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Biosynthesis of Cardiolipin from Phosphatidylglycerol in *Staphylococcus aureus*

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Cardiolipin (CL) synthetase from *Staphylococcus aureus* catalyzes the complete conversion of two molecules of phosphatidylglycerol (PG) to one molecule of CL and one molecule of glycerol. The fatty acids and phosphates of the two PG molecules can be quantitatively recovered in the CL. The enzyme is membrane-bound, shows a linear relationship with the product formed between 10 and 125 μg of membrane protein, has a *p*H optimum at 4.4, a temperature optimum between 37 and 45 C, a K_m for PG of 2.1×10^{-4} M, a V_{max} of 200 nmoles of CL per min per mg of membrane protein, and does not require monovalent or divalent metals for activity. The enzyme has no nucleotide requirement and is not affected by prolonged dialysis, and treatment of the enzyme with charcoal has no effect on its activity. The enzyme has no phosphomonoesterase or phosphodiesterase activity, does not act on CL, is specific for PG, and CL and glycerol are the sole products of its activity. Other lipids do not stimulate or inhibit its activity. The enzyme is inhibited by organic solvents and some detergents. There is sufficient CL synthetase activity to account for CL synthesis during exponential growth. Inhibition of CL hydrolysis during growth results in an increase in CL that is balanced by a loss of PG. The activity of CL synthetase is not affected by cytidine diphosphate diglyceride but is inhibited competitively by the product, CL.

The phospholipid cardiolipin (CL) is unique among the glycerol phosphatides in that it contains a hydrophilic glycerol diphosphate bridge between the hydrophobic diacylated glycerols at each end of the molecule. Not only is the structure unique, but CL is localized almost exclusively in the inner membrane of the mitochondria in eukaryotic cells (4) and seems to be associated with the cytochrome oxidase of the electron transport chain (1). In the bacterial membranes, CL is usually a relatively minor component, but under conditions of stress, i.e., colicin treatment (3), incubation under unfavorable growth conditions (18, 20), inhibition of cell division (25), or in auxotrophs deprived of essential lipid components (9, 12), CL can accumulate from 2 to 25% of the lipid in many bacteria. The finding of a CL-specific phospholipase D in the membrane of *Haemophilus parainfluenzae* (15) led to the detection of a highly reactive pool of CL which is made and hydrolyzed in minutes (27). Using isolated CL-specific phospholipase D, the metabolism of the CL molecule was shown to be complex during exponential growth. The hydrophobic diglyceride ends seem to be metabolically stable, but the glycerol diphosphate in the middle undergoes rapid synthesis and hydrolysis from a small portion of the phosphatidylglycerol (PG) pool in *H. parainfluenzae* and *Staphylococcus aureus* (20, 27). Inhibitors of oxidative phosphorylation and proton gradient formation, like carbonylcyanide-chlorophenolhydrazone (m-CCCP) or tetrachlorosalicylanilide, inhibited an activity coupled to the rapid metabolism of CL but had no effect on the isolated phospholipase D in vitro (16). The unique localization of CL in the inner membrane of the mitochondria and the fact that the rapid metabolism of CL in bacteria was coupled to components in the membrane involved in oxidative phosphorylation suggested some possible role for CL in the electron transport system. During exponential growth, the CL content of the membrane remained constant (20, 28) even though there was rapid metabolism. This indicated that there must be rapid synthesis of CL in the membrane which could account for the rapid metabolism of the glycerol diphosphate in the middle of the mole-

cule. In this paper the synthesis of CL from two molecules of PG (Fig. 1) will be established, and it provides evidence for another unique feature of CL metabolism. All of the other major phospholipids are formed from a cytidine diphosphate (CDP) derivative of the alcohol that is esterified with the glycerol phosphate in the phospholipid or by reaction of the alcohol with CDP diglyceride (CDP-DiGly; Fed. Proc. **20**:934-940, 1961). Since PG was shown to be formed from CDP-DiGly and glycerol phosphate [with the intermediate formation of PG phosphate (PGP)], it was suggested that CL was formed by the addition of CDP-DiGly to PG (11). Such a reaction was detected in very low yields in *Escherichia coli* (23) and supposedly with endogenous CDP-DiGly in mitochondria (5). Addition of CDP-DiGly to mitochondrial preparations yielded PG and PGP in one set of experiments (5) or a very low rate of 6 pmoles of CL formed per mg of protein per hr in another (10).

