Oxidative phosphorylation in cardiolipin-lacking yeast mitochondria

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The role of cardiolipin in mitochondrial energy transformation was studied by comparing oxidative phosphorylation in the *Saccharomyces cerevisiae* cardiolipin synthase null mutant *crd1Δ*, and in isogenic wild type. Oxygen consumption experiments and membrane potential kinetics during the phosphorylation cycle in isolated mitochondria indicated that the absence of cardiolipin causes only a moderate deficiency of mitochondrial energy-transforming machinery at 25 °C. However, at 40 °C, respiration was completely uncoupled from phosphorylation for the mutant mitochondria, in contrast with that for the wild-type. Membrane potential kinetics demonstrated an increased susceptibility of

the mutant mitochondria to gradual deterioration during *in vitro* incubation. These results suggest that cardiolipin, although normally associated with several of the major enzymes of oxidative phosphorylation and required *in vitro* for their maximal activity, is not absolutely necessary for mitochondrial energy transformation under optimal conditions. The role of cardiolipin is, rather, to improve efficiency of oxidative phosphorylation and its resistance to unfavourable conditions, such as increased temperature.

Key words: energetic coupling, phospholipids, respiration.

INTRODUCTION

Cardiolipin is a unique phospholipid with dimer-like structure that is almost exclusively located in the inner mitochondrial membrane. It interacts with many of the respiratory and ATP-synthesizing enzymes, which require it for full activity [1,2]. However, its role in the overall oxidative phosphorylation process has been obscure, to a large extent because of the lack of a suitable experimental model in which cardiolipin levels could be manipulated. Recently, we and others identified the *Saccharomyces cerevisiae* gene encoding cardiolipin synthase, *CRD1* [3–5]. The *crd1Δ* mutant, which has no cardiolipin in its mitochondrial membranes, provides an excellent system *in vivo* in which to characterize the physiological role of cardiolipin. We have determined that cardiolipin is necessary for optimal mitochondrial function (F. Jiang, M. T. Ryan, M. Schlame, M. Zhao, Z. Gu, M. Klingenberg, N. Pfanner and M. L. Greenberg, unpublished work). Nevertheless, the *crd1Δ* mutant appears to grow normally in a non-fermentable medium at 16–30 °C, which implies that its bioenergetic apparatus performs satisfactorily under these conditions [3]. To clarify this apparent paradox, a detailed study of oxidative phosphorylation in cardiolipin-lacking mitochondria was performed. In the present report we show that, under optimal conditions, the absence of cardiolipin does not significantly affect mitochondrial function. However, absence of cardiolipin does lead to significantly weakened mitochondrial stability, especially at an elevated temperature.

MATERIALS AND METHODS

Isolation of mitochondria and measurement of oxidative phosphorylation

The strains used in the present study were FGY3 (wild type) and FGY2 (*crd1Δ*), as described previously in [3]. Yeast cultures were grown as described previously [3] in the presence of fermentable (2% glucose) and non-fermentable (3% glycerol or 3% glycerol/1% ethanol) carbon sources. Isolation of mitochondria was performed essentially as described in [6], with minor modifications. The medium used for isolation was 0.6 M

mannitol, 20 mM Hepes/KOH, pH 7.3, 1 mM EGTA, 0.2% (w/v) BSA. The final mitochondrial pellet was suspended in the isolation medium at approximately 10 mg of protein/ml. The reaction medium for oxidative phosphorylation used was 0.6 M mannitol, 20 mM Hepes/KOH, pH 7.4, 10 mM potassium phosphate, pH 7.4, 2 mM MgCl₂, 1 mM EGTA, 0.1% (w/v) BSA, and ethanol (20 mM) was used as a respiratory substrate. Respiration rates were measured with the Clark-type electrode. Phosphorylation was estimated from ADP-stimulated respiration [7] using the total oxygen consumption or, independently, with the hexokinase/glucose-6-phosphate dehydrogenase assay system [8]. In the latter case, the medium was supplemented with 20 mM glucose, 0.3 mM NADP and saturating amounts of hexokinase (8 units/ml) and glucose-6-phosphate dehydrogenase (4 units/ml).

Enzymic and membrane potential assays

Cytochrome *c* oxidase activity was measured polarographically with 5 mM ascorbate/0.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) in the presence of antimycin A [9].

