated by high phosphate, Triton X-100 (TX-100) and bovine serum albumin (BSA). However, their results do not explain how CL synthase is regulated. To study the enzymatic regulation, a homogeneous preparation and a defined assay system were desired.

The present study reports the amplification and isolation of homogeneous CL synthase. Furthermore, the influence of various phosphoglycerides on the pure enzyme were examined. An initial step in the purification of CL synthase was the amplification of the enzyme. The *cls* gene was subcloned into an over-expression vector, pET3.[37] Plasmid pET3, containing the T7 RNA promoter, will actively and selectively transcribe any gene inserted downstream of the T7 promoter. T7 RNA polymerase is supplied by the host strain, BL21(DE3), and is under the control of a lacUV5 promoter. Therefore, when induced with isopropyl β-D-thiogalactopyranoside (IPTG), BL21(DE3) synthesizes T7 RNA polymerase which binds to the T7

promoter and transcribes the *cls* gene.

Since CL synthase is associated with the cell membrane it needs to be solubilized. Detergents are commonly used to solubilize membrane enzymes.[38] Triton X-114 (TX-114) was chosen because of its low cloud point. At a temperature of about 30°C TX-114 will form aggregates including hydrophobic proteins. These aggregates are easily pelleted by centrifugation and, after resuspension, usually result in a good purification step. Once soluble, the mixture of hydrophobic proteins were chromatographed on a DEAE-cellulose column and a homogeneous preparation obtained. This pure enzyme preparation was used to study CL synthase regulation.

CHAPTER 2

MATERIALS AND METHODS

Chemicals: Ampicillin; isopropyl β-D-thiogalactopyranoside (IPTG); acrylamide; *bis*-acrylamide; bovine serum albumin (BSA) (Fraction V powder); trichloroacetic acid (TCA); Sepharose CL-6B; Triton X-114; DEAE cellulose; glycerokinase (from *Escherichia coli*); carbonic anhydrase; egg albumin; N,N,N',N'-Tetramethylethylenediamine (TEMED); cardiolipin (CL) from *E. coli*, sodium salt; *L*-α-phosphatidyl-*DL*-glycerol (PG) from egg yolk lecithin, sodium salt; *L*-α-phosphatidylethanolamine (PE) from *E. coli*; *L*-α-phosphatidic acids (PA): dipalmitoyl (C16:0), distearoyl (C18:0), dioleoyl (C18:1) [cis]-9, and dimyristoyl (C14:0), all sodium salts; *L*-α-phosphatidylinositol (PI) from soybean, sodium salt; *L*-α-phosphatidyl-*L*-serine (PS), dipalmitoyl; adenosine-5'-triphosphate (ATP); adenosine 5'-diphosphate (ADP); adenosine-5'-monophosphate (AMP); cytidine-5'-diphosphate (CDP); 2-[N-morpholino]ethanesulfonic acid (MES); Folin & Ciocalteu's phenol reagent, 2.0 N; bromophenol blue; deoxycholic acid,

sodium salt; n-octyl-β-D-glucopyranoside; Tween-80; sodium cholate; and *DL*-α-glycerophosphate, disodium salt (hexahydrate) were purchased from the Sigma Chemical Co., St. Louis, MO. Cytidine 5'-diglyceride (CDP-DAG), dipalmitoyl and *bis*-phosphatidic acid (*bis*-PA), tetrapalmitoyl were purchased from Serdary Research Laboratories, Ontario, Canada. Bacto-tryptone, Bacto-yeast extract, and Bacto-agar are products of Difco Laboratories, Detroit, MI. Agarose, low melting point agarose, ethidium bromide, sodium dodecyl sulfate (SDS), restriction endonucleases, restriction endonuclease buffers, T4 DNA ligase and buffer, were acquired from BRL, Gaithersburg, MD or IBI, New Haven, CT. Triton X-100 and calf-intestine alkaline phosphatase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. GENECLEAN® kit was purchased from BIO 101 Inc, La Jolla, CA. [^{3}H]Glycerol and [^{35}S]methionine were obtained from New England Nuclear, Wilmington, DE. Carrier free [^{32}P]phosphate is a product of ICN, Irvine, CA. FAST STAIN® was purchased from Zoion Research Inc., Allston, MA. Polygram® Sil G thin-layer chromatography plates were obtained from Brinkmann Instruments, Inc, Westbury, NY. All other chemicals were reagent grade or better.

Media and Growth conditions: M9ZB broth[37] consists of 0.1% ammonium chloride, 0.3% potassium phosphate, 0.6% sodium phosphate, 1.0% bacto-tryptone, and 0.5% sodium chloride. Sodium hydroxide was added to adjust the pH to 7.4, and after autoclaving, the broth was brought to 0.4% glucose, and 1.0 mM magnesium sulfate. The medium was supplemented with 200 μg/ml of ampicillin, and where indicated, IPTG was added to 0.8 mM. LB broth[39] consists of 1.0% bacto-tryptone, 0.5% yeast extract, and 0.5% sodium chloride. Cell growth was monitored by measuring turbidity with a Klett-Summerson photometer (red filter). One Klett unit corresponds to approximately 5 x 10^6 cells/ml.

Bacteria and Plasmids: The *E. coli* strains used are described in Table 1. Strain BL21(DE3) and the pET vector were a gift from William Studier. BL21(DE3) has the gene for T7 RNA polymerase under control of a lacUV5 promoter. When this strain is induced with 0.8 mM IPTG, the bacteria produces T7 RNA polymerase. HB101, a highly competent strain, will transform at a high frequency,[40] and was used as a host to accept constructed plasmids.

Table 2 describes all of the plasmids used in this study. Plasmid pET3 allows transcription of a gene inserted between the T7 promoter (ϕ10) and

the transcription terminator (Tϕ). A gene inserted after the strong ϕ10 promoter will be minimally transcribed by *E. coli* RNA polymerase but selectively and actively transcribed by T7 RNA polymerase.[37]

Plasmid pPGL2019, contains the pgsA gene, for PG synthase,[8] and was used to convert [2-^{3}H]glycerol-3-phosphate and CDP-diacylglycerol to phosphatidyl[2-^{3}H]glycerol.

Construction of Cardiolipin Synthase Overproducer: Plasmid pSH111, containing the cloned *cls* gene, was isolated from HB101/pSH111 by the SDS-alkaline lysis method.[41] Cells were cultured in 5 ml of M9ZB broth, supplemented with 200 μg/ml ampicillin, shaking at 250 rpm overnight at 37°C. The following morning, 1.5 ml of cells were harvested, resuspended in 100 μl of GTE buffer (50 mM glucose, 25 mM Tris-HCl, and 10 mM EDTA; pH 8.0), and incubated in a microfuge tube at room temperature for 5 min. Then, 200 μl of freshly prepared NaOH/SDS (1.0% SDS in 0.2 N NaOH) were added, and the microfuge tube was inverted 6 times. The tube was incubated on ice for 5 min, after which time 150 μl of KOAc buffer (prepared by mixing 60 ml of 5 M KOAc, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water) at 4°C was added. The mixture was incubated on ice for 5 min. and then centrifuged for 5 min at 4°C. The supernatant was

divided into two fractions containing 300 µl, and each was transferred to a fresh microfuge tube. After adding 300 µl of CPI (chloroform: Tris-buffered phenol: isoamyl alcohol [24:25:1]), tubes were inverted ten times, and then centrifuged for 4 min at room temperature. Being careful not to include any interface, the top aqueous layer was transferred to a fresh microfuge tube, and 600 µl of absolute ethanol were added. The tube was inverted 10 times and incubated for 2 min at room temperature. After a 5 min centrifugation, the supernatant was removed and the tubes were inverted on a paper towel to remove any remaining solvent. A 1.0 ml solution of 70% ethanol was added to the precipitate, followed by agitation with a vortex mixer, and another 5 min centrifugation. The supernatant was discarded and the tubes were inverted on a paper towel. The pellet was dried briefly in a Savant condenser under vacuum, and resuspended in 50 µl of TE buffer (10 mM Tris-Base, 1.0 mM EDTA; pH 8.0), containing 20 µg/ml boiled RNAse. Plasmid DNA was stored at -20°C.

Plasmid pSH111 has a BamHI site on only one side of the *cls* gene. A BamHI site had to be introduced on the other side of *cls* before the gene could be introduced into the pET3 vector. The second BamHI site was derived from the polylinker in pIBI20 (Figure 5). Restriction endonuclease

digests, typically 20 µl, contained approximately 1-10 µg DNA (as determined by ethidium bromide staining of DNA standards), and were incubated, at 37°C for 2 h, in restriction endonuclease buffer which contained: 25 mM Tris-HCl; (pH 7.8), 10 mM $MgCl_2$, 100 µg/ml BSA, 2 mM β-mercaptoethanol, and depending upon which restriction enzyme was being used, either 50 mM or 150 mM NaCl. These buffers were supplied by the manufacturer at 10X. The reactions were terminated with the addition of 2 µl of EndoR stop buffer, which contained: 0.5 M EDTA (pH7.5), 0.025% bromophenol blue, 0.025% xylene cyanol, and 40% sucrose. Plasmid pSH111 was digested with a combination of 10 units of PvuII and 10 units of EcoRI, while pIBI20 was digested with a combination of 10 units of EcoRV and 10 units of EcoRI. To prevent self ligation of pIBI20, 1.0 unit of calf-intestine alkaline phosphatase was incubated with the digested plasmid for 30 min at 37°C prior to the addition of EndoR stop buffer. The alkaline phosphatase was inactivated by heating the mixture at 65°C for 45 min prior to phenol extraction (see below). After electrophoresis, the 2.2 kb fragment from pSH111 was cut out of the low melting point agarose and purified using GENECLEAN® (see below). This fragment, was ligated to digested pIBI20 (3:1, insert:vector) at room temperature for 2 h, in a 20 µl

reaction mixture containing T4 DNA ligase buffer (25 mM Tris-HCl (pH 7.6), 10 mM magnesium chloride, 1.0 mM dithiothreitol, 0.4 mM ATP), and 2 units T4 DNA ligase to form pLR111. HB101 was transformed (see below) with pLR111, and spread on selective M9ZB plates containing ampicillin. After amplification, pLR111 was isolated by the SDS-alkaline lysis method and the correct construction was verified by restriction endonuclease digests. Plasmid pLR111 was digested with BamHI, and the fragments produced were separated by electrophoresis. The 1.8 kb fragment, containing *cls*, was excised from soft agarose and purified by GENECLEAN®. This 1.8 kb fragment was ligated to a BamHI digested pET3 plasmid, by the same procedure as above, to form pLR3. Insertion in the correct orientation was verified by analysis of restriction endonuclease digests.

Phenol Extraction: Phenol extraction was used to purify DNA. One volume of water-saturated phenol was added to a DNA sample and the mixure inverted several times. After a brief centrifugation the aqueous (upper) phase was removed without disturbing the interface. This procedure was repeated several times until no material appeared at the interface. Then, one-half volume of 7.5 M ammonium acetate was added along with two

volumes of 95% ethanol. After a 30 min incubation at -70°C, the material was centrifuged and the pellet resuspended in 3 M ammonium acetate. Two volumes of cold 95% ethanol were added and the mixture centrifuged for 5 min. Tubes were inverted on a paper towel until dry, and the pellet resuspended in the desired buffer.

Agarose Gel Electrophoresis: Agarose gel electrophoresis was used to separate DNA fragments. Agarose (0.8%) or low melting point agarose (1.0%), was prepared in either TAE buffer (40 mM Tris-base (pH 8.0), 20 mM sodium acetate, 2 mM EDTA) for overnight gels (15 volts) or TBE buffer (80 mM Tris-base (pH 8.0), 40 mM boric acid, 2mM EDTA) for overday gels (50 volts). The agarose gels were stained with 5 μg/ml ethidium bromide in gel buffer for 20 min and destained in tank buffer for 30 min. Photographs of the stained gels were scanned with a Model M-6000 CG (Marstek Inc, Irvine, CA) hand scanner.

DNA Purification: Plasmid DNA fragments were purified using a commercially available GENECLEAN® kit. DNA was excised from the gel using a razor blade under long wave UV light and transferred into a microfuge tube. Approximately 2-3 volumes of saturated sodium iodide solution was added and the tube was placed in a 50°C water bath. The

microfuge tube was inverted every minute until the gel was fully dissolved. A glassmilk bead suspension, provided by manufacturer, (5 µl for the first 5 µg of DNA and 1.0 µl for every 0.5 µg thereafter) was mixed with the solution and the suspension was incubated at 4°C for 5 min to allow the DNA to bind to the glassmilk. Then, the matrix, including the bound DNA, was centrifuged for 5 s and washed with 300 µl of saturated sodium iodide. The tube was again placed at 50°C for 3 min, followed by a brief centrifugation. The pellet was washed three times with 500 µl of ice cold NEW WASH, using care to remove all of the supernatant after the third wash. DNA was eluted from the glassmilk by adding 300 µl of TE buffer, centrifuging, and collecting the supernatant. After washing the glassmilk once more with 150 µl of TE buffer, the supernatants, containing the DNA, were pooled and stored at -20 °C.

DNA Transformation: Plasmid DNA was introduced into bacteria by a low efficiency, colony transformation procedure.[40] Several colonies were picked off a petri plate with a sterile toothpick and dispersed in 200 µl of TFB buffer (a filter sterilized solution containing 10 mM MES (pH 6.3), 45 mM manganese chloride, 10 mM calcium chloride, 100 mM potassium chloride, and 3 mM hexamine cobalt chloride; $Cl_3CoH_{18}N_6$) at 4°C. The cells

were vortexed vigorously and incubated on ice for 10 min. Approximately 10 μg of plasmid DNA (1 μg/μl) were added to the cell suspension and incubated on ice for 10 min. The suspension was then heat shocked at 37°C for 90 s, followed by the addition of 1.0 ml of LB broth. After a 90 min outgrowth, at 37°C, the cells were centrifuged briefly, resuspended in 0.1 ml of fresh LB broth, and spread on a plate with selective media.

SDS-PAGE: A discontinuous buffer system as described by Laemmli[42] was used to qualitatively monitor cardiolipin synthase purification, and to determine molecular weight. The polyacrylamide gel was cast in two stages. To cast the resolving gel: An acrylamide stock solution was made containing 30% (w/v) acrylamide and 2.7% (w/v) bis-acrylamide. Then, 15 ml of this acrylamide stock solution, was added to 11.25 ml of 1.5 M Tris-base (pH 8.8), 18 ml of distilled water, and 450 μl of a 10% SDS. The solution was polymerized with the sequential addition of 225 μl 10% ammonium persulfate, and 40 μl TEMED. The acrylamide gel was quickly pipetted onto a Hoeffer Scientific Instruments, San Francisco, CA, Vertical Slab Gel Unit, leaving about 5 cm of space for the stacking gel. Distilled water was carefully layered onto the top of the solidifying gel. Once solidified, the polyacrylamide resolving gel could be used immediately or stored at room

temperature for a few days. On the day of the experiment the stacking gel was cast on top of the resolving gel and contained: 1.33 ml of the acrylamide stock solution, 2.5 ml of 0.5 M Tris-HCl (pH 6.8), 6.1 ml of distilled water, and 0.1 ml of a 10% SDS solution. This solution was polymerized with the sequential addition of 50 µl of 10% ammonium persulfate and 20 µl of TEMED. Tank buffer contained 0.025 M Tris-base (pH 8.3), 0.1% SDS, and 0.192 M glycine. One volume of sample was added to one volume of a 2X buffer containing 0.125M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.025% bromophenol blue. The combined mixture was placed in a boiling water bath for 5 min, cooled, and then loaded on the gel. A typical sample was 50 µl and contained between 10-100 µg of protein. Electrophoresis was preformed either overnight or overday. When the electrophoresis was preformed overnight, 55 V was maintained for both the stacking and the resolving gels. Alternatively, when the electrophoresis was preformed overday, 125 V was maintained for the stacking gel, and then shifted to 200 V for the resolving gel. The power pack was from Buchler Instruments Inc, Fort Lee, NJ. In order to remove detergent, the gel was rinsed three times with 300 ml of a solution which was 45% (v/v) in methanol and 10% (v/v) glacial acetic acid. Each rinse

was for 20 min with gentle shaking. The gel was stained with 150 ml of FASTSTAIN® for 20 min and destained with multiple washings of 10% (v/v) aqueous glacial acetic acid.

[^{35}S]Methionine Pulse Labeling and SDS-PAGE of Whole Cells: BL21(DE3)/pLR3 was incubated with [^{35}S]methionine so that the molecular weight of the overproduced protein could be determined. A 5 ml culture of BL21(DE3)/pLR3 and BL21(DE3)/pET3 in M9ZB broth, supplemented with ampicillin (200 μg/ml) was incubated at 37°C with shaking at 250 rpm, until the turbidity reached 200 Klett units. Then, the indicated cultures were made 0.8 mM in IPTG to induce T7 RNA polymerase formation. After a 30 min incubation, normal *E. coli* mRNA synthesis was inhibited by adding 200 μg/ml rifampicin to the indicated cultures for 5 min. Then, 200 μl of cells were removed from the culture and transferred to a sterile microfuge tube. These cells were pulse labeled for 5 min with 4.0 μCi of [^{35}S]methionine (1180 Ci/mmol), centrifuged for 30 s, and resuspended in 200 μl of PAGE buffer containing (50 mM Tris-HCl (pH 6.5), 2 mM EDTA, 1% β-mercaptoethanol, 1% SDS, 8% glycerol, 0.025% bromophenol blue). This suspension was then placed in a boiling water bath for 5 min, and loaded on an SDS polyacrylamide gel (see below).

After electrophoresis, the polyacrylamide gel was dried using a BIORAD®, Melville, NY-model 535 gel drier, set at 80°C for 2 h. under vacuum. Figure 6 illustrates the relative positions of the different layers within the drying system. A sheet of Kodak® XAR film was placed over the dried gel and was exposed for 1 week.

Lipid Analysis of BL21(DE3)/pLR3: The lipid distribution in cells harboring plasmid pLR3 was determined with and without IPTG induction. A 5 ml culture of either BL21(DE3)/pLR3 or BL21(DE3)/pET3 in M9ZB broth, supplemented with ampicillin, was incubated at 37°C with shaking. At approximately 40 Klett units, IPTG was added to 0.8 mM, along with 5 μCi/ml of carrier-free [^{32}P]phosphate, and growth continued for 2 h. Cells were harvested by centrifugation and resuspended in 1.0 ml of distilled water. Phospholipids were isolated as previously described[23], and separated by one-dimensional thin layer chromatography on Polygram® Sil G plates. The solvent system used to separate lipids was chloroform-methanol-acetic acid (65:25:8).[43] The R_f values of the major lipids are PE (0.69), PG (0.78), and CL (0.87). Radioactive spots were identified by autoradiography, and quantified by cutting radioactive spots from the TLC plate and counting them in an ISOCAP® 300 liquid scintillation counter.

