Cardiolipin Biosynthesis and Mitochondrial Respiratory Chain Function Are Interdependent*  

Received for publication, March 5, 2004, and in revised form, July 22, 2004  
Published, JBC Papers in Press, July 29, 2004, DOI 10.1074/jbc.M402545200  

Vishal M. Gohil‡, Paulette Hayes§, Shigemi Matsuyama§, Hermann Schägger¶, Michael Schlame¶, and Miriam L. Greenberg‡***  

From the ‡Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202, §Blood Research Institute, The Blood Center of South Eastern Wisconsin and Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, ¶Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, D-60590 Frankfurt, Germany, and ¶Department of Anesthesiology, New York University, School of Medicine, New York, New York 10016

Cardiolipin (CL) is an acidic phospholipid present almost exclusively in membranes harboring respiratory chain complexes. We have previously shown that, in Saccharomyces cerevisiae, CL provides stability to respiratory chain supercomplexes and CL synthase enzyme activity is reduced in several respiratory complex assembly mutants. In the current study, we investigated the interdependence of the mitochondrial respiratory chain and CL biosynthesis. Pulse-labeling experiments showed that in vivo CL biosynthesis was reduced in respiratory complexes III (ubiquinolcytochrome c oxidoreductase) and IV (cytochrome c oxidase) and oxidative phosphorylation complex V (ATP synthase) assembly mutants. CL synthesis was decreased in the presence of CCCP, an inhibitor of oxidative phosphorylation that reduces the pH gradient but not by valinomycin or oligomycin, both of which reduce the membrane potential and inhibit ATP synthase, respectively. The inhibitors had no effect on phosphatidylglycerol biosynthesis or CRD1 gene expression. These results are consistent with the hypothesis that in vivo CL biosynthesis is regulated at the level of CL synthase activity by the ΔpH component of the proton-motive force generated by the functional electron transport chain. This is the first report of regulation of phospholipid biosynthesis by alteration of subcellular compartment pH.

Cardiolipin (CL) is an acidic glycerophospholipid with a unique dimeric structure consisting of four fatty acyl chains (1). It is almost exclusively present in membranes designed to generate an electrochemical potential gradient for ATP synthesis, including the mitochondrial inner membrane and bacterial plasma membrane. CL plays a vital role in mitochondrial structure and function by providing osmotic stability to mitochondrial membranes (2) and by specifically interacting with many inner membrane proteins (reviewed in Refs. 3 and 4). Normal levels of CL are required for optimal mitochondrial bioenergetic functions (5, 6). Decreased CL levels are observed in cells undergoing apoptosis (7–9) and aging (10, 11) and in fibroblasts of Barth syndrome patients (12). Thus, a detailed understanding of the regulation of CL levels will provide important insights into the manifestation of these conditions.

CL is associated with major proteins of the mitochondrial respiratory chain including NADH dehydrogenase (complex I) (13), ubiquinol:cytochrome c reductase (complex III) (13–17), and cytochrome c oxidase (complex IV) (18, 19), the ATP synthase (complex V) (20), and the carrier proteins for phosphate (21) and adenine nucleotides (22). CL modulates the catalytic activities of proteins, as seen in the case of the ADP-ATP carrier (5, 23, 24) and complex IV (25, and/or provides stability as reported for complex III (17) and complex IV (19). CL binds specifically and irreversibly to cytochrome c (26), providing a membrane attachment site for cytochrome c and limiting the soluble pool of the protein. X-ray crystallography studies have further confirmed the specific interaction of CL with integral membrane proteins such as the bacterial photoreaction center of the photosynthetic bacterium Rhodopseudomonas sphaeroides (27) and Saccharomyces cerevisiae respiratory complex III (28). The mitochondrial energy-generating system thus is clearly dependent on CL. Interestingly, the characterization of yeast CL synthase indicated that its activity was decreased in respiratory complex assembly mutants (29), suggesting that CL synthesis was dependent upon a functionally assembled respiratory chain. The current study addresses the interdependence of CL and the mitochondrial respiratory chain.

The CL biosynthetic pathway is well characterized in the yeast S. cerevisiae. The first step catalyzed by phosphatidylglycerophosphate synthase (PGP) is the synthesis of PGP from CDP-diacylglycerol and glycero-3-phosphate (Glc-3-P). PGP phosphatase dephosphorylates PGP to phosphatidylglycerol (PG). In the final step, CL synthase catalyzes the formation of CL from PG and CDP-diaclylglycerol (30, 31). All of the enzymes of this pathway are associated with the mitochondrial membrane (32). PGP synthase and CL synthase have been characterized in yeast, and the genes, PGS1 (33, 34) and CRD1 (35–37), encoding these enzymes have been identified. An early study by Jakovcic et al. (38) shows that CL levels were correlated with mitochondrial development. CL was more abundant in the stationary than logarithmic growth phase and in aerobic versus anaerobic conditions. Respiratory-deficient p0 cells had reduced CL. Similar results were obtained by Gaynor et al. (39) and Gallet et al. (40). These studies pointed to an important role for CL in mitochondrial biogenesis and sug-
gested that mitochondrial development and CL synthesis may be coordinate regulated. The characterization of purified CL synthase from yeast and mammalian mitochondria showed strong pH dependence of reaction velocity around mitochondrial matrix pH. Sclame and Hostetler (41) propose that the trans-membrane pH gradient across the inner mitochondrial membrane offered a possible mechanism of regulation. However, this hypothesis was not previously tested.

The optimization of methods for quantification of CL enabled us to address the following questions. 1) What is the effect of mitochondrial respiratory chain complex assembly mutants on in vivo CL biosynthesis and steady-state CL levels? 2) What is the effect of mitochondrial trans-membrane electrochemical potential gradient and ATP synthesis on in vivo CL biosynthesis? We carried out pulse-labeling and steady-state labeling experiments that showed that the functional assembly of the mitochondrial respiratory complexes positively regulates in vivo CL biosynthesis. Our data are consistent with the hypothesis that the trans-membrane pH gradient acts as a regulatory mechanism that controls CL biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

Glucose, yeast extract, and peptone were purchased from Difco Laboratories (Detroit, MI). Galactose, valinomycin, oligomycin, CCCP, and all of the phospholipid standards, phosphatidic acid (PA), phosphatidylethanolamine, CL, phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol, and phosphatidylcholine, were from Sigma. 33P, and [α-32P]UTP were from PerkinElmer Life Sciences. 10-N-Nonyl-3,6-bis(dimethylamino)-acridine (NAO) was purchased from Molecular Probes (Eugene, OR). Zymolyase was from ICN Biomedicals Inc. (Aurora, OH). Thin layer chromatography plates (LKB Silica Gel 150 A) were purchased from Whatman Inc. (Clifton, NJ). Riboprobe system kit was from Promega. Chloroform, ethanol, methanol, glycerol, boric acid, and triethylamine were from Fisher Scientific (Fair Lawn, NJ). All of the other chemicals used were reagent grade or better.

**Methods**

Yeast Strains and Growth Media—The yeast S. cerevisiae strains used in this work are listed in Table 1. Yeast strains were grown at 30°C. Complex medium (YP dextrose) for liquid cultures contained yeast extract, 1% w/v, peptone, 2% w/v, and glucose 2% w/v. Solid medium contained 2% w/v agar in addition to the above. Glucose was replaced as carbon source by galactose (2%) or ethanol (1%) and glycerol (3%) where indicated. Semi-synthetic medium contained 5 g of (NH₄)₂SO₄, 1 g of KH₂PO₄, 0.5 g of MgSO₄•7H₂O, 0.1 g of NaCl, 0.1 g of CaCl₂, 5 g of peptone, 3.75 g of yeast extract, 20 g of galactose, or 3% glycerol and 1% ethanol per 1 liter of medium. The pH of the medium was adjusted to 5.8 by 50 mM sodium citrate/sodium phosphate buffer as per Kovac et al. (42). The respiratory complex assembly mutants were verified for genetic markers and their inability to grow on non-fermentable medium by replica plating on synthetic dropout and YPG (yeast extract, peptone, 3% glycerol, and 1% ethanol) plates, respectively.

Direct CL Determination in Yeast using NAO—CL quantification using NAO was essentially as described by Gallet et al. (43). Yeast cells at the indicated growth phase were fixed in cold ethanol (70%) and stored at −20°C. Fixed cells were washed three times with cold buffer (10 mM Tris/HCl, pH 7), vortexed vigorously to eliminate aggregates, and counted using a hemocytometer. Yeast cells (0–2.5 × 10⁶) were added to 45 μl NAO and incubated for 15 min at 20°C. Cells were then centrifuged (3000 × g, 5 min, 20°C), washed three times in buffer to remove unbound dye, and resuspended in 3 ml of buffer. Red fluorescence emission of NAO bound to yeast cells was measured at 640 nm with an excitation wavelength of 450 nm using a Hitachi fluorescence spectrophotometer (Model F-2000) (emission and excitation bandwidths were set at 10 nm). We observed that NAO did bind to yeast cells in the concentrations reported. However, binding was not specific to CL, because fluorescence emission from CL-deficient crd1Δ cells was similar to that of wild-type cells. Thus, this method was not used for CL quantification.

In Vivo Pulse Labeling of Phospholipids—10-ml cultures were pulse-labeled with [33P]P (0.5 μCi) for 15 min. Cells were harvested, washed once with sterile water, and digested with zymolyase (Zymolyase-20T) (2.5 mg/ml in 50 mM Tris-SCN buffer, pH 7.5, containing 1.2 mM glycerol and 100 mM sodium thioglycolate) at room temperature for 15 min to yield spheroplasts. Phospholipids were extracted from spheroplasts by 2.1 chloroform/methanol (44), partitioned in sterile water, and separated by one-dimensional thin layer chromatography (45) on LKB Silica Gel 150 A plates in chloroform/ethanol/water/triethy lamine (30/35/7/35, v/v). Phospholipids were identified by co-migration with known standards. 32P, in individual phospholipids was visualized by phospha-ror imaging and quantified by ImageQuant software (Amersham Biosciences). Incorporation of the radiolabel ([33P]) into individual phospholipid is expressed as the percentage of radiolabel incorporated into total phospholipids.

**Steady-state Phospholipid Determination**—Yeast strains were grown at 30°C in YP dextrose or YP galactose media with a starting A₅₅₀ of 0.1. Immediately after inoculation, cultures were supplemented with 10 μg/ml of CCCP (Sigma) and 200 μg/ml of oligomycin (Boehringer-Mannheim) for the entire duration of the experiment (47). For valinomycin treatment, cells were grown in semi-synthetic medium (42) with galactose or glycerol−ethanol as carbon source to the early logarithmic growth phase (A₅₅₀ = 0.8). Valinomycin was added to a final concentration of 5 μg/ml. The time of treatment was 1.5 h for CCCP and 2 h for oligomycin and valinomycin. Cells were then pulse-labeled with [33P]P for phospholipid analysis or harvested for RNA isolation.

**Estimation of Mitochondrial Matrix pH**—For in vivo experiments that showed that the functional assembly of the CL biosynthesis and steady-state CL levels? 2) What is the effect of mitochondrial trans-membrane electrochemical potential gradient and ATP synthesis on in vivo CL biosynthesis? We carried out pulse-labeling and steady-state labeling experiments that showed that the functional assembly of the mitochondrial respiratory complexes positively regulates in vivo CL biosynthesis. Our data are consistent with the hypothesis that the trans-membrane pH gradient acts as a regulatory mechanism that controls CL biosynthesis.

**Table 1**

| Strain | Genotype | Source/reference |
|--------|----------|------------------|
| W303-1A | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1 | 29 |
| aW303ΔCOR1 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1, cor1Δ::HIS3 | 29 |
| aW303ΔCOR2 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1, cor2Δ::HIS3 | 29 |
| aW303ΔCOR6 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1, cor6Δ::URA3 | 29 |
| aW303ΔATP10 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1, atp10Δ::LEU2 | 29 |
| aW303ΔATP11 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1, atp11Δ::HIS3 | 29 |
| aW303ΔATP12 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1, atp12Δ::LEU2 | 29 |
| aW305Δ29 | MAT a, ade2-1, trpl-1, leu2-3, 112, his3-1, 15, ura3-1 | 29 |
| JHY1–2Ca | MAT a, leu2-3, leu-2-122, ura3-52, his4-519, ade6, trp1 | 29 |
| DL1Δ | MAT a, his3, leu2, ura3 | 29 |
| FY1679 | MAT a, ura3–3, trpΔ63, leu2Δ1, hisΔ200, cls1::CanMX4 | G. Daum |
| FY1679CΔR1 | MAT a, ura3–52, trpΔ63, hisΔ200, cls1::CanMX4 | G. Daum |
| FGY3 | MAT a, ura3–52, trplΔ1, leu2Δ1, hisΔ200, ade2–101, lys2–801 | 35 |
| FGY2 | MAT a, ura3–52, trplΔ1, leu2Δ1, hisΔ200, ade2–101, lys2–80, crd1Δ::URA3 | 35 |
| D275–10B/A/HV | MAT a, met, ura3 | 56 |
| PYV10 | MAT a, met, atpl::URA3 | 56 |
Oligomycin is an inhibitor of the F₀ component of the F₀-F₁-phore that disrupts the trans-membrane electrical gradient.

8.2) containing 10 semi-synthetic galactose medium of various pH values (6.5, 7.0, 7.6, and 8.2) containing 10 µM CCCP. The pH estimation was done on cells after treatment with CCCP, oligomycin, and valinomycin as described above, and the data are expressed as relative change in MFI upon treatment with inhibitors compared with control.

DNA Analysis—DNA was isolated by hot phenol extraction (49), separated on an agarose gel, and transferred to a nylon membrane. The blots were hybridized with a 32P-labeled CRD1 riboprobe followed by a riboprobe for the constitutively expressed gene ACT1 to normalize for loading variation. RNA probes were synthesized using the Promega Riboprobe System. Plasmids used as templates for riboprobe synthesis with pCDB7 (50) and pPLG (51). These plasmids were linearized upon digestion with HindIII and BamHI, respectively. Riboprobes were synthesized using RNA polymerase T7 for CRD1 and SP6 for ACT1. The results were visualized by phosphorimaging and quantified using ImageQuant software.

RESULTS

In Vivo CL Biosynthesis Is Regulated by Carbon Source and Growth Curve—Previous studies have shown that growth phase and carbon source regulate steady-state CL levels (38–40). CL levels are higher in growth medium, which favors mitochondrial biogenesis including YP galactose and non-fermentable medium such as YPGE (38, 39). Because our mitochondrial respiratory complex assembly mutants and ATP synthase mutants cannot grow in non-fermentable medium, we decided to use fermentable medium with glucose or galactose as carbon source. The aim was to identify growth medium and growth phase in which CL synthesis is maximum, such that quantification of CL is more reliable. As seen in Fig. 1a, the maximum labeling of CL during pulse labeling occurs in the mid-logarithmic phase in YP galactose medium, whereas maximum steady-state labeling of CL occurs in early stationary phase (Fig. 1b). Quantification of CL under these conditions results in a strong CL-specific signal upon radiolabeling.

Decreased CL Biosynthesis in Respiratory Chain Assembly Mutants—CL synthesis was measured in respiratory chain and ATP synthase mutant cells grown in YP galactose or YP dextrose medium (Table II). Pulse labeling and steady-state labeling of CL decreased in most of the assembly mutants of complexes III, IV, and V. In pulse-labeling experiments, CL was decreased in all of the mutants with the exception of coxΔ. Steady-state CL levels decreased in all of the mutants with the exception of coxΔ in which case CL actually increased slightly in the stationary phase. As expected, in the ρ0 cells, the steady-state CL decreased. In summary, in almost all of the cases, disruption of the mitochondrial respiratory chain and ATP synthase by genetic mutation led to a decrease in biosynthesis and steady-state levels of CL.

Effect of Oxidative Phosphorylation Inhibitors on CL Synthesis—The absence of a functional electron transport chain in the respiratory complex assembly mutants leads to a decrease in the trans-membrane pH gradient, trans-membrane electrical gradient, and mitochondrial ATP synthesis. Any or all of these factors may be responsible for the observed decrease in CL synthesis in these mutants. To determine the role of each of these factors in CL regulation, we perturbed them individually with specific inhibitors of oxidative phosphorylation, including CCCP, valinomycin, and oligomycin. CCCP disrupts the trans-membrane pH gradient by allowing free movement of protons across the inner mitochondrial membrane. This results in a reduced mitochondrial matrix pH. Valinomycin is a K⁺ ionophore that disrupts the trans-membrane electrical gradient. Oligomycin is an inhibitor of the F₀ component of the F₀-F₁ ATP synthase complex and inhibits mitochondrial ATP synthesis. We observed that CCCP (4.1 µg/ml), oligomycin (3 µg/ml), and valinomycin (5 µg/ml) inhibited the growth of wild-type cells in non-fermentable media (Fig. 2, a and b), suggesting that the concentration of inhibitors used is effective in perturbing mitochondrial oxidative phosphorylation. Inhibition was observed after 1.5 h with CCCP treatment and 2 h for oligomycin or valinomycin treatment (Fig. 2, a and b). These inhibitors did not dramatically affect growth in fermentable media (Fig. 2, c and d) in which inhibition was observed much later. Thus, in vivo CL biosynthesis was determined in fermentable medium since inhibitor treatment in non-fermentable medium
resulted in growth arrest and inefficient incorporation of the radiolabel.

To test the effect of these inhibitors on mitochondrial matrix pH, we used pH-sensitive GFP expressed in mitochondria matrix (pH-GFPmito), which gives higher MFI with increasing pH (Fig. 3a). In FY1679 wild-type cells, the treatment by oligomycin or valinomycin results in a small increase in the GFP intensity, whereas CCCP treatment results in a large drop in the intensity (Fig. 3b). These results confirmed that oligomycin and, to a lesser degree, valinomycin increase mitochondrial pH and that CCCP treatment decreases mitochondrial pH.

Pulse-labeling experiments carried out under similar growth conditions showed that CCCP reduced CL by almost 45%, oligomycin resulted in small increase in CL (~20%), and valinomycin treatment showed no effect, although the mean $^{32}\text{P}$ incorporation in CL increased (Fig. 3c). These results are consistent with the change in mitochondrial matrix pH (Fig. 3b), such that an increase in mitochondrial matrix pH resulted in an increased incorporation of $^{32}\text{P}$ in CL, whereas a decrease in pH resulted in the decreased biosynthesis of CL. To determine whether the effect of these inhibitors was on the first step of the CL biosynthetic pathway, we quantified PG in crd1/H9004 cells after treatment with inhibitors. PG biosynthesis was tested in crd1/H9004 cells, because PG levels in wild-type cells are very low and cannot be measured accurately. The high resolution separation of yeast phospholipids by one dimensional thin layer chroma-

---

| Strain          | Deletion | Affected complex | Pulse-labeled CL | Steady-state CL | Steady-state CL |
|-----------------|----------|------------------|------------------|-----------------|-----------------|
|                 |          |                  | Mid-log phase    | Early-stationary phase | % of control |
| aW303/cor1Δ     | cor1Δ    | III              | 54.8 ± 3.71      | 40.6 ± 0.70      | 95.48 ± 0.72   |
| aW303/cor2Δ     | cor2Δ    | III              | 24.0 ± 0.74      | 43.3 ± 2.43      | 49.57 ± 2.15   |
| WD1             | cor4Δ    | IV               | 93.7 ± 9.4       | 65.6 ± 8.9       | 37.34 ± 1.85   |
| aW303/cox6Δ     | cox6Δ    | IV               | 74.2 ± 5.79      | 100.5 ± 2.2      | 117.75 ± 2.21  |
| JDΔ             | cox7Δ    | IV               | 21.9 ± 8.21      | 41.7 ± 7.0       | 42.59 ± 5.97   |
| aW303/atp10Δ    | atp10Δ   | V                | 27.1 ± 2.59      | 49.3 ± 0.89      | 58.82 ± 1.17   |
| aW303/ATP11     | atp11Δ   | V                | 33.1 ± 1.76      | 57.5 ± 1.47      | 75.88 ± 1.03   |
| aW303/ATP12     | atp12Δ   | V                | 34.0 ± 1.80      | 66.3 ± 1.01      | 72.70 ± 3.19   |
| PVY10           | atp4Δ    | V                | 43.4 ± 7.37      | 51.3 ± 13.05     | 49.81 ± 4.37   |
| aW303pΔ         | Mitochondrial DNA | III, IV, and V | 51.5 ± 2.05      | 56.1 ± 2.45      | 73.01 ± 1.44   |

---

**Fig. 2.** Cell growth in the presence of oxidative phosphorylation inhibitors. Wild-type cells (FY1679) were grown in non-fermentable media (a, YPGE; b, semi-synthetic GE) and fermentable media (c, YP galactose; d, semi-synthetic galactose) at 30 °C. Cells were grown to mid-logarithmic phase (a and c) or to an early logarithmic phase (b and d) and then divided into separate tubes and treated with oligomycin (3 μg/ml), CCCP (4.1 μg/ml) (a and c), and valinomycin (5 μg/ml) (b and d) at the indicated times (arrow). In control tubes, an equal volume of solvent used for dissolving the inhibitor was used. Cell growth was monitored by measuring $A_{550}$ at the indicated times. The data are representative of two independent experiments.

---

**Table II**

Cardiolipin quantification in respiratory chain complexes III and IV and oxidative phosphorylation complex V assembly mutants

Cells were grown in YP galactose medium to the indicated growth phase (asterisk indicates that galactose was replaced by glucose). Pulse labeling and steady-state labeling were carried out as described under “Experimental Procedures.” The amount of CL in each mutant is expressed as a percent of CL in the isogenic wild-type strain. The data represent an average of three experiments.

| Strain          | Deletion | Affected complex | Pulse-labeled CL | Steady-state CL | Steady-state CL |
|-----------------|----------|------------------|------------------|-----------------|-----------------|
|                 |          |                  | Mid-log phase    | Early-stationary phase | % of control |
| aW303/cor1Δ     | cor1Δ    | III              | 54.8 ± 3.71      | 40.6 ± 0.70      | 95.48 ± 0.72   |
| aW303/cor2Δ     | cor2Δ    | III              | 24.0 ± 0.74      | 43.3 ± 2.43      | 49.57 ± 2.15   |
| WD1             | cor4Δ    | IV               | 93.7 ± 9.4       | 65.6 ± 8.9       | 37.34 ± 1.85   |
| aW303/cox6Δ     | cox6Δ    | IV               | 74.2 ± 5.79      | 100.5 ± 2.2      | 117.75 ± 2.21  |
| JDΔ             | cox7Δ    | IV               | 21.9 ± 8.21      | 41.7 ± 7.0       | 42.59 ± 5.97   |
| aW303/ATP10     | atp10Δ   | V                | 27.1 ± 2.59      | 49.3 ± 0.89      | 58.82 ± 1.17   |
| aW303/ATP11     | atp11Δ   | V                | 33.1 ± 1.76      | 57.5 ± 1.47      | 75.88 ± 1.03   |
| aW303/ATP12     | atp12Δ   | V                | 34.0 ± 1.80      | 66.3 ± 1.01      | 72.70 ± 3.19   |
| PVY10           | atp4Δ    | V                | 43.4 ± 7.37      | 51.3 ± 13.05     | 49.81 ± 4.37   |
| aW303pΔ         | Mitochondrial DNA | III, IV, and V | 51.5 ± 2.05      | 56.1 ± 2.45      | 73.01 ± 1.44   |
tography (Fig. 5) allowed reliable quantification of PG and CL in contrast to the NAO-based fluorescence assay in which case wild-type and crd1Δ cells showed similar fluorescence emission at 640 nm. None of the inhibitors affected synthesis of PG in crd1Δ cells (Fig. 4b), suggesting that CL synthesis was reduced by the inhibition of CL synthase. To determine whether CCCP inhibited CL synthase by affecting CRD1 expression, we measured the effect of inhibitors on the CRD1 mRNA level by Northern analysis. As seen in Fig. 6, none of the inhibitors affected CRD1 mRNA levels. These results are consistent with the hypothesis that a decrease in mitochondrial pH leads to decreased CL synthesis by decreasing CL synthase enzyme activity.

**DISCUSSION**

In this work, we addressed the hypothesis that CL biosynthesis is regulated by the mitochondrial trans-membrane pH gradient. We used pulse labeling to directly measure in vivo CL biosynthesis. Consistent with the hypothesis, inhibition of the mitochondrial respiratory chain by genetic mutation led to decreased CL synthesis. More specifically, the disruption of the pH gradient, but not membrane potential or ATP synthesis, resulted in decreased CL synthesis. These results support the hypothesis that the pathway for CL biosynthesis is regulated by the trans-membrane pH component of the proton-motive force generated by the mitochondrial respiratory chain. This is the first report that suggests the alteration of subcellular compartment pH as a mechanism of regulation of phospholipid biosynthesis.

The enzymes of the CL biosynthetic pathway, PGP synthase, and CL synthase and the genes (PGS1 and CRD1) encoding these enzymes are regulated by factors affecting mitochondrial development such as growth phase, carbon source, and mitochondrial genome (29, 39, 50, 52). These mitochondrial development factors regulated CL levels (Fig. 1) with the maximum pulse-labeled CL observed in the mid-logarithmic phase and maximum steady-state labeled CL in early stationary phase in YP galactose medium (Fig. 1). Our results corroborated earlier studies showing a doubling of CL levels in YP dextrose medium from logarithmic to stationary phase (38, 39). The gradual
increase in steady-state CL from early logarithmic to stationary phase is consistent with the expression profiles of CL pathway genes (50, 52) and the mitochondrial development pattern in YP dextrose medium (53). The growth phase regulation of pulse-labeled CL in YP dextrose (Fig. 1a) followed a pattern similar to the regulation of respiration in this medium (53), suggesting that in vivo CL biosynthesis is linked to respiration.

This link was further confirmed by the results showing a decrease in CL synthesis in almost all of the mitochondrial respiratory chain assembly mutants (Table II). This is consistent with the previous finding that CL synthase activity is reduced in respiratory chain assembly mutants (29).

To understand the mechanism underlying the decrease in CL biosynthesis during respiratory deficiency, we used inhibitors of oxidative phosphorylation that specifically perturb the trans-membrane pH gradient (CCCP), trans-membrane electrical gradient (valinomycin), and mitochondrial ATP generation (oligomycin). Matsuyama et al. (48) have shown that CCCP reduces the matrix pH by 0.5 units and that oligomycin increases matrix pH by 0.15 units in mammalian cells. We observed similar effects of these inhibitors in yeast cells with oligomycin treatment resulting in a small increase and CCCP resulting in a relatively large decrease in mitochondrial matrix pH (Fig. 3b). Consistent with the pH change, CCCP inhibited CL synthesis and oligomycin treatment resulted in a small increase in CL synthesis (Fig. 4a). PG biosynthesis (Fig. 4b) and CRD1 gene expression (Fig. 6) were unaffected upon treatment with inhibitors, indicating that the pH effect is not at the level of PGP synthase or CRD1 gene expression. These findings indicate that CL biosynthesis is specifically regulated by the mitochondrial matrix pH at the level of CL synthase, which has a pH optimum of 9.0 (30) and an active site facing the mitochondrial matrix (40). Interestingly, mammalian (31) and plant CL synthases (54) display a strong pH dependence of reaction velocity around the mitochondrial matrix pH with the catalytic site facing the mitochondrial matrix (55). Therefore, pH regulation of CL biosynthesis may be a universal mode of regulating CL levels in the eukaryotic kingdom.

In summary, we have shown that in vivo CL biosynthesis is regulated by the pH component of the functional respiratory chain and that the effect is most probable at the level of CL synthase enzyme activity. This is the first demonstration of
regulation of phospholipid biosynthesis by alteration of the mitochondrial matrix pH.

Acknowledgments—We thank Gunther Daum for yeast strains and John M. Lopes for providing the pPLG plasmid. We thank Deirdre Vaden, Daobin Ding, Zhiming Gu, Shulin Ju, and Quan Zhong for valuable discussion and advice.

REFERENCES

1. LeCoq, J., and Ballou, C. C. (1964) Biochemistry 155, 976–980
2. Koshkin, V., and Greenberg, M. L. (2002) Biochem. J. 364, 317–322
3. Hoch, F. L. (1992) Biochim. Biophys. Acta 1113, 71–133
4. Schleif, M., Rua, D., and Greenberg, M. L. (2000) Programm Lipid Res. 39, 257–288
5. Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfeiffer, K., and Greenberg, M. L. (2000) J. Biol. Chem. 278, 22987–22994
6. Hager, M., Scha¨gger, H., Hagen, T., Roth, B., Brandt, U., Link, T. A., and von Jagow, G. (2003) J. Biol. Chem. 278, 9829–9836
7. Gomez, B., and Robinson, N. C. (1999) Biochim. Biophys. Acta 1428, 1–15
8. Robinson, N. C., Zborowski, M., and Talbert, L. H. (1999) Biochim. Biophys. Acta 1428, 1–15
9. Sedlak, E., and Robinson, N. C. (1999) Biochemistry 38, 14966–14972
10. Eble, K. S., Coleman, W. B., Hantgan, R. R., and Cunningham, C. C. (1999) J. Biol. Chem. 265, 19434–19440
11. Kadenbach, B., Mende, P., Kelbe, H. V., Stipani, I., and Palmieri, F. (1982) FEBS Lett. 129, 109–112
12. Beyer, K., and Klingenberg, M. (1985) Biochemistry 24, 3821–3826
13. Beyer, K., and Yusuf, M. (1990) Biochemistry 29, 5784–5790
14. Depp, K., and Beyer, K. (1989) Biochemistry 27, 8554–8558
15. Pfeiffer, K., Kohl, V., Stuart, R. A., Hunte, C., Brandt, U., Greenberg, M. L., and Schae¨gger, H. (2003) J. Biol. Chem. 278, 52873–52880
16. Rice, L., and Kinnunen, P. K. J. (1994) J. Biol. Chem. 269, 1770–1774
17. McAuley, K. E., Fyle, P. K., Ridge, J. P., Isaacs, N. W., Copiedel, R. J., and Jones, M. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14706–14711
18. Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) EMBO J. 20, 6591–6600
19. Zhao, M., Schleif, M., Rua, D., and Greenberg, M. L. (1998) J. Biol. Chem. 273, 2402–2408
20. Tamai, K. T., and Greenberg, M. L. (1990) Biochim. Biophys. Acta 1046, 214–222
21. Schleif, M., and Hostetler, K. Y. (1991) J. Biol. Chem. 266, 22388–22403
22. Kuchler, K., Daum, G., and Paltz, F. (1986) J. Bacteriol. 165, 901–910
23. Chang, S.-C., Heacox, P. N., Clancy, C. J., and Dowhan, W. (1998) J. Biol. Chem. 273, 9629–9636
24. Dzugasova, V., Obermaierova, M., Horvatova, K., Vachova, M., Zakova, M., and Subik, J. (1998) Curr. Genet. 34, 297–302
25. Jiang, F., Rizavi, H. S., and Greenberg, M. L. (1997) Mol. Microbiol. 26, 481–491
26. Tuller, G., Hrastnik, C., Aichberger, G., Schieffer, U., Klein, F., and Daum, G. (1998) FEBS Lett. 421, 15–18
27. Chang, S.-C., Heacox, P. N., Mileikowskaya, E., Voolker, D., and Dowhan, W. (1998) J. Biol. Chem. 273, 14933–14941
28. Jakovice, S., Getz, G. S., Rabkowitz, M., Jakob, H., and Swit, M. (1971) J. Cell Biol. 48, 490–502
29. Gaynor, P. M., Hubbell, S., Schmidt, A. J., Lina, R. A., Minsky, S. F., and Greenberg, M. L. (1991) J. Biol. Chem. 266, 6124–6131
30. Gallet, P. F., Peitl, J. M., Maftah, A., Zachowski, A., and Julien, R. (1997) Biochem. J. 324, 627–634
31. Schleif, M., and Hostetler, K. Y. (1997) Biochim. Biophys. Acta 1348, 207–213
32. Kroc, L., Bohmurova, E., and Butko, P. (1982) Biochim. Biophys. Acta 721, 341–348
33. Gallet, P. F., Maftah, A., Petit, J. M., Denis-Gay, M., and Julien, R. (1995) Eur. J. Biochem. 238, 113–119
34. Felch, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–508
35. Leary, C., Pelletier, X., Hemmendinger, S., and Cazeneuve, J. P. (1987) J. Chromatogr. 420, 411–416
36. Atkinson, K. D., Jensen, B., Kost, A. L., Storm, E. M., Henry, S. A., and Fogel, S. (1980) J. Bacteriol. 141, 558–564
37. Epstein, C. B., Waddle, J. A., Hale, W., Dave, V., Thornton, J., Macatee, T. L., Garner, H. R., and Butow, R. A. (2001) Mol. Biol. Cell 12, 297–308
38. Matsumoto, S.-L., Lipis, J., Deveraux, Q. J., Tsien, R. Y., and Reed, J. C. (2000) Nat. Cell Biol. 2, 318–325
39. Collart, M. A., and Oliviero, S. (1994) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 13.12.1–13.12.2, John Wiley & Sons, Inc., New York
40. Jiang, F., Go, Z., Granger, J. E., and Greenberg, M. L. (1999) Mol. Microbiol. 31, 373–379
41. Anderson, M. S., and Lopes, J. M. (1996) J. Biol. Chem. 271, 26596–26601
42. Zhang, Q., and Greenberg, M. L. (2003) J. Biol. Chem. 278, 33978–33984
43. Yotsuyanagi, Y. (1962) J. Ultrastruct. Res. 7, 121–140
44. Frenzen, M., and Griebel, R. (1994) Plant Physiol. 106, 1527–1532
45. Schla¨me, M., and Haldar, D. (1995) J. Biol. Chem. 269, 74–79
46. Velez, J., Arvold, G., Paul, M. F., Galante, M., Durrens, P., Aigle, M., and Guerin, B. (1989) Biochimie (Paris) 71, 963–915