Oligomycin-sensitive ATPase was assayed in the presence of 10 mM Tricine/KOH, pH 8.5, 2 mM MgCl₂, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.2 mM NADH, 5 units/ml pyruvate kinase and 5 units/ml lactate dehydrogenase [10].

Cytochrome content was measured from the differential reduced–oxidized spectra of the mitochondrial preparations diluted in 0.1 M phosphate buffer, pH 7.4, 2% (v/v) Triton X-100, using absorption coefficients as described previously [11].

Mitochondrial membrane potential was monitored using the fluorescence of the cyanine dye 3,3'-dipropylthiadicarbocyanine iodide [diSC₃(5)], with excitation and emission wavelengths set at 616 and 676 nm respectively [12]. The final dye concentration was lowered to 0.33 μM in order to minimize its damaging effect on mitochondria. The responsiveness of the dye fluorescence to a membrane potential was calibrated with a K⁺-diffusion potential by the gradual method [13]. Calibration was performed in Na⁺-containing medium with 80 ng/ml valinomycin; K⁺-diffusion potential was varied by successive additions of KCl

Abbreviations used: RCR, respiratory control ratio; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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(equivalent additions of NaCl did not change fluorescence intensity, indicating that the change of ionic strength did not affect calibration). Calculations were performed as described previously [14].

RESULTS

Mitochondria from cells grown in respiratory carbon sources

Respiration and ATP synthesis

Mitochondria from *crd1Δ* mutant cells grown in non-fermentable carbon sources were found to lack cardiolipin, but not its precursor, phosphatidylglycerol (F. Jiang, personal communication, and [4]). Therefore these mutant mitochondria allow for the detection of functional alterations caused specifically by the lack of cardiolipin in mitochondrial membranes. Oxidative phosphorylation in native and mutant mitochondria was characterized by the following parameters: State 3 (induced by ADP and uncoupler) and State 4 respiration rates, coupling efficiency of oxidative phosphorylation (denoted by the ratio of ADP/O), respiratory control ratio (RCR) and membrane potential kinetics

Figure 1 Respiration in wild-type (WT) and *crd1Δ* mutant mitochondria

The direct oxygraph tracings, typical of 15 WT and 15 *crd1Δ* mitochondrial batches, are shown. Mitochondria from WT and *crd1Δ* cells were added to the incubation medium to a final concentration of 0.18 mg of protein/ml (indicated by Mit). Phosphorylating and uncoupled respiration were initiated by addition of 200 μ M ADP (indicated by ADP) and 20 μ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (indicated by FCCP) respectively; for the latter, the concentration used was that necessary to overcome interference of 0.1% BSA.

Table 1 Parameters of oxidative phosphorylation in wild-type (WT) and *crd1Δ* yeast cells grown in a glycerol/ethanol medium

For all experiments, the number of mitochondrial batches tested (*n*) = 15. *, significantly different from corresponding value in WT mitochondria (*P* < 0.05).

Yeast	Parameter		
	State 3 respiration (nmol of O ₂ /min per mg)	ADP/O	RCR
WT	181.5 ± 59.9	1.63 ± 0.15	2.40 ± 0.31
<i>crd1Δ</i>	129.0 ± 47.9*	1.21 ± 0.26*	1.52 ± 0.36*

Figure 2 Titration of the whole respiratory flux and cytochrome *c* oxidase step with azide

Mitochondrial respiration in State 3 with 20 mM ethanol (●) or 5 mM ascorbate/0.1 mM TMPD in the presence of 10 μ M antimycin A (○) were titrated with sodium azide. Wild-type (WT) and mutant (*crd1Δ*) mitochondria concentrations were 0.15 mg of protein/ml; the ADP concentration was 0.8 mM. Data were collected from two WT and two *crd1Δ* mitochondrial batches.

during the phosphorylation cycle. Ethanol (20 mM), which is actively oxidized by yeast mitochondria [15], was used as a substrate for respiration.