Amplification of Cardiolipin Synthase: For enzyme purification, BL21(DE3)/pLR3 was cultured in 400 ml of M9ZB broth supplemented with 200 μg/ml of ampicillin, shaking (250 rpm) at 37°C on the floor shaker. When the culture reached a turbidity of approximately 150 Klett units, IPTG was added to a final concentration of 0.8mM and cells were incubated for an additional 3 h. Unless stated otherwise all further procedures were performed at 4°C. Cells were harvested by centrifugation in an SS-34 rotor at 5000x*g* for 5 min, washed once with 0.1 M Tris-HCl (pH 7.8) containing 5.0 mM EDTA and 10 mM β-mercaptoethanol, and resuspended in 200 ml of the same buffer. The cells were then disrupted by sonication using a model-W140 (Heat Systems-Ultra Sonics Inc, Plainview, NY) sonicator with six 30 s pulses, at a setting of 8, pausing 15 s between pulses. Unbroken cells and cell debris were removed by centrifugation at 5000x*g* for 5 min. The supernatant was collected and centrifuged in a Sorvall RC80 ultracentrifuge fitted with a T865 rotor, at 150,000x*g* for 1 h. The pellet, crude membrane, which contained approximately 36 mg of protein,[44] was stored frozen at -70°C with no appreciable loss of enzymatic activity for at least 6 months.

Protein Expression vs. Time of IPTG Induction: In order to determine the amount of time which would yield the maximum amount of cardiolipin synthase, activity was measured at different intervals of IPTG induction. Strain BL21(DE3)/pLR3 was cultured at 37°C in 20 ml of M9ZB supplemented with 200 μg/ml ampicillin, shaking at 250 rpm. At a turbidity of 150 Klett units, IPTG was added to 0.8 mM and 2 ml aliquots were removed every hour, for 5 h. An aliquot was also removed at the time of IPTG induction for comparison.

Once the cells were removed from the culture, they were washed with 2 ml of Buffer I which contained: 100 mM potassium phosphate (pH 7.5), and 10 mM β-mercaptoethanol. The cells were resuspended in 2 ml of the same buffer and disrupted by sonication using a Model-W140 (Heat Systems-Ultra Sonics Inc, Plainview, NY) sonicator with six 30 s pulses, at a setting of 8, pausing 15 s between pulses. Unbroken cells and cell debris were removed by centrifugation at 5000x*g* for 5 min. The supernatant was collected, and centrifuged in a Sorvall RC80 ultracentrifuge fitted with a T865 rotor, at 150,000x*g* for 1 h. The resulting pellets were assayed as described below.

Protein Determination: Protein concentration was determined by a modification of the Folin-Ciocalteu reagent method as given by Lowry *et al.* The procedure, modified by Peterson, permitted the analysis of very dilute protein solutions and overcame the problems associated with interfering substances.[44]

Stock Reagents: [1]Copper-tartrate-carbonate (CTC); A 250 ml solution of 20% sodium carbonate was added slowly with stirring to a 250 ml solution which was 0.2% (w/v) copper (II) sulfate (pentahydrate) and 0.4% tartrate to give final concentrations of 0.1% copper (II) sulfate, 0.2% potassium tartrate, and 10% sodium carbonate. This solution is stable for at least 6 months at room temperature. [2] 10% sodium dodecyl sulfate (SDS). [3] 0.8 N Sodium hydroxide. [4] 2.0 N Folin-Ciocalteu reagent.

Standard assays contained between 1-80 μg of protein in a total volume of 1.0 ml. The first step was to add 0.1 ml of 0.15% deoxycholate to each tube. Then, tubes were vortexed, and allowed to stand for 10 min at room temperature. Next, 0.1 ml of 72% TCA was added, the tubes were vortexed, and then centrifuged at 3000xg for 15 min. The supernatants were discarded and the tubes inverted on paper towels. Special attention was given to assure the complete removal of any remaining fluid. Pellets were resuspended in

1.0 ml of distilled water. Reagent A, which contained equal volumes of stock solutions [1], [2], [3], and distilled water, was prepared immediately before using, and 1.0 ml added to each tube. The tubes were mixed, and allowed to stand 10 min at room temperature. Reagent B, which contained one volume of 2.0 N Folin-Ciocalteu phenol reagent mixed with five volumes of distilled water, was also prepared fresh, and 0.5 ml was added to each tube. After 30 min, the absorbance was read at 750 nm. The color was stable for approximately 2 h at room temperature. BSA was used to obtain a standard curve.

Purification Scheme: Approximately 36 mg of thawed crude membrane was resuspended with a Teflon® homogenizer in 12 ml Buffer I, which contained 100 mM potassium phosphate (pH 7.5) and 10 mM β-mercaptoethanol. The final protein concentration was adjusted to 3.0 mg/ml. The suspension, was stirred gently for 15 min, centrifuged at 150,000x*g* for 1 h, and resuspended in an equal volume of Buffer I containing 1.0% TX-114. After 1 h of mixing, the insoluble material was removed by centrifugation at 150,000x*g* and the supernatant (E-114) was carefully layered onto 12 ml of a buffered sucrose solution which had been incubated previously at 30°C. The buffered sucrose solution contained 6.0% sucrose,

0.06% TX-114, 100 mM potassium phosphate (pH 7.5), and 10 mM β-mercaptoethanol. TX-114 aggregates, which included hydrophobic proteins, were formed by placing the tube in a 30°C water bath for 5 min. Then, the tube was centrifuged at 300x*g* for 5 min at room temperature. The supernatant was removed, and the oily droplet (P-114) was washed with 2 ml of Buffer I at 30°C. Proteins were solubilized by diluting the oily droplet, with 12 ml of buffer containing 10 mM Tris-HCl (pH7.5), and 10 mM β-mercaptoethanol at 4°C (S-114).

Approximately 3.5 g of DEAE-cellulose (fine) was suspended in 100 ml of 0.5 N HCl for 30 min. The HCl was removed by vacuum filtration, followed by several washes with distilled water, until the filtrate had a pH of approximately 7. The process was repeated with 100 ml of 0.5 N NaOH. In order to remove extremely fine particles, the matrix was resuspended in distilled water and left to settle under gravity for about 5 min. This process was repeated until all of the material settled within a 5 min period. A DEAE-cellulose (fine) column (1cm x 8 cm) was equilibrated with Buffer A, which contained 10 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 20% sucrose, and 1.0% TX-100. The column was then charged with 12 ml of solubilized protein (S-114; 0.17 mg/ml) and washed with four solutions

(12 ml each): (i) Buffer A without sucrose; (ii) Buffer A without TX-100 or sucrose; (iii) Buffer I, and (iv) 5.0 mM potassium phosphate (pH 7.5) containing, 10 mM β-mercaptoethanol. Enzyme activity was eluted using a linear gradient. One chamber of the gradient mixer contained 25 ml of 5.0 mM potassium phosphate (pH 7.5), 0.2% TX-100, and 10 mM β-mercaptoethanol. The other chamber contained 25 ml of 400 mM potassium phosphate (pH 7.5), 0.2% TX-100, and 10 mM β-mercaptoethanol. The eluate was collected in 3 ml fractions and assayed for protein and enzymatic activity. Active fractions, typically fractions 6 through 11 (total 18 ml), were pooled, mixed with an equal volume of 40% sucrose, and stored as frozen aliquots at -70°C with no appreciable loss of enzymatic activity for at least 6 months.

HW55/pPGL2019 Crude Membrane Preparation: Crude membrane from HW55/pPGL2019 was prepared for use in converting [2-^{3}H]glycerol 3-phosphate and CDP-diacylglycerol into phosphatidyl[2-^{3}H]glycerol. A 20 ml culture of HW55/pPGL2019 in LB broth supplemented with 200 μg/ml ampicillin, was incubated at 37°C with shaking at 250 rpm until the culture reached a turbidity of approximately 250 Klett units. Unless stated otherwise all further procedures were performed at 4°C. Cells were harvested by

centrifugation in an SS-34 rotor at 5000x*g* for 5 min, washed once with 20 ml of 0.1 M Tris-HCl (pH 7.8) containing 5.0 mM EDTA and 10 mM β-mercaptoethanol, and resuspended in 20 ml of the same buffer. The cells were then disrupted by sonication, with six 30 s pulses, at a setting of 8, pausing 15 s between pulses. Unbroken cells and cell debris were removed by centrifugation at 5000x*g* for 5 min. The supernatant was collected and centrifuged in a Sorvall RC80 ultracentrifuge fitted with a T865 rotor, at 150,000x*g* for 1 h. The pellet was stored frozen at -70°C with no appreciable loss of enzymatic activity over a period of at least 6 months.

Substrate Preparation: Phosphatidyl[2-^{3}H]glycerol was synthesized enzymatically from [2-^{3}H]glycerol in two steps. In the first step, *E. coli* glycerokinase converted [2-^{3}H]glycerol into [2-^{3}H]glycerol-3-phosphate. In the second step, crude *E. coli* membrane converted [2-^{3}H]glycerol-3-phosphate and CDP-diacylglycerol to phosphatidyl[2-^{3}H]glycerol.

[2-^{3}H]Glycerol-3-phosphate synthesis: A 50 µl reaction mixture consisting of 50 mM glycine (pH 9.8), 5 mM magnesium chloride, 10 mM ATP (pH 7.0, adjusted with KOH), 2.0 Units glycerokinase, and 0.15 mM [2-^{3}H]glycerol (50 µCi, 6.67 mCi/µmole), was incubated at 22°C for 1.0 h. A DEAE-cellulose (coarse, and washed as described above) column was

packed in a 5 ml disposable Pasteur pipet to a height of 4 cm, and equilibrated with distilled water. The reaction mixture was then placed on the DEAE-cellulose column to purify the [2-^{3}H]glycerol-3-phosphate. The column was washed with 5 ml of water followed by 5 ml of 2 mM ammonium bicarbonate. [2-^{3}H]Glycerol-3-phosphate was eluted with 5 ml of 60 mM ammonium bicarbonate. The eluate was mixed with about 0.5 g of Dowex 50WX8 (H^+ form), and the aqueous portion was collected by centrifugation, and neutralized with 5 µl of 8 M ammonium hydroxide. The material was concentrated to 250 µl, under a stream of nitrogen gas. Usually about 46 µCi of [2-^{3}H]glycerol-3-phosphate was recovered.

Phosphatidyl[2-^{3}H]glycerol synthesis: A 1.0 ml reaction mixture contained, 80 mM Tris-HCl (pH 7.4), 10 mM magnesium chloride, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.2% TX-100, 0.5 mM CDP-diacylglycerol, 0.2 mM [2-^{3}H]glycerol-3-phosphate (46 µCi, 0.23 mCi/µmol), and crude membrane extract, corresponding to approximately 150 µg of protein, from HW55/pPGL2019. The reaction proceeded for 3.5 h at 30°C with gentle shaking.

The reaction was terminated and the lipids extracted by the addition of 3.6 ml methanol-HCl : chloroform (2:1). Methanol-HCl is 0.1 N in HCl.

Then, 1.2 ml of chloroform and 1.2 ml of 2 M magnesium chloride were added. The top aqueous layer was discarded and the chloroform layer was washed twice with 1.2 ml of distilled water and evaporated under a stream of nitrogen gas. After resuspending in 100 µl of chloroform, lipids were spotted onto a Polygram® Sil G thin-layer chromatography plate that had been heated in a 115°C oven for 40 min. The plate was developed with chloroform-methanol-glacial acetic acid (100:41.7:16.7, v/v).[43] The band corresponding to phosphatidyl[2-^{3}H]glycerol was cut out, placed in a 50 ml polypropylene tube, and the silica gel scraped off the plastic backing. After removing the plastic backing, 5 ml of chloroform-methanol-water (5:10:4) was added, and the tube was agitated vigorously on a vortex mixer. The suspension was centrifuged briefly, and the solvent saved. The silica gel residue was extracted with 5 ml of the same solvent two additional times. The chloroform extracts were combined and 3.6 ml each of chloroform and distilled water were added. Then the mixture was agitated on a vortex mixer, and centrifuged. The bottom chloroform layer was collected and 25 µl of methanolic sodium hydroxide (prepared by mixing 100 µl methanol with 20 µl 6 N NaOH) was added to it. The mixture was then washed twice with 2.0 ml of distilled water. Phosphatidyl[2-^{3}H]glycerol was evaporated

to a film under a stream of nitrogen gas and resuspended in 7 ml of chloroform-methanol (2:1). Typically, a yield of approximately 15 μCi of phosphatidyl[2-^{3}H]glycerol was obtained. The specific activity was adjusted, by the addition of unlabeled PG to approximately 20,000 dpm/ nmol of *L*-α-phosphatidyl-*DL*-glycerol.

Cardiolipin Synthase Assay: *E. coli* CL synthase was assayed by following the release of [2-^{3}H]glycerol, one of the reaction products formed when two PG molecules combine to form CL.

The 50 μl reaction mixture contained 20 μl of 800 mM potassium phosphate (pH 7.1), 5 μl of 100 mM β-mercaptoethanol, 5 μl of 400 μM phosphatidyl[2-^{3}H]glycerol (20,000 dpm/nmol) suspended in 0.3% TX-100, and 15 μl of distilled water. The tubes were preincubated at 37°C for 5 min and the reaction started by adding 5 μl of enzyme (see above). Typically, the purified enzyme was diluted to between 2-32 ng, and the crude envelope was diluted to between 30-50 μg. The dilutions were made with 100 mM potassium phosphate, 10 mM β-mercaptoethanol (pH 7.1).

Unless stated otherwise, reactions proceeded for 5 min at 37°C and were terminated by adding 50 μl of a solution which contained 20 mg/ml BSA and 10 mg/ml glycerol, followed by the quick addition of 100 μl of 10% TCA.

After a brief agitation on a vortex mixer, the material was centrifuged at 12,000 rpm, for 5 min in a microfuge at room temperature. To a vial containing 6 ml of Ecoscint A® scintillation fluid, 150 µl of the supernatant was added, and radioactivity determined in an ISOCAP® 300 liquid scintillation counter. The results reported reflect incorporation by 75% of the assay mixture. One unit of enzyme activity is defined as 1 nmol of [2-^{3}H]glycerol released per min.

Characterization of the Reaction Product: The product of the reaction was characterized by thin-layer chromatography to ensure the formation of cardiolipin. The assay was scaled up twenty-fold and the 1 ml reaction mixture contained: 400 µl of 800 mM potassium phosphate (pH 7.1), 100 µl of 100 mM β-mercaptoethanol, 100 µl of 400 µM phosphatidyl[2-^{3}H]glycerol (20,000 dpm/nmol) suspended in 0.3% TX-100, and 300 µl of distilled water. The tubes were preincubated at 37°C for 5 min, and the reaction started by adding 100 µl of enzyme (6 ng/µl). After 10 min at 37°C, the lipids were extracted as previously described,[23] and separated by one-dimensional thin-layer chromatography on Polygram Sil G plates. The solvent system used to separate the lipids was tetrahydrofuran-methylal-methanol-4.0 N ammonium hydroxide (50:25:25:5).[45] This solvent system was used because

it resolves CL from PG, phosphatidic acid (PA), and *bis*-phosphatidic acid (*bis*-PA). R_f values of the lipids are 0.25, 0.5, 0.97, and 0.0, respectively.[45] After the plate was developed, and dried, spots corresponding to the migration of standard CL, PG, PA, and *bis*-PA, were cut out and counted with 6 ml of Ecoscint A® in an Isocap® 300 liquid scintillation counter.

Mixed Micelle Formation: A Sepharose CL-6B column assay was used to show that the lipids under study form mixed micelles with TX-100.[38] The components within the assay were chromatographed through the column alone, and in combination. The migration of each was determined.

Sepharose CL-6B was packed in a glass column 1.5 cm wide (inner diameter) to a height of 35 cm and washed with 500 ml of equilibration buffer containing 350 mM potassium phosphate (pH 7.0), 0.02% TX-100, and 10 mM β-mercaptoethanol. The void volume and inclusion volume were determined with a sample of 3 mg/ml Blue Dextran and 0.3 mg/ml DNP-aspartate in 0.5 ml, respectively. The flow rate through the column was approximately 0.5 ml/min. A Fractomette 200 fraction collector (Buchler Instruments, Fort Lee, NJ) was set up and fractions of about 0.8 ml were collected. Each of the following were chromatographed alone on a Sepharose CL-6B column in a volume of 0.5 ml: 1.0 mM TX-100 (500

nmol), 0.125 mM phosphatidyl[2-^{3}H]glycerol [650 dpm/nmol] (62.5 nmol), and 0.075 mM CL (diphosphatidyl[2-^{3}H]glycerol) [650 dpm/nmol] (37.5 nmol). A combination of TX-100, phosphatidyl[2-^{3}H]glycerol and CL; as well as TX-100, PG and (diphosphatidyl[2-^{3}H]glycerol) at the above concentrations, were also analyzed by gel filtration. TX-100 elution was monitored by absorbance at 275 nm. Radioactive PG and CL elution were followed by counting 200 µl of each fraction with 6 ml of Ecoscint A® in an ISOCAP® liquid scintillation counter.

Effect of Time and Enzyme Concentration on Cardiolipin Formation: The rate of cardiolipin production was determined at various enzyme concentrations. The standard reaction mixture (final volume 0.5 ml) contained the following: 200 µl of 800 mM potassium phosphate (pH 7.1); 50 µl of 100 mM β-mercaptoethanol; 50 µl of 400 µM phosphatidyl[2-^{3}H]glycerol (20,000 dpm/nmol) in 0.3% TX-100; and 150 µl of distilled water. When the reaction mixture contained phosphatidylethanolamine (PE), 50 µl of 400 µM phosphatidyl[2-^{3}H]glycerol was added in 0.2% TX-100 and the PE was added in 0.05% TX-100. Additional TX-100 was used to bring the final concentration of TX-100 to 0.03%. All tubes were preincubated at 37°C for 5 min, and the reaction initiated by adding 50 µl of an enzyme

preparation (see above) diluted with 100 mM potassium phosphate, and 10 mM β-mercaptoethanol. Final enzyme concentrations were 40 ng, 80 ng, or 160 ng per ml. At 3, 6, 9, 12, 15, 30, 45, 60, 75, and 90 min, 50 µl of reaction mixture were transferred from the assay mixture, to tubes containing 50 µl of (20 mg/ml BSA, and 10 mg/ml glycerol), immediately followed by the addition of 100 µl of 10% TCA. The tubes were centrifuged for 5 min at 12,000 rpm in a microfuge at room temperature, and 150 µl of supernatant were added to a vial containing 6 ml of Ecoscint A®. Vials were counted in an ISOCAP® 300 liquid scintillation counter.

An attempt was made to determine the limiting reagent. At 60 min, when the reaction was complete, 2 nmol of phosphatidyl[2-^{3}H]glycerol, 100 mM β-mercaptoethanol, 1.5 mg/ml BSA, 2 nmol of PE, or 2 ng of enzyme were added to a portion of the reaction mixture. Further activity was measured every 5 min for an additional 20 min.