Evidence has been accumulating that CL biosynthesis which was sufficiently rapid to account for the metabolic activities of this molecule did not require a nucleotide precursor. In *Micrococcus cerificans*, CDP-DiGly generated endogenously produced only PG and PGP but no CL (W. R. Finnerty, Bacteriol. Proc., p. 151, 1971). In *M. lysodeikticus*, a membrane-bound enzyme formed CL from PG in high yield in the absence of detectable CDP-DiGly or degradation of PG (6). The accumulation of CL was balanced by the loss of PG in *E. coli* incubated in the absence of an energy source where the CDP-DiGly presumably could not be formed (17). Transesterification reactions with cabbage phospholipase D gave a very low yield of CL from PG without the involvement of nucleotides (23). In this study, an active membrane enzyme forms CL rapidly and has neither a nucleotide requirement nor phospholipase activities.

MATERIALS AND METHODS

Materials. Sodium acetate-*1*-^{14}C, glycerol-*1,3*-^{14}C, and $H_3{}^{32}PO_4$ were purchased from New England Nuclear Corp., Boston, Mass. Beef heart CL was obtained from General Biochemicals, Chagrin Falls, Ohio. Lysostaphin was purchased from Schwarz/Mann, Orangeburg, New York. The sources of the other materials used have been previously reported (19).

Growth of S. aureus. The strain, culture conditions, methods of preservation, preparation of inocula, and harvesting procedures were previously described (29). The growth medium was prepared as described (19).

Preparation of the enzyme. The bacteria were harvested from late exponential-phase cultures by centrifugation at 4 C. The cells were washed once with 50 mM phosphate buffer (PB buffer), *p*H 7.4, and resuspended in PB buffer containing 4.1 M NaCl. Then lysostaphin [0.08 mg/g (wet weight) of cells] was added, and the cell suspension was incubated for 20 min at 37 C in a water-bath shaker. The protoplasts were collected by centrifugation and resuspended in cold PB buffer by vigorous homogenization with a Teflon glass homogenizer, and the lysis mixture was sonically treated for 3 min. The homogenate was centrifuged three times at 5,090 × *g* to remove whole cells. The 5,090 × *g* pellet was suspended in cold PB buffer, sonically treated, and recentrifuged as above. The 5,090 × *g* supernatant fluids were combined and centrifuged at 60,000 × *g* for 60 min. The high-speed pellet was resuspended in cold PB buffer and frozen at −20 C. Protein was measured by the method of Lowry et al. with bovine serine albumin as standard (13).

Extraction and deacylation of the lipid. The phospholipid was extracted from *S. aureus* by the procedure of Bligh and Dyer (2). The diacyl phospholipids were deacylated by mild alkaline methanolysis as described previously (21).

Chromatography. The phospholipids were separated on Whatman silica gel-impregnated paper (SG-81) with solvents I and III as described by Wurthier (19, 30). The glycerol phosphate esters derived from the diacyl phospholipids were separated and identified by either chromatography on aminocellulose paper or by anion exchange chromatography by using Dowex-1 columns with an ammonium formate gradient containing sodium borate (7, 21). The free glycerol obtained from the aqueous phase of the reaction mixture was chromatographed on Eastman cellulose chromagrams in a solvent of *n*-butanol-pyridine-water (6:3:4, v/v; reference 26).

Preparation of phospholipid substrates. ^{14}C- or ^{32}P-labeled phospholipid was extracted from cells of *S. aureus* that had been grown with $H_3{}^{32}PO_4$ or glycerol-*1,3*-^{14}C for 16 hr. Following the separation of the diacyl phospholipids on SG-81 paper, the PG was extracted from the paper by the procedure of

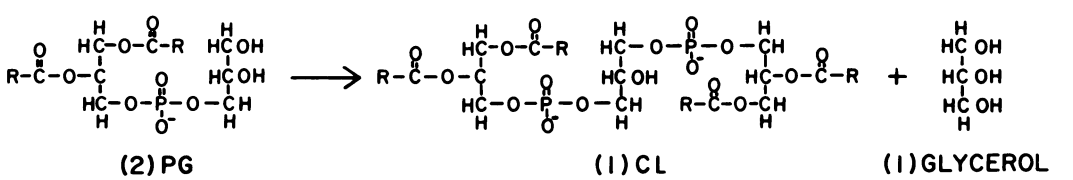

FIG. 1. *Synthesis of cardiolipin (CL) from phosphatidylglycerol (PG).*

Bligh and Dyer. The PG was rechromatographed on SG-81 paper in two dimensions and extracted as above. The purity of the PG was determined by mild alkaline methanolysis and chromatography of the glycerol phosphate ester on aminocellulose paper and Dowex-1 columns.

To prepare ^{14}C- and ^{32}P-labeled PG, *H. parainfluenzae* was pulsed for one generation with sodium acetate-*1*-^{14}C and $H_3{}^{32}PO_4$, the lipids were extracted and separated, and the PG was purified as described for *S. aureus*.

Enzyme assay. CL synthetase activity was assayed in 0.5-ml reaction mixtures containing 40 mM acetate buffer, *p*H 4.4; 10 mM $MgCl_2$; 50 to 100 μg of protein; and PG suspended in Triton X-100. The final concentration of Triton in the reaction mixture was 0.14%. The reaction mixtures were incubated at 37 C for 15 min unless otherwise indicated in the figure legends. The reaction was terminated by the addition of chloroform, methanol, and water to give Bligh and Dyer (2) proportions. The reaction mixtures were placed in the refrigerator overnight and centrifuged, the upper phase was aspirated, and the chloroform phase was evaporated under a stream of nitrogen. The lipids in the chloroform phase were dissolved in chloroform-methanol (2:1, v/v), and a sample was removed for chromatography on SG-81 paper in solvent I as described by Wurthier (30). After the one-dimension chromatography, the papers were placed on Kodak no-screen X-ray film. The areas of the chromatogram corresponding to the darkened areas of the film were cut out and their radioactivities were determined. CDP-DiGly was synthesized and purified as described by McCaman and Finnerty (14). The phosphatidic acid cytidyl transferase was the generous gift of W. R. Finnerty. CDP-^{14}C-DiGly was prepared from PA that was labeled both in the fatty acids and glycerol backbone. Cytidine monophosphate (CMP)-^{32}P-DiGly was prepared from ^{32}P-PA.

RESULTS

An enzyme located in the membrane fraction prepared by *S. aureus* protoplasts synthesized CL from PG without the addition of nucleotide. This enzyme was found almost exclusively in the 60,000 $\times$ *g* pellet of the lysate obtained from ultrasonic disruption of lysostaphin protoplasts. Attempts to solubilize this enzyme have thus far been unsuccessful. The activity of *S. aureus* cardiolipin synthetase was inhibited by Triton X-114 and X-405, Sarkosyl NL97 and NL30, Brij 35 and 58, Tween 80, Nonidet P-40, sodium lauryl sulfate, sodium deoxycholate, and hexadecylpyridinium chloride but not diminished significantly by the detergents Triton X-100, Triton N-101 or Cutscum. Detergents were tested at 0.1% and 1.0% (w/v). The enzymatic activity was completely destroyed by incubating the membrane preparation at 75 C for 5 min. Phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, and CL added to the reaction mixture were recovered quantitatively after incubation for 60 min with the enzyme. The enzyme preparation did not contain any phosphomonoesterase or phosphodiesterase activity as evidenced by the absence of ^{32}P or ^{14}C from the fatty acids or glycerols in the aqueous phase of the Bligh and Dyer extraction of the reaction mixture.

Properties of the enzyme. The activity of cardiolipin synthetase was stimulated 12% by the addition of 10 mM $MgCl_2$ to the reaction mixture. Other divalent metals such as Ca^{2+}, Zn^{2+}, CO^{2+}, Ba^{2+}, and Mn^{2+} inhibited the activity of the enzyme. The monovalent metals, Na^+ and K^+, had no effect on the enzyme activity. In accordance with the lack of an added metal requirement, cardiolipin synthesis was unaffected by ethylenediaminetetraacetic acid (EDTA). Dithiothreitol did not stimulate and *p*-hydroxymercuribenzoate did not inhibit enzyme activity. The addition of the nucleotides adenosine triphosphate (ATP), cytidine triphosphate (CTP), CDP, or combinations of these compounds had no affect upon the total amount of CL synthesized by the enzyme preparation. Furthermore, the activity of the enzyme preparation was unaffected by prolonged dialysis and treatment of the preparation with activated charcoal.

Unlike many of the other phospholipid synthesizing-enzymes reported in the literature, the *S. aureus* CL synthetase had a *p*H optimum of 4.4 in acetate buffer (Fig. 2). Moreover, the synthesis of CL was inhibited 90% by incubation of the enzyme, *p*H 7.4, in several different buffers. The temperature optimum for cardiolipin synthetase was between 37 and 45 C.

The synthesis of cardiolipin was linear with protein concentration in the range of 10 to 125 μg of protein. The CL synthesized was related linearly with the PG added to the reaction mixture at subsaturating PG concentrations (Fig. 3). Cardiolipin synthesis by the *S. aureus* enzyme proceeded rapidly with approximately 78% of the total CL formed in 1 hr being made in the first 15 min of incubation (Fig. 4). The data presented in Fig. 2 and 3 demonstrate that there was a stoichiometric conversion of substrate, PG, to CL.

Identification of CL. CL was the only phosphate-containing product formed during the CL synthetase reaction. The acylated lipid product obtained from the reaction mixture co-chromatographed with authentic *S. aureus* CL. When the lipid from the entire chloroform phase was collected and deacylated by mild

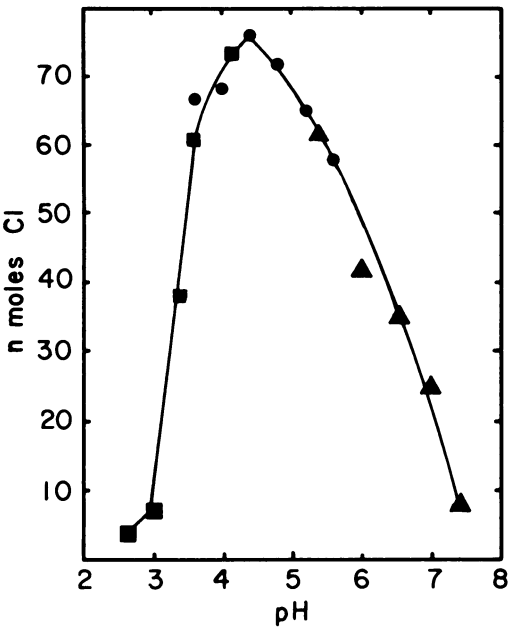

FIG. 2. *The pH optimum for cardiolipin synthetase. The enzymatic activity was measured in 40 mM buffers containing 10 mM $MgCl_2$, 50 μg (protein) of S. aureus membrane, and 100 nmoles of phosphatidylglycerol (50,000 counts per min) suspended in 0.14% Triton X-100 in 0.5 ml. After 30 min at 37 C, chloroform and methanol were added, and the lipids were extracted and separated chromatographically.*

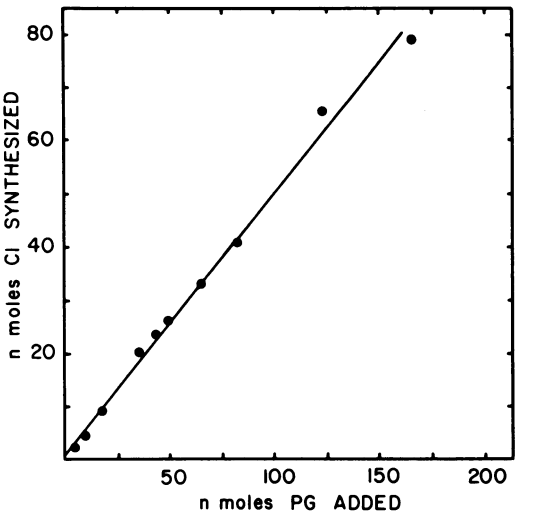

FIG. 3. *Conversion of phosphatidylglycerol (PG) to cardiolipin (CL) at subsaturating substrate concentrations. The enzyme was assayed at pH 4.4 in 40 mM acetate buffer as in Fig. 2 except that 125 μg of membrane protein was used.*

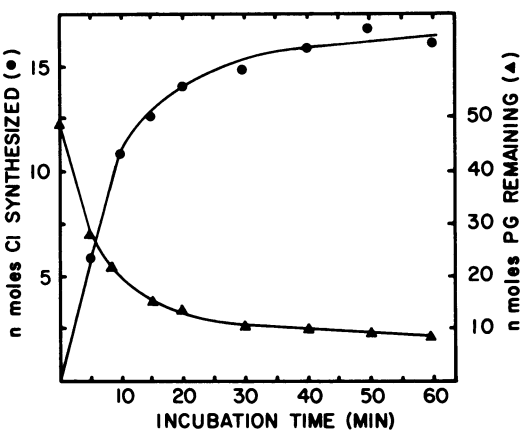

FIG. 4. *Kinetics of conversion of phosphatidylglycerol (▲) to cardiolipin (●). Enzyme was assayed as in Fig. 2 with 50 nmoles of phosphatidylglycerol.*

alkaline methanolysis, the glycerol phosphate ester of CL, bis-glycerylphosphorylglycerol (GPGPG), was the only ^{32}P-containing product obtained. This GPGPG co-chromatographed in two dimensions on aminocellulose paper with authentic GPGPG derived from beef heart CL. The glycerol ester of the CL also had the same elution volume from Dowex-1 columns as did authentic GPGPG.

CDP-DiGly and CL synthesis. Since the involvement of CDP-DiGly has been postulated for CL synthesis in *E. coli* (2), the role of this compound in the synthesis of CL and its relation to the *S. aureus* CL synthetase were examined. The CDP-DiGly was synthesized from phosphatidic acid and CTP in a reaction catalyzed by *M. cerificans* phosphatidic acid cytidyl transferase (14). The compound isolated from the reaction mixture had the same chromatographic mobility as authentic CDP-DiGly and had molar ratios for cytidine-phosphate-fatty acid of 1:2:2. When CDP-^{14}C-DiGly was incubated with cardiolipin synthetase at *p*H 4.4 in the presence of ^{32}P-PG, the CL isolated from the reaction mixture was labeled only with ^{32}P. Examination of the rate of CDP-DiGly hydrolysis during an incubation period of 15 min revealed that this compound was not metabolized by the enzyme preparation at *p*H 4.4. Furthermore, incubation of *S. aureus* cardiolipin synthetase at *p*H 7.4 with CMP-^{32}P-DiGly or CDP-^{14}C-DiGly did not result in the synthesis of CL. When sn-glycero-3-P was added to the above reaction mixture, PG was synthesized. However, the synthesis of CL could not be detected. A membrane preparation with 13 mg of protein per ml showed less than 0.001 absorbance at 260 nm, indicating that the nucleotide content was less

than 5 nmoles per mg of membrane protein. Membranes prepared from *S. aureus* with the CL synthetase activity have been shown to contain no detectable ATP (23).

Stoichiometry of the reaction. The mechanism by which CL was synthesized from PG was investigated using ^{14}C- and ^{32}P-labeled PG. The doubly labeled PG was obtained from *H. parainfluenzae* that had been pulsed for one generation with ^{14}C-acetate and $H_3{}^{32}PO_4$. The PG thus obtained was labeled with ^{14}C only in the fatty acid portion of the molecule as evidenced by the absence of the ^{14}C label in the glycerylphosphorylglycerol (GPG) derived from the diacyl PG and with ^{32}P in the phosphate moiety of the molecule. When this ^{14}C, ^{32}P-PG was used as the substrate for cardiolipin synthetase, the CL isolated was also labeled with both ^{14}C and ^{32}P. Moreover, when the $^{14}C/^{32}P$ ratio calculated for the substrate PG was compared with that calculated for the product CL, the ratios for both lipids were identical (Table 1). As predicted from the methods of labeling the PG, no ^{14}C was found in the aqueous phase of the Bligh and Dyer extraction.

The stoichiometry of the reaction was examined using ^{14}C-glycerol-labeled PG as the substrate (Table 2). If CL is indeed synthesized from two molecules of PG with the elimination of one molecule of glycerol, then 75% of the total ^{14}C in the glycerol phosphate ester of PG should be recovered in GPGPG and 25% of the initial ^{14}C recovered as free glycerol. The radioactivity of the ^{14}C-GPG obtained from the zero time controls was taken as 100% of the ^{14}C present in the reaction mixture. The data presented in Table 2 show that 74.5% of the ^{14}C present in the glycerol phosphate backbone of the substrate PG was recovered as GPGPG. The aqueous phase from the reaction extract accounted for 24.8% of the initial ^{14}C in the reaction mixture. The ^{14}C-labeled compound recovered from the aqueous phase was identified as free glycerol by co-chromatography with authentic standards.

