Localization and characterization of the *sn*-glycerol-3-phosphate acyltransferase in *Rhodopseudomonas sphaeroides*

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Abstract The membrane localization and properties of the *Rhodopseudomonas sphaeroides sn*-glycerol-3-phosphate acyltransferase have been examined utilizing enzymatically prepared acyl-acyl carrier protein (acyl-ACP) substrates as acyl donors for *sn*-glycerol-3-phosphate acylation. Studies conducted with membranes prepared from chemotrophically and phototrophically grown cells show that *sn*-glycerol-3-phosphate acyltransferase activity is predominantly (>80%) associated with the cell's cytoplasmic membrane. Enzyme activity associated with the intracytoplasmic membranes present in phototrophically grown *R. sphaeroides* was within the range attributable to cytoplasmic membrane contamination of this membrane fraction. Enzyme activity was optimal at 40°C and pH 7.0 to 7.5, and required the presence of magnesium. No enzyme activity was observed with any of the long-chain acyl-CoA substrates examined. Vaccenoyl-ACP was the preferred acyl-ACP substrate and vaccenoyl-ACP and palmitoyl-ACP were independently utilized to produce lysophosphatidic and phosphatidic acids. With either vaccenoyl-ACP or palmitoyl-ACP as sole acyl donor substrate, the lysophosphatidic acid formed was primarily 1-acylglycerol-3-phosphate and the $K_{m(app)}$ for *sn*-glycerol-3-phosphate utilization was 96 μM. The implications of these results to the mode and regulation of phospholipid synthesis in *R. sphaeroides* are discussed.—**Cooper, C. L., and D. R. Lueking.** Localization and characterization of the *sn*-glycerol-3-phosphate acyltransferase in *Rhodopseudomonas sphaeroides*. *J. Lipid Res.* 1984. **25:** 1222–1232.

Supplementary key words membrane biogenesis • acyl-acyl carrier protein • phospholipid synthesis

The gram-negative, facultatively phototrophic bacterium *Rhodopseudomonas sphaeroides* has been widely employed for studies on the mode and regulation of membrane assembly (1–4). When grown phototrophically, this organism produces a specialized intracytoplasmic membrane (ICM) that harbors the proteins and photopigments required for photosynthetic growth. ICM formation occurs under conditions of low oxygen partial pressure via invagination and differentiation of the cytoplasmic membrane and, during phototrophic growth, the quantity of ICM produced is governed by the incident light intensity (1, 5–13).

The results of studies conducted with synchronously dividing cell populations of *R. sphaeroides* (2) have shown that discontinuous increases in the net accumulation of cellular phospholipids occur during the cell cycle. The cellular basis for this pattern of phospholipid accumulation was subsequently shown to be due to temporal control being exerted upon the rate of total cellular phospholipid synthesis (12) and this control was further shown to uniformly affect the rates of synthesis of the major phospholipid species possessed by *R. sphaeroides* (14). Lueking, Fraley, and Kaplan (2) further showed that the insertion of ICM protein and phospholipid constituents into the ICM was not coordinated and Fraley, Lueking, and Kaplan (12) proposed that the discontinuous appearance of ICM phospholipids, with attendant changes in ICM composition and physical state (2, 12, 15–17), could be due to discontinuous phospholipid synthesis by ICM-localized phospholipid biosynthetic enzymes, or to a discontinuous insertion of phospholipids into the ICM from a site of synthesis outside of the ICM.

The present report specifically examines the membrane localization and properties of the *R. sphaeroides sn*-glycerol-3-phosphate acyltransferase, the enzyme that catalyzes the first committed step in bacterial phospholipid synthesis (18). Lueking and Goldfine (19) have previously demonstrated this enzyme activity in cell-free extracts of *R. sphaeroides* and studies conducted with *Escherichia coli* have implicated the *sn*-glycerol-3-phosphate acyltransferase as a site of regulation for bacterial phospholipid synthesis (20–23). The present results show that greater than 80% of the *sn*-glycerol-3-phosphate acyltransferase activity present in phototrophically and chemotrophically grown cells of *R. sphaeroides* is specifi-

Abbreviations: ICM, intracytoplasmic membrane; ICM, cytoplasmic membrane; OM, outer membrane; PBP, penicillin-binding protein; SDS, sodium dodecyl sulfate; ACP, acyl carrier protein; DTT, dithiothreitol; DCPIP, 2,6-dichlorophenolindophenol.

cally localized in the cell's cytoplasmic membrane. In addition, a reevaluation of the kinetic properties of the *R. sphaeroides* glycerophosphate acyltransferase employing native acyl-ACP substrates is presented.

EXPERIMENTAL PROCEDURES

Organism, media, and growth conditions

Rhodopseudomonas sphaeroides strains 2.4.1 (wild type) and M29-5 (Leu$^-$, Met$^-$), derived from strain 2.4.7, were obtained from Samuel Kaplan, University of Illinois, Urbana, IL. Strain M29-5 was grown in a succinic acid minimal medium supplemented with 50 μg/ml each of L-leucine and L-methionine as described by Lueking et al. (2). Strain 2.4.1, which was utilized for the production of [^{3}H]vaccenic acid, was grown in a succinic acid minimal medium supplemented with 2.0% (w/v) casamino acids (Difco), 40 μg/ml of acetic acid, added as the sodium salt, and 5 μCi/ml of [^{3}H]acetate (26 Ci/mmol) as described by Cohen, Lueking, and Kaplan (24). Stock cultures were maintained at −25°C in succinic acid minimal medium adjusted to 10% (w/v) glycerol. Phototrophic growth was conducted in completely filled and sealed flat-walled vessels at 31°C with saturating illumination (5,380 lx) provided by a bank of Lumiline lamps (Sylvania). Chemotrophic growth was conducted at 31°C in the dark with constant mixing. Aerobically grown cultures were constantly sparged with a mixture of nitrogen–oxygen–carbon dioxide (74:25:1) (25) and additional oxygen sparging was utilized to maintain the total dissolved oxygen concentration between 12 and 20 ppm. Culture growth was monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 66 filter and cells adapted to the logarithmic phase of growth were the inoculum source for all studies.