Optimization of Assay Conditions: The assay system was a scaled down version of the standard assay. The final assay volume was 50 µl and the concentration of the component under study was varied. Reactions were incubated for 10 min at 37°C in separate 1.5 ml microfuge tubes, using 3.2 ng of enzyme. Reactions were terminated by the addition of 50 µl of 20

mg/ml BSA and 10 mg/ml glycerol, immediately followed by the addition of 100 µl of 10% TCA. The tubes were centrifuged for 5 min at 12,000 rpm in a microfuge at room temperature and 150 µl of supernatant were added to a vial containing 6 ml of Ecoscint A®. Vials were counted in an ISOCAP® 300 liquid scintillation counter.

The TX-100 concentrations were varied from 0.0% to 0.05% in 0.005% increments to determine the optimum detergent concentration. A pH profile was established from pH 6.0 to 8.0, with 0.5 pH unit increments using potassium phosphate buffer. The effect of various salts, including potassium phosphate, sodium phosphate, potassium chloride, and sodium sulfate were studied over a range of 0.0 mM to 560 mM in 80 mM increments. Variation of salts at a constant ionic strength were studied. The ionic strength, $(\mu) = \frac{1}{2} \Sigma C Z^2$, where C, is the concentration in molarity, and Z is the charge of the ion.

BSA, which enhances the rate of reaction, was left out of the final assay system because it might have complicated interpretation of the mixed micelle assay. An optimization profile for BSA was, however, determined over a range of 0.0 mg/ml to 3.5 mg/ml in 0.5 mg/ml increments.

Effect of Nucleotides on Enzymatic Activity: The effects of various nucleotides, including ATP, ADP, and AMP at 10 mM, were investigated in the absence and presence of phosphate buffer.

Effects of Various Phospholipids on Enzyme Activity: CL synthase activity was studied in the presence of various phospholipids. Included among these were CL, CDP-diacylglycerol, *bis*-PA, PA with saturated and unsaturated fatty acids, PE, phosphatidylinositol, and PS. The assay system was altered slightly to allow for the addition of phospholipid into the assay mixture. A 50 µl reaction mixture contained the following: 20 µl of 800 mM potassium phosphate (pH 7.1); 5 µl of 100 mM β-mercaptoethanol; 5 µl of 400 µM phosphatidyl[2-^{3}H]glycerol (20,000 dpm/nmol) in 0.2% TX-100; up to 10 µl of the indicated phospholipid from a stock solution (0.15 nmol/µl) in 0.05% Trition X-100; and additional 0.05% TX-100 to 10 µl, in order to bring the final concentration of TX-100 to 0.03%. The tubes were preincubated at 37°C for 5 min, and the reaction initiated by adding 30 ng of enzyme in 5 µl. The reaction was terminated after 5 min with the addition of 50 µl of 20 mg/ml BSA in 10 mg/ml glycerol followed by the quick addition of 100 µl of 10% TCA. After a brief agitation with a vortex mixer, the material was centrifuged at 12,000 rpm for 5 min in a microfuge

at room temperature. Then 150 µl of the supernatant were added to a vial containing 6 ml of Ecoscint A® scintillation fluid and the radioactivity was determined in an ISOCAP® 300 liquid scintillation counter.

The effect of PE on CL inhibition was studied. Combinations of 0.9 nmol CL or 0.9 nmol PA, with PE varying from 0.0 nmol to 1.2 nmol in 0.3 nmol increments, were investigated.

Effect of Magnesium on Enzymatic Activity: The effect of magnesium on CL and PA inhibition were studied. To examine the effects of magnesium on CL synthase, the chloride salt was diluted to 5, 10, 50, and 100 mM, in the presence of 0.9 nmol of either CL or PA.

CHAPTER 3

RESULTS

Construction of Cardiolipin Synthase Overproducer: The 1.8 Kb fragment of plasmid pSH111, which includes the *cls* gene, was inserted into the pET3 vector as described in Chapter 2 (Figure 5). The resulting plasmid, pLR3, was introduced into strain BL21(DE3) by transformation. Seven ampicillin resistant colonies were isolated and purified. To verify that the *cls* gene was in the correct orientation and functional, pLR3 was digested with either HindIII or BamHI. In each case, fragments of 1.8 kb and 4.8 kb in length were observed (Figure 7). This corresponded to a *cls* gene that was inserted into pET3 in the correct orientation. Insertions with *cls* in the opposite orientation were also obtained. However, HindIII and BamHI restriction digests produced fragments of 1.0 Kb and 1.8 Kb, respectively.

Growth Studies of Cardiolipin Synthase Overproducer: The effects of IPTG induction on the growth of BL21(DE3)/pET3 and BL21(DE3)/pLR3 were compared. As illustrated in Figure 8A, IPTG has an almost immediate growth inhibitory effect upon BL21(DE3)/pLR3. The effect of IPTG on the growth of BL21(DE3)/pET3 was minimal (Figure 8B). After 1 h of IPTG

induction only 7% of BL21(DE3)/pLR3 remained viable, as compared to BL21(DE3)/pET3 in which 80% of the cells remained viable (Table 3). This immediate effect was consistent with the findings of Hiraoka *et al.* working with a strain that also overproduces cardiolipin synthase.[36]

[^{35}S]Methionine Pulse Labeling and SDS-PAGE of Whole Cells: When subjected to treatment with IPTG, rifampicin, and [^{35}S]methionine, BL21(DE3)/pLR3 synthesizes only one radioactive protein (MW = 46,000 Da) which is not synthesized by BL21(DE3)/pET3 (Figure 9). This protein is found primarily in the crude membrane fraction and not in the initial high speed supernatant. Since this was the sole overproduced protein, and the molecular weight corresponded to the approximate size of the coding region of the *cls* gene,[46] this must be the *cls* gene product.

Lipid Analysis of BL21(DE3)/pLR3 and BL21(DE3)/pET3: The phospholipid distribution of BL21(DE3)/pLR3 clearly demonstrates that a functional *cls* gene is present in pLR3 (Table 4). The CL level found in the membrane of IPTG induced BL21(DE3)/pLR3, was over four times that of BL21(DE3)/pET3. The increased level of CL was at the expense of PG, while PE levels remained fairly constant. The elevated CL level is very significant, since *E. coli* CL levels rise only slightly in proportion to gene

dosage in strains harboring the *cls* gene on a multicopy plasmid.[46] The increased CL level was probably due to a combination of increased enzyme production and arrested cell growth.

Amplification of Cardiolipin Synthase: The effects of IPTG induction on cardiolipin synthase activity were determined for BL21(DE3)/pLR3 and BL21(DE3)/pET3. Crude membrane extracts from BL21(DE3)/pLR3 which had been induced with 0.8 mM IPTG, had an enzymatic activity nearly 30 times that of uninduced BL21(DE3)/pLR3, and over 1200 times the enzymatic activity of IPTG induced BL21(DE3)/pET3 (Table 5). The assay developed for cardiolipin synthase activity was a modification of a published procedure.[36] Unreacted phosphatidyl[2-^{3}H]glycerol was removed by TCA precipitation instead of chloroform extraction.

Effect of IPTG Induction Time on CL Synthase Activity: The effect of IPTG induction time, on cardiolipin synthase activity in the crude membrane of BL21(DE3)/pLR3 was determined. The specific activity of crude membrane extracts from BL21(DE3)/pLR3 increased for two consecutive hours of IPTG induction (Table 6). Incubation of strain BL21(DE3)/pLR3 with IPTG for periods longer than 2 hours, resulted in no further increase in specific activity for the crude membrane fractions.

Therefore, an induction time of 3 hours was chosen for all future experiments, to ensure maximum production of cardiolipin synthase.

Purification of Cardiolipin Synthase: Several different detergents such as deoxycholate, n-octyl-β-D-glucopyranoside, Tween-80, and sodium cholate were tested to see which was most effective for extracting CL synthase activity from the crude membrane preparation. Both TX-100 and sodium cholate provided sufficient solubilization of CL synthase but further purification of these extracts was unsuccessful. TX-114 was selected because of its ability to solubilize the CL synthase activity and its low cloud point of 20°C. When TX-114 is incubated at 30°C, ten degrees above its cloud point, oil droplets are formed. These oil droplets contains hydrophobic proteins, including CL synthase, which are easily pelleted. This cloud out step resulted in a 5-fold purification (Table 7). Once CL synthase was solubilized and partially purified by clouding out, the material was purified by ion-exchange chromatography. Exhaustive attempts were made to reproduce the binding of CL synthase to Whatman P11 phosphocellulose by the procedure of Hiraoka *et al.*[36] Exact conditions were duplicated, including the use of TX-100 for enzyme solubilization. The CL synthase failed to bind to the column. However, preparations of CL synthase solubilized in 1% TX-

114 did bind to DEAE-cellulose. Elution with a linear phosphate gradient yielded a homogeneous protein in fractions 6-12 (Figure 10). Earlier fractions had a small amount of enzyme activity but also contained other proteins (Figure 10).

At each stage of the purification, proteins were analyzed by SDS-PAGE and the results are shown in Figure 11. As summarized in Table 7, cardiolipin synthase was purified nearly 17-fold by this purification scheme, corresponding to an approximate 40,000-fold increase in the purity of the enzyme as compared to unamplified whole cells.

Characterization of Cardiolipin Synthase Product: The reaction product was confirmed by thin-layer chromatography. Lipids produced by the CL synthase assay were extracted and chromatographed on Polygram Sil G plates. Two major radioactive lipids were observed. One of these was unreacted PG and the other co-chromatographed with CL (Table 8 & Figure 27).

Mixed Micelle Determination: The Sepharose CL-6B column was useful for demonstrating that the lipids studied were present in mixed micelles. As shown in Figure 12, PG alone elutes in the void volume and PG in 0.03% TX-100 elutes in the same position as TX-100.

Since PG is converted to CL, CL's influence on mixed micelle formation was also studied. As shown in Figure 13, CL alone elutes in the void volume and CL in 0.03% TX-100 elutes in the same position as TX-100. Furthermore, CL, PG, and TX-100 form a mixed micelle when present in the same mixture. This can be seen in Figure 12D which shows a chromatogram for a mixture of [^{3}H]PG, CL, and TX-100 and in Figure 13D which shows a chromatogram for a mixture of PG, [^{3}H]CL, and TX-100.

Characteristics of Cardiolipin Synthase: An attempt was made to optimize the assay conditions. TX-100, which is critical for activity, was varied over a wide concentration range (Figure 14). Optimum activity was observed at 0.015%. This is what would be expected for mixed micelle formation since TX-100's critical micelle concentration is about 0.015%. The standard assay uses 0.03% TX-100 to ensure micelle formation. Although activity is somewhat lower at 0.03% TX-100 than at 0.015% TX-100, it helps to ensure mixed micelle formation.

Cardiolipin synthase has a pH optimum of 7.0 when either potassium or sodium phosphate (not shown) is used as a buffer (Figure 15). Phosphate stimulates enzyme activity, with optimal activity observed at 320 mM (Figure 16). This stimulation was specific for phosphate, either the sodium

or the potassium salt. Neither sodium sulfate nor potassium chloride at the same ionic strength as the optimal potassium phosphate, could reproduce the phosphate stimulation of enzymatic activity (Figure 16). CL synthase activity is stimulated by some sort of specific interaction with phosphate and it appears to be more than just an ionic effect.

BSA stimulated the activity of CL synthase, although to a lesser extent (Figure 17). A concentration of 1.5 mg/ml BSA gave maximum activity. BSA was not included in later kinetic experiments because of a concern that it would interfere with the interpretation of results from the mixed micelle assay.

CL formation was followed as a function of time at three different enzyme concentrations (Figure 18). In the reaction catalyzed by 4 ng of CL synthase, the standard reaction condition, CL formation was linear for about 10 min. However, all three reactions came to a halt well before all of the PG was converted to CL. At each enzyme concentration, the reaction ceases when approximately 3.5×10^3 mol of CL are formed per mol of enzyme. The cardiolipin produced at the plateau appeared proportional to the initial enzyme concentration (Figure 19). This result suggested that either reaction product, CL or glycerol, might be responsible for enzyme inhibition.

Therefore, CL synthase was assayed in the presence of various concentrations of glycerol and CL.

Glycerol has no effect on CL synthase even when concentrations as high as 160 mM are used (Figure 20). In contrast, CL has a marked inhibitory effect (Figure 21A) when present at a concentration of 25 μM (3.6×10^3 mol of CL/mol CL synthase). This clearly shows that CL synthase is inhibited by its product CL.

Other anionic phosphoglycerides were also tested to determine whether they can inhibit CL synthase. As shown in Figure 21B, dipalmitoyl PA also has a strong inhibitory effect. Nearly identical inhibition was observed when dimyristoyl, dioleoyl, or distearoyl PA were tested (data not shown). *bis*-PA and CDP-diacylglycerol have virtually no inhibitory effect, and phosphatidylinositol has a slight stimulatory effect (Figure 21B). Zwitterionic lipids were also tested. PE and PS have slight stimulatory effects (Figure 22). Since *E. coli* membranes have a fairly high PE concentration, the effect of this lipid on CL synthase activity was studied in more detail.

As seen in Figures 23a, 23b and 23c, when PE was already present in the assay mixture, activity was enhanced up to 4-fold, after a 90 min

incubation. As shown in Figure 24, PE not only influences the rate of CL formation but also the extent of CL formation. At 24 μM PE, activity peaked and the addition of additional PE had no greater effect (Figure 24). PE has a stimulatory effect when added at the start of the reaction but has no effect once the reaction is complete (Figure 26). β-Mercaptoethanol and BSA also do not stimulate the completed reaction (data not shown) while PG has a slight effect (Figure 26). The addition of another 2 ng of enzyme does, however, result in more CL synthesis (Figure 26). This, along with analysis of lipids in the reaction mixture (Figure 27), demonstrates that the reaction mixture still contains unreacted PG.

CL and PA inhibition appear to be different. PE offsets CL inhibition but has no effect on PA inhibition (Figure 25). Magnesium chloride has the opposite effect; it reverses PA inhibition but not CL inhibition (Figure 28).

Effects of Nucleotides on Cardiolipin Synthase Activity: Because the energy state of the bacteria seems to influence CL levels,[29] the effects of various nucleotides on CL synthase activity were examined. ATP, ADP, and AMP have no effect on CL synthase activity when added at 10 mM in the presence of 320 mM phosphate. A very slight stimulatory effect on CL synthase activity, in the absence of phosphate, is observed with 10 mM ATP

(Table 9). This can be attributed to hydrolysis of ATP which would provide phosphate to the reaction mixture.

CHAPTER 4

Discussion

Hiraoka and coworkers have reported the partial purification of CL synthase from a strain of *E. coli* carrying a recombinant plasmid in which the *cls* gene is under the control of the *tac* promoter.[36] In the present study, the *cls* gene from a different *E. coli* strain was inserted into the pET3 vector to produce pLR3 in which *cls* is under the control of the T7 promoter. When strain BL21(DE3)/pLR3 are incubated with IPTG, they produce more than 1200 times the CL synthase activity of wild type cells and cell growth is almost immediately arrested (Figure 8A).

Attempts to purify CL synthase from these cells by the procedure of Hiraoka and coworkers were unsuccessful because CL synthase did not bind to Whatman P11 phosphocellulose. Several different batches of Whatman P11 phosphocellulose, as well as phosphocellulose from other manufacturers, all gave similar results. The reason for the inability of the BL21(DE3)/pLR3 CL synthase to bind to phosphocellulose is not clear. Maybe the lipid environments of the two enzymes are different. Perhaps the CL synthase

produced by BL21(DE3)/pLR3 has a slightly different amino acid sequence from the enzyme isolated by Hiraoka *et al.* This seems possible since the CL synthase isolations involved different strains of *E. coli.* Because CL synthase did not bind to phosphocellulose, and since Hiraoka *et al.* had only partially purified the enzyme, an alternate purification procedure was devised. This procedure, which takes advantage of TX-114's ability to form aggregates with integral membrane proteins, as well as DEAE-cellulose ion-exchange chromatography, resulted in an apparent 17-fold purification (Table 7). The true purification cannot be calculated because enzyme activity is influenced by the lipids present. Therefore, estimates of enzyme activity in the crude membranes are probably too low because the high level of CL inhibits enzyme activity and unlabeled PG lowers the specific activity of the added phosphatidyl[2-^{3}H]glycerol.

The combination of TX-114 cloud out and DEAE-cellulose proved to be a very effective purification scheme. The enzyme obtained was homogeneous, yielding a single electrophoretic band at 46 kD when tested by SDS-PAGE, and had a specific activity of 8400 units/mg. The intact protein was subjected to Edman degradation (Dr. Yu-wen Hwang). No amino acid derivative was detected, suggesting that the amino terminus is

blocked. Further attempts to sequence the amino acids of CL synthase will be made using mass spectroscopy. The enzyme produced by BL21(DE3)/pLR3 is similar to that isolated by Hiraoka *et al.* in several respects. Both enzymes have a pH optimum of 7.0, maximum activity in the presence of 0.015% TX-100, and are stimulated by bovine serum albumin and potassium phosphate.

The present studies also support the notion that *cls* is the structural gene for CL synthase. When BL21(DE3)/pLR3 and BL21(DE3)/pET3 were cultured in the presence of [^{35}S]methionine, IPTG and rifampicin, only one radioactive protein (46 kD) was present in the membranes of the former strain but not in those of the latter (Figure 9). CL synthase activity co-purifies with this 46 kD protein.

The 0.015% TX-100 optimum suggested that PG, the substrate for CL synthase, is in a mixed micelle. The Sepharose CL-6B gel filtration data (Figures 12 & 13) indicates that CL and PG form mixed micelles with TX-100 at ratios of detergent to phospholipid of 8:1 or higher. This means that CL synthase can be studied under the defined conditions of a mixed micelle assay.[38] In addition, mixed micelles have been reported to mimic the biological membrane surface.[47,48] Although BSA stimulates CL synthase

activity, it was omitted from the standard assay because it might interfere with the interpretation of the mixed micelle data.

An unexpected result was observed when the CL synthase reaction was studied at different enzyme concentrations and times (Figure 18). At three different enzyme concentrations, the reaction reached a plateau when approximately 3.5 x 10^3 CL molecules were formed per enzyme molecule. This result suggested that one of the reaction products, either CL or glycerol, might be an inhibitor of the reaction.