Typical oxygen kinetic traces of the wild-type and mutant mitochondrial preparations shown in Figure 1 demonstrate that cardiolipin-lacking mitochondria retain clear State 3–State 4 transitions (i.e. respiratory control) and quite efficient coupling. The rate of the ‘phosphorylating’ respiration in native mitochondria was somewhat higher than in mutant preparations (Table 1). A similar difference was observed with uncoupler-induced respiration (results not shown). Some of the decrease in respiration rate in the mutant mitochondria can be ascribed to the well-known activating effects of cardiolipin on the respiratory enzymes [1]. There was no significant difference in cytochrome *c* content between wild-type and mutant mitochondria (results not shown). Control over the respiration rate is shared among respiratory enzymes, with cytochrome *c* oxidase being the most powerful regulator in the yeast mitochondria (control strength ≥ 0.5) [9]. Since a pronounced dependence of this enzyme on cardiolipin was demonstrated [1], we compared cytochrome *c* oxidase control strength in the native and mutant mitochondria. For this purpose, the respiratory flux and isolated cytochrome *c* oxidase step (oxidation of ascorbate + TMPD in the presence of antimycin A) was titrated with the cytochrome *c* oxidase inhibitor azide (Figure 2), and control strength coefficients were calculated from initial slopes of the inhibition curves [9]. The control strength coefficient of cytochrome *c* oxidase in wild-type mitochondria was 0.44 ± 0.06 (*n* = 2), close to that reported previously [9]. Any specific depression of cytochrome *c* oxidase capacity, caused by the absence of cardiolipin, would increase limitation of respiratory flux exerted by this step and, consequently, its control

Figure 3 Membrane potential kinetics during phosphorylation cycle in wild-type (WT) and *crd1*Δ mitochondria

The tracings, typical of eleven WT and 5 *crd1*Δ mitochondrial batches, represent the fluorescence of 3,3'-dipropylthiadicarbocyanine iodide [diSC₃(5)] (0.33 μM) added to incubation medium containing 0.11 mg of protein/ml of the WT or mutant (*crd1*Δ) mitochondria. Additions of 20 mM ethanol as the respiratory substrate (Resp.sub.), 200 μM ADP and 80 ng/ml valinomycin (val)/2 mM KCN (to dissipate the membrane potential) are indicated by arrows. The line represented by dots shows a calibrating record, with additions of 0.16, 1.37, 2.34, 5.26 and 8.18 mM KCl.

strength. However, an increase in cytochrome *c* oxidase control strength was not observed in *crd1*Δ mitochondria (control strength coefficient of 0.33 ± 0.05 , $n = 2$). This suggests that, in

Figure 4 Effect of atractyloside on the respiration and membrane potential kinetics of *crd1*Δ mutant mitochondria

The tracings, typical of three mitochondrial batches, show the effect of addition of 10 μM atractyloside (attract) or 3 μg/ml oligomycin (oligo) on respiration (**A**) and membrane potential (**B**) in *crd1*Δ mutant mitochondria after the phosphorylation cycle. The conditions used are as described in the legend to Figure 3. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Resp.sub., respiratory substrate; val, valinomycin.

Figure 5 Effect of temperature on the respiration of wild-type (WT) and *crd1*Δ mutant mitochondria

The tracings, typical of four WT and four *crd1*Δ mitochondrial batches, show State 4 and State 3 respiration of WT and *crd1*Δ mitochondria at 25 ° and 40 °C. Additions: Mit, mitochondria at 0.11 mg of protein/ml; ADP, 202 μM; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 20 μM.

Table 2 Effect of temperature on energetic coupling in wild-type (WT) and *crd1*Δ mitochondria

In all experiments, the number of mitochondrial batches tested (n) = 4. n.d., not detectable.

Yeast	Temperature (°C) ...	ADP/O		RCR	
		25	40	25	40
WT		1.78 ± 0.16	1.39 ± 0.16	2.71 ± 0.34	1.76 ± 0.19
<i>crd1</i> Δ		1.51 ± 0.28	n.d.	1.94 ± 0.41	n.d.

mutant mitochondria, cytochrome *c* oxidase did not undergo stronger inactivation than the rest of the respiratory chain.

Parameters of energetic coupling (ADP/O and RCR) determined from the oxygen consumption kinetics were in agreement with phosphorylation rates assayed independently with the hexokinase/glucose-6-phosphate dehydrogenase system. Table 1 shows that ADP/O and RCR coefficients are within the usual range for well-coupled yeast mitochondria [6] in the wild-type strain, and appreciably lowered in *crd1*Δ mutant mitochondria.

Membrane potential ($\Delta\Psi$)

An example of membrane potential kinetics during the phosphorylation cycle in the wild-type and mutant mitochondria is shown in Figure 3. Both preparations generate a State 4 potential of similar magnitude (about 170 mV) on addition of respiratory substrate, and exhibit significant depolarization (40–50 mV) during the State 4–State 3 transition on addition of ADP. However, they differed greatly in the return to State 4 after the phosphorylation cycle. After completion of phosphorylation, the membrane potential returned to its State 4 value in the wild-type mitochondria. In contrast, mutant mitochondria demonstrated variable behaviour. Some preparations were unable to

restore the State 4 membrane potential, as shown in Figure 3. Under the conditions employed (10 mM phosphate), membrane potential both constitutes the main part of, and provides a reasonable estimate of, the total protonmotive force [16,17]. In addition, the measurements were performed in the presence of nigericin, which converts ΔpH into $\Delta\Psi$ so that the entire protonmotive force is represented by its electrical component [17,18]. With nigericin, the wild-type membrane potential returned to State 4 after completion of the phosphorylation cycle. In contrast, mutant mitochondria showed a hampered return to the State 4 level.

To determine whether ATP synthase or the adenine nucleotide carrier plays a role in poor recovery of membrane potential, inhibitors of these enzymes (oligomycin and atractyloside) were added after completion of the phosphorylation cycle. Addition of oligomycin to the mutant mitochondria in State 4 had no effect. In contrast, atractyloside restored membrane potential and decreased the respiration rate (Figure 4). These data suggest that the adenine nucleotide transporter, but not ATP synthase, is involved in incomplete recovery of the membrane potential in mutant mitochondria.

Temperature effect

Because the mutant has a temperature-sensitivity phenotype, it was of interest to study the effect of temperature on oxidative phosphorylation in the parent and mutant mitochondria. Figure 5 demonstrates oxygen consumption by the parent and mutant mitochondria at 25 and 40 °C. At 25 °C, the mutant preparation is relatively well coupled, and its State 3–State 4 transitions do not differ significantly from those of the wild type. However, a dramatic difference between mutant and wild-type mitochondria is observed at 40 °C. Mutant mitochondria are completely uncoupled, and the respiration rate decreases gradually. In contrast, wild-type mitochondria are still coupled, with only slightly lowered parameters (Table 2), typical of oxidative phosphorylation at elevated temperatures [19]. This suggests that an important function of cardiolipin is to support mitochondrial resistance to high temperature. This may partly account for the temperature-sensitivity of the *crd1Δ* mutant in non-fermentable carbon sources [2].

Mitochondria from cells grown in the fermentable carbon source

We have shown previously that the *crd1Δ* mutant lacks cardiolipin in all conditions tested. On non-fermentable medium, it does possess the precursor phosphatidylglycerol; however, in glucose, this is lacking as well (F. Jiang, personal communication, and [4]). To determine how the more severe defect (lack of both cardiolipin and phosphatidylglycerol) affected mitochondrial function, respiration was measured in mitochondria from glucose-grown cells. The effect of an absence of both phosphatidylglycerol and cardiolipin on oxidative phosphorylation was qualitatively similar, but more pronounced than the effect of a lack of only cardiolipin. The RCR was decreased [2.29 ± 0.30 ($n = 4$) in wild-type; 1.29 ± 0.32 ($n = 4$) in mutant mitochondria]. Mitochondrial ability to restore membrane potential after a phosphorylation cycle was weakened as a result of the mutation (results not shown). The respiratory rate in State 3 was reduced by 40 % (for an average of four mitochondrial preparations) and, in many cases, respiration in State 4 could not be accelerated by the second addition of ADP or uncoupler. This suggests that the lack of both cardiolipin and phosphatidylglycerol has a more deleterious effect on mitochondrial function

than lack of cardiolipin alone, not only in terms of energy coupling, but also on the respiratory chain. As with glycerol-grown cells, mitochondria from the *crd1Δ* mutant were much less resistant to increased temperature than the wild-type mitochondria (results not shown).

DISCUSSION

The experiments presented here show that a lack of cardiolipin in mitochondrial membranes leads to reduced respiratory control and coupling efficiency of oxidative phosphorylation, decreased resistance to elevated temperature, and a compromised ability to restore membrane potential at the State 4 level after phosphorylation. Since the magnitude of the ADP-induced depolarization of the membrane potential can serve as an indicator of activity of the phosphorylating system as a whole [20], a membrane potential kinetic trace for *crd1Δ* mitochondria (Figure 3) suggests that the energy-transforming system is functionally competent in the initial period of incubation, but undergoes deterioration by the end of the phosphorylation cycle.

At least three hypotheses could account for these effects. One possibility is that modification of the adenine nucleotide carrier in the absence of cardiolipin leads to the observed defects. We observed a recoupling effect on mutant mitochondria in the presence of the adenine nucleotide carrier inhibitor atractyloside (Figure 4). This effect is usually attributed to inhibition of adenine nucleotide carrier-mediated translocation of fatty acids across the mitochondrial membrane [21]. However, in the present study, the presence of BSA in all media render this possibility unlikely, since BSA prevents the effects of endogenous [22] and even exogenously added [23] fatty acids. It is more plausible that the effect of the adenine nucleotide carrier on the mitochondrial megachannel is to play a role in decreasing the membrane potential. Even though the adenine nucleotide carrier probably does not play a direct role in the yeast mitochondrial megachannel [24], a relationship between them is suggested by the observation that atractyloside can inhibit opening of the yeast megachannel, probably by affecting the conformational state of the adenine nucleotide carrier [25]. This protein interacts tightly with, and requires, cardiolipin for maximal activity [26]. One can surmise that its conformation in cardiolipin-lacking mitochondria is modified in such a way that the probability of the megachannel opening (in the low-conductance mode) is increased. This increase, and consequent decrease in membrane potential in State 4, is counteracted by atractyloside.

Even though the data may be explained in part by the effects of cardiolipin on enzyme activity, the effects of cardiolipin on the membrane itself might also explain the observed defects in the cardiolipin-lacking mutant. This is consistent with earlier reports suggesting that cardiolipin can stabilize the membrane bilayer [27] and decrease membrane fluidity [28]. Variable behaviour and faster deterioration of the mutant mitochondria suggest that cardiolipin provides improved stability to the mitochondrial energy-transforming machinery. The absolute necessity of cardiolipin for oxidative phosphorylation at the elevated temperature (Figure 5) further supports this hypothesis.

A third possibility is that the lowered coupling in cardiolipin-lacking mitochondria might be ascribed to the participation of cardiolipin in the direct coupling proposed to occur between the respiratory chain and ATP synthase [29]. Some data suggest that direct coupling, in parallel with the classic chemiosmotic coupling, contributes considerably to oxidative phosphorylation in yeast mitochondria [30]. Cardiolipin could be involved in this process, because its acidic head-groups are able to perform an

intramembrane proton transfer [1]. Cardiolipin-lacking yeast would be defective in this component of coupling.

The unique phospholipid composition of the *crd1Δ* mutant offers an opportunity to address the question: to what extent can phosphatidylglycerol substitute functionally for cardiolipin? In respiratory carbon sources, mutant mitochondria contain phosphatidylglycerol, but no cardiolipin. Reduced coupling and respiration in these mitochondria indicate that cardiolipin plays a role in energetic coupling. In fermentable carbon sources, mutant mitochondria lack both phosphatidylglycerol and cardiolipin [4], and exhibit more severe defects in coupling and respiration. How might phosphatidylglycerol substitute for cardiolipin? Cardiolipin possesses unique features in both the polar and hydrophobic regions of the molecule. The polar regions of both cardiolipin and phosphatidylglycerol are anionic, although phosphatidylglycerol contains only one phosphate group, in contrast with the two found in cardiolipin. The hydrophobic regions differ greatly: that of cardiolipin appears 'dimeric', and contains two sets of acyl chains, whereas phosphatidylglycerol has only one set. Both the head and fatty acid groups of cardiolipin can contribute to its effects on mitochondrial enzymes [31]. The relative contribution of these structural moieties of cardiolipin probably varies with the affected enzyme. It is likely that phosphatidylglycerol can partially substitute for cardiolipin when the effect of the anionic headgroup is dominant.

Can the thermal sensitivity of the bioenergetic functions in cardiolipin-lacking mitochondria account for the decreased viability of the *crd1Δ* mutant at an elevated temperature? Thermal sensitivity of the bioenergetic functions might explain temperature-sensitivity in a non-fermentable medium, in which these functions are essential. However, it is not the most probable explanation for temperature-sensitivity in glucose medium, in which respiration is not essential. It is more likely that non-energetic mitochondrial functions, such as participation in Ca^{2+} homeostasis or supply of the oxidized forms of nicotinamide nucleotides, are involved in the temperature-sensitivity of the *crd1Δ* mutant in glucose.

In summary, these results suggest that, although cardiolipin is required *in vitro* for many enzymes of oxidative phosphorