Absence of Cardiolipin in the crd1 Null Mutant Results in Decreased Mitochondrial Membrane Potential and Reduced Mitochondrial Function

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Cardiolipin (CL) is a unique phospholipid which is present throughout the eukaryotic kingdom and is localized in mitochondrial membranes. Saccharomyces cerevisiae cells containing a disruption of CRD1, the structural gene encoding CL synthase, have no CL in mitochondrial membranes. To elucidate the physiological role of CL, we compared mitochondrial functions in the crd1Δ mutant and isogenic wild type. The crd1Δ mutant loses viability at elevated temperature, and prolonged culture at 37 °C leads to loss of the mitochondrial genome. Mutant membranes have increased phosphatidyglycerol (PG) when grown in a nonfermentable carbon source but have almost no detectable PG in medium containing glucose. In glucose-grown cells, maximum respiratory rate, ATPase and cytochrome oxidase activities, and protein import are deficient in the mutant. The ADP/ATP carrier is defective even during growth in a nonfermentable carbon source. The mitochondrial membrane potential is decreased in mutant cells. The decrease is more pronounced in glucose-grown cells, which lack PG, but is also apparent in membranes containing PG (i.e. in nonfermentable carbon sources). We propose that CL is required for maintaining the mitochondrial membrane potential and that reduced membrane potential in the absence of CL leads to defects in protein import and other mitochondrial functions.

Cardiolipin (1,3-bis (1’, 2’-diacyl-3’-phosphoryl-sn-glycerol)-sn-glycerol (CL)) is a structurally unique phospholipid that carries four acyl groups and two negative charges. It is thus highly hydrophobic and acidic. The biosynthesis of CL occurs in three enzymatic steps (1–3). Phosphatidylglycerolphosphate (PGP) synthase catalyzes the formation of PGP from phosphatidyl-CMP (CDP-diacylglycerol; CDP-DG) and glycerol 3-phosphate. PGP is then dephosphorylated to phosphatidylglycerol (PG) by PG phosphatase. Eukaryotes and bacteria utilize different reactions to convert PG to CL. In prokaryotes, CL synthase catalyzes a phosphatidyl transfer between two PG molecules (4). This is a near-equilibrium (transesterification) reaction that is mainly controlled by substrate availability. In contrast, eukaryotic CL synthase catalyzes a phosphatidyl transfer from CDP-DG to PG (5–7). This is an irreversible reaction that involves cleavage of a high energy anhydride bond. This reaction can take place in the presence of low substrate concentration and is mainly regulated by CL synthase activity. The differences in these reactions probably reflect different functions of PG and CL in prokaryotes and mitochondria.

In Escherichia coli, the enzymes that catalyze the synthesis of CL have been characterized biochemically, and the genes encoding these enzymes have been cloned. Although disruption of the cls gene (encoding CL synthase) is not lethal, bacterial strains bearing a null allele of pgsA (encoding PGP synthase) are inviable (8, 9). Interestingly, bacterial cls null mutants do synthesize CL, presumably by another enzyme. These experiments suggest that the anionic phospholipids PG and/or CL are essential for bacterial viability.

In eukaryotic cells, CL is found primarily in mitochondrial membranes (10). Because of its acidic and hydrophobic nature, CL has the ability to interact with many different proteins (10, 11). It is associated with the major proteins of oxidative phosphorylation, including complex V (ATP synthase), respiratory complexes I, III, and IV, as well as the carrier proteins for phosphate and adenine nucleotides (12–17). Trivedi et al. (18) showed that a temperature-sensitive yeast mutant that had reduced CL at the elevated temperature had a concomitant decrease in cytochrome oxidase activity. In vitro experiments suggest that mitochondrial inner membrane integrity may depend specifically on CL, because enzymatic digestion of CL, but not PE or PC, correlates with the disruption of structure (19). Evidence also suggests that CL may be required for import of proteins into the mitochondria, because doxorubicin (which binds irreversibly to CL) inhibits protein import (20, 21).

Despite the obvious importance of CL, in vivo experiments to elucidate the role of this lipid and the mechanisms of its regulation have not been previously possible, because of the lack of a model system in which CL levels could be genetically manipulated. The molecular tools are now available to carry out these experiments in the yeast Saccharomyces cerevisiae. We identi-
ried the \textit{S. cerevisiae} structural gene encoding CL synthase (\textit{CRD1}, originally named \textit{CLS1}) and showed that disruption of the \textit{CRD1} gene eliminates CL from mitochondrial membranes (22). These findings were confirmed by Tuller et al. (23) and Chang et al. (24). Growth of the \textit{crd1} mutant in glucose or nonfermentable carbon sources is largely unaffected at 30 °C; however, at elevated temperature, the mutant loses viability, even in glucose (25). Expression of \textit{CRD1} is highly regulated by factors affecting mitochondrial development, including carbon source, growth stage, and the presence of a mitochondrial genome (25). These results point to the involvement of CL in critical cellular functions. In this paper, we investigated the role of CL in mitochondrial function by characterizing the physiological effects of eliminating CL from the membrane.

**EXPERIMENTAL PROCEDURES**

\textbf{DNA Manipulations—Plasmid purification from \textit{E. coli} was performed using the Wizard Plus Miniprep DNA Purification system (Promega, Madison, WI). \textit{CRD1} fragments were generated by polymerase chain reaction and ligated into the expression vector pYES2.0 as described (26). Transformants of \textit{E. coli} cells were performed using an electro cell manipulator at 2.45 kV.}

\textbf{Yeast Genetic Techniques—\textit{S. cerevisiae} strains used in this work are listed in Table I. Complex media for liquid cultures used in all experiments except measurement of ADP/ATP carrier (AAC) (see Table IV) contained 1% Bacto yeast extract and 2% Bacto peptone (Difco Laboratories, Detroit, MI), with 2% glucose (YPD), 2% galactose, 2% raffinose (YPR), 3% glycerol (YPG), or 3% glycerol plus 0.95% ethanol (YPGE) as carbon source. Complex media for experiments to measure AAC (Table IV) contained 0.3% yeast extract, 0.05% peptone, 2% lactate, and 0.1% glucose. Synthetic medium was prepared as described previously (27). Solid medium contained 2% agar in addition to the above. Yeast transformations were performed with an electro cell manipulator (BTX, San Diego, CA) as described previously (22), and transformants were selected on synthetic medium lacking specific nutrients as required. Induction of \( \rho^0 \) mutants was carried out using ethidium bromide treatment as described (28). Haploid cells were cultured for 48 h in the presence of 25 \( \mu \)g/ml ethidium bromide in minimal medium containing 2% glucose, 0.2% ammonium sulfate, 0.69 mg/ml vitamin-free yeast base and required nutrients. Cells that formed colonies on YPD but not YPGE plates were selected. Putative \( \rho^0 \) mutants were crossed to several \( \rho^- \) mutants bearing mitochondrial DNA lesions \textit{oxi1}, \textit{oxi2}, \textit{oxi3}, or \textit{cob}. After overnight growth on YPD plates at 30 °C, the diploids were replicated to solid YPGE medium. The absence of a mitochondrial genome in the induced petite mutants was indicated by the inability of the diploids to grow on YPGE medium.

\textbf{Viable Cell Determination—Yeast cells from a 20-ml preculture in the logarithmic phase of growth were resuspended in 200 ml of prewarmed YPD or YPGE medium. Cultures were incubated with continuous agitation for 6–7 days. Aliquots were serially diluted, spread on YPD plates, and incubated at 30 °C until colonies formed.}

\textbf{Isolation of Mitochondria—Intact mitochondria were isolated as described by Daum et al. (29) using 2.5 \( \mu \)g of Zymolyase 20T (ICN Biomedicals, Aurora, OH) as required to form spheroplasts. The extent of spheroplast formation was monitored with a spectrophotometer. The reaction was terminated when a 1:100 water-diluted reaction mixture reached an \( A_{600} \) of about 20% of the starting value. Alternatively, when integrity of mitochondria was not required, mitochondria were isolated using the glass bead method as described earlier and purified by differential centrifugation (27).}

\textbf{Analysis of Total Mitochondrial Phospholipids—Mitochondria were isolated as described above and resuspended in isolation buffer at a protein concentration of 5 mg/ml. Total mitochondrial phospholipids were extracted and purified as described (30). Briefly, the mitochondrial suspension was extracted twice with 20 volumes of chloroform/methanol (2:1). The extract was then washed sequentially with 0.2 volumes of 0.9% NaCl and water, followed by low speed centrifugation. The organic phase was transferred to a glass tube, dried under a nitrogen current, and resuspended in 150 \( \mu \)l of chloroform/methanol (2:1). Isolated total phospholipids were then applied to silica gel 60 plates (EM Separations Technology, Gibbstown, NJ), which were developed with chloroform/methanol/25% ammonium hydroxide (65:35:5) in the first dimension and chloroform/acetonemethanol/acetic acid/water (50:20:10:10.5) in the second dimension. The developed TLC plates were dried and exposed to iodine vapor to visualize the phospholipid spots, which were labeled with a pencil and subjected to sulfuric acid combustion and phosphorus quantitation as described (31). The relative percentage of phosphorus in the individual phospholipid species is presented as a percentage of the total mitochondrial phospholipid phosphorus. Assays of Mitochondrial Enzyme Activity and Content—Yeast cells were grown in fermentable or nonfermentable medium with agitation. Mitochondria were isolated from cells harvested at the early stationary growth stage. Mitochondrial ATPase and cytochrome oxidase were assayed as described (32, 33). The isolation of AAC from mitochondria and the reconstitution into phospholipid vesicles were performed as described (34). The exchange rates were measured according to the rapid mixing and removal procedure and evaluated to a first order rate law using a computerized program. As a control, inhibition by prior addition of 5 \( \mu \)M bongkrekate (BKA) and 10 \( \mu \)M carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was carried out using ethidium bromide treatment as described (28). Haploid cells were cultured for 48 h in the presence of 25 \( \mu \)g/ml ethidium bromide in minimal medium containing 2% glucose, 0.2% ammonium sulfate, 0.69 mg/ml vitamin-free yeast base and required nutrients. Cells that formed colonies on YPD but not YPGE plates were selected. Putative \( \rho^0 \) mutants were crossed to several \( \rho^- \) mutants bearing mitochondrial DNA lesions \textit{oxi1}, \textit{oxi2}, \textit{oxi3}, or \textit{cob}. After overnight growth on YPD plates at 30 °C, the diploids were replicated to solid YPGE medium. The absence of a mitochondrial genome in the induced petite mutants was indicated by the inability of the diploids to grow on YPGE medium.
0.02 ml of 45% HClO₄ at the following time intervals: wild type mitochondria at 10, 20, 40, 60, and 120 s and mutant mitochondria at 0.5, 1, 2, 4, 8, and 12 min. After neutralization with KOH and removal of KCIO₄ by centrifugation, ATP was assayed by the hexokinase-glucose-6-phosphate-dehydrogenase assay. The initial phosphorylation rate, \( V_{\text{corr}} \), was calculated from the increase in ATP content. For obtaining the AAC-dependent rate, \( V_{\text{corr}} \), a correction factor was calculated from the ATP content with and without CAT + BKA taken 2 min after the start, according to the formula: \( V_{\text{corr}} = V_{\text{ATP}}(1 - \text{ATP} + \text{BKA})/\text{ATP} \), where \( V_{\text{ATP}} \) is the rate of ATP without inhibitor, and [ATP] and [ATP + BKA] are the amount of ATP found without and with BKA 150 s after start of oxidative phosphorylation, respectively.