Glycerol has no effect upon enzyme activity, even when used at concentrations as high as 160 mM (Figure 20). In contrast, CL has an inhibitory effect when added to the assay mixture at a concentration of 25 μM (about 3.6 x 10^3 molecules of CL per molecule of CL synthase) (Figure 21A). The inhibition by CL was quite specific. Only one other phosphoglyceride tested, PA, significantly inhibits CL synthase (Figure 21B). Since PE partially offsets CL inhibition but not PA inhibition (Figure 25) and magnesium chloride has the opposite effect (Figure 28), the inhibition by CL and PA are probably different. Two other anionic phosphoglycerides, *bis*-PA and phosphatidylinositol, have little or no effect. CDP-diacylglycerol is also without effect. Two zwitterionic lipids, PE and PS, have a slight

stimulatory effect (Figure 22). PE's stimulatory effect is only apparent when PE is added at the start of the reaction (Figures 24 & 26). PE has no stimulatory effect after the reaction reaches a plateau (Figure 26). PG addition has only a slight stimulatory effect after the reaction reaches a plateau (Figure 26). The addition of more enzyme does, however, stimulate the reaction after it plateaus (Figure 26). This clearly demonstrates that all other necessary enzyme requirements are still present. CL levels are known to increase when cells have difficulty in producing energy.[29] Neither ATP, ADP, nor AMP has any significant influence on CL synthase activity (Table 9).

In *E. coli* certain cell events, such as the replication and segregation of chromosomes, and cell division, occur with remarkable precision. This suggests some sort of regulation mechanism, a "biological clock". The nature of this clock in *E. coli* is unknown. The effects phosphoglycerides have on cell cycle events are beginning to become more evident. An increase in the rate of phosphoglyceride synthesis has been associated with chromosome replication,[49] cell division,[50] and termination of chromosome replication in *E. coli*.[51] Uninterrupted phosphoglyceride synthesis is required for unperturbed growth.[3] If phosphoglyceride synthesis is interrupted, a

decrease in chromosome replication and cell division is observed.[3] The dnaA protein, involved in the initiation of chromosome replication, is activated by a specific binding to PG or CL which contain unsaturated fatty acids.[12] Since *E. coli* cells progressing from early to late log have an increased level of saturated fatty acids within phosphoglycerides[18], the binding to dnaA may decrease and lead to a decrease of chromosome replication. This activation process has an absolute dependence upon a fluid membrane.[12]

One factor influencing the fluidity of the membrane is a process known as transmembrane movement (flip-flop).[52] Flip-flop occurs fairly rapidly in bacteria, with a half-time of approximately 3 min.[53] This rate is much slower for acidic phospholipids, which are believed to accumulate in the inner monolayer of the cytoplasmic membrane and, at a critical density, are flipped to the outer monolayer.[53] This is believed to occur once per cell cycle.[53] The phosphoglyceride composition is another factor which influences the fluidity of the membrane. Changes in phosphoglyceride composition will lead to changes in the ratio of bilayer to non-bilayer lipids. Certain localized deviations from the bilayer phase are believed to facilitate cell division,[54] the formation of membrane adhesion sites,[55] and protein

translocation.[10] Recently, polymorphic regulation of phosphoglyceride composition, in order to preserve H_{II} phase possibilities, has been demonstrated in *E. coli.*[17]

The experiments presented in this thesis show for the first time that CL, the product of the CL synthase catalyzed reaction, is an inhibitor of the reaction. This inhibition probably plays a physiological role in regulating the PG to CL ratio in *E. coli.* CL synthase, located in the cytoplasmic membrane, may be regulated by changes in its lipid environment. The specific interaction with CL, or possibly, the ratio of non-bilayer phosphoglyceride (CL) to bilayer phosphoglyceride (PG) may be an important factor regulating this enzyme. This would explain why significant levels of PG are still found within the membrane even though CL synthase is present. This may also explain why *pss* mutants make up for the lost PE (non-bilayer phosphoglyceride) in the membrane with an over production of CL, another non-bilayer phosphoglyceride. Acidic phosphoglyceride flip-flop in late log phase may cause a change in the phosphoglyceride packing density around CL synthase, and be responsible for the increase in the CL level as cells progress into late log phase. Since an increase in the CL to PG ratio is associated with a decrease in cell division,[56] the balance of these two

phosphoglycerides has important cellular consequences. The increased CL level in IPTG induced BL21(DE3)/pLR3 causes arrest of cell growth. The very high CL to PG ratio tolerated is probably due to the fact that a very high CL concentration is required to inhibit the extraordinary high CL synthase level present in the induced cells. The results discussed here suggest that protein interaction with lipid, appears to play an important role regulating the phosphoglyceride composition, and may therefore influence membrane functions.

TABLES

Table 1.

Strain List

Strain	Geneotype	Reference
HB101	F' recA13 supE44 rpsL20(sm^r) hsds20 (r_B^-, m_B^-) ara14 galK2 lacY1 proA2 xyl15 leu mtl1 λ^-	40
BL21(DE3)	F^- ompT T7 RNA polymerase unde control of lacUV5 promoter	37
HW55	HfrC glpR cls-1	23

Table 2.

Plasmid List

Plasmid	Relevant Properties	Reference
pET3	Over-expression vector with T7 promotor.	37
pIBI20	BamHI site within polylinker.	a.
pLR3	*cls* inserted within overexpression vector.	This study
pLR111	Intermediate plasmid with the *cls* gene flanked by two BamHI sites.	This study
pPGL2019	pgsA gene, ampicillin resistant.	8
pSH111	amp^r cls^+.	This study

[a.] Product of IBI, New Haven, CT.

Table 3.

Effect of IPTG Induction on Cell Viability

Strain	M9ZB (Cells/ml)	M9ZB + Ampicillin (Cells/ml)
BL21(DE3)/pET3	~$1.5x10^9$	~$1.5x10^9$
BL21(DE3)/PET3 + IPTG	~$1.2x10^9$	~$1.2x10^9$
BL21(DE3)/pLR3	~$1.0x10^9$	~$1.0x10^9$
BL21(DE3)/pLR3 + IPTG	~$7.0x10^7$	~$6.8x10^7$

Table 3. Bacteria were cultured in M9ZB medium supplemented with 200 μg/ml ampicillin, shaking at 37°C and 250 rpm. At a turbidity of 150 Klett units cells were induced, where indicated, with 0.8 mM IPTG. After 1 h of induction cells were removed, diluted 10^6-fold, and spread on the indicated plates. The plates were incubated at 37°C for 18 h and colonies counted.

Table 4.

Phospholipid Distributions

	Mole %		
Strain	**PE**	**PG**	**CL**
BL21(DE3)/pET3	75.8	19.1	5.1
BL21(DE3)/pET3 (0.8 mM IPTG)	77.6	18.0	4.4
BL21(DE3)/pLR3	77.2	16.2	6.6
BL21(DE3)/pLR3 (0.8 mM IPTG)	71.9	6.3	21.8

Table 4. Bacteria were cultured in 5.0 ml of M9ZB supplemented with 200 μg/ml ampicillin, shaking at 37°C and 250 rpm. At a turbidity of 40 Klett units IPTG was added, where indicated, to 0.8 mM along with 5 μCi/ml of carrier-free [^{32}P]phosphate, and the culture was incubated for an additional 2 h. Phospholipids were isolated and chromatographed as described in Chapter 2 on Polygram® Sil G plates in a solvent system of tetrahydrofuran: methylal: methanol: 4.0 N ammonium hydroxide (50:25:25:5).

Table 5.

Cardiolipin Synthase Activity of Crude Membranes

Strain	(Units/mg)
BL21(DE3)/pET3	0.2
BL21(DE3)/pET3+ IPTG	0.4
BL21(DE3)/pLR3	17.5
BL21(DE3)/pLR3+ IPTG	507

Table 5. Bacteria were cultured in M9ZB medium, supplemented with 200 μg/ml ampicillin, shaking at 37°C and 250 rpm. At a turbidity of 150 Klett units cells were induced, where indicated, with 0.8 mM IPTG and incubated for an additional 2 hours. Crude membranes were prepared and activity determined as described in Chapter 2. A Unit of cardiolipin synthase activity is defined as 1 nmol of glycerol released per min.

TABLE 6.

Effect of IPTG Induction Time on Cardiolipin Synthase Activity

Cardiolipin synthase activity (Units/mg)

Fraction	0 hour	1 hour	2 hours	3 hours	4 hours	5 hours
Crude membrane	19	339	421	506	500	509

Table 6. Strain BL21(DE3)/pLR3 was cultured in M9ZB medium supplemented with 200 μg/ml ampicillin, shaking at 37°C and 250 rpm. At a turbidity of 150 Klett units 0.8 mM IPTG was added to the growing culture. Crude membranes were isolated, as described in Chapter 2, at the indicated times and cardiolipin synthase activity determined. A Unit of activity corresponds to 1 nmol of glycerol released per min.

Table 7.

Solubilization and Purification of Cardiolipin Synthase

Fraction	**Total Enzyme** (units)	**Protein** (mg)	**Sp. Activity** (units/mg protein)	**Yield** (%)	**Purification** (-fold)
Crude membrane	21,000	30	507	100	1
S-114	17,100	2.2	2,380	81	4.7
DEAE	3,400	0.8	8,400	16	16.6

Table 7. Strain BL21(DE3)/pLR3 was induced with 0.8 mM IPTG and cardiolipin synthase purified as described in Chapter 2. Crude membrane, S-114, and DEAE fractions correspond to the first high speed pellet, the TX-114 oil droplet dissolved in buffer (10 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol), and active fractions from the DEAE-cellulose column, respectively. A Unit of activity corresponds to 1 nmol of glycerol release per min.

Table 8.

Characterization of Reaction Product

% of Radioactivity

Lipid Product	Control	Enzyme
bis-Phosphatidate*	0.5	1.6
Phosphatidylglycerol	97.0	55.3
Cardiolipin	2.5	43.1
Phosphatidate	0.0	0.0

* Product co-chromatographs with bis-PA (True identity unknown)

Table 8. A 1.0 ml standard assay mixture was incubated with 1 μg of enzyme for 15 min, and the phospholipids isolated and chromatographed as described in Chapter 2. The Polygram® Sil G plate was cut into 1 cm sections, counted, and compared to the migration of unlabeled standards.

Table 9.

Effect of Nucleotides on Cardiolipin Synthase

Nucleotide	Cardiolipin Synthase Units	
	(w/ Phosphate)	(w/o Phosphate)
Control	82	4
AMP (10 mM)	85	6
ADP (10 mM)	84	6
ATP (10 mM)	84	17

Table 9. Cardiolipin synthase activity was measured for 5 min at 37°C, using 32 ng of enzyme. Nucleotides were added at a concentration of 10 mM, and the control was an equal volume of distilled water. A Unit of activity corresponds to 1 μmol of glycerol released per min.

FIGURES

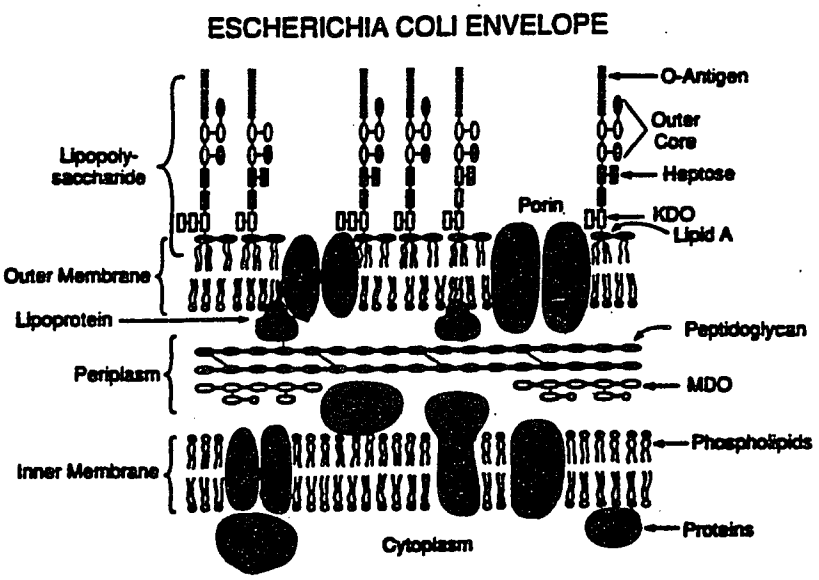
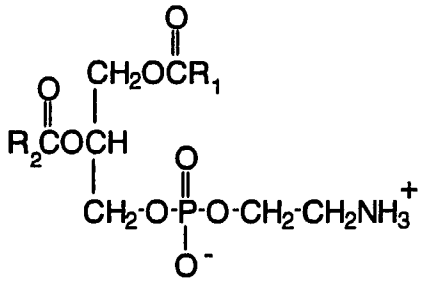
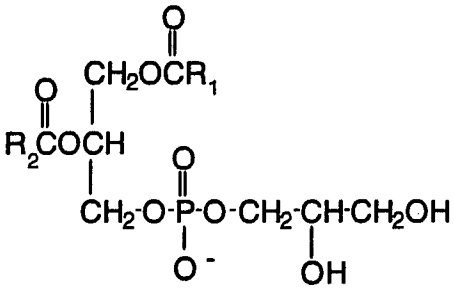


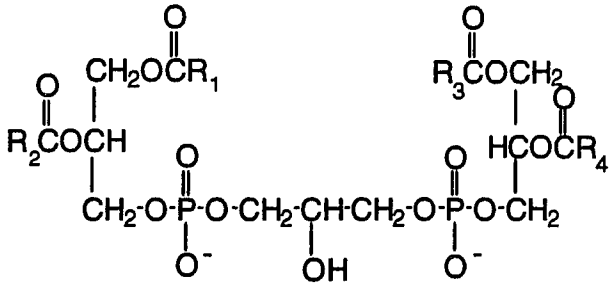
Figure 1. The *Escherichia coli* cell envelope.



Phosphatidylethanolamine



Phosphatidylglycerol



Cardiolipin

Figure 2. Structures of PE, PG, and CL.

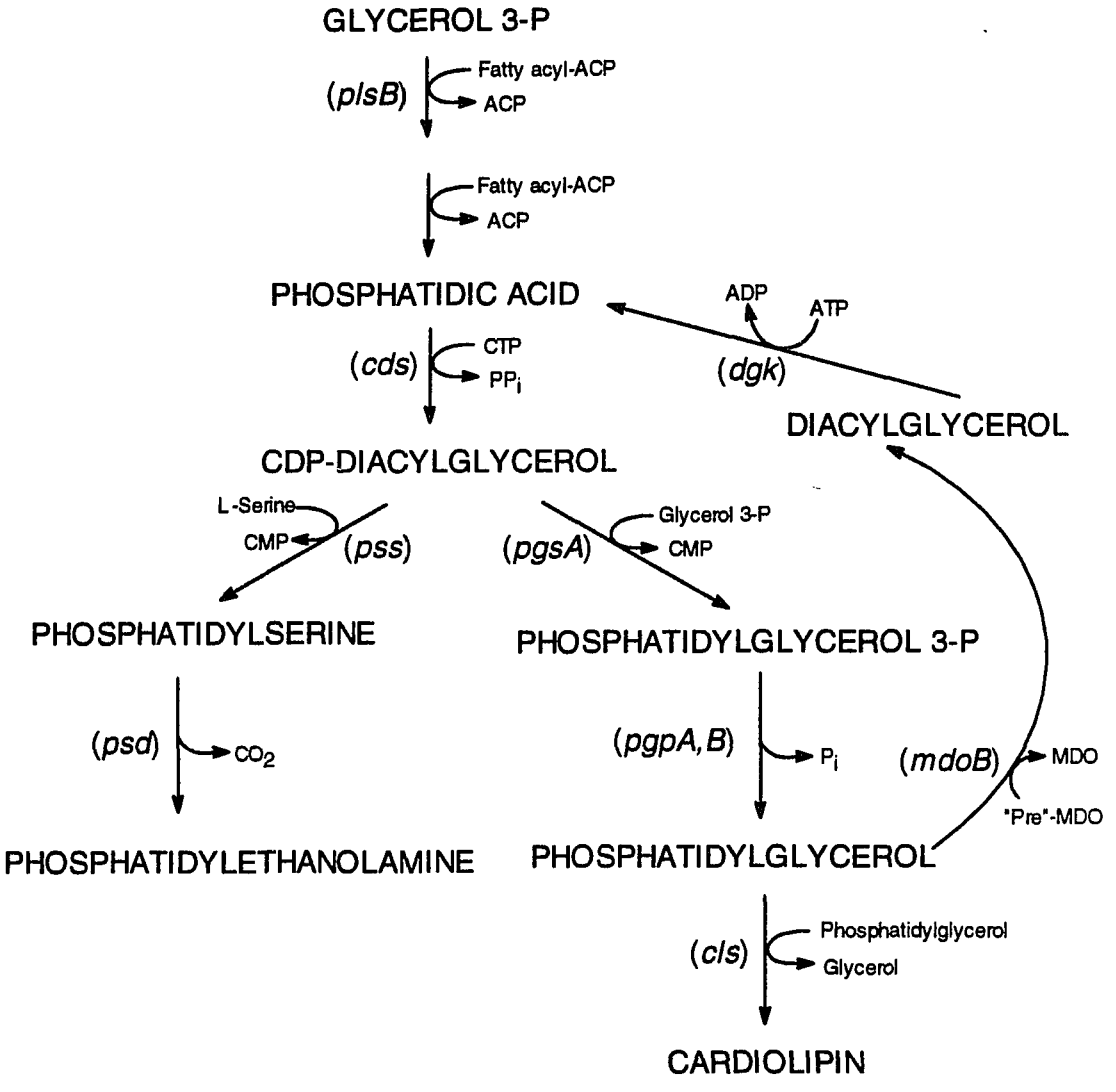


Figure 3. The *Escherichia coli* phosphoglyceride biosynthetic pathway.