TABLE 1. *Conservation of fatty acids and phosphate in cardiolipin synthesis*[a]

$^{14}C/^{32}P$ ratios		
PG	CL	Glycerol
1.783	1.746	0
7.058	7.097	0

[a] Phosphatidylglycerol (PG), labeled with ^{14}C in the fatty acids but not in the glycerols and with ^{32}P, was incubated with the enzyme as in Fig. 2. The $^{14}C/^{32}P$ ratios for the substrate (PG) and the product cardiolipin (CL) were calculated. The values given represent the average of 12 replications for each experiment.

TABLE 2. *Stoichiometry of the cardiolipin synthetase reaction*[a]

Phosphatidylglycerol		Cardiolipin		Glycerol	
^{14}C	%	^{14}C	%	^{14}C	%
16,200	100	12,100	74.8	4,070	25.1
37,300	100	27,200	72.9	9,020	24.2
64,100	100	47,800	74.5	15,900	24.8

[a] The phosphatidylglycerol labeled with ^{14}C in the glycerols and fatty acids was added to the reaction mixtures described in Fig. 2. The reaction was incubated for 1 hr at 37 C and the lipids were extracted. The aqueous phase was transferred to a weighed tube and the chloroform was dried in a stream of nitrogen. The lipids recovered in the chloroform phase were deacylated by mild alkaline methanolysis. The fatty acid methyl esters were extracted and the water-soluble glycerol phosphate esters of phosphatidylglycerol and cardiolipin were separated on aminocellulose paper. Samples of the aqueous phase of the reaction mixture extract contained glycerol which was identified by chromatography on Eastman cellulose chromagrams and on Dowex-1 columns. Each value given represents the average obtained from eight reaction mixtures for each experiment.

Kinetics of CL formation. When the initial velocities for the formation of CL were plotted according to the method of Lineweaver and Burk, the apparent K_m of CL synthetase for PG was 2.13×10^{-4} M and the V_{max} was 200 nmoles per min per mg of membrane protein (Fig. 5). The addition of beef heart or *S. aureus* CL to give final concentrations of 6×10^{-5} M and 1.2×10^{-4} M increased the apparent K_m without altering the value calculated for the V_{max} of the reaction (Fig. 5).

CL synthesis in vivo. To investigate the possible role of CL synthetase in the synthesis of CL in vivo, an inhibitor, m-CCCP, was used to block CL catabolism but not its synthesis. Addition of 10^{-4} M m-CCCP to a culture which had been grown for eight generations in $H_3{}^{32}PO_4$ and then transferred to medium containing no radioactivity resulted in immediate cessation of growth. The proportion of the ^{32}P in each phospholipid was then examined. The proportion of phospholipid ^{32}P for phosphatidic acid remained constant and that for the lysyl ester of PG increased slightly (from 12 to 16%). The most dramatic changes were observed in the percentages of PG and CL (Fig. 6). The proportion of PG decreased by 24% and was approximately balanced by an increase of 21% in CL. During the period of the

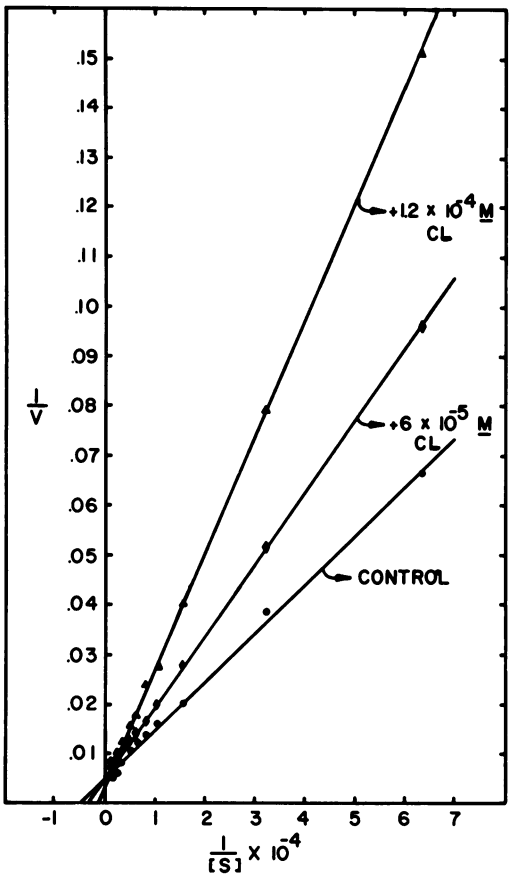

FIG. 5. *Double reciprocal plot of the conversion of phosphatidylglycerol to cardiolipin (CL). Enzyme was assayed as in Fig. 3 after 3 min of incubation. Beef heart CL was added to give final concentrations of 0.12 mM and 0.06 mM in 0.14% Triton X-100 as indicated.*

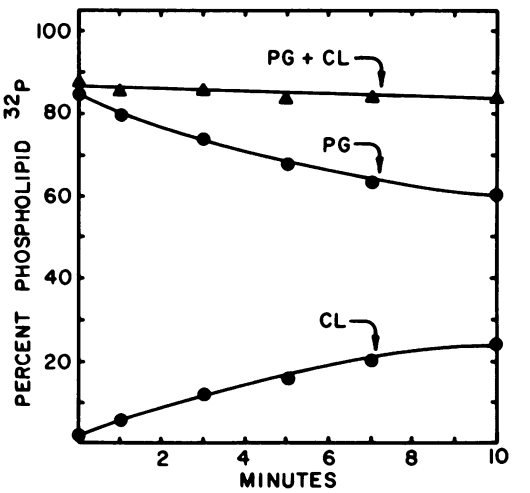

FIG. 6. *Effect of 0.1 mM carbonylcyanide m-chlorophenolhydrazone (m-CCCP) on phosphatidylglycerol (PG) and cardiolipin (CL) metabolism. Exponentially growing cells were grown for eight doublings with $H_3{}^{32}PO_4$, washed and transferred to nonradioactive medium containing m-CCCP, and sampled. The lipid composition was then analyzed (19). Rate was measured in nanomoles per minute per milligram of protein.*

experiment, the total proportion of PG plus CL remained constant at 87% of the total phospholipid ^{32}P.

DISCUSSION

From this and previous studies, it appeared that the membrane of *S. aureus* formed PG from CDP-DiGly just as in other bacteria (11). Two molecules of PG then formed one molecule of CL and one molecule of glycerol (Table 2) with both the diacylated glycerols being incorporated into the molecule (Table 1). Several properties of the enzyme were unusual. (i) The reaction went to completion which may be due to the removal of the water-soluble glycerol from the lipid-membrane complex (Tables 1 and 2). (ii) The reaction had a *p*H optimum of 4.4 (Fig. 2) which might reflect the problem of presenting a lipid substrate-detergent complex to a membrane-bound enzyme in aqueous suspension. Perhaps the charges on the phosphates must be partially neutralized for the reaction with added PG to be detectable. (iii) The kinetics (Fig. 5) suggest a much greater affinity for one of the two PG molecules that react. The unimolecular kinetics (Fig. 5) was like that for porphobilinogen synthetase (8). In this reaction two molecules of δ-aminolevulinic acid condense to form one molecule of porphobilinogen (8). The kinetics also suggest that increasing the *S. aureus* or beef heart CL concentration inhibited CL formation competitively. (iv) The synthesis of CL by the membranes apparently required no involvement of cytidine nucleotides. The absorbance of the membrane preparation at 260 nm indicated a nucleotide content of less than 5 nmoles per mg of protein. Since the V_{max} of the CL synthetase is 200 nmoles per min per mg of protein (Fig. 5), any endogenous nucleotide involved in CL synthesis would have a turnover of at least 40 times that of the enzyme. Dialyzing the enzyme or adsorption on charcoal did not decrease the specific activity. In this respect the formation of CL was unique among the phospholipids. (v) The enzyme was unlikely to be a nonspecific phosphatase as it will not react with phosphatidic acid, phosphatidylserine, or phosphatidylethanolamine and was inhibited but will not react with CL.

In an attempt to determine whether an enzyme like CL synthetase could account for CL synthesis in *S. aureus*, the inhibition by m-CCCP was utilized (Fig. 6). In *H. parainfluenzae*, m-CCCP inhibits a reaction coupled to the hydrolysis of CL by phospholipase D, and the synthetic reactions converted some of the PG to CL very rapidly (16). In *S. aureus* m-CCCP inhibited the catabolism of CL and causes the accumulation of CL with an almost stochiometric loss of PG (Fig. 6). These data coupled with the incorporation and turnover data (21, 28) suggested strongly that CL was formed from a small portion of the PG pool with a significantly different specific activity and indicated that PG was indeed the precursor of CL in both *S. aureus* and *H. parainfluenzae*.

ACKNOWLEDGMENTS

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