**Import Assay:** Mitochondria were isolated from yeast cells grown in YPG or YPD according to Daum et al. (29) and Hartl et al. (37). Radiolabeled preproteins were obtained by in vitro transcription and translation reactions using rabbit reticulocyte lysate (Amersham Pharmacia Biotech) in the presence of [35S]methionine/cysteine (Sigma). Mitochondrial in vitro import reactions were performed in BSA-containing buffer (3% (w/v) fatty acid-free BSA, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS/KOH, pH 7.2) in the presence of 2 mM ATP and 2 mM NADH. To dissipate the membrane potential, 8 mM fluoroethylestrene sulfonamide, and samples were incubated for a further 10 min at 4 °C before SDS-PAGE analysis.

**Mitochondrial Membrane Potential Determination—**The Δψ of isolated mitochondria was determined by recording the fluorescence decrease of the voltage-sensitive dye 3,3'-dipropylthiadicarbocyanine ion (Molecular Probes Inc., Eugene, OR) as described previously (39). The method is based on the potential-dependent partitioning of the dye between mitochondria and the medium, leading to a decrease in fluorescence with increasing Δψ (40). The addition of mitochondria results in some quenching of the fluorescent dye, and the Δψ is assessed by comparing the fluorescence observed after addition of mitochondria and the fluorescence observed after the Δψ is dissipated by the addition of the uncouplers valinomycin and KCN. Mitochondria possessing a higher Δψ have a greater uptake of the dye, resulting in a lower fluorescence. An aliquot of 25 μg of mitochondria was used per assay. Assays were performed at a temperature of 25 °C.

**Electrophoresis—**SDS-PAGE was performed on 10 × 7 cm slab minigels with a thickness of 0.75 mm at an acrylamide concentration of 12.5%. The electrophoresis was carried out using the procedure described by Laemmli (41). Blue native gel electrophoresis of total mitochondrial proteins was carried out using the procedure described by Laemmli (41), with isoelectric focusing as the first dimension and SDS-PAGE as the second. The gels were stained with either Coomassie Brilliant Blue R-250 or silver stain (Bio-Rad).

**Protein Assay—**Protein concentration was determined by the method of Bradford (45) with BSA as the standard. Buffers identical to those containing the protein samples were used as blanks.

**RESULTS**

The crd1Δ Mutant Has Aberrant Composition of Acidic Phospholipids in Its Mitochondrial Membranes—Previous results indicating that the crd1Δ mutant lacked CL were based on TLC analysis of mitochondrial membrane phospholipids (22–24). More recently, utilizing a technique specific for detection of CL, i.e., HPLC analysis of CL derivatives, to detect CL in the null mutant (46), we observed no detectable CL in all conditions tested (data not shown). We also compared mutant and isogenic strains in fermentable (YPD) and nonfermentable (YPGE) media. In fermentable medium, many mitochondrial functions such as respiration are not required. Interestingly, when cells were grown in YPGE, the crd1Δ mutant FGY2 had significantly increased PG in its mitochondrial membranes, which accounted for about 10% of total phospholipid phosphorus (Table II). Under these growth conditions, the wild type CL content is approximately 12% of phospholipid phosphorus. In striking contrast, no increase in PG was observed in mutant cells grown in YPD. Throughout the logarithmic phase of growth, PG levels in the mutant mitochondrial membranes remained nearly undetectable (data not shown). PG content increased only slightly as the mutant cells entered the stationary phase of growth, accounting for about 1% of total phospholipid phosphorus in the mitochondria. CL content in wild type cells in this growth condition was approximately 8%. Levels of other major phospholipids, including phosphatidic acid, remained largely unaltered in the mutant.

In summary, these experiments indicate that the crd1Δ mutant FGY2 is completely lacking in CL. During growth in YPGE, the mutant membrane contains 10% PG, slightly less acidic phospholipid than the wild type (12% CL). However, during growth in YPD, the mutant has almost no acidic phospholipid, whereas the wild type has 8% CL. These data predict that functions dependent upon acidic mitochondrial phospholipids PG and CL are defective in the mutant, and the defects may be more apparent during growth in glucose than in nonfermentable carbon sources.

The crd1Δ Mutant Loses Viability during Growth at Elevated Temperature—When cells of the crd1Δ mutant are patched onto plates and incubated at 37 °C, the mutant exhibits no apparent growth defect. However, we have shown that the crd1Δ mutant strain FGY2 could not form colonies at 37 °C from single cells seeded on YPD or YPGE plates (25). Crd1Δ mutants in two other strain backgrounds, including the one described by Tuller et al. (23), also exhibit the inability to form colonies at 37 °C (data not shown). To further elucidate the effect of the crd1 allele on growth at the elevated temperature, we compared the viability of cells grown at 30 and 37 °C. At 30 °C, no decrease in viability was observed in the mutant grown in YPGE, and only a slight decrease in viability was observed in mutant cells entering stationary phase in YPD (Fig. 1). In contrast, at 37 °C, the mutant cells had significantly decreased viability compared with the wild type in both YPD and YPGE medium.

Crd1Δ mutant cells grown in YPD or YPGE segregated large numbers of petites (respiratory incompetent cells) after prolonged culture at elevated temperature (data not shown). Diploids formed by mating the crd1Δ petites with a rho₀ mutant (which lacks mitochondrial DNA) were incapable of growing on glycerol plates, indicating that the petite phenotype was caused by cytoplasmic mutation (loss of mitochondrial DNA). The crd1Δ petite mutants could not complement oxi1, oxi2, oxi3, or cob mutants, which contain mutations in mitochondrial genes, for growth on glycerol, suggesting that they were very likely rho₀ mutants. The crd1Δ petites had a temperature sensitivity phenotype similar to the parent crd1 FGY2 strain on glucose plates, in contrast to an isogenic CRD1 rho₀ mutant (data not shown). These data indicate that the crd1Δ allele leads to loss of viability and loss of mitochondrial DNA during growth at elevated temperatures, in both fermentable and nonfermentable carbon sources.

**Protein Import Is Partially Defective in the crd1Δ Mutant—**To determine whether mitochondria lacking CL exhibited preprotein import defects, mitochondria were isolated from both wild type and crd1Δ mutant cells grown in both nonfermentable (YPD) or fermentable (YPG) medium and then subjected to in vitro import analysis. Two preproteins were employed: the precursor of the β subunit of the F₁-ATPase (F₁β) that is targeted to the matrix face of the inner membrane and a preprotein consisting of the presequence of F₆-ATPase sub-
unit 9 fused to dihydrofolate reductase (Su9-DHFR) that is targeted to the matrix. The preproteins were synthesized in vitro in rabbit reticulocyte lysate in the presence of [35S]methionine/cysteine and incubated with isolated yeast wild type or crd1∆ mitochondria at 25 °C for increasing times. Following import, samples were treated with proteinase K to remove nonimported preprotein. In the presence of a membrane potential, import was only slightly reduced when the cells were grown on YPD medium (Fig. 2B). The import of both preproteins was only slightly reduced when the cells were grown on YPG (Fig. 2A).

To assess whether the import defect observed in vitro affected the steady state mitochondrial protein profile, we carried out one- and two-dimensional gel electrophoresis of total mitochondrial proteins. No obvious changes were observed in cells grown in YPD or YPGE medium, indicating that the observed import defect was probably not severe enough to affect the steady state protein level at 30 °C (data not shown).

The crd1∆ Mutant Has Decreased Mitochondrial Membrane Potential—The import inhibition in crd1∆ mitochondria from YPD-grown cells was greater for the F1β preprotein than for Su9-DHFR. It was previously shown that the import of F1β shows a stronger dependence on the mitochondrial membrane potential, ∆ψ, than that of Su9-DHFR (47). We thus wondered whether the decrease in import in CL-lacking mitochondria was due to a lower ∆ψ. We assayed the ∆ψ of wild type mitochondria and crd1∆ mitochondria using the fluorescent dye, 3,3′-dipropylthiadicarbocyanine iodide. As shown (Fig. 3), mitochondria lacking CL possessed a decreased ∆ψ (higher fluorescence) in comparison with wild type mitochondria from cells grown in either YPG or YPD preparations. Moreover, this decreased ∆ψ was more pronounced in mutant mitochonrida isolated from cells grown in YPD medium. These results suggest that the decrease in ∆ψ is the likely explanation for the partial inhibition of protein import.

Respiratory Enzyme Activity and Maximum Respiration Rate Are Decreased in the crd1∆ Mutant in YPD—Cytochrome c oxidase catalyzes the last step of the respiratory chain, the transfer of four electrons to molecular oxygen. As a result, proton and ion gradients are generated across the mitochondrial inner membrane, which drive the ATPase reaction. These reactions play a key role in energy production. In vitro studies have shown that ATPase and cytochrome oxidase activities are strongly CL-dependent. We investigated the effect of the crd1∆ mutation on in vivo activities of these enzymes. As shown in Table III, when cells were grown in YPG, no differences between the mutant and wild type ATPase or cytochrome c oxidase activities were observed. In contrast, when cells were grown in YPD, activities of both enzymes were significantly decreased in the mutant mitochondria compared with those of the isogenic wild type. The mutant exhibited a corresponding decrease in respiration. As shown in Table III, mitochondria from mutant cells grown in YPD had a significantly decreased maximum respiratory rate in comparison with the wild type. No such difference was observed for mitochondria isolated from cells grown in YPGE. These data show that in the absence of both PG and CL, respiration is defective. However, it is likely that PG can substitute for CL to some extent, because the maximum respiratory rate in YPGE is not reduced in the mutant.

Oxidative Phosphorylation and AAC Activity Are Decreased in the crd1∆ Mutant—To further characterize the respiratory capacity of crd1∆ mutant mitochondria, oxidative phosphorylation was measured in the presence of the more active substrates α-glycerol phosphate and ethanol (Table IV). Cells were
FIG. 2. 

**crd1Δ mitochondria have a partial defect in preprotein import efficiency.** Mitochondria were isolated from wild type (WT) and 
crd1Δ cells grown in YPG (A) or YPD (B) medium and subjected to preprotein import analysis (A and B) in the presence (lanes 1–4 and 6–9) or absence (lanes 5 and 10) of Δψ. Following import at 25 °C, samples were treated with protease K prior to SDS-PAGE and phosphorimage analysis. Imported preprotein was quantified where 100% represents the amount of preprotein imported in wild type mitochondria after 15 min (A and B, lower panels).

FIG. 3. 

**crd1Δ mitochondria have a reduced membrane potential.** The Δψ of mitochondria (Mito.) isolated from wild type and crd1Δ cells grown in YPG (A) or YPD (B) medium was assayed using the 
potential-sensitive dye 3,3′,5′,5′-tetrachlorodicyanomethylene-3H,3′H-dipropylthiadicarbocyanine iodide. A more 
pronounced fluorescence quenching (decrease) after addition of mito-
dochondria indicates a higher Δψ, whereas the addition of 1 μM valino-
mycin (Val.) 1 mM KCN dissipates Δψ.

grown in the nonfermentable carbon source lactate, which, like 
glycerol/ethanol, is derepressing for PG (23) and is the carbon 
source used in previous experiments characterizing the ADP/ 
ATP carrier (34–35). Oxidative phosphorylation activity of iso-
lated mitochondria was measured directly by the rate of ATP 
synthesis from added ADP (35). The ATP synthesis was started 
by initiating electron transport upon addition of α-glycerol 
phosphate. The time progress of ATP formation was deter-
mined in samples withdrawn at increasing time intervals. The 
rates of oxidative phosphorylation are given in Table IV. Ox-
dative phosphorylation is strongly decreased in the CL-defi-
cient mitochondria. The rates in the mutant are only about 20% 
of the parent strain. Respiration is decreased to a lesser extent. 
The ATP synthesis is nearly fully blocked by the combined 
addition of BKA and CAT, indicating that all the ATP passes 
through the AAC. The amount of AAC contained in these 
mitochondria is assayed by [3H]CAT binding (48). There is no 
difference between the CL-deficient and parent strain, indicat-
ing that the amount of AAC taken up in the mitochondria is not 
affected by the lack of CL but that the AAC present is much less 
active.

AAC was isolated from the mitochondria and reconstituted 
into phospholipid vesicles to determine the actual transport 
rates (34). For this purpose, the “basic” ADP/ADP exchange 
was measured, because it is the most indifferent to Δψ among 
the four exchange modes. Vesicles were prepared with and 
without 8% CL. The transport activity was evaluated from the 
time progress of [14C]ADP uptake. It is clearly evident that the 
exchange activity is dramatically dependent on the presence 
of CL. The results from two separate reconstitution experiments 
are listed in Table IV. Without CL addition, AAC from the 
CL-deficient yeast has virtually no activity, whereas the AAC 
from the parent strain still shows a low but definite exchange. 
Obviously, residual endogenous CL still enables some trans-
port activity. Upon CL addition, the stimulated exchange ac-
tivity of the AAC from the CL-deficient yeast reaches only half 
(or even less) the activity observed in the parent strain. Pres-
umably, the AAC from the deficient strain remains less optimi-
ally folded, even with excess of added CL.

AAC exists as a dimer of 34.5-kDa subunits (49). When 
mitochondria are solubilized in digitonin-containing buffer and 
are subjected to blue native PAGE, wild type AAC is predomi-
nantly found at a position of ~80 kDa (50). The position 
when native AAC on this PAGE system may reflect phospholipid 
molecules that are still tightly bound to the AAC dimer. Indeed 
when mitochondria lacking CL were subjected to blue native 
PAGE and probed with AAC antibodies, it was found that AAC 
showed a slight but significant increase in its mobility (Fig. 4, 
lanes 2 and 4) compared with wild type mitochondria (Fig. 4, 
lanes 1 and 3). This change correlates well with the absence of 
bound CL molecules in the mutant protein. The mobility of 
AAC was affected in mutant mitochondria containing or lack-
ing PG. Additionally, larger AAC oligomers of low abundance 
were visible in wild type but not in crd1Δ mitochondria. Viewed 
with the decreased AAC activity in mitochondria containing 
PG, the data indicate that PG cannot substitute for CL in 
supporting AAC function.