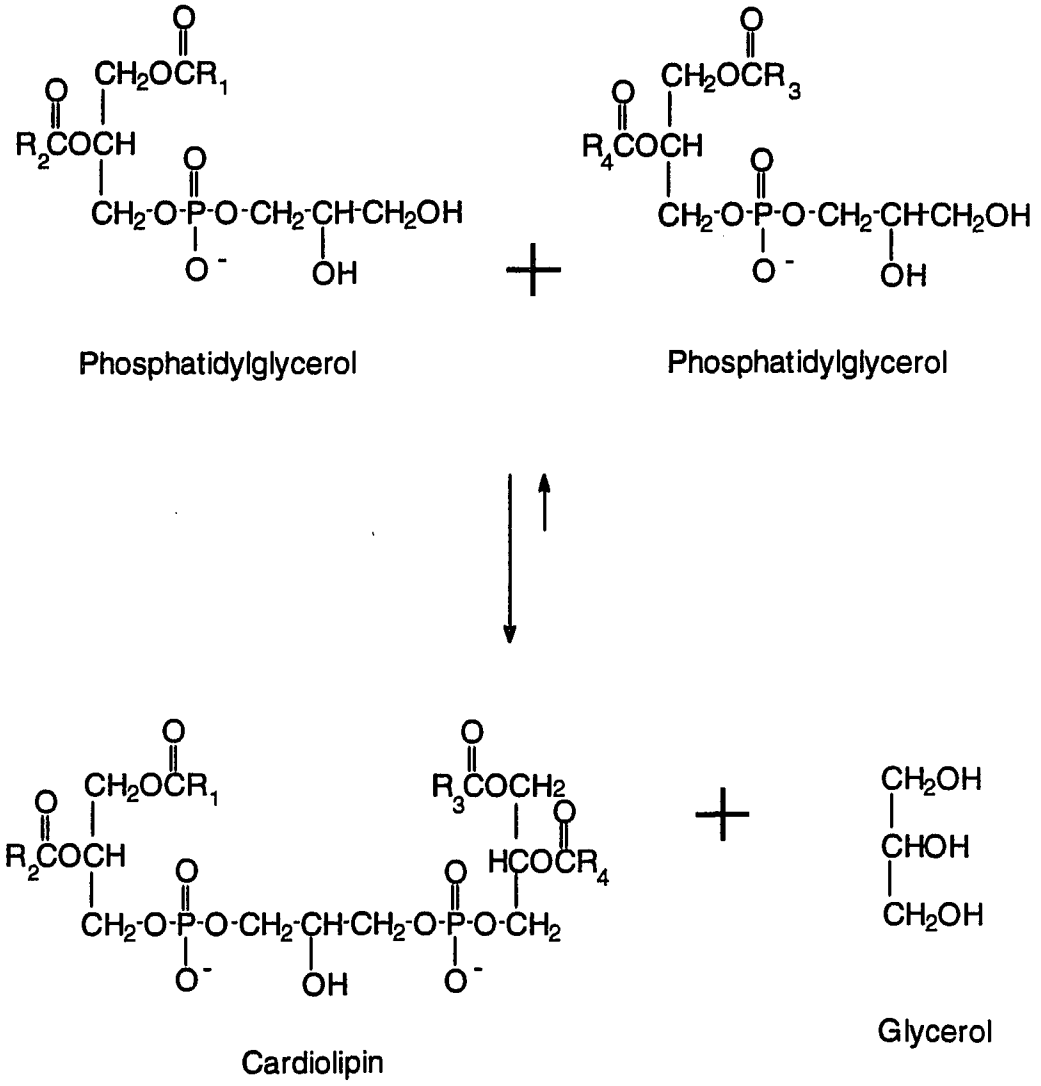


Figure 4. The CL synthase reaction.

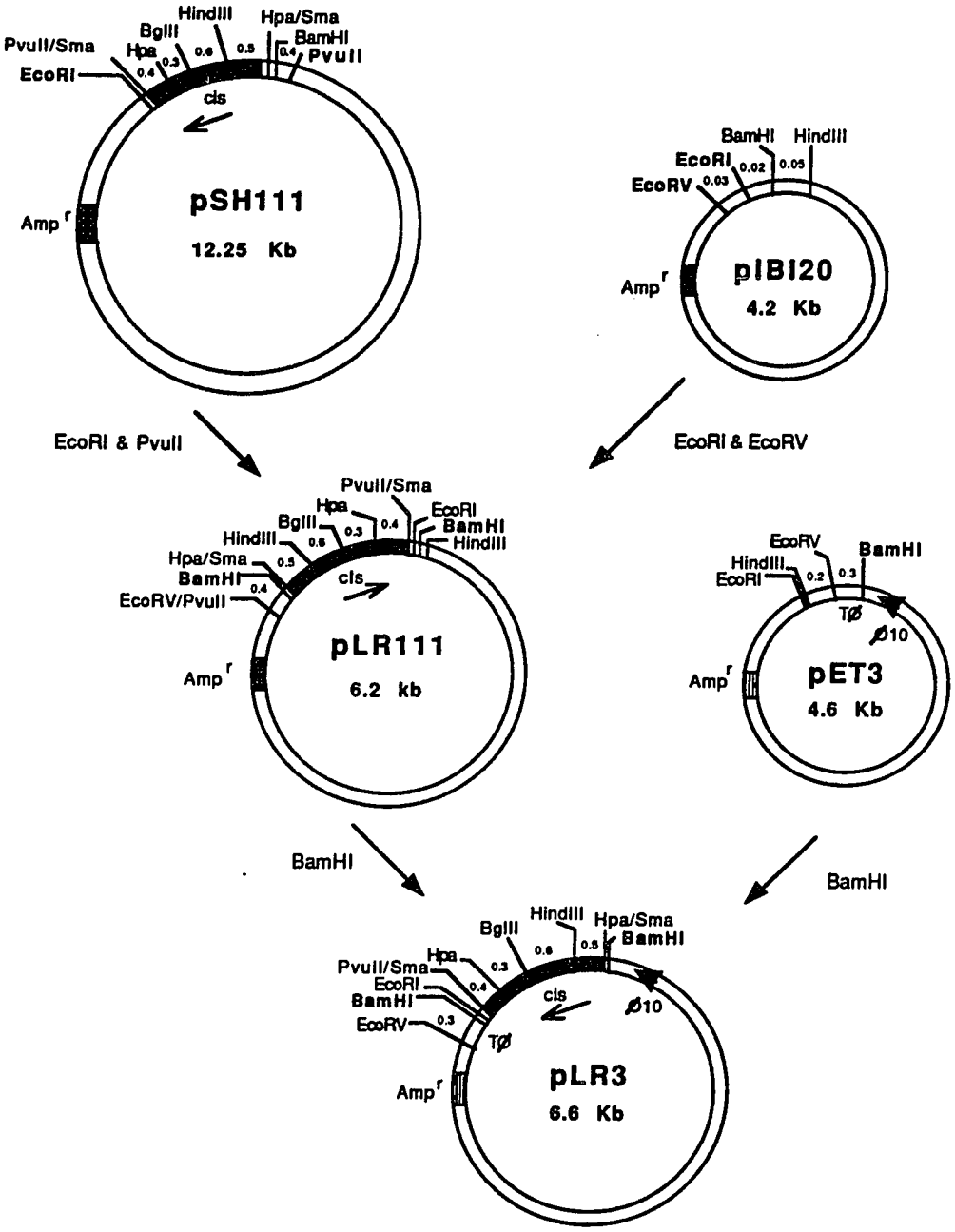

pSH111
12.25 Kb
PvuII/Sma
EcoRI
Hpa
BglII
HindIII
Hpa/Sma
BamHI
PvuII
cis
Amp r
pIBI20
4.2 Kb
BamHI
HindIII
EcoRI
EcoRV
Amp r
EcoRI & PvuII
EcoRI & EcoRV
pLR111
6.2 kb
PvuII/Sma
Hpa
EcoRI
BglII
BamHI
HindIII
Hpa/Sma
EcoRV/PvuII
cis
Amp r
pET3
4.6 Kb
EcoRV
HindIII
EcoRI
BamHI
TØ
Ø10
Amp r
BamHI
BamHI
pLR3
6.6 Kb
BglII
HindIII
Hpa/Sma
BamHI
Hpa
PvuII/Sma
EcoRI
BamHI
EcoRV
cis
Ø10
TØ
Amp r

Figure 5.

Figure 5. Construction of pLR3. Plasmid pLR111 was constructed by inserting the 2.2 kb EcoRI-PvuII fragment, from pSH111, into the EcoRI-EcoRV site of pIBI20. Calf-intestine alkaline phosphatase was used to prevent the ligation of two pIBI20 plasmids. Selection for pLR111 was based upon ampicillin resistance. Plasmid pLR3 was constructed by inserting the 1.8 kb BamHI fragment, containing the *cls* gene, from pSH111, into the BamHI site of pET3. To prevent the ligation of two pET3 plasmids, calf-intestine alkaline phosphatase was used. Ampicillin resistance was used for selection. The *cls* gene is represented by (▬). Tϕ represents the T7 transcription termination sequence and ϕ10, the T7 promoter (◂). Distances are in kilobases.

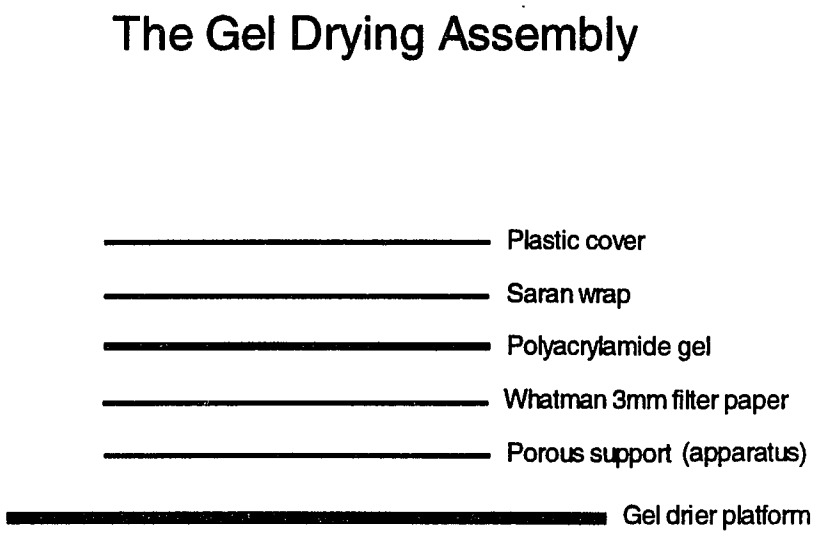


Figure 6. The gel drying assembly.

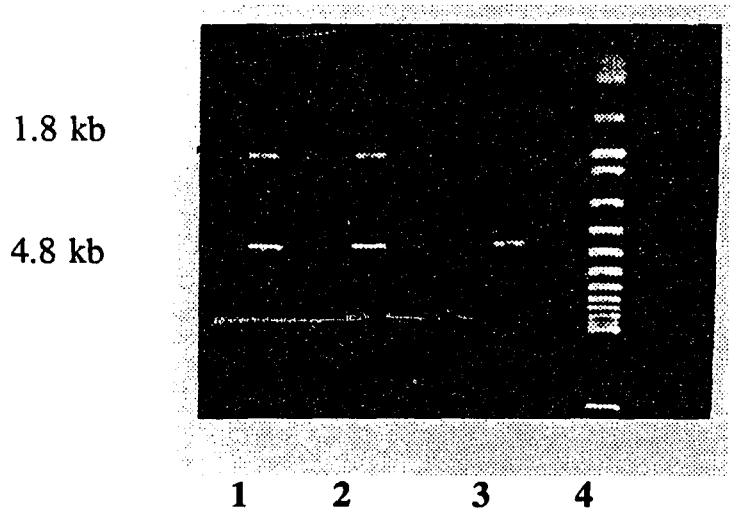


Figure 7. Restriction endonuclease digestion of pLR3 and pET3. Ethidium bromide-stained agarose gel of pLR3 and pET3. Lane 1 corresponds to a BamHI digestion of pLR3; lane 2, HindIII digestion of pLR3; lane 3, BamHI digestion of pET3; and lane 4, HindIII λ DNA ladder.

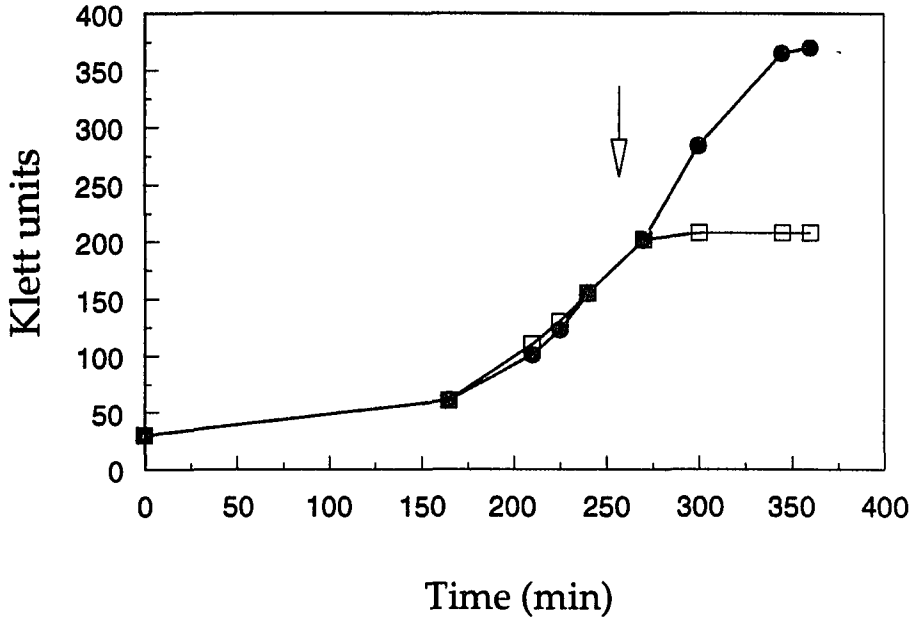


Figure 8A. Effect of IPTG induction on the growth of BL21(DE3)/pLR3. Strain BL21(DE3)/pLR3 was grown in M9ZB medium supplemented with 200 μg/ml ampicillin, shaking at 37°C and 250 rpm. At 250 min (↓) either water (●) or IPTG to 0.8 mM (□) was added. Cell growth was measured using a Klett-Summerson photometer fitted with a red filter. One Klett unit corresponds to approximately 5 x 10^6 cells/ml.

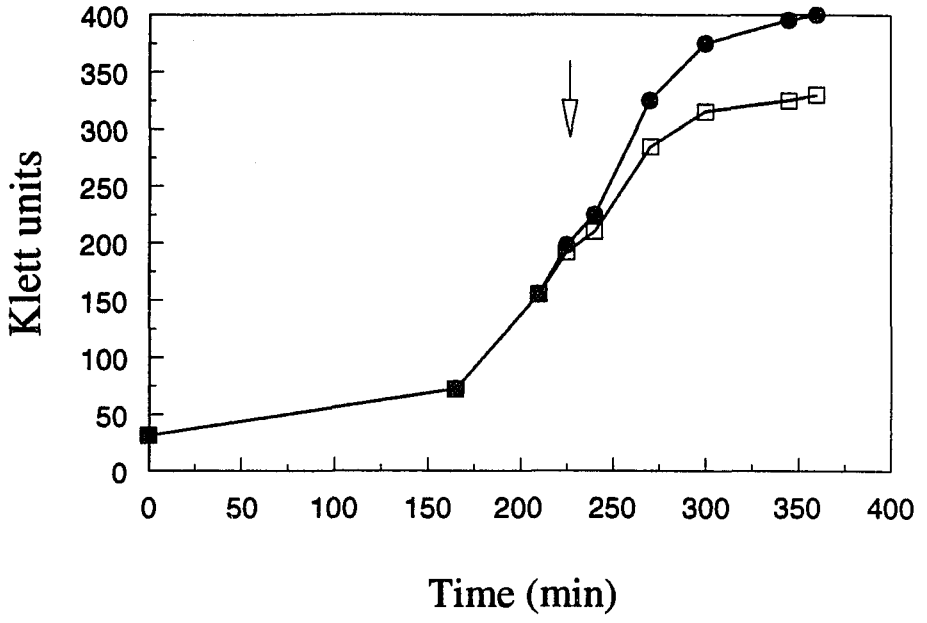


Figure 8B. Effect of IPTG induction on the growth of BL21(DE3)/pET3. Strain BL21(DE3)/pET3 was grown in M9ZB medium supplemented with 200 μg/ml ampicillin, shaking at 37°C and 250 rpm. At 225 min (↓) either water (●) or IPTG to 0.8 mM (□) was added. Cell growth was measured using a Klett-Summerson photometer fitted with a red filter. One Klett unit corresponds to approximately 5 x 10^6 cells/ml.

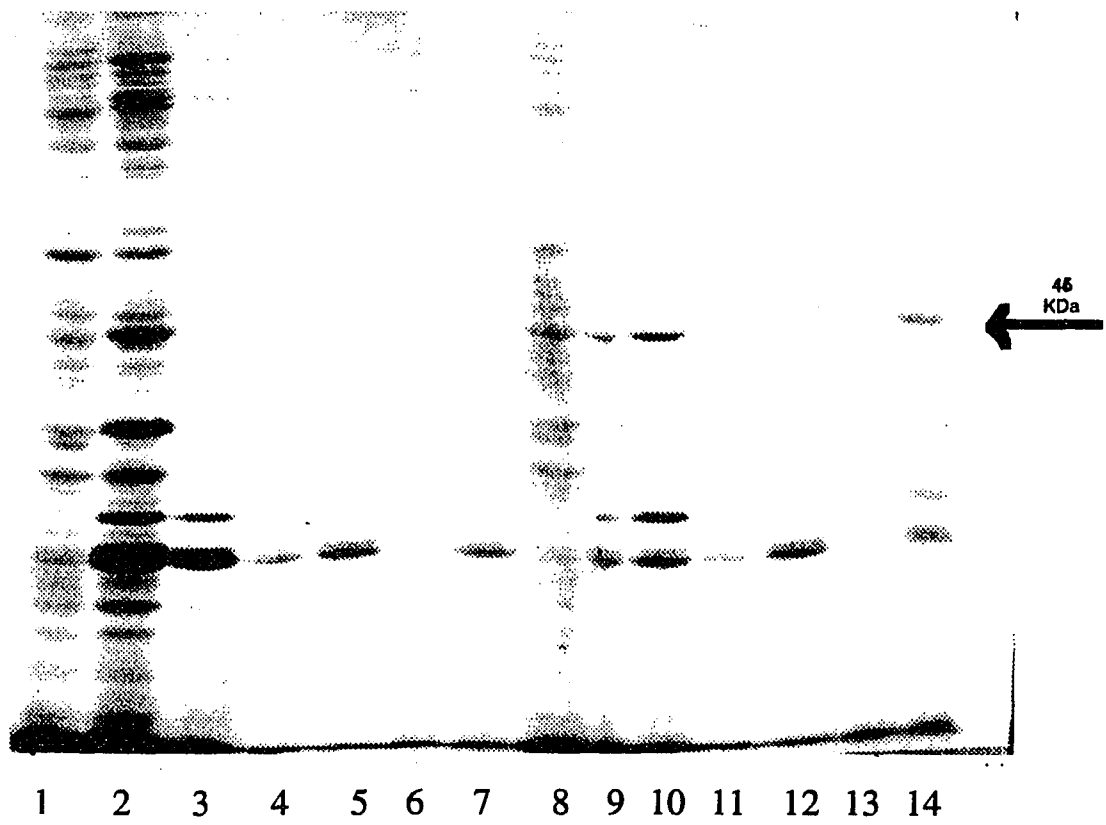


Figure 9.

Figure 9. SDS-PAGE on BL21(DE3)/pLR3 whole cells. Strain BL21(DE3)/pLR3 was grown in M9ZB medium, supplemented with 200 μg/ml ampicillin, shaking at 37°C. At 200 Klett units, cells were induced with 0.8 mM IPTG, followed by the addition of 200 μg/ml rifampicin and 4.0 μCi of [^{35}S]methionine (1180 Ci/mmol). After a centrifugation for 30 s, the cells were treated either as: (i) Whole cells and resuspended in 200 μl of sample buffer containing 50 mM Tris-HCl (pH 6.5), 2 mM EDTA, 1% β-mercaptoethanol, 1% SDS, 8% glycerol, 0.025% bromophenol blue or (ii) Alternatively cells were separated into crude membrane and supernatant. The cells were resuspended in 100 μl of a buffer containing 50 mM Tris-base (pH 8.0), 2.0 mM EDTA, and 100 μg/ml lysozyme and incubated for 15 min at 30°C. Then, the mixture was centrifuged at 12,000xg for 15 min and separated into supernatant (S) and crude membrane (P) and 100 μl of the sample buffer added to each. All suspensions were boiled for 5 min, and loaded on an SDS polyacrylamide gel. A sheet of Kodak® XAR film was placed over the dried gel and was exposed for 1 week. Lanes 1-7 correspond to BL21(DE3)/pET3; lane 1, no IPTG induction (whole cells); lane 2, IPTG induced (whole cells); lane 3, IPTG and rifampicin (whole cells); lane 4, IPTG induced (S); lane 5, IPTG and rifampicin (S); lane 6, IPTG induced (P); and lane 7, IPTG and rifampicin (P). Lanes 8-14 correspond to BL21(DE3)/pLR3; lane 8, no IPTG induction (whole cells); lane 9, IPTG induced (whole cells); lane 10, IPTG and rifampicin (whole cells); lane 11, IPTG induced (S); lane 12, IPTG and rifampicin (S); lane 13, IPTG induced (P); and lane 14, IPTG and rifampicin (P).

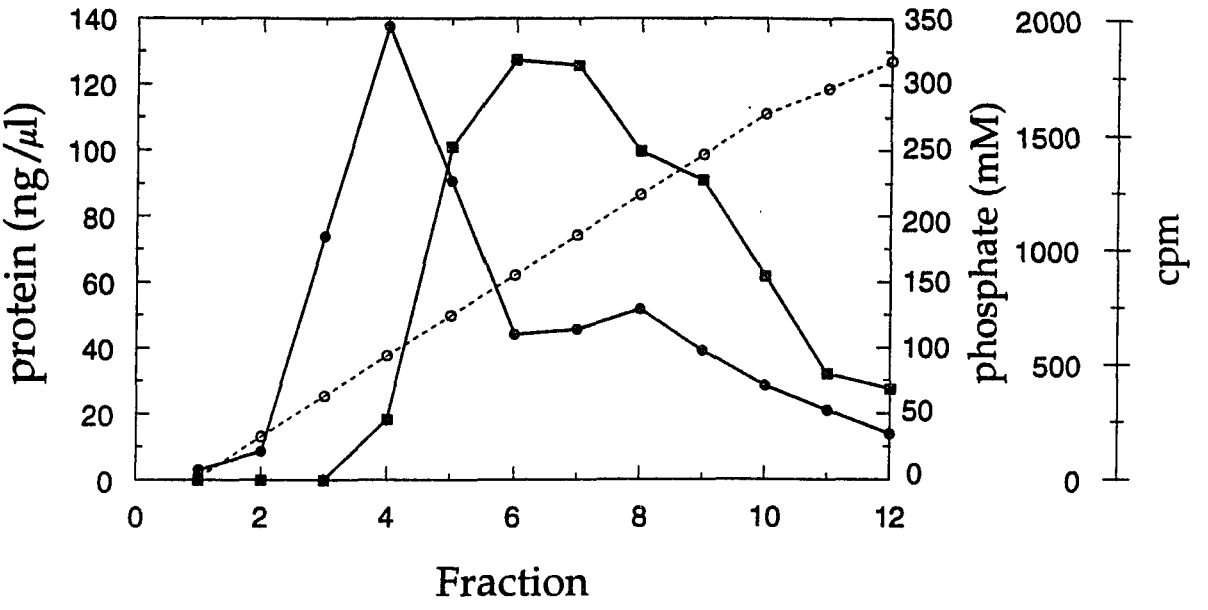


Figure 10. DEAE-cellulose elution profile. The TX-114 droplet was dissolved in buffer (10 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol) and the solution was chromatographed on a DEAE-cellulose column (1 x 8 cm) as described in Chapter 2. Enzyme was eluted with a linear gradient from 5.0 mM to 400 mM potassium phosphate (pH 7.5), containing 0.2% TX-100, and 10 mM β-mercaptoethanol. The eluate was collected in 3 ml fractions and subjected to assays for protein (●), cardiolipin synthase activity (■), and phosphate (O).

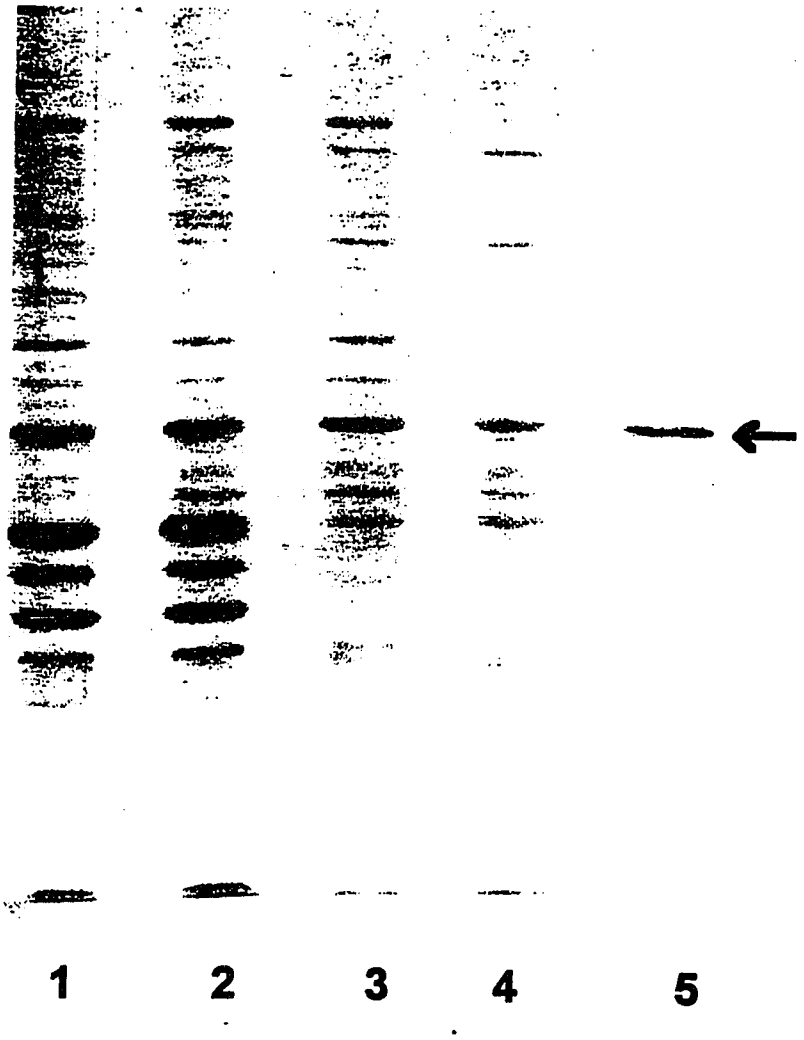


Figure 11.

Figure 11. SDS-PAGE analysis of fractions during purification. At each stage of purification, a sample containing between 10-100 μg was analyzed by SDS-PAGE. Lane 1, corresponds to the crude membrane fraction; lane 2, to the proteins after the potassium phosphate wash; lane 3, to the TX-114 extract (E); lane 4, to the oil droplet dissolved in 10 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol (S-114); and lane 5, to the pure DEAE-cellulose fraction. The arrow indicates the *cls* gene product of 46 kD.

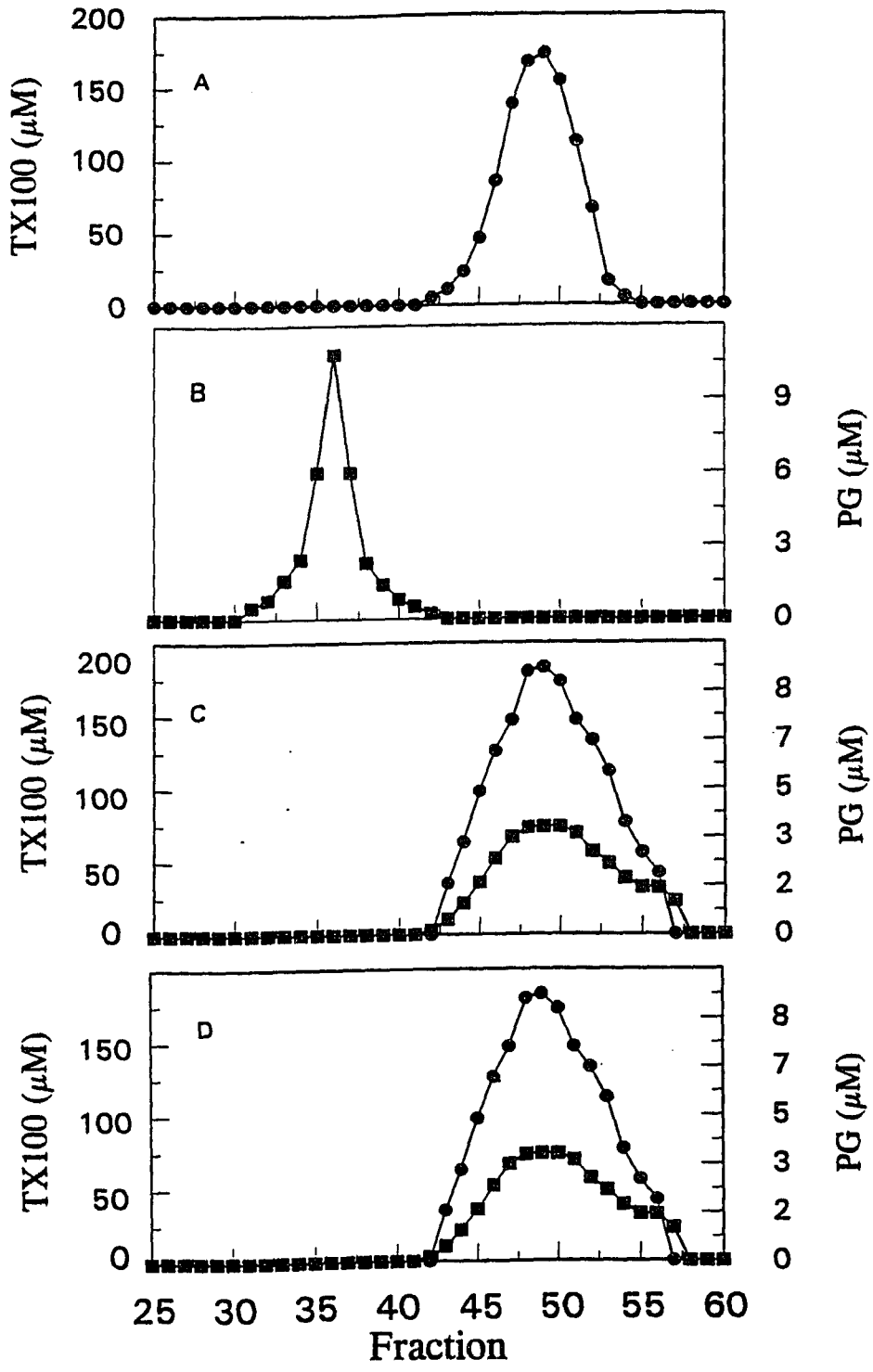

A
B
C
D
TX100 (μM)
PG (μM)
200
150
100
50
0
9
6
3
8
7
5
2
25 30 35 40 45 50 55 60
Fraction

Figure 12. Sepharose CL-6B chromatography of TX-100, cardiolipin, and phosphatidyl[2-^{3}H]glycerol. The column (1.5 x 35 cm) was equilibrated and eluted at room temperature (0.8 ml/min) with buffer consisting of 350 mM potassium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 0.02% sodium azide, and 0.02% TX-100 in A, C and D, and no TX-100 in B. The following samples (0.5 ml) were applied: A, 1.0 mM TX-100; B, 0.125 mM phosphatidyl[2-^{3}H]glycerol (650 dpm/nmol); C, 1.0 mM TX-100 and 0.125 mM phosphatidyl[2-^{3}H]glycerol; and D, 1.0 mM TX-100, 0.125 mM phosphatidyl[2-^{3}H]glycerol, and 0.075 mM CL. The concentration of TX-100 was determined from its molar extinction coefficient (1.41 x 10^3 at 275 nm), and the concentrations of both phosphatidyl[2-^{3}H]glycerol and diphosphatidyl[2-^{3}H]glycerol were determined by liquid scintillation counting. The symbols used are (●) TX-100, and (■) PG.

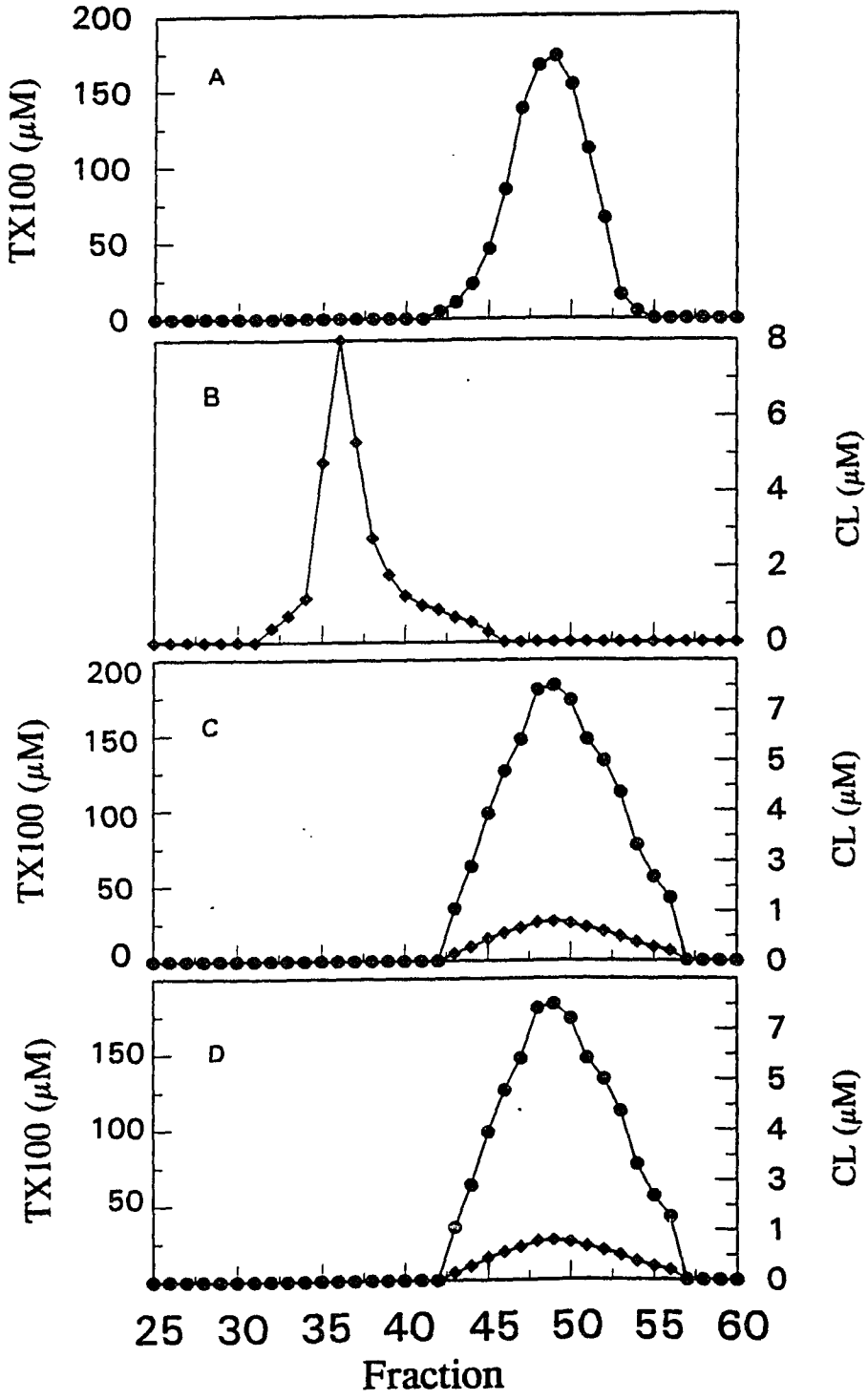

A
B
C
D
TX100 (μM)
CL (μM)
200
150
100
50
0
8
6
4
2
7
5
3
1
25 30 35 40 45 50 55 60
Fraction

Figure 13. Sepharose CL-6B chromatography of TX-100, phosphatidylglycerol, and diphosphatidyl[2-^{3}H]glycerol. The column (1.5 x 35 cm) was equilibrated and eluted at room temperature (0.8 ml/min) with buffer consisting of 350 mM potassium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 0.02% sodium azide, and 0.02% TX-100 in A, C and D, and no TX-100 in B. The following samples (0.5 ml) were applied: A, 1.0 mM TX-100; B, 0.075 mM CL (diphosphatidyl[2-^{3}H]glycerol (650 dpm/nmol); C, 1.0 mM TX-100 and 0.075 mM CL (diphosphatidyl[2-^{3}H]glycerol; 650 dpm/nmol); and D, 1.0 mM TX-100, 0.125 mM PG, and 0.075 mM diphosphatidyl[2-^{3}H]glycerol. The concentration of TX-100 was determined from its molar extinction coefficient (1.41 x 10^3 at 275 nm), and the concentrations of both phosphatidyl[2-^{3}H]glycerol and diphosphatidyl[2-^{3}H]glycerol were determined by liquid scintillation counting. The symbols used are (●) TX-100 and (♦) CL.

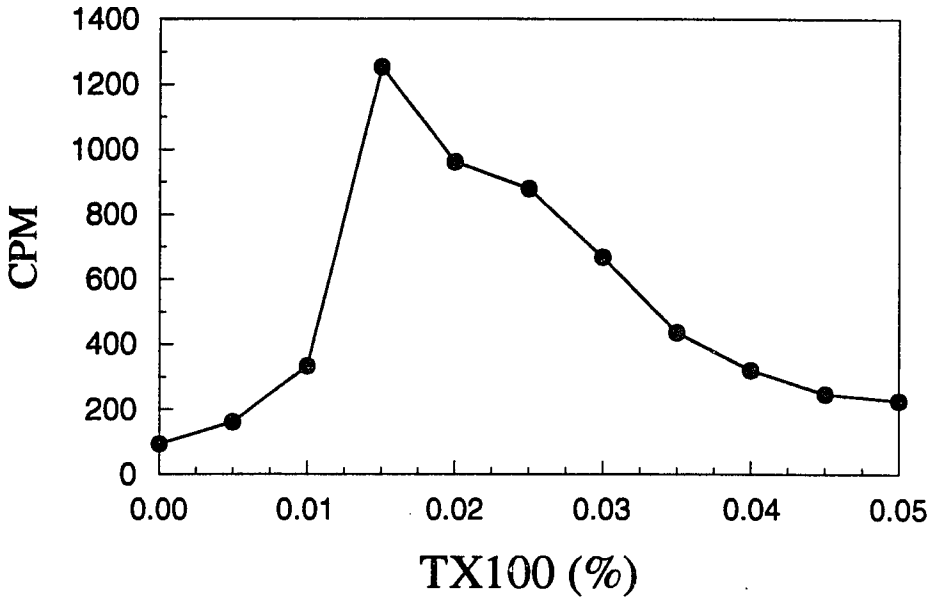


Figure 14. TX-100 profile of cardiolipin synthase. Cardiolipin synthase activities were measured after 5 min at 37°C for an enzyme preparation, at a concentration of 64 ng/ml, purified by DEAE-cellulose column chromatography as described in Chapter 2, except that TX-100 concentration was altered as indicated on the x-axis.