Preparation of cellular membrane fractions

The membranes from phototrophically grown cells were prepared essentially as described by Fraker and Kaplan (26). Cells of *R. sphaeroides* strain M29-5 were harvested by centrifugation at 14,500 *g* in the logarithmic phase of growth (1.2×10^9 cells/ml) and the cell pellets were washed and resuspended in PEM buffer (0.1 M NaH_2PO_4, pH 7.6, 5 mM ethylenediaminetetraacetic acid, and 5 mM 2-mercaptoethanol). The washed cells (2.6 g wet weight) were disrupted by two passages through a French pressure cell at 18,000 lb/in^2 and the resulting cellular extract was treated with DNase and RNase (5 μg/ml) for 30 min at 4°C. Unbroken cells and cellular debris were removed by centrifugation at 14,500 *g* for 10 min, and the supernatant obtained was centrifuged at 106,000 *g* (Beckman Ti 60 rotor) for 2 hr. The resulting crude particulate fraction was washed and resuspended by hand-homogenization in 4 to 5 ml of PEM buffer and, if not immediately used, was stored at 4°C. Cell-free membrane fractions employed for kinetic studies and product analyses were never stored frozen or for more than 7 days at 4°C. Unless otherwise noted, all procedures were conducted at 4°C.

The initial resolution of cellular envelope and ICM membrane fractions was achieved by gel filtration of the crude particulate preparation on a 2.5 × 67 cm column of Sepharose 2B equilibrated with PEM buffer. The crude particles were clarified by centrifugation at 14,500 *g* for 10 min prior to loading onto the column and were chromatographed with PEM buffer. Column fractions containing ICM and cellular envelope membranes were pooled separately and the membranes were collected by centrifugation at 106,000 *g* for 2 hr. Both membrane fractions were resuspended in PEM buffer by hand homogenization and were further purified by rate-zonal sucrose density gradient centrifugation on linear (15–55%, w/w), preformed sucrose gradients. Gradients (4.5 ml) were centrifuged for 5 hr at 84,000 *g* in a Beckman SW50.1 rotor. Gradients were hand-fractionated and fractions containing ICM and cellular envelope membranes were individually pooled, the membranes were collected by centrifugation, and the washed membranes were resuspended in PEM buffer. Purified outer membrane preparations were obtained from phototrophically grown cells by the contained, discontinuous sucrose gradient centrifugation procedure exactly as described by Shepherd, Kaplan, and Park (27).

Cytoplasmic and outer membrane preparations were obtained from chemotrophically grown cells by the procedure of Ding and Kaplan (28) as modified by Cain et al. (29). Cells in the logarithmic phase of growth (9×10^8 cells/ml) were harvested by centrifugation, washed in 0.1 M KH_2PO_4 (pH 7.6) buffer containing 10 mM EDTA, and the washed whole cell pellet was stored at −20°C. Cells were disrupted as described above for the preparation of membranes from phototrophically grown cells and a crude envelope fraction was prepared and resolved into CM and OM fractions as described by Ding and Kaplan (28), with the exception that the crude, Tris-washed particles were not sonicated prior to being subjected to sucrose density gradient centrifugation. Discontinuous (20/40/60%, w/w) sucrose density gradients were centrifuged at 63,000 *g* for 16 hr and were fractionated by upward displacement with 60% w/w sucrose. Material banding at the 20/40 (CM) and 40/60 (OM) gradient interfaces was localized by monitoring the absorbancies at 280 nm of individual fractions. The fractions containing CM and OM were pooled separately, diluted with buffer, and the membranes were collected by centrifugation at 106,000 *g* for 3 hr. The resulting pellets were resuspended in PEM buffer and were stored

at 4°C prior to assay. This procedure yielded a sharp separation of CM and OM with no evidence of membranous material of intermediate density.

Purification of acyl carrier protein (ACP)

ACP was purified from cells of *E. coli* B (Grain Processing Co., Muscatine, IA) by the procedure of Rock and Cronan (30) and the purity of the ACP was monitored by conformationally sensitive polyacrylamide gel electrophoresis (30, 31).

Preparation of acyl-ACP substrates

Long-chain acyl thiolester derivatives of ACP were prepared by an enzymatic procedure utilizing *E. coli* acyl-ACP synthetase (32). *E. coli* acyl-ACP synthetase was obtained from cells of *E. coli* B as described by Rock and Cronan (33) except that the enzyme preparation was loaded onto the final hydroxylapatite column at a higher flow rate (60 ml/hr) than that previously utilized.

Acyl-ACP substrates were prepared using immobilized preparations of acyl-ACP synthetase essentially as described by Green, Merrill, and Bell (34). Purified acyl-ACP synthetase (6 mg) was loaded onto a column (1 × 4 cm) of Matrex Gel Red A equilibrated with 0.1 M Tris-HCl (pH 8.0), 0.1% Triton X-100, and 5 mM 2-mercaptoethanol and a reaction mixture (20 ml) containing 0.1 M Tris-HCl (pH 8.0), 0.4 M LiCl, 10 mM ATP, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.2% Triton X-100, 60 to 100 μM ACP, and 160 μM [^{3}H]palmitic acid (0.31 to 1.0 Ci/mol), or [^{3}H]vaccenic acid (1.0 Ci/mol), was cycled (37°C for 4 hr or room temperature for 24 hr) over the column at a flow rate of 30 to 50 ml/hr. Acyl-ACP substrates were recovered from reaction mixtures and freed of unesterified ACP by chromatography on columns of DEAE-52 and octyl-Sepharose CL-4B exactly as described by Rock, Garwin, and Cronan (32). The purity of acyl-ACP substrates was determined by conformationally sensitive polyacrylamide gel electrophoresis (30). The quantitation of acyl-ACP derivatives was accomplished by monitoring the amount of ^{3}H-labeled fatty acid released following treatment of the derivatives with neutral hydroxylamine (35). The ^{3}H-labeled hydroxamic acid produced was extracted with ethyl ether and the radioactivity was determined by scintillation counting (36).