**DISCUSSION**

Numerous in vitro experiments have pointed to the impor-
tance of CL in cellular and mitochondrial function (reviewed in 
Ref. 10). The availability of a yeast mutant that cannot syn-
thesize CL provides us with an experimental vehicle to ascer-
tain the role of this lipid. We previously cloned the CRD1 gene
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**TABLE III**

| YPD | Cytochrome c oxidase | ATPase | Respiration rate | YPD | Cytochrome c oxidase | ATPase | Respiration rate |
|-----|---------------------|--------|-----------------|-----|---------------------|--------|-----------------|
|     | μmol/min/mg         | μmol/min/mg | nmol O/min/mg   |     | μmol/min/mg         | μmol/min/mg | nmol O/min/mg   |
| Wild type | 0.022 ± 0.002     | 11.7 ± 0.8     | 52.7 ± 5.7     | crd1 | 0.993 ± 0.096     | 12.3 ± 1.0     | 243.0 ± 23.3    |
| crd1 | 0.011 ± 0.001     | 6.3 ± 1.2     | 18.5 ± 6.6     | crd1 | 0.975 ± 0.125     | 11.7 ± 1.3     | 253.4 ± 32.3    |

**TABLE IV**

Oxidative phosphorylation, respiration, and content and transport activity of the AAC

Wild type and crd1Δ mutant cells were grown in the presence of the nonfermentable carbon source lactate. Oxidative phosphorylation in isolated mitochondria was measured by following the kinetics of ATP synthesis, as described under “Experimental Procedures.” AAC content was determined by the kinetics of 3H CAT binding.

| Strain | V<sub>ATP</sub> | Respiration (25 °C) | 3H CAT binding | V<sub>3H CAT</sub><sup>a</sup> |
|--------|----------------|-------------------|----------------|-----------------|
|        | μmol/g prot/min | μAtom O/g prot/min | μmol/g protein | μmol/g prot/min |
| Wild type (FGY3) | 158 | 174 | 5 | 320 | 0.096 | 12.3 |
| crd1Δ (FGY2) | 35 | 46 | 2 | 192 | 0.22 |

<sup>a</sup> Initial exchange rates of external 14C ADP against internal ADP at 10 °C. The experiments were performed according to the procedure described by Heidkämper et al. (34). Experiments with two different mitochondrial preparations and vesicles are given.

FIG. 4. Altered mobility of ADP/ATP carrier on blue native electrophoresis in crd1Δ mutant. Mitochondria (50 μg) isolated from wild type (WT) or CL-deficient (crd1Δ) cells grown in either YPG or YPD medium as indicated were solubilized in digitonin buffer prior to blue native electrophoresis and immunoblotting with antibodies against AAC.

rate, oxidative phosphorylation, and protein import. The defects were less apparent in conditions under which mitochondrial membranes contained PG, suggesting that PG can compensate for lack of CL in some, but not all, cellular functions. 2) Mitochondria lacking CL have a significantly decreased membrane potential. The decrease is less pronounced when the membranes contain PG. 3) At elevated temperatures, CL is required for (an) essential cellular function(s) and for maintenance of mitochondrial DNA.

Previous studies showed that crd1Δ mutant cells did not exhibit severe growth defects at 30 °C in fermentable or non-fermentable carbon sources (22–24). At least two explanations can account for this observation. One is that, despite its unique structure and localization, CL is largely dispensable for cellular and mitochondrial function. Alternatively, it is possible that CL is essential for optimal mitochondrial function, but PG can compensate to some extent for the loss of CL. The crd1Δ mutant strain FGY2 provides an ideal experimental vehicle with which to distinguish between these possibilities. The mutant is completely lacking in CL, and the presence of PG can be manipulated by growth conditions. Thus, mutant mitochondrial membranes contain PG in nonfermentable carbon sources, whereas in fermentable carbon sources, PG is undetectable throughout the logarithmic growth phase and only slightly detectable in early stationary phase (Table II and data not shown). Tuller et al. (23) also reported the absence of PG in glucose-grown crd1Δ mutant cells. (The growth stage in which their phospholipid analyses were carried out was not indicated.) Controlling PG content in this manner, we determined that, in the absence of PG, the maximum respiration rate and activities of respiratory enzymes are decreased in the mutant (Table III). In the presence of PG, these activities are comparable with wild type. Thus, it appears that some acidic phospholipid is needed for these functions and that PG can compensate for lack of CL. It is possible that other phospholipids may be able to compensate for the lack of CL. Phosphatidic acid might be a potential CL substitute, owing to its acidic nature. In vitro studies have shown that phosphatidic acid could activate some CL-dependent enzymes, including yeast PGP synthase (51) and beef heart cytochrome c oxidase (52). Tuller et al. (23) did observe an increase in phosphatidic acid in the crd1Δ
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mutant mitochondria. However, in our strain, no dramatic changes in phosphatidic acid level were apparent in the mutant under any condition tested. Therefore, it is more likely that PG compensates for the lack of CL. This conclusion is further supported by the fact that although disruption of CRD1 led to no severe phenotypes in nonfermentable medium at 30 °C, disruption of PGS1, the structural gene for PGP synthase, caused severe mitochondrial dysfunction (53, 54).

The decrease in the rate of protein import correlates with a decreased membrane potential in CL-lacking mitochondria, suggesting that one or more components of the respiratory complexes or inner membrane transporters is impaired. Indeed, the activities of cytochrome oxidase and the AAC were reduced in crd1Δ mitochondria. Moreover the activity of the AAC purified from mutant cells grown in nonfermentable medium (and thus containing PG) is reduced compared with wild type (Table IV), indicating that not all CL-requiring functions can be fully compensated by PG. Among the mitochondrial components so far known, the AAC has by far the strongest requirement for CL (17, 55, 56). NMR analysis of isolated bovine heart AAC indicates that six CL molecules are tightly bound to the AAC and can only be released by denaturation (17). Similar high CL content was found in the isolated yeast AAC2 (55). Two additional molecules of CL can be bound loosely but with high specificity (56). Because of the tight binding, the dependence of bovine heart AAC transport on CL could not be shown, because CL was carried over into the reconstituted phospholipid vesicles. However, the yeast AAC (AAC2) has an absolute dependence on CL addition for transport. This is particularly evident in the various cysteine mutants in which Cys is replaced by Ser without any effect on the transport activity (55). In AAC from these mutants, NMR measurements indicate that the bound CL is reduced to about 2–3 mol CL/AAC dimer. The specificity requirement for CL in the activation of AAC in the reconstituted system was very high, and no other acidic phospholipid could replace CL.