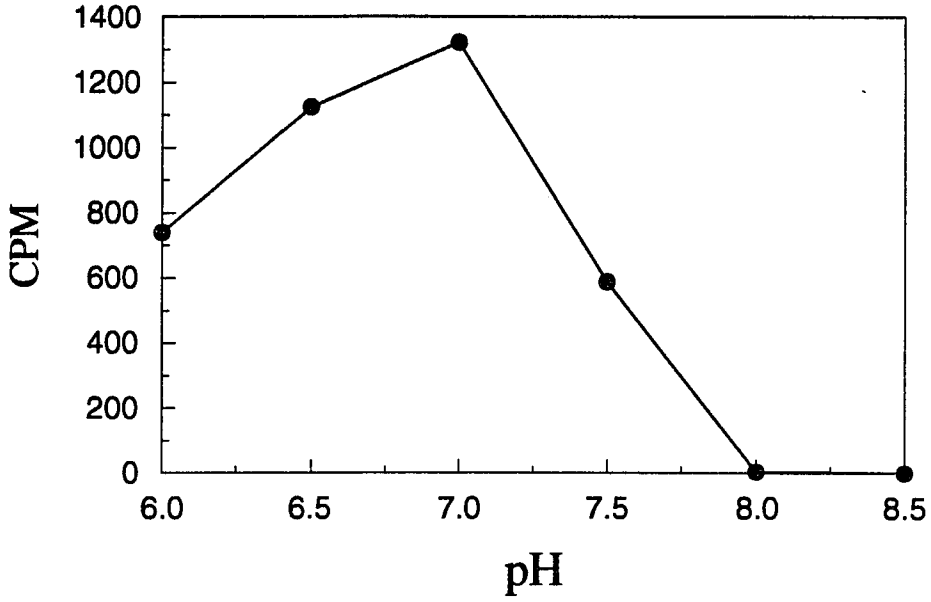


Figure 15. pH profile of cardiolipin synthase. Cardiolipin synthase activities were measured after 5 min at 37°C for an enzyme preparation, at a concentration of 64 ng/ml, purified by DEAE-cellulose column chromatography as described in Chapter 2, except that the pH was altered as indicated on the x-axis. A concentration of 320 mM potassium phosphate was used in this experiment.

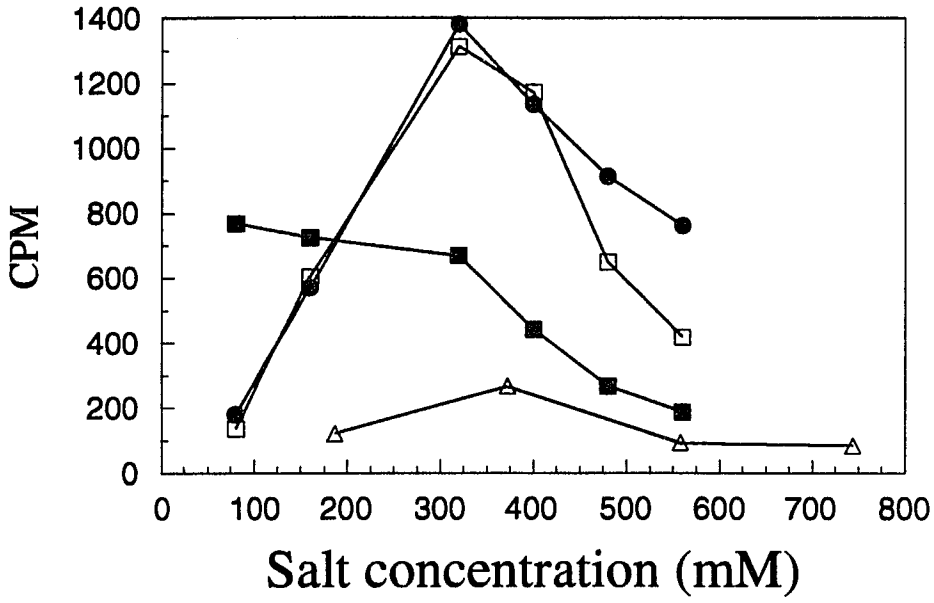


Figure 16. The effect of salt on cardiolipin synthase activity. Cardiolipin synthase activities were measured after 5 min at 37°C for an enzyme preparation, at a concentration of 64 ng/ml, purified by DEAE cellulose column chromatography as described in Chapter 2, except that the buffers were altered as follows: potassium phosphate; pH 7.0 (●), sodium phosphate; pH 7.0 (□); sodium sulfate; pH 7.0 (■), and potassium chloride; pH 7.0 (△).

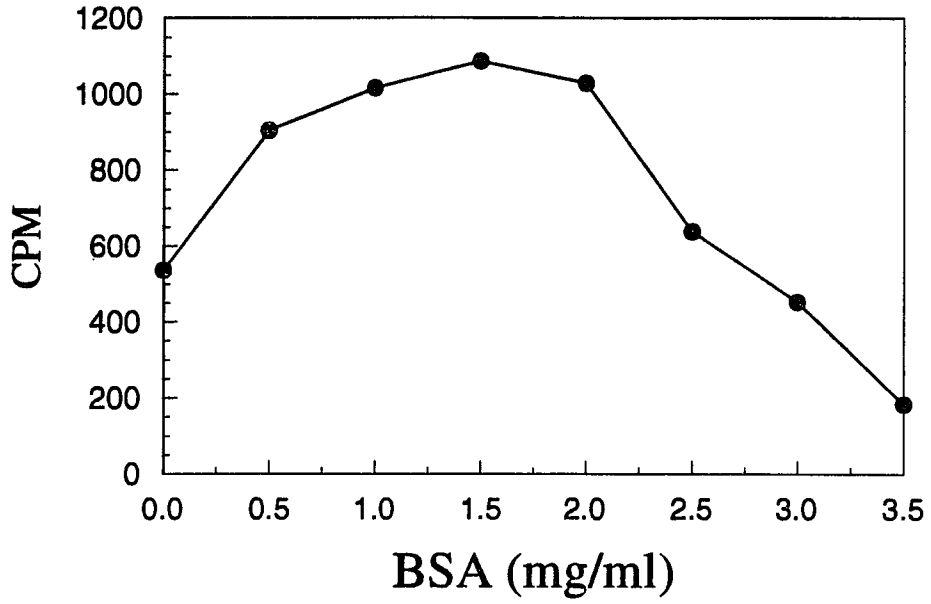


Figure 17. Bovine serum albumin profile of cardiolipin synthase. Cardiolipin synthase activities were measured after 5 min at 37°C for an enzyme preparation, at a concentration of 64 ng/ml, purified by DEAE-cellulose column chromatography as described in Chapter 2, except that BSA was altered as indicated on the x-axis.

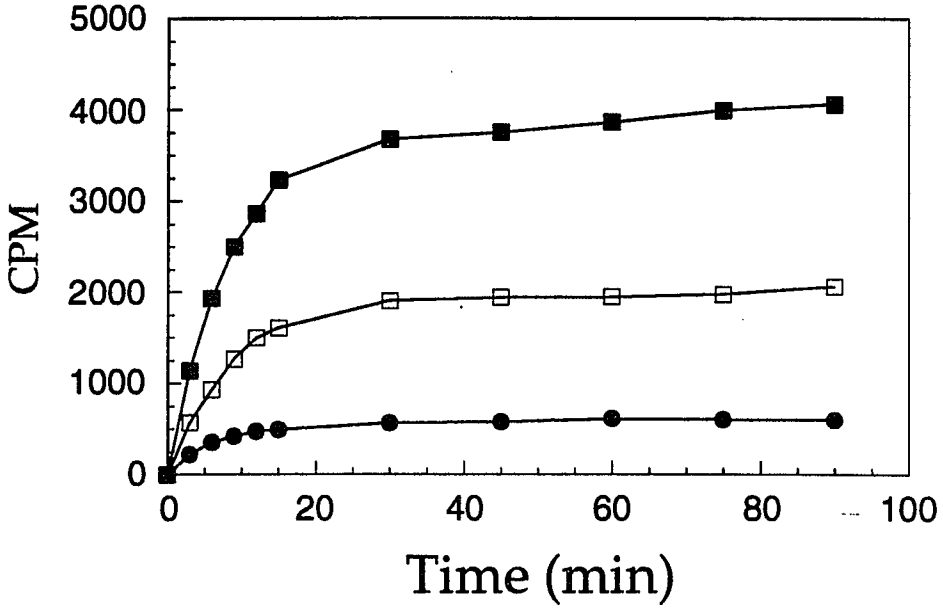


Figure 18. Effect of enzyme concentration on cardiolipin formation. Cardiolipin synthase assays were performed as described in Chapter 2, with enzyme concentrations of 40 ng/ml (●), 80 ng/ml (□), and 160 ng/ml (■). Activity was measured over 90 min.

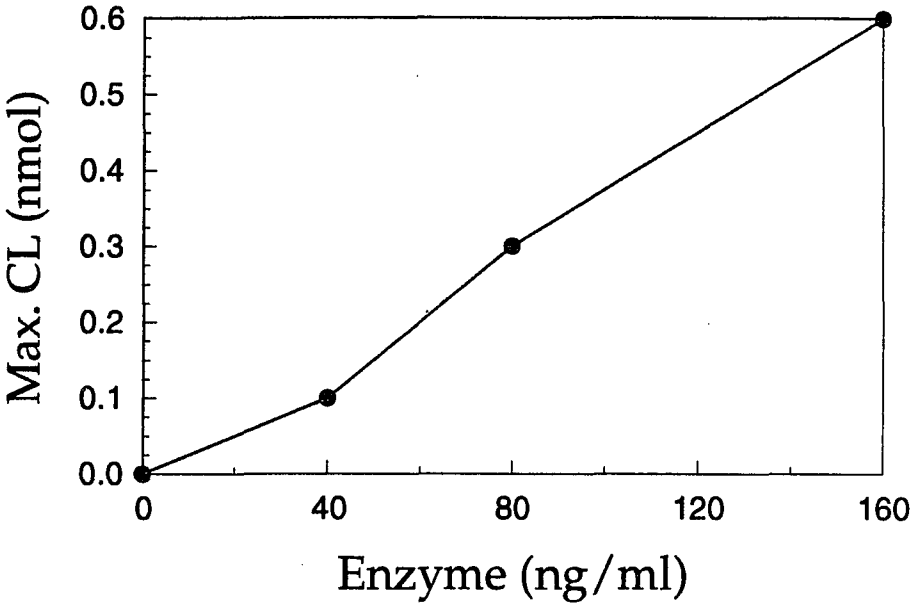


Figure 19. Effect of enzyme concentration on maximum cardiolipin formation. Cardiolipin synthase assays were performed as described in Chapter 2, with enzyme concentrations ranging from 40-160 ng/ml. Activity was measured after 60 min at 37 °C.

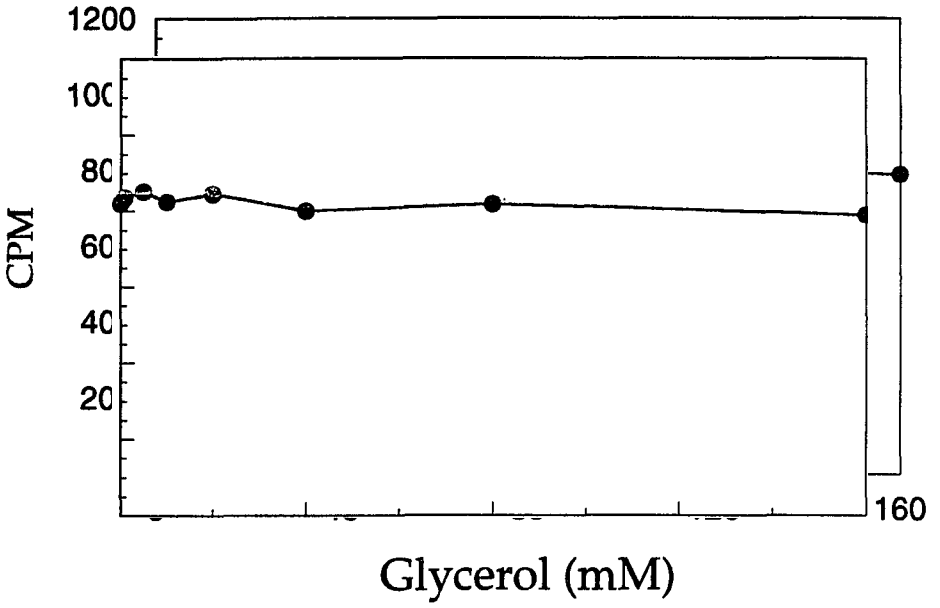


Figure 20. Effect of glycerol on cardiolipin synthase activity. Cardiolipin synthase activities were measured after 5 min at 37°C for an enzyme preparation, at a concentration of 40 ng/ml, purified by DEAE-cellulose column chromatography as described in Chapter 2, except that glycerol was altered as indicated on the x-axis.

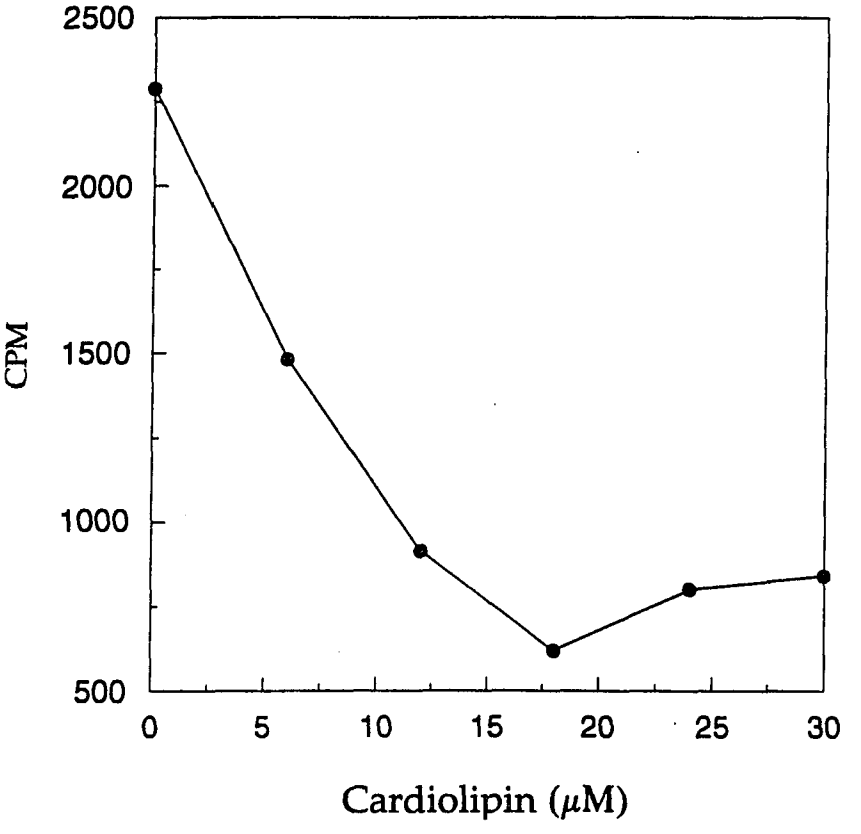


Figure 21A. Effect of cardiolipin on cardiolipin synthase activity. CL synthase assays were performed as described in Chapter 2, except that cardiolipin was added at the indicated concentrations. Glycerol release was measured after 5 min at 37 °C with a purified enzyme concentration of 320 ng/ml.

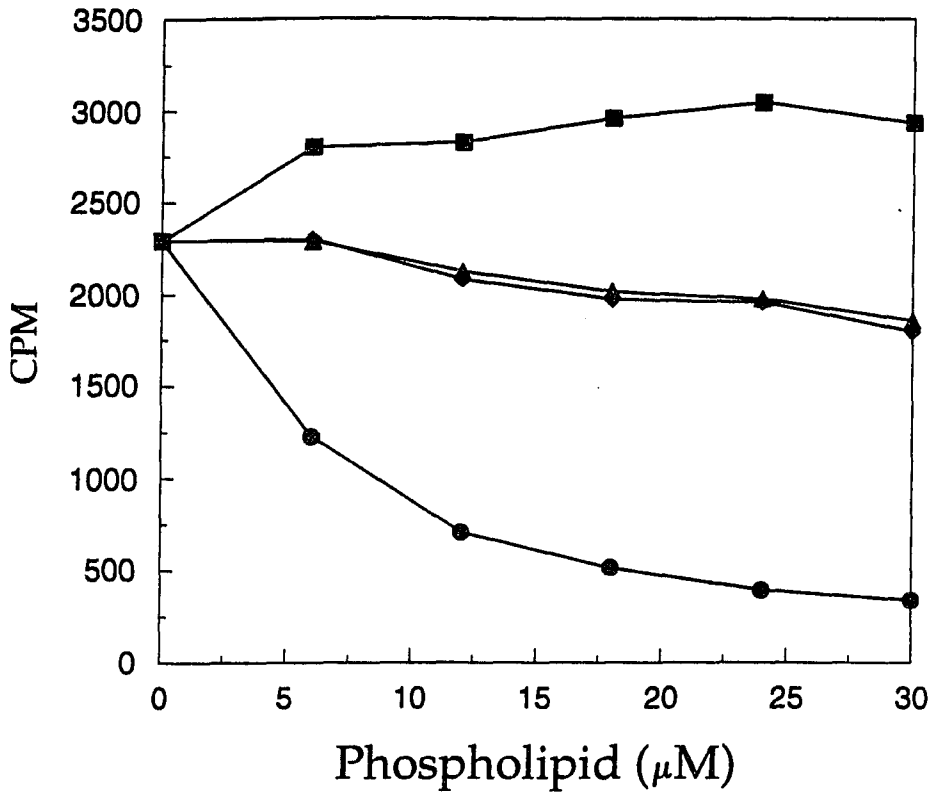


Figure 21B. Effect of anionic phosphoglycerides on cardiolipin synthase activity. Cardiolipin synthase assays were performed as described in Chapter 2, except that additional PA (●), CDP-diacylglycerol (▲), PI (■), or *bis*-PA (◆) were added. Glycerol release was measured after 5 min at 37°C with a purified enzyme concentration of 320 ng/ml.

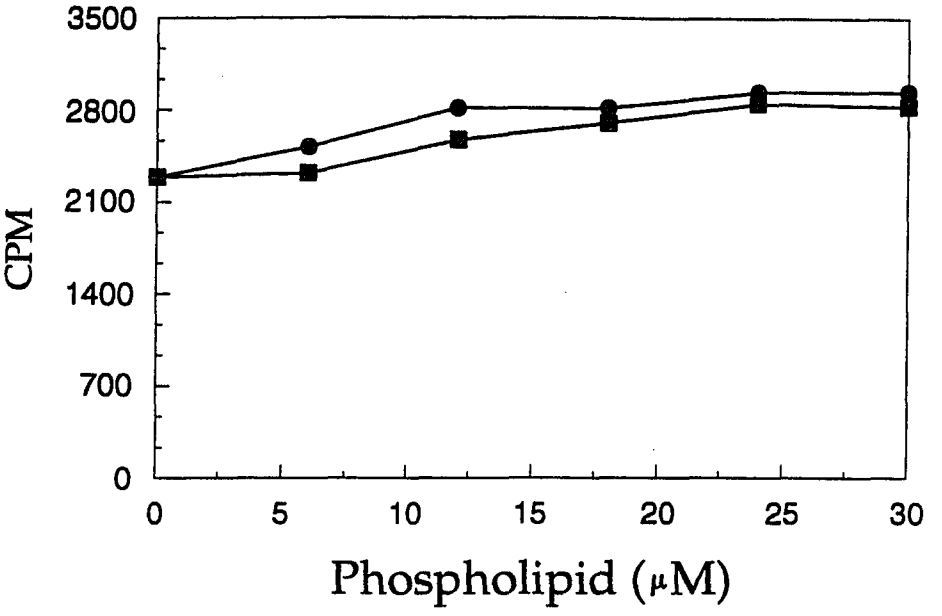


Figure 22. Effect of zwitterionic phosphoglycerides on cardiolipin synthase. Cardiolipin synthase assays were performed as described in Chapter 2, except that additional PS (■) or PE (●), were added. Glycerol release was measured after 5 min at 37°C with a purified enzyme concentration of 320 ng/ml.

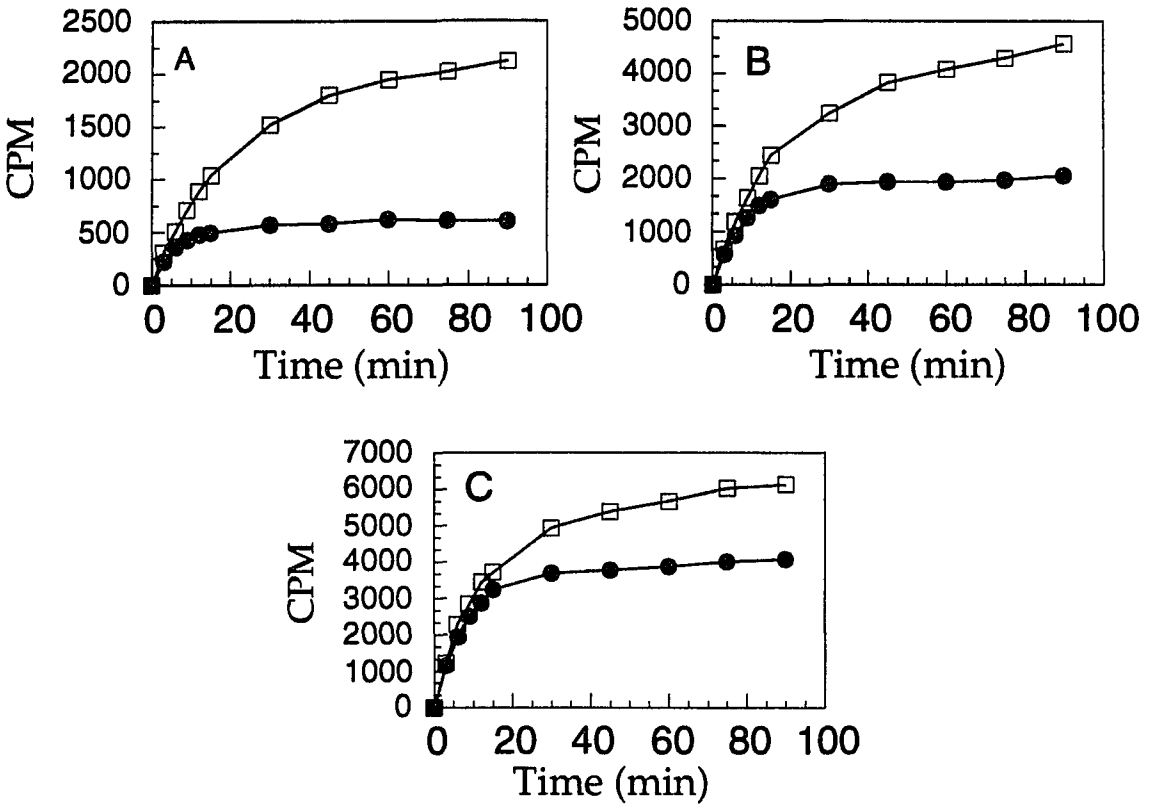


Figure 23. Effect of phosphatidylethanolamine on enzyme inhibition over time. Cardiolipin synthase assays were performed as described in Chapter 2. Enzyme concentrations were varied: A, 40 ng/ml; B, 80 ng/ml; and C, 160 ng/ml, in the presence (□), or absence (●) of 24 μM phosphatidylethanolamine, and activity was measured over time.

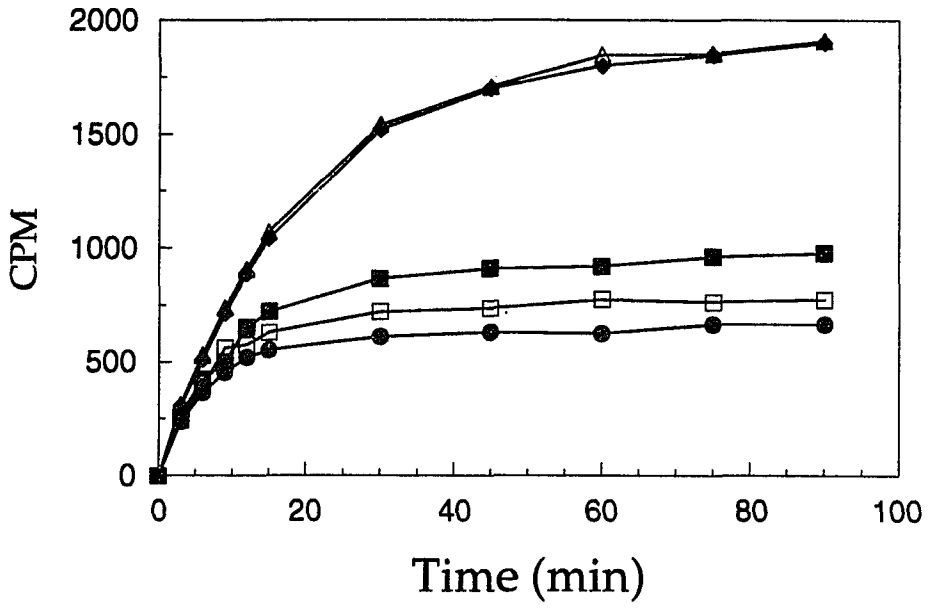


Figure 24. Effect of phosphatidylethanolamine on cardiolipin synthase activity. Cardiolipin synthase assays were performed as described in Chapter 2, except that tubes were pre-incubated with various amounts of phosphatidylethanolamine. The phosphatidylethanolamine: absent (●); 6 μM (□); 12 μM (■); 24 μM (♦); and 30 μM (Δ), was resuspended in 0.05% TX-100, and activity was followed over time with 40 ng/ml of enzyme.

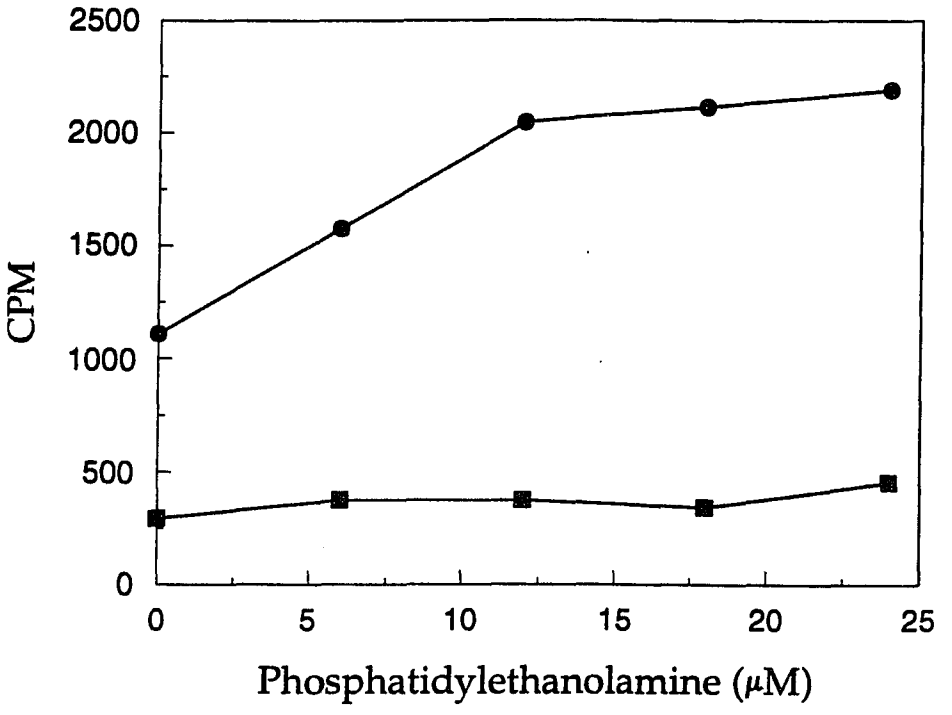


Figure 25. Effect of phosphatidylethanolamine on the anionic phosphoglyceride inhibition of cardiolipin synthase. Purified CL synthase (320 ng/ml) was assayed for 5 min as described in Chapter 2. All tubes were pre-incubated with varying amounts of phosphatidylethanolamine, resuspended in 0.05% TX-100, and an initial concentration of either 12 μM cardiolipin (●), or 12 μM phosphatidic acid (■).

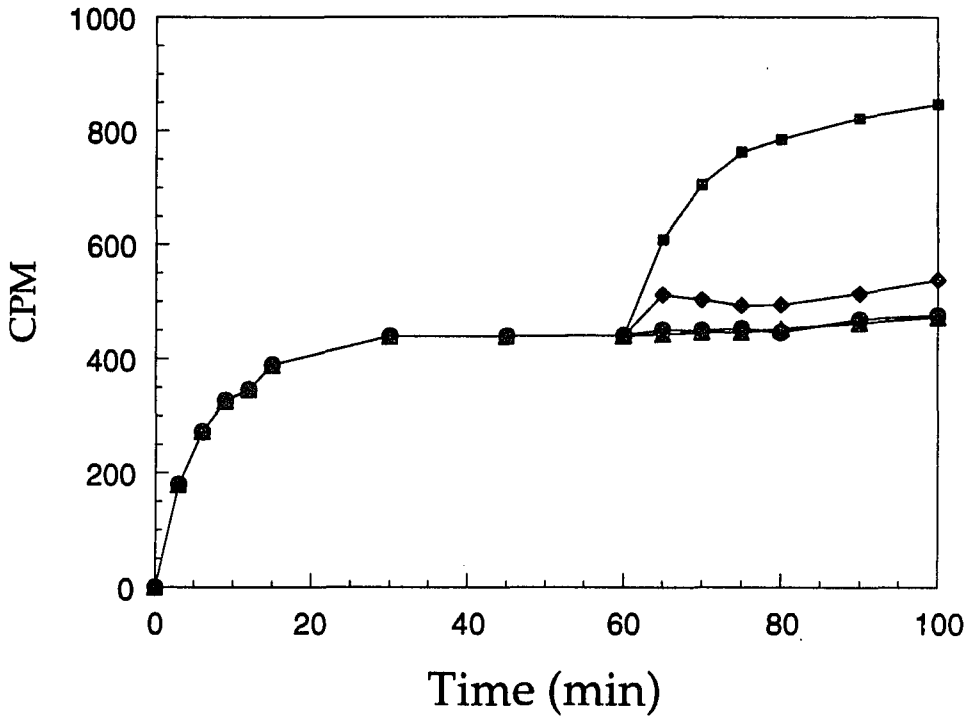


Figure 26. Reversal of cardiolipin synthase inhibition. Purified CL synthase was assayed as described in Chapter 2. At 60 min, when the reaction was complete, the reaction mixture was divided into four equal portions. Then, either distilled water (●), (2.0 nmol PE (▲), 2.0 nmol phosphatidyl[2-^{3}H]glycerol (♦), or 2.0 ng CL synthase (■) were added and glycerol release determined at the indicated times.

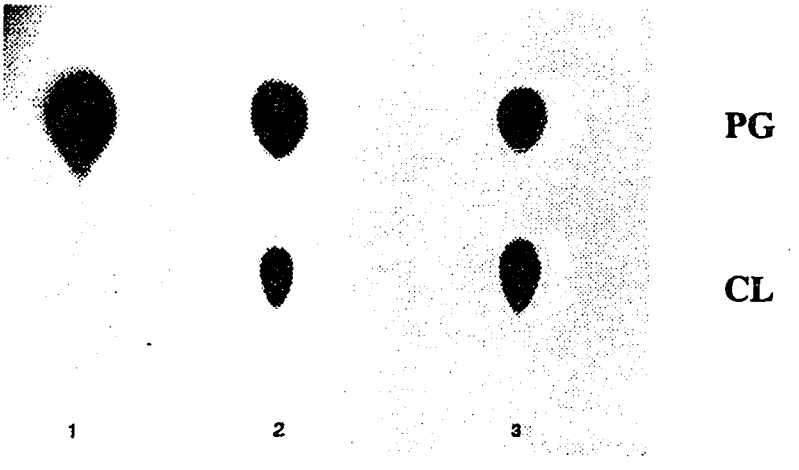


Figure 27. Autoradiogram of reaction products. [^{3}H]Lipids from a 1 ml reaction mixture were extracted and chromatographed on a Polygram Sil G thin-layer chromatography plate in a solvent system of tetrahydrofuran-methylal-methanol-4.0 N ammonium hydroxide (50:25:25:5). The plate was dried and exposed to film. Lane 1, no enzyme; lane 2, 10 min incubation; and lane 3, 20 min incubation.

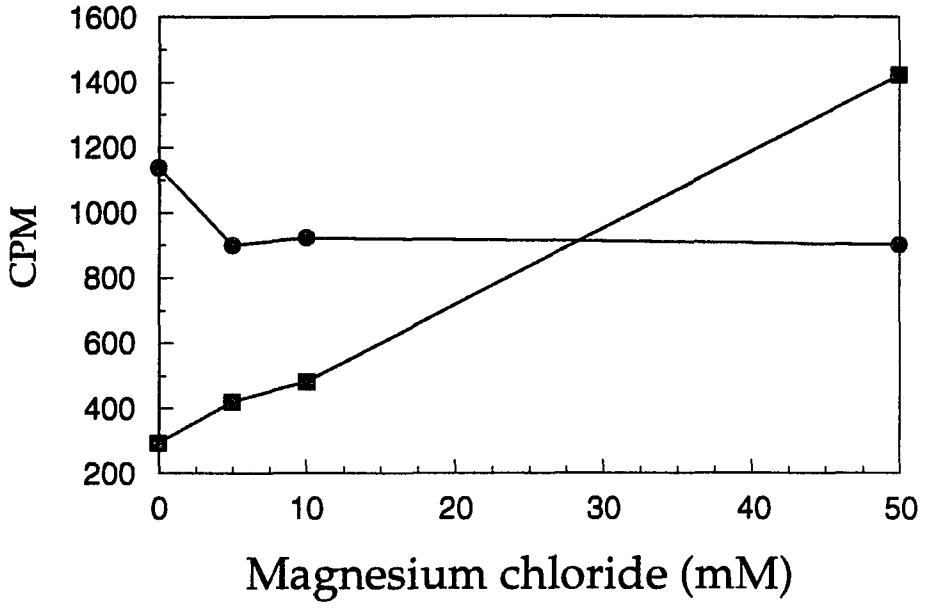


Figure 28. Effect of magnesium on the inhibition of cardiolipin synthase by anionic phosphoglycerides. Purified CL synthase (320 ng/ml) was assayed for 5 min as described in Chapter 2. All tubes were pre-incubated with varying amounts of magnesium chloride and an initial concentration of either 12 μM cardiolipin (●), or 12 μM phosphatidic acid (■).

Appendix

MEDIA & BUFFERS

M9ZB:	Ammonium chloride 0.1% Potassium phosphate 0.3% Sodium phosphate 0.6% Bacto-tryptone 1.0% Sodium chloride 0.5% Glucose 1.0% Magnesium sulfate 1.0 mM
L-Broth:	Bacto-tryptone 1.0% Yeast extract 0.5% Sodium chloride 0.5%
GTE Buffer:	Glucose 50 mM Tris-HCl (pH 8.0) 25 mM EDTA 10 mM
KOAc Buffer:	Potassium acetate 3.0 mM Glacial acetic acid 2.0 M
TE Buffer:	Tris-base (pH8.0) 10 mM EDTA 1.0 mM
Ligase Buffer:	Tris-HCl (pH 7.6) 25 mM Magnesium chloride 10 mM Dithiothreitol 1.0 mM ATP 0.4 mM
TAE Buffer:	Tris-base (pH 8.0) 40 mM Sodium acetate 20 mM EDTA 2.0 mM
TBE Buffer:	Tris-base (pH8.0) 80 mM Boric acid 40 mM EDTA 2.0 mM

TFB Buffer:	MES (pH7.3) 10 mM Magnesium chloride 45 mM Calcium chloride 10 mM Potassium chloride 100 mM Hexamine cobalt chloride 3 mM
PAGE Buffer:	Tris-HCl (pH 6.5) 50 mM EDTA 2.0 mM β-mercaptoethanol 1.0% SDS 1.0% Glycerol 8.0% Bromophenol blue 0.025%
Buffer I:	Potassium phosphate (pH7.5) 100 mM β-mercaptoethanol 10 mM
Sol. Buffer:	Potassium phosphate (pH 7.5) 100 mM β-mercaptoethanol 10 mM TX-114 1.0%
Buffered Sucrose:	Potassium phosphate (pH 7.5) 100 mM Sucrose 6.0% TX-114 0.06% β-mercaptoethanol 10 mM
Buffer A:	Tris-HCl (pH 7.5) 10 mM β-mercaptoethanol 10 mM Sucrose 20% TX-100 1.0%
EndoR Stop:	EDTA 0.5 M Bromophenol blue 0.025% Xylene cyanol 0.025% Sucrose 40%

Restriction Endonulcease Buffer:	Tris-HCl (pH 7.8) 25 mM Magnesium chloride 10 mM BSA 100 μg/ml β-mercaptoethanol 2.0 mM Sodium chloride 50 mM-150 mM

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