sn-Glycerol-3-phosphate acyltransferase assay

Glycerophosphate acyltransferase activity was measured as described by Lueking and Goldfine (19) by monitoring the incorporation of *sn*-[U-^{14}C]glycerol-3-phosphate into trichloroacetic acid-precipitable material employing a nonspecific filter paper disc assay (37). Unless otherwise noted, incubation mixtures contained: 100 mM KH_2PO_4 buffer, (pH 7.2); 1.0–1.5 mM *sn*-[U-^{14}C]glycerol-3-phosphate (10 Ci/mol); 25 μM acyl-ACP; 10 mM $MgCl_2$; 1.0 mM dithiothreitol; and 0.15 to 0.50 mg/ml of particulate protein in a final volume of 0.040 ml. Incubations were conducted at 40°C for 5 min and 0.035 ml of the incubation mixture was utilized for the measurement of glycerophosphate incorporation. Comparable rates of enzyme activity were observed when the lipid products of glycerophosphate acylation were directly extracted and quantitated by scintillation counting (19). A unit of enzyme activity is the amount of enzyme required to esterify 1 nmol of *sn*-glycerol-3-phosphate per minute. Glycerophosphate acyltransferase activity was measured in the same manner for the enzymes from both phototrophically and chemotrophically grown cells.

Extraction, identification and quantitation of lipid products

Lipids were extracted from membrane preparations and incubation mixtures by the method of Bligh and Dyer (38) as described by Ames (39). Phase partitioning and washing of the chloroform extracts was routinely chloroform–methanol–0.1 M $MgCl_2$ 1:2:0.80 (v/v) and Burghardt (40). For the identification and quantitation of lysophosphatidic (LPA) and phosphatidic (PA) acids, incubation mixtures (0.10 ml) were treated with an equal volume of 4 N HCl, combined with authentic lipid standards (50 μg each), and extracted with 3.8 ml of chloroform–methanol–0.1 M $MgCl_2$ 1:2:0.80 (v/v) and the chloroform phases were washed with 0.1 M $MgCl_2$ (41). The chloroform extracts were taken to dryness under a stream of nitrogen and the lipids were redissolved in 0.10 ml of chloroform. LPA and PA were resolved by thin-layer chromatography on commercial Silica Gel H Redi plates (Analtech) with chloroform–methanol–acetic acid–water 62.5:37.5:10:5 (v/v) as developing solvent (42). Neutral lipids (mono- and diglycerides) were separated on plates of Silica Gel G developed in diethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2 (v/v) (43). Lipids were visualized by exposure of the plates to iodine vapor and the silica gel lanes were divided into 1-cm sections and scraped directly into scintillation vials. Water (0.5 ml) and Redi Solv EP (Beckman) scintillation fluid (10 ml) were then added and the radioactivity was quantitated by scintillation counting (34).

The positional specificity of the *sn*-glycerol-3-phosphate acyltransferase was examined by the analysis of reaction products generated and extracted in the presence of 40 mM borate, which served to minimize acyl group migration (34, 44). Neutral lipids were generated by the enzymatic conversion of labeled LPA to the respective 1- or 2-monoacylglycerol by treatment with 45 μg of *E. coli* alkaline phosphatase (45). The neutral

lipids generated were extracted with hexane–diethyl ether 1:1 (v/v) (45) and the 1- and 2-monoacylglycerols were resolved on boric acid (0.5 M)-impregnated plates (34) of Silica Gel H developed in chloroform–acetone–acetic acid–methanol 145:50:1:4 (v/v) (44). Lipids were visualized with iodine vapor and quantitated as described above. Diglycerides were not produced at detectable levels by this procedure.

Analytical procedures

Protein was determined by the method of Lowry et al. (46) employing bovine serum albumin as standard. Protein samples were treated for 15 min at 60°C in 0.5 N NaOH prior to assay. Samples utilized for lipid phosphorus determinations were hydrolyzed as described by Goldfine et al. (47) and phosphorus was determined by the method of Bartlett (48). Membrane-associated penicillin-binding proteins were quantitated by the method of Shepherd et al. (27) using benzyl-[^{14}C]-penicillin (54 Ci/mol). Bacteriochlorophyll was quantitated spectrophotometrically in acetone–methanol 7:2 (v/v) extracts as described by Wraight et al. (13) using an absorption coefficient at 775 nm of 75 $mM^{-1}cm^{-1}$ (49). Membrane fractions were qualitatively evaluated by comparison of the protein profiles resulting from SDS-polyacrylamide gel electrophoresis of the preparations as described by Baumgardner, Deal, and Kaplan (50). Succinate dehydrogenase activity was measured by the method of Kasahara and Anraku (51) using 0.1 and 0.05 mg protein in 1.0 ml assays as described by Wraight et al. for *R. sphaeroides* (13). One unit of enzyme activity is the amount of enzyme required to reduce 1 nmol of 2,6-dichlorophenolindophenol (DCPIP) per minute.

Chemicals

Palmitoyl-CoA, bovine serum albumin, DNase, RNase, penicillin G, monopalmitin, palmitic acid, *cis*-vaccenic acid, linoleic acid, linolenic acid, diolein, and dipalmitin were obtained from Sigma Chemical Co. Monooleins (1 and 2 positions), dioleoyl phosphatidic acid, and 1-oleoyl-lysophosphatidic acid were purchased from Sedary Research Labs. Acyl-CoA thiolester derivatives of vaccenic, linoleic, and linolenic acids were prepared by the enzymatic procedure of Merrill, Gidwitz, and Bell (52) employing resin-bound rat liver fatty acyl-CoA ligase. These substrates were shown to be greater than 95% pure by the chromatographic procedure of Shapiro and Prescott (53). *sn*-[U-^{14}C]Glycerol-3-phosphate (144 Ci/mol) and [9,10-^{3}H]palmitic acid (11 Ci/mmol) were purchased from New England Nuclear Corp. Benzyl-[^{14}C]penicillin (54 Ci/mol) was purchased from Amersham. [^{3}H]Acetic acid (26 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. [^{3}H]Vaccenic acid was purified as its methyl ester derivative (54) from cells of *R. sphaeroides* strain 2.4.1 grown in medium supplemented with [^{3}H]acetic acid as described above. Vaccenoyl methyl esters were purified by argentation chromatography (54, 55), washed with Tris buffer (56), and were saponified, and the free fatty acids were recovered as described by Christie (57). The specific activity of the vaccenoyl-methyl esters was determined by gas chromatography on a Hewlett Packard Series 5790A gas chromatograph in conjunction with scintillation counting.

RESULTS

Properties of the glycerophosphate acyltransferase

As was previously observed with *R. sphaeroides* strain 2.4.1 Ga by Lueking and Goldfine (19), crude particulate preparations derived from phototrophically grown cells of *R. sphaeroides* strain M29-5 (parental strain 2.4.7) displayed a high level of glycerophosphate acyltransferase activity that was dependent upon acyl-ACP substrates as acyl donors for the reaction. Enzyme activity was optimal at 40°C and pH 7.0–7.5, and was stimulated 1.7-fold in the presence of dithiothreitol. When assayed under the conditions described in Experimental Procedures, enzyme activity was linear with respect to time (5 min) and the concentration of particulate protein (0.06 to 0.5 mg/ml) and exhibited an obligate dependence upon the presence of magnesium.[1] This is in marked contrast to the enzyme from *R. sphaeroides* strain 2.4.1 Ga which did not require a divalent cation for its activity and was only slightly (10%) affected by the presence of 10 mM $MgCl_2$ (19). In agreement with a prevous report (19), long-chain acyl-CoA substrates were found to be unable to replace acyl thiolester derivatives of ACP as acyl donor substrates for the *R. sphaeroides* glycerophosphate acyltransferase. Substitution of palmitoyl-, vaccenoyl-, linolenoyl-, or linoleoyl-CoA substrates for acyl-ACP resulted in a complete loss of enzyme activity and this loss was not recoverable by the addition of BSA (1 mg/ml) to incubation mixtures. Thus, as was discussed by Lueking and Goldfine (19), it does not appear that the inability of the *R. sphaeroides* glycerophosphate acyltransferase to utilize acyl-CoA substrates can be trivially explained by enzyme sensitivity to the detergent properties of these molecules.

[1] Vaccenoyl- and palmitoyl-ACP were routinely utilized at concentrations (25 μM) which supported 20 and 55%, respectively, of the maximal velocities observed at saturating levels of these substrates. Due to the high level of enzyme activity supported by vaccenoyl-ACP, results obtained by a direct measurement of the V_{max} at saturating levels of this substrate would be unreliable. Values for maximal enzyme activities are those determined by extrapolation of the data presented in Fig. 1b.

The kinetics of enzyme activity with saturated and unsaturated acyl-ACP substrates as acyl donors for *sn*-glycerol-3-phosphate acylation is presented in Fig. 1. With either palmitoyl-ACP or vaccenoyl-ACP as acyl substrate, the apparent K_m for *sn*-glycerol-3-phosphate utilization was 96 μM (Fig. 1a). This value agrees favorably with the value (90 μM) reported by Rock, Goelz, and Cronan (58) for the *E. coli* (low K_m) glycerophosphate acyltransferase, but is considerably (12-fold) lower than the value of 1.2 mM determined for the enzyme from *R. sphaeroides* strain 2.4.1 Ga by Lueking and Goldfine (19). The basis for this difference is unknown. At saturating (1.5 mM) levels of *sn*-glycerol-3-phosphate, vaccenoyl-ACP supported the highest rate of enzyme activity, 28 nmol/min per mg protein, and had an apparent K_m of 70 μM (Fig. 1b). Although the apparent K_m, 20 μM, for palmitoyl-ACP utilization was significantly lower than the K_m determined for vaccenoyl-ACP, the V_{max} value observed with palmitoyl-ACP as substrate, 3.7 nmol/min per mg, was markedly lower than that observed with vaccenoyl-ACP as acyl substrate (Fig. 1b). The maximal velocity observed with vaccenoyl-ACP was comparable to that reported for the strain 2.4.1 Ga enzyme with oleoyl-ACP as substrate (27 nmol/min per mg), however, the maximal activity observed with palmitoyl-ACP as acyl substrate differed significantly for the two strains of *R. sphaeroides*. With the strain 2.4.1 Ga enzyme, oleoyl-ACP and palmitoyl-ACP gave comparable maximal velocities for the initial acylation of glycerophosphate (19).

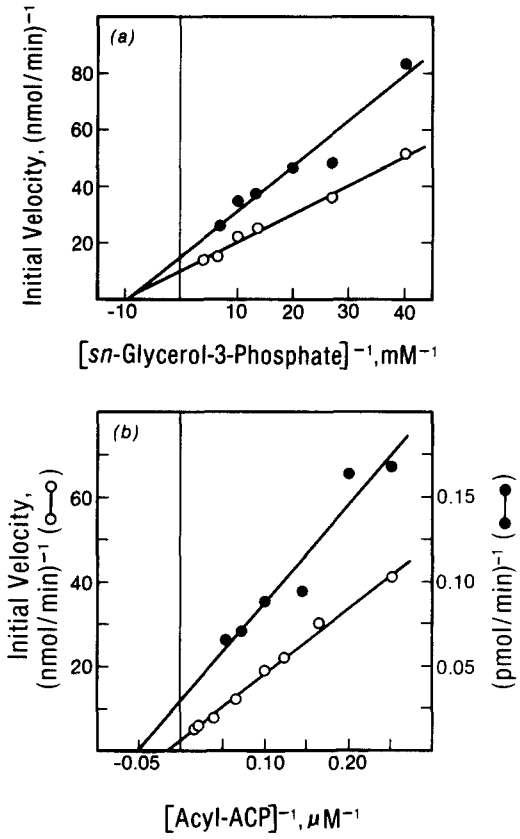

Fig. 1. Kinetic analysis of the *sn*-glycerol-3-phosphate acyltransferase reaction. Assay mixtures contained 0.1 M KH_2PO_4, pH 7.2, 10 mM $MgCl_2$, and 1 mM DTT in a final volume of 0.04 ml, and were conducted in the linear region of time dependence (2 and 4 min). (a) Glycerol-3-phosphate was varied as indicated using 0.4 mg/ml particulate protein with vaccenoyl-ACP at 25 μM (○——○); and 0.3 mg/ml particulate protein was used with palmitoyl-ACP at 25 μM (●——●). (b) The dependence of initial velocities on vaccenoyl-ACP (○——○) and palmitoyl-ACP (●——●) was determined in assays containing 0.4 and 0.2 mg/ml particulate protein, respectively, with glycerol-3-phosphate held constant at 1.5 mM. For purposes of presentation, the data shown for initial velocities are from individual assays. The values for the K_m with vaccenoyl-ACP as substrate ranged from 65 to 86 μM.

Analysis of the products of glycerophosphate acylation

The extractable lipid products of glycerophosphate acylation were identified and quantitated as described in Experimental Procedures. With either vaccenoyl-ACP, or palmitoyl-ACP as sole acyl substrate, the only labeled products recoverable from reaction mixtures were lysophosphatidic acid and phosphatidic acid. Detectable quantities of neutral lipids (monoglycerides or diglycerides) were not produced under the incubation conditions employed. Although vaccenoyl-ACP supported the highest rates of both lysophosphatidic acid and phosphatidic acid production, the percent distribution of these two lipid products in reaction mixtures containing either vaccenoyl-ACP or palmitoyl-ACP as acyl substrate were similar (**Table 1**). Thus, following a 4-min incubation period, approximately 50% of the lysophosphatidic acid produced had been further acylated to phosphatidic acid, irrespective of the nature of the acyl-ACP substrate (Table 1). It should be mentioned however, that the extent of phosphatidic acid production varied between different membrane preparations and was influenced by the age and method of storage of the membranes. The data presented in Table 1 are those observed when newly prepared membranes are employed for assays.

The positional specificity of the *R. sphaeroides* glycerophosphate acyltransferase was examined by treating incubation mixtures containing ^{14}C-labeled lysophosphatidic acid with alkaline phosphatase (45) and resolving and quantitating the resulting 1- and 2-monoacylglycerols (44). Phosphatidic acid was not hydrolyzed by this procedure and, with either palmitoyl-ACP or vaccenoyl-ACP as acyl substrate, greater than 90% of the monoglyceride produced was recovered as the 1-acyl derivative (Table 1). These results are in agreement with those reported for the positional specificity of the *E. coli* enzyme (34, 58) and indicate that the acylation specificity

TABLE 1. Incorporation of *sn*-[^{14}C]glycerol-3-phosphate into lipid products of the acyltransferase reaction

Acyl Donor	Lysophosphatidic Acid	Phosphatidic Acid	Monoacylglycerol 1-Isomer/2-Isomer
		nmol/ml[a]	
cis-Vaccenoyl-ACP			
2 min	1.29	1.04	N.D.[b]
4 min	1.91	2.17	1.74/0.17
Palmitoyl-ACP			
2 min	0.80	0.41	N.D.
4 min	1.12	1.11	1.04/0.08

[a] Product recoveries from the thin-layer plates ranged between 73 and 82% for LPA and PA and between 66 and 71% for the 1- and 2-isomers of monoacylglycerol. The values for the species of monoacylglycerol have been normalized for observed recoveries during product analyses. Product analyses were performed as described in Experimental Procedures.

[b] Not determined.

of the *R. sphaeroides* acyltransferase is for the primary hydroxyl of *sn*-glycerol-3-phosphate. As was previously discussed by Green et al. (34) and Rock et al. (58), however, due to the occurrence of some (15–20%) acyl group migration during product analysis, the employed procedure is unable to demonstrate exclusive acylation at the C-1′ position of glycerophosphate.

Membrane localization of glycerophosphate acyltransferase activity

The localization of glycerophosphate acyltransferase activity in membranes obtained from phototrophically grown cells was determined by monitoring the distribution of units of enzyme activity in membrane fractions resolved by Sepharose 2B chromatography and sucrose density gradient centrifugation. As is shown in **Fig. 2a,** Sepharose 2B chromatography resolves crude particulate preparations of *R. sphaeroides* into two separate membrane fractions. One fraction (Peak 1; Fig. 2a) elutes in the column void volume, is photopigment-depleted and consists primarily of the cell's CM-OM fraction with some contaminating ICM (26). A second fraction (Peak 2; Fig. 2a) is retained by the column, is photopigment-enriched, and consists of the majority of the ICM and some contaminating CM. The distribution of glycerophosphate acyltransferase activity in these two membrane fractions is shown in Fig. 2b. The CM-OM fraction (fractions 24–37) contained 60% of the recovered enzyme activity, 23% of the bacteriochlorophyll, and 57% of the membrane protein (**Table 2**). The ICM fraction (Peak 2; fractions 38–69) possessed 40% of the units of enzyme activity, 77% of the bacteriochlorophyll, and 43% of the membrane protein. Thus, the bulk of the glycerophosphate acyltransferase activity comigrated with the CM-OM fraction, which was significantly depleted of bacteriochlorophyll, a specific ICM marker.

To determine if the enzyme activity associated with the crude ICM fraction was due to the presence of contaminating CM, the distribution of penicillin-binding protein (PBP) activity in CM-OM and ICM fractions was evaluated. As was shown by Shepherd et al. (27), *R. sphaeroides* possesses a spectrum of PBP's that are specifically associated with the CM in both chemotrophically and phototrophically grown cells. As is shown in Table 2, the distribution of PBP activity in the two membrane fractions almost exactly mimicked the distribution of enzyme activity in these preparations. This finding suggested that the enzyme activity associated with the ICM could possibly be completely attributable to the presence of contaminating CM. To evaluate this possibility, the CM-OM and ICM fractions resolved by gel chromatography were further purified by rate-zonal gradient centrifugation through preformed, linear sucrose gradients (Experimental Procedures). The results of these analyses are presented in **Fig. 3.** Gradient fractionation of the pooled CM-OM material revealed that the enzyme activity associated with these membranes migrated as a

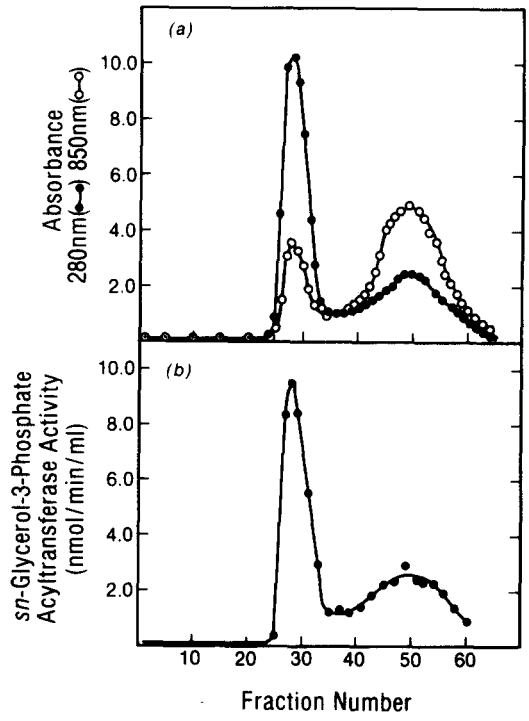

Fig. 2. Resolution of *R. sphaeroides* membrane fractions by gel filtration chromatography. Washed, crude particles (82 mg) were chromatographed with equilibration buffer (PEM) over Sepharose 2B (2.5 × 67 cm). Five-ml fractions were collected at a flow rate of 20 ml/hr. (a) Absorbancies at 280 nm (● —— ●) and 850 nm (○ —— ○) represent protein and bacteriochlorophyll, respectively. (b) Glycerol-3-phosphate acyltransferase activity (nmol/min per ml) was assayed as described in Experimental Procedures using 0.225 mg protein/ml. The distribution of membrane components in Peak 1 (fractions 24–37) and Peak 2 (fractions 38–69) is shown in Table 2.

TABLE 2. Distribution of membrane components in membranes derived from phototrophically grown *R. sphaeroides* as resolved by gel filtration chromatography

Membrane Component[a]	Distribution	
	Peak 1[b]	Peak 2[c]
Protein (mg)	34.2 (57)	26.1 (43)
Phospholipid (mg)	11.0 (49)	11.3 (51)
Bacteriochlorophyll (mg)	0.70 (23)	2.28 (77)
Glycerol-3-phosphate acyltransferase (units)	139.0 (60)	91.8 (40)
Penicillin-binding proteins (CPM)	8.87×10^4 (66)	4.57×10^4 (34)

[a] All analyses were performed as described in Experimental Procedures. Recoveries of all components following Sepharose 2B chromatography ranged between 70 and 80%. Gel filtration chromatography was conducted as described in Experimental Procedures and the legend to Fig. 2 employing a crude particulate preparation (82 mg protein) of membranes derived from phototrophically grown cells. The values in parentheses are the percent distributions of the recovered material present in the pooled fractions of Peak 1 and Peak 2 (Fig. 2).

[b] Peak 1 represents pooled fractions 24 to 37 in Fig. 2.

[c] Peak 2 represents pooled fractions 38 to 69 in Fig. 2.

symmetrical peak of activity at a gradient position identical to that of bulk CM-OM (Fig. 3a and b). No enzyme activity was associated with the small amount of ICM (A_{850}; Fig. 3a) resolved by this procedure. In contrast, an identical treatment of the crude ICM fraction (Peak 2; Fig. 2a) clearly resolved two, distinct peaks of enzyme activity that comigrated with CM-OM and ICM (Fig. 3c and d). Furthermore, since the recovered enzyme activity was equally distributed between these two membrane fractions, these results indicate that no more than 20% of the cellular glycerophosphate acyltransferase can be associated with the ICM. In addition, the density gradient purified ICM was also shown to possess 23% of the total PBP activity (Fig. 3, legend) which suggests that possibly all of the glycerophosphate acyltransferase activity which co-purifies with the ICM may reflect CM contamination of this membrane fraction. The outer membrane from phototrophically grown cells was purified by the procedure of Shepherd et al. (27), and the enzyme specific activity associated with this membrane ranged from 5 to 20% of the enzyme specific activity observed in the pooled Peak 1 material obtained from gel filtration chromatography (Fig. 2).

