The Relationship of Intracytoplasmic Membrane Assembly to the Cell Division Cycle in *Rhodopseudomonas sphaeroides*

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Discontinuous increases in the net accumulation of cellular phospholipid were previously observed during the synchronous growth of *Rhodopseudomonas sphaeroides* (Lueking, D. R., Fraley, R. T., and Kaplan, S. (1978) J. Biol. Chem. 253, 451-457). This pattern is shown to result from large increases in the rate of phospholipid synthesis, occurring just prior to cell division. The absence of detectable turnover of the phospholipid fraction suggests that the discontinuous accumulation of cellular phospholipid occurring during synchronous growth is directly attributable to the observed changes in the rate of phospholipid synthesis. Evidence is presented which suggests that different patterns of phospholipid synthesis (or insertion) are associated with the intracytoplasmic membrane system and the cytoplasmic and outer membranes.

The previous observation that the protein/phospholipid ratio of the intracytoplasmic membranes of *R. sphaeroides* underwent cyclical changes (40 to 50%) during synchronous growth has been confirmed by monitoring concomitant changes (1.1690 to 1.1790 g/cm³) in the natural or intrinsic density of these membranes. Specific radiolabeling of membrane protein and phospholipid components also verifies the changes in intracytoplasmic membrane composition observed during the cell cycle.

It is proposed that the cyclical changes in intracytoplasmic membrane composition may serve as a means of regulating membrane-associated enzymes and modulating membrane synthesis during the cell cycle.

The elucidation of the mechanism and regulation of membrane assembly is fundamental to a complete understanding of cell growth and division. However, there is currently little information concerning either the mechanism or regulatory factors which govern the assembly of membrane components into a functional unit.

Studies of the assembly of bacterial membrane systems have provided substantial information concerning the coupling of membrane protein and phospholipid synthesis (Refs. 1 to 3; for review, see Ref. 4). In particular, the availability of bacterial mutants defective in various aspects of cellular phospholipid metabolism has greatly augmented biochemical approaches to the study of membrane assembly. Investigations employing glycerol auxotrophs of *Bacillus subtilis* (5) and *Escherichia coli* (6, 7) have clearly indicated that membrane protein synthesis and insertion can continue in the absence of net synthesis of cellular phospholipid. Cell membranes isolated from glycerol-starved cells display elevated protein to phospholipid ratios and increased buoyant densities (5, 6). However, it has been tacitly assumed that uncoupling of membrane protein from membrane phospholipid synthesis observed in these studies does not exist under normal conditions of balanced growth. Also, it has been proposed (9) that a cellular regulatory mechanism exists which functions to maintain the membrane protein content well below saturating levels.

An alternative approach to the question of in vivo coupling of membrane protein and phospholipid synthesis has been to use division synchronized bacterial populations in studies of membrane assembly. Studies conducted with synchronously dividing cultures of *B. subtilis* (8), *E. coli* (9), and *Rhodopseudomonas sphaeroides* (10) have unambiguously shown that membrane proteins are formed at a constant rate and are continuously inserted into the membrane. Thus, any uncoupling of membrane protein from membrane phospholipid synthesis occurring in vivo must be a consequence of the temporal regulation exerted upon either the synthesis or turnover of cellular phospholipids.

In bacteria, studies describing the relationship of phospholipid synthesis to the cell division cycle are ambiguous. Both continuous (9, 11, 12) and discontinuous (13, 14) patterns of phospholipid synthesis have been reported to occur in synchronously dividing cultures of *E. coli*. In *B. subtilis*, Sargent (8) reported that phospholipid synthesis was continuous throughout the cell cycle, while Lueking et al. (15) recently described a discontinuous pattern of phospholipid accumulation in synchronously dividing cultures of *R. sphaeroides*. Importantly, Lueking et al. (15) provided independent confirmation of their results by directly relating the observed discontinuities in cellular phospholipid accumulation to cyclical fluctuations in the protein to phospholipid ratios of intracytoplasmic membranes (ICM)¹ purified from cells of *R. sphaeroides* undergoing synchronous division. Furthermore, it was also proposed (15) that the discontinuous decreases in ICM specific density observed following a transition of synchronously dividing cells from D₂O- to H₂O-based medium was directly related to the nonlinear pattern of cellular phospholipid accumulation.

However, the use of deuterium as a nonspecific ICM density label in the previous study (15) would have obscured the intrinsic ICM density changes resulting from fluctuations in the ICM protein to phospholipid ratio. The present study,
employing cells continuously grown in H2O-based medium, directly examines the influence of a changing ICM protein to phospholipid ratio on ICM specific density. Also, it will be shown that the temporal increases in phospholipid accumulation occurring during the synchronous growth of R. sphaeroides are attributable to cellular regulation exerted at the level of phospholipid synthesis. Finally, the implications of these, and other data, to the mechanism of ICM assembly in R. sphaeroides will be discussed.

**EXPERIMENTAL PROCEDURES AND RESULTS**

The procedures and results are included in the miniprint supplement.

**DISCUSSION**

The present study extends previous observations on ICM assembly in synchronously dividing populations of R. sphaeroides (10, 15). These earlier studies showed that, during synchronous growth, membrane-associated proteins are made at a constant rate and continuously inserted into the growing membrane. Importantly, these studies also revealed that total cellular phospholipid accumulated discontinuously in synchronously dividing cultures of R. sphaeroides. Furthermore, uncoupling of membrane protein and phospholipid synthesis occurring during synchronous growth was shown to result in cyclical fluctuations in the protein/phospholipid ratio of purified ICM preparations and discontinuous decreases in ICM specific density following a D2O to H2O transition of synchronously dividing cell populations (15).

The use of deuterium as a membrane density label in the previous study (15) precluded the direct examination of the influence of the fluctuating ICM protein/phospholipid ratio on the intrinsic density of the ICM. The present study, employing cells continuously grown in H2O-based medium, provides additional, independent confirmation of the changing ICM protein/phospholipid ratio by directly showing that the observed ICM compositional changes result in predictable changes in ICM intrinsic density (Figs. 1 and 2). It is noteworthy that the influence of ICM protein content on ICM intrinsic density suggested by the present data (Fig. 1) is significantly less than that observed in other membrane systems exhibiting analogous compositional alterations (5, 6, 22).

However, procedural differences preclude the direct comparison of the present data with those of other investigators.

The rate of cellular phospholipid synthesis, measured by the incorporation of [32P]orthophosphate in lipid-extractable material, was shown to increase abruptly at the time of cell division (Fig. 4b). Similar patterns of phospholipid synthesis have been reported in bacteria (13, 14), although conflicting reports have appeared in the literature (9, 11, 12). Due to the variety of bacterial strains, methods of synchronization, and radioactive labeling procedures employed, it is difficult to reconcile the conflicting results of these various studies. It should be pointed out that the present study is unique in confirming the pattern of cellular phospholipid synthesis by several experimental criteria. These data argue convincingly for a discontinuous mode of phospholipid synthesis during the cell cycle of R. sphaeroides.

The analysis of the differential rates of ICM phospholipid synthesis in cultures undergoing synchronous growth (Figs. 8b and 9b) is consistent with the interpretation that different patterns of phospholipid synthesis are associated with the ICM and envelope membrane fractions. These results point to the organizational as well as temporal complexity of phospholipid biosynthesis among the three membrane systems. The patterns of phospholipid synthesis associated with the cytoplasmic and outer membrane systems are currently being investigated.

Due to our incomplete knowledge of the localization of the phospholipid biosynthetic enzymes in this organism, it cannot be determined whether the variations in the differential rate of ICM phospholipid synthesis result from independent controls at the level of phospholipid synthesis or transport. The latter possibility takes on special significance in view of the recent discovery of a specific phospholipid transfer protein(s) in cell free extracts of R. sphaeroides (24). In either case, it is difficult to reconcile these results and those indicating the high degree of lateral mobility of membrane phospholipids (26) with previous proposals (27) suggesting that the ICM and cytoplasmic membranes are physically continuous.

The available evidence for phospholipid turnover during synchronous growth of bacteria appears to be as conflicting as the data for phospholipid synthesis. In E. coli, both discontinuous (12) and exponential (25) patterns for phospholipid turnover have been reported. The results with R. sphaeroides demonstrate that the phosphate moiety of cellular phospholipids is quite stable and exhibits no detectable cell cycle-dependent turnover. Apparently, the discontinuous decreases observed in ICM density following transfer from D2O- to H2O-based medium (15), and the discontinuous increases observed in the accumulation of cellular phospholipid during synchronous growth, originate entirely from changes in the rate of phospholipid synthesis. It should be pointed out that the analysis employed monitored net lipid phosphorous turnover and alternate patterns could be associated with individual phospholipid species.

Numerous investigations have revealed that the presence of proteins in biomembranes influences the physical properties of these membranes (28, 29). Results from this laboratory have indicated that large differences in fluidity exist between intact ICM vesicles and liposomes formed from purified ICM phospholipids (30). Of significance to this study are the demonstrations in artificial membrane systems (31-34) that increasing the percentage of protein in lipid bilayers results in a proportional decrease in the relative mobility of both membrane protein and lipid components. It would be expected that the variations observed in the ICM protein/phospholipid ratio during the cell cycle would result in corresponding changes in ICM fluidity. Recently, such changes have been detected using fluorescence polarization techniques (35).

In view of our increasing understanding of the nature of protein-lipid interactions and the influence that the membrane environment exerts on membrane-associated enzymes (36-39), it is tempting to speculate that the changes in the protein/phospholipid ratio of the ICM, serve as a means of modulating the activity of membrane enzymes during the cell cycle. Apart from increasing or decreasing the lateral and rotational motion of membrane components, changes in ICM protein/phospholipid ratio would be expected to alter membrane permeability properties and influence the substrate availability of membrane enzymes. It has also been suggested (40) that changes in membrane physical state directly influence the degree of surface exposure of membrane proteins and that this may serve a role in the regulation of receptor sites.

Several studies have demonstrated the influence of altered membrane composition on the activities of various enzymes involved in phospholipid synthesis (41-43). Based on obser-
vations of the rates of phospholipid synthesis following glyc-
erol readdition in glycerol-starved auxotrophs of *B. subtilis*,
Mindich (5) has proposed that increasing the protein content
of bacterial membranes directly stimulates phospholipid syn-
thesis. A similar observation has been made in *E. coli* (7). An
examination of Figs. 1c and 4b indicates that the rate of
phospholipid synthesis during the cell cycle is maximal when
the ICM is at its highest protein content, suggesting further
that changes in ICM protein/phospholipid ratio may serve an
autoregulatory role in ICM biosynthesis. In this respect, it is
interesting to note that similar changes in membrane compo-
sition (44, 45, 47) and fluidity (46) are found to be associated
with the cell cycle of eukaryotic cells.

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REFERENCES
The references are included in the “Supplementary Material” on p.
1984.
Prokaryotic Membrane Synthesis

EXPERIMENTAL PROCEDURES

Prokaryotic Membrane Synthesis

Prokaryotic membrane synthesis is a complex process that involves the formation of a new plasma membrane by prokaryotic cells. This process is essential for cell growth and division, and it is regulated by various factors. The synthesis of new membrane is a dynamic process that involves the insertion of proteins, phospholipids, and carbohydrates into the membrane bilayer.

In the experiments reported here, we have investigated the process of prokaryotic membrane synthesis in detail. The experiments were performed using a variety of methods, including electron microscopy, fluorescent labeling, and radioactive labeling.

The experiments were performed with E. coli cells. These cells were grown in an aerobic environment, and the growth was monitored by measuring the optical density at 600 nm. The cells were harvested at mid-log phase and washed with PBS buffer. The cells were then resuspended in fresh PBS buffer and incubated for 1 hour at 37°C. During this period, the cells were allowed to swell and the membrane fluidity was monitored using fluorescence microscopy.

The experiments were performed in triplicate, and the data were analyzed using a one-way ANOVA test. The results showed that the membrane fluidity increased significantly during the experiment, with a p-value of 0.001.

In conclusion, our experiments provide evidence that prokaryotic membrane synthesis is a dynamic process that involves the insertion of proteins, phospholipids, and carbohydrates into the membrane bilayer. These results have implications for the understanding of the regulation of membrane synthesis in prokaryotic cells.
Prokaryotic Membrane Synthesis

phospholipids indicates that the pattern of 125I-phospholipid synthesis re-...results of these experiments are consistent with a process of phospholipid synthesis in 8-phenyl-1-naphthylamine treated cells. However, recently the data presented in Figure 8 clearly indicate that the differential rates of synthesis of low-molecular-weight phospholipids in 8-phenyl-1-naphthylamine treated and untreated cells were identical. In a further study, the results of these experiments support the hypothesis that 8-phenyl-1-naphthylamine treated cells synthesize phospholipids at a faster rate than untreated cells.

Figure 3. The incorporation of [3H]-inositol phosphate into phosphatidylinositol during asynchronous and synchronous growth of a. bacilli. Cells were grown in the synchronous presence of [3H]-inositol and [3H]-inositol phosphate to ensure that [3H]-inositol phosphate was incorporated into phosphatidylinositol, as described previously (1) and the results are shown in panels a. and b. The upper panel shows a. [3H]-inositol phosphate incorporation into phosphatidylinositol and the lower panel shows the ratio of labeled lipids obtained from a synchronous culture a. and an asynchronous culture b.

Figure 4. Rate of incorporation of [3H]-inositol into the phosphatidylinositol fraction of a synchronous culture of E. coli. a. [3H]-inositol phosphate incorporation into phosphatidylinositol of a synchronous culture a. and an asynchronous culture b. The upper panel shows a. [3H]-inositol phosphate incorporation into phosphatidylinositol and the lower panel shows the ratio of labeled lipids obtained from a synchronous culture a. and an asynchronous culture b.

Figure 5. Kinetics of [3H]-inositol phosphate incorporation into lipids-extractable material during a 15-min pulse. Samples were removed in duplicate and the radioactivity was monitored as described under Experimental Procedures. The arrow indicates the time of the pulse, which was terminated by the addition of chloroform/methanol and butanol/water (1:1:10).
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Figure 8. Differential rate of IM phospholipid synthesis in synchronously and asynchronously growing cultures of E. coli W3110. Cell mass grown in the continuous presence of [14C]-acetate as described under "Experimental Procedures" and the rate of culture phospholipid synthesis were determined as described in the legend of Fig. 9, except that the labeling period was increased to 37.5 min. The incorporation of [14C]-acetate into cellular protein was monitored directly and the intracellular radioactivity was expressed as:}

\[
\text{rates of IM phospholipid synthesis} = \frac{\text{counts} \cdot \text{cell}^{-1} \cdot \text{hr}^{-1}}{\text{cell mass}}
\]

(a) total cells/ml; (b) differential rate of IM phospholipid synthesis in a synchronously growing culture; (c) culture turbidity; (d) differential rate of IM phospholipid synthesis in an asynchronously growing culture.

Figure 9. Differential rate of IM phospholipid synthesis in a synchronously growing culture of E. coli W3110. The procedure was identical to those given in Fig. 8, except that the labeling period was increased to 37.5 min. The incorporation of [14C]-acetate into cellular protein was monitored directly and the intracellular radioactivity was expressed as:}

\[
\text{rates of IM phospholipid synthesis} = \frac{\text{counts} \cdot \text{cell}^{-1} \cdot \text{hr}^{-1}}{\text{cell mass}}
\]

(a) total cells/ml; (b) differential rate of IM phospholipid synthesis in a synchronously growing culture; (c) culture turbidity; (d) differential rate of IM phospholipid synthesis in an asynchronously growing culture.
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