Viewed in the background of these results, it is not surprising that oxidative phosphorylation is strongly reduced in the mitochondria from CL-deficient cells. This oxidative phosphorylation activity was entirely dependent on the ADP/ATP exchange by the AAC, as shown by the inhibition with BKA and CAT. Respiration is less affected by CL deficiency, probably in line with the lower dependence of the respiratory components on CL. Interestingly, the content of AAC in the deficient mitochondria appears not to be affected, indicating that the AAC present is largely inactive. The distinct residual activity of the AAC in these mitochondria suggests that the endogenous high content of acidic phospholipids can replace CL to some extent, in contrast to the in vitro situation with reconstituted AAC (55). It seems that, in vivo, AAC is somehow better adapted by subtle rearrangements to the high content of PG, which thus allows for a minimum transport activity. After isolation and reconstitution, the AAC from CL-deficient mitochondria has virtually an absolute dependence on CL. Addition of PG instead of CL did not enhance activity.

A key finding in this study is that the mitochondrial inner membrane lacking acidic phospholipids PG and CL has a reduced membrane potential. The decrease is less pronounced in membranes containing PG than in membranes lacking both PG and CL (Fig. 3). The lack of CL may impair the generation of a proton gradient across the inner membrane (by inhibition of the activity of respiratory chain complexes), and/or the maintenance of a proton gradient (by affecting the membrane barrier either directly or via impairment of inner membrane carrier proteins). Either defect may result in a reduction of Δψ. Because oxidative phosphorylation and protein import depend on the presence of a membrane potential, it is likely that their reduced function in the crd1Δ mutant can be attributed either partly or wholly to the reduced membrane potential.

Why is there no detectable PG in mitochondria from glucose-grown crd1Δ mutant cells? One possible explanation may be that PGP synthase activity is defective in the membrane. We did not detect a difference between wild type and mutant specific activity in glucose-grown cells (22), although Tuller et al. (23) found a 70% decrease and Chang and co-workers (24) observed a 35% decrease in activity in glucose-grown mutant mitochondria. The variation among these three laboratories may be due to strain differences, growth stage, and/or growth conditions. Strain differences clearly play a role in both PG content and temperature sensitivity, because some crd1 null mutant strains constructed in other laboratories are not temperature-sensitive. Alternatively, the assay in mitochondrial extracts may not reflect the true in vivo activity. The enzyme present in the mutant membrane may be incorrectly folded and, thus, inactive. This would be missed in enzyme assays carried out under optimal conditions in the presence of Triton X-100.

The loss of viability of the crd1Δ mutant at the elevated temperature suggests that CL is required for some essential cellular function(s). Respiration is not essential for viability of yeast cells during growth in glucose. Protein import is essential; however, the observed decrease in the rate of import is probably not sufficient to account for loss of viability. The temperature sensitivity may be related to loss of AAC activity or to other CL-requiring activities not yet identified. Current experiments are aimed at identifying the essential functions that require CL.

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REFERENCES
1. Cohen, G. S., Crowfoot, P. D., and Linnane, A. W. (1974) Biochem. J. 144, 265–275
2. Steiner, M. R., and Lester, R. I. (1972) Biochim. Biophys. Acta 250, 222–243
3. van den Bosch, H., van Golde, L. M. G., and van Deenen, L. L. M. (1972) Rec. Physiol. Biochem. Exp. Pharmacol. 66, 13–145
4. Hirschberg, C. B., and Kennedy, E. P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 648–651
5. Hostetler, K. Y., van den Bosch, H., and van Deenen, L. L. M. (1972) Biochim. Biophys. Acta 250, 597–513
6. Tamai, K. T., and Greenberg, M. L. (1990) Biochim. Biophys. Acta 1046, 211–224
7. Schlamme, M., Brody, S., and Hostetler, K. Y. (1993) Eur. J. Biochem. 212, 727–735
8. Nishijima, S., Asami, Y., Uetake, N., Yamagoe, S., Ohta, A., and Shibusawa, I. (1988) J. Bacteriol. 170, 775–780
9. Heacock, P. N., and Dowhan, W. (1989) J. Biol. Chem. 264, 14972–14977
10. Hoch, F. L. (1992) Biochim. Biophys. Acta 1113, 71–133
11. Schlamme, M., and Greenberg, M. L. (1997) Biochim. Biophys. Acta 1348, 201–206
12. Fry, M., and Green, D. E. (1981) J. Biol. Chem. 256, 1874–1880
13. Hayer-Hartl, M., Schagger, H., von Jagow, G., and Beyer, K. (1992) Eur. J. Biochem. 209, 423–430
14. Robinson, N. C. (1993) J. Bioenerg. Biomembr. 25, 153–162
15. Eble, K. S., Coleman, W. B., Hantgan, R. G., and Cunningham, C. C. (1990) J. Biol. Chem. 265, 1944–1949
16. Kadenbach, B., Mende, P., Kolbe, H. V. J., Stipani, I., and Palmieri, P. (1982) FEBS Lett. 139, 109–112
17. Beyer, K., and Klingenberg, M. (1985) in Integration of Mitochondrial Function (Lemasters, J. J., Hacker- brock, C. R., Thurner, R. G., and Weterhoff, H. V., eds) pp. 87–94, Plenum Publishing, New York
18. Awashty, T. Y., Berezney, R., Ruzicka, F. J., and Crane, F. L. (1969) Biochim. Biophys. Acta 189, 457–460
19. Eilers, M., Ender, and Schatz, G. (1989) J. Biol. Chem. 264, 2945–2950
20. Ender, M., Eilers, M., and Schatz, G. (1989) J. Biol. Chem. 264, 2951–2956
21. Jiang, F., Rizavi, H. S., and Greenberg, M. L. (1997) Mol. Microbiol. 26, 481–489
22. Tuller, G., Hrašnik, C., Alešteiner, G., Schießhalter, U., Klein, F., and Daum, G. (1998) FEBS Lett. 421, 15–18
23. Chang, S.-C., Heacock, P. N., Milejkovskaya, E., Voolker, D. R., and Dowhan, W. (1998) J. Biol. Chem. 273, 14933–14941
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25. Jiang, F., Gu, Z., Granger, J., and Greenberg, M. L. (1999) *Mol. Microbiol.* 31, 373–379
26. Sambrook, F., Fritsch, E. P., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Greenberg, M. L., Hubbell, S., and Lam, C. (1988) *Mol. Cell. Biol.* 8, 4773–4779
28. Goldring, E. S., Grossman, L. I., Krupsick, D., Cryer, D. R., and Marmur, J. (1970) *J. Mol. Biol.* 52, 323–335
29. Daum, G., Boehni, P. C., and Schatz, G. (1982) *J. Biol. Chem.* 257, 13028–13033
30. Christiansen, K., and Jensen, P. K. (1972) *Biochem. Biophys. Acta* 260, 449–459
31. Bartlett, G. R. (1959) *J. Biol. Chem.* 234, 466–468
32. Tzagoloff, A., Akai, A., and Needleman, R. B. (1975) *J. Bacteriol.* 123, 826–831
33. Tzagoloff, A., Akai, A., Needleman, R. B., and Zulch, G. (1975) *J. Biol. Chem.* 250, 8236–8242
34. Heidkamper, D., Muller, V., Nelson, D. R., and Klingenberg, M. (1996) *Biochem.* 35, 16144–16152
35. Muller, V., Basset, G., Nelson, D. R., and Klingenberg, M. (1996) *Biochem.* 35, 16132–16143
36. Yaffe, M. P. (1991) *Methods Enzymol.* 194, 627–643
37. Hartl, F. U., Ostermann, J., Guiard, B., and Neupert, W. (1987) *Cell* 51, 1027–1037
38. Soliner, T., Rassow, J., and Pfanner, N. (1991) *Methods Cell Biol.* 3, 345–358
39. Gartner, F., Voos, W., Querol, A., Miller, B. R., Craig, E. A., Cunsky, M. G., and Pfanner, N. (1995) *J. Biol. Chem.* 270, 3788–3795
40. Sims, P. J., Waggner, A. S., Wang, C.-H., and Hoffman, J. F. (1974) *Biochemistry* 13, 3315–3330
41. Laemmli, U. K. (1970) *Nature* 227, 680–685
42. Blom, J., Dekker, P. J. T., and Meijer, M. (1995) *Eur. J. Biochem.* 232, 309–314
43. Dekker, P. J. T., Martin, F., Maarse, A. C., Bomer, U., Muller, H., Guiard, B., Meijer, M., Rassow, J., and Pfanner, N. (1997) *EMBO J.* 16, 5408–5419
44. O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P. H. (1977) *Cell* 12, 1133–1141
45. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
46. Schlame, M., Shanske, S., Doty, S., Konig, T., Seulce, S., DiMauro, S., and Blanek, T. J. (1999) *J. Lipid. Res.* 40, 1555–1592
47. Martin, J., Mahlke, K., and Pfanner, N. (1991) *J. Biol. Chem.* 266, 18051–18057
48. Klingenberg, M., Grebe, K., and Scherer, B. (1975) *Eur. J. Biochem.* 52, 351–363
49. Lawson, J. E., and Douglas, M. G. (1988) *J. Biol. Chem.* 263, 14812–14818
50. Ryan, M. T., Muller, H., and Pfanner, N. (1999) *J. Biol. Chem.* 274, 20619–20627
51. Jiang, F., Kelly, B. L., Hagopian, K., and Greenberg, M. L. (1998) *J. Biol. Chem.* 273, 4681–4688
52. Goormaghtigh, E., Brasseur, R., and Ruysschaert, J.-M. (1982) *Biochem. Biophys. Res. Commun.* 104, 314–320
53. Subik, J. (1974) *FEBS Lett.* 421, 309–312
54. Chang, S.-C., Heacock, P. N., Clancey, C. J., and Dowhan, W. (1998) *J. Biol. Chem.* 273, 9829–9836
55. Hoffmann, B., Stockl, A., Schlame, M., Beyer, K., and Klingenberg, M. (1994) *J. Biol. Chem.* 269, 1940–1944
56. Drees, M., and Beyer, K. (1988) *Biochemistry* 27, 8584–8591