Membrane fractions derived from chemotrophically grown cells were prepared as described in Experimental Procedures. The CM and OM fractions obtained followed three washes in Tris-buffer, pH 8.8, and sucrose density gradient centrifugation of a crude membrane preparation contained 24.6 and 12.4%, respectively, of the protein present in unfractionated membranes. Similar values for protein recoveries have been reported by Ding and Kaplan (28) and Cain et al. (29). In agreement with Ding and Kaplan (28), succinate dehydrogenase activity was found to be 9-fold higher in the CM versus OM and was significantly decreased (83%) in the OM relative to unfractionated membranes (**Table 3**). The specific activity of the *sn*-glycerol-3-phosphate acyltransferase was approximately 2-fold higher in the CM (23.3 nmol/min per mg) as compared to unfractionated membranes (12.9 nmol/min per mg) and was 5.3-fold higher than the OM associated activity (4.4 nmol/min per mg). As was observed with the succinate dehydrogenase activity, the activity of the glycerophosphate acyltransferase was markedly reduced (66%) in the OM relative to that observed in the unfractionated membranes. The net distribution of glycerophosphate acyltransferase activity between the CM and OM of chemotrophically grown cells was found to be 92% and 8%, respectively, and was in good agreement with the distribution of 95% and 5% (CM and OM, respectively) observed for succinate dehydrogenase activity; a CM marker (28) (Table 3). Importantly, the 5.3-fold enrichment of glycerophosphate acyltransferase activity in the CM versus OM is in good agreement with the values of 5.5:1 and 5.7:1 observed for the CM to OM enrichment of phosphatidylglycerophosphate synthetase and phosphatidylserine decarboxylase activities, respectively, by Cain et al. (29).

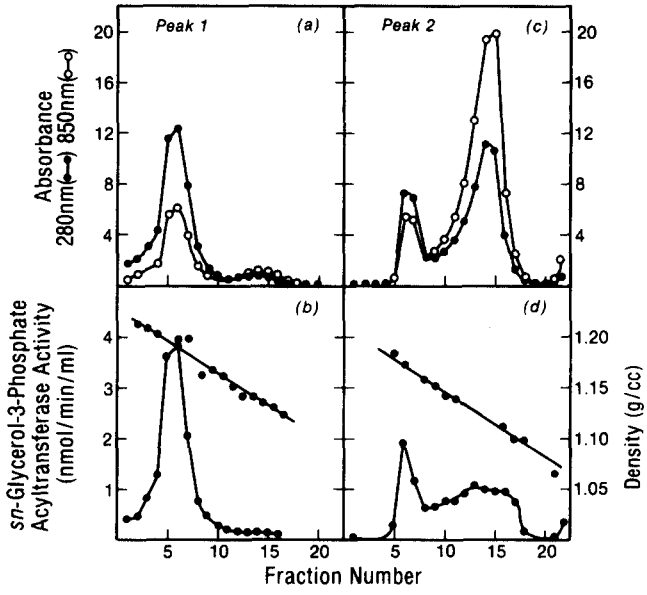

Fig. 3. Resolution of membrane vesicles by rate zonal density gradient centrifugation. Panels (a) and (b) are the results obtained from 1.5 mg of pooled material from fractions 24–37 of Fig. 2. Panels (c) and (d) are the results obtained from 1.5 mg of pooled material from fractions 38–69 of Fig. 2. Five-ml gradients were centrifuged at 84,000 *g* for 5 hr and were fractionated dropwise. Fractions were monitored for absorbancies at 280 and 850 nm, refractive index to obtain densities, and glycerol-3-phosphate acyltransferase activity, using 0.15 mg/ml protein as described in Experimental Procedures. The fractions containing purified ICM contained 23% of the total PBP activity initially present in the crude membranes subjected to Sepharose 2B chromatography.

TABLE 3. Membrane localization of *sn*-glycerol-3-phosphate acyltransferase activity in chemotrophically grown *R. sphaeroides*

Membrane Component[a]	Crude Particles	CM	OM
Protein (mg)	40.0	9.83	4.95
Succinate dehydrogenase activity (units)	3380	1300	72.3
Glycerol-3-phosphate acyltransferase activity (units)	516	229	21.8

Cytoplasmic and outer membrane fractions were prepared from crude particulate preparations of chemotrophically grown cells as described in Experimental Procedures. The three successive washings in Tris-HCl buffer, pH 8.8, removed 34% of the protein present in the unwashed particles and 7% and 39%, respectively, of the glycerophosphate acyltransferase and succinate dehydrogenase activities. The recoveries of protein and units of enzyme activities following density gradient centrifugation ranged between 52 and 67%.

[a] Protein determinations were conducted on trichloroacetic acid precipitates of the designated fractions. Succinate dehydrogenase activities were determined as described in Experimental Procedures with 1 unit of enzyme activity corresponding to the reduction of 1 nmole of DCPIP per min. Glycerol-3-phosphate acyltransferase activities were determined employing the assay conditions described for the enzyme from phototrophically grown cells. Vaccenoyl-ACP (15 μM) was employed as acyl donor substrate and incubations were for 2 min at 0.15 mg particulate protein per ml.

DISCUSSION

Lueking and Goldfine (19) previously reported that *R. sphaeroides* strain 2.4.1 Ga possessed an *sn*-glycerol-3-phosphate acyltransferase that, in contrast to the *E. coli* enzyme, displayed a strict requirement for acyl-ACP substrates for the initial acylation of glycerophosphate. These investigators only examined the enzyme present in phototrophically grown cells and the exact membrane localization of enzyme activity was not determined. Although the results of extensive kinetic studies were presented, these studies utilized acyl-ACP substrates prepared by a chemical procedure (36) that has subsequently been shown to result in alterations of ACP secondary structure (59). The present study thus examines the membrane localization and properties of the *R. sphaeroides* glycerophosphate acyltransferase employing enzymatically prepared acyl-ACP substrates that possess a native conformation (60).

The glycerophosphate acyltransferase from both chemotrophically and phototrophically grown *R. sphaeroides* strain M29-5 (derived from strain 2.4.7) were found to be unable to utilize long-chain acyl-CoA substrates as acyl donors for glycerophosphate acylation. Vaccenoyl-ACP was the preferred acyl-ACP substrate and was shown to support a maximal activity (28 nmol/min per mg protein) 7.5-fold higher than the activity (3.7 nmol/min per mg protein) observed with palmitoyl-ACP as substrate (Fig. 1). Interestingly, however, the $K_{m(app)}$ determined for vaccenoyl-ACP (70 μM) was considerably higher than the $K_{m(app)}$ observed for palmitoyl-ACP (20 μM). Since vaccenic acid and palmitic acid constitute 90% and 4%, respectively, of the total esterified fatty acids possessed by *R. sphaeroides* (54, 61), it is tempting to speculate that the low $K_{m(app)}$ displayed by palmitoyl-ACP allows this substrate to compete, albeit poorly, with vaccenoyl-ACP for utilization by the glycerophosphate acyltransferase. Studies recently conducted with *E. coli* (62) suggest that competition for esterification among endogenous acyl-ACP substrates does not occur in vivo and the glycerophosphate acyltransferase may not play a role in determining membrane fatty acid composition. However, these conclusions were based largely upon the results of analyses of the endogenous acyl-ACP pool possessed by *E. coli* which was found to be devoid of acyl-ACP species that are known to serve as substrates for the glycerophosphate acyltransferase. The size and composition of the endogenous acyl-ACP pool in *R. sphaeroides* is currently under investigation.

The finding that vaccenoyl-, linoleoyl-, and linolenoyl-CoA were not utilized as acyl donor substrates by the *R. sphaeroides* glycerophosphate acyltransferase supports the recent proposal by Boyce and Lueking (63) that the utilization of exogenously supplied fatty acids for phospholipid synthesis in *R. sphaeroides* requires their prior esterification to ACP. Campbell and Lueking (54) have previously shown that exogenously supplied vaccenic, linoleic, and linolenic acids are directly utilized for phospholipid synthesis by *R. sphaeroides* and vaccenoyl-ACP and linoleoyl-ACP (data not shown) have been found to serve as substrates for this organism's glycerophosphate acyltransferase.

The properties of the *R. sphaeroides* strain M29-5 enzyme described in the present study differed significantly from those reported for the enzyme from strain 2.4.1 Ga (19). However, it is unclear whether these differences reflect strain differences, or result from the use of chemically versus enzymatically prepared acyl-

ACP substrates. The strain 2.4.1 Ga enzyme displayed a markedly higher activity with acetylated palmitoyl-ACP than was observed with the strain M29-5 enzyme utilizing native palmitoyl-ACP as its substrate, and the strain 2.4.1 Ga enzyme lacked a magnesium requirement. Neither of these properties are consistent with existing information (59, 64) concerning the use of acetylated versus native acyl-ACP substrates. Furthermore, although the apparent discrepancy between the $K_{m(app)}$ values for glycerophosphate utilization determined for the strain M29-5 (96 μM) and strain 2.4.1 Ga (1.2 mM) enzymes may very well reflect the use of native versus acetylated acyl-ACP substrates, respectively, an alternative explanation for this difference is possible. The low K_m value determined for glycerophosphate utilization in strain M29-5 with either vaccenoyl- or palmitoyl-ACP as acyl substrate, and the high K_m value observed with strain 2.4.1 Ga with oleoyl-ACP as acyl donor substrate, may indicate the existence of distinct glycerophosphate acyltransferases in *R. sphaeroides* as has been proposed for *E. coli* by Rock et al. (58).

Cain et al. (29, 65) have recently obtained substantial in vivo and in vitro evidence that the production of phosphatidylserine and phosphatidylglycerol by cells of *R. sphaeroides* occurs via reaction sequences analogous to those utilized by other gram-negative organisms and that the enzymes, CDP-diglyceride synthetase and phosphatidylglycerolphosphate synthetase, are predominantly localized in the cytoplasmic membranes of both chemotrophically and phototrophically grown cells. The results of the present study correlate with these findings by showing that 80% of the glycerophosphate acyltransferase activity present in chemotrophically and phototrophically grown *R. sphaeroides* is localized in the cell's cytoplasmic membrane. The enzyme activity present in crude membrane fractions derived from phototrophically grown cells comigrated with penicillin-binding protein activity (Table 2), a specific cytoplasmic membrane marker (27), during Sepharose 2B chromatography (Fig. 2) and sucrose density gradient centrifugation (Fig. 3), and the residual enzyme activity remaining associated with the purified ICM fraction was found to be within the range attributable to CM contamination of this membrane fraction. The data presented in Table 3 show that the distribution of glycerophosphate acyltransferase activity in the cytoplasmic and outer membranes prepared from chemotrophically grown cells mimicked the distribution of succinate dehydrogenase activity (percent distribution of recovered activity) in these membranes and the CM to OM ratio (5.3:1) of glycerophosphate acyltransferase specific activities was in good agreement (±10%) with the values observed by Cain et al. (29) for the CM to OM ratios of phosphatidylglycerophosphate synthetase and phosphatidylserine decarboxylase activities. Thus, the results of the present study, together with the results reported by Cain et al. (29), provide substantive evidence that as in other gram-negative organisms, de novo phospholipid biosynthesis occurs predominantly in association with the CM in *R. sphaeroides*. At present, studies are being conducted to evaluate the role of the *sn*-glycerol-3-phosphate acyltransferase in the temporal (11, 12, 14) and light-mediated control (35) of phospholipid synthesis by cells of *R. sphaeroides*.

We would like to thank Drs. Samuel Kaplan, Robert M. Bell, Charles O. Rock, and John E. Cronan, Jr. for kindly providing preprints of unpublished manuscripts. We are also indebted to Drs. Charles O. Rock, John E. Cronan, Jr., and Alfred H. Merrill, Jr. for helpful discussions concerning the preparation of acyl-ACP substrates. Finally, we acknowledge Mr. Stephen G. Boyce for providing enzymatically synthesized derivatives of acyl-CoA. A preliminary report of this work was presented at the American Society of Biological Chemists meeting, San Francisco, CA, June 1983. The work described here was included in a dissertation submitted to the Graduate College of Texas A & M University by C.L.C. in partial fulfillment of the requirements for the PhD degree. C.L.C. was the recipient of a Robert A. Welch Predoctoral Fellowship. D.R.L. was the recipient of Grant GM-28036 from the National Institutes of Health and Grant A-817 from the Robert A. Welch Foundation.

Manuscript received 16 April 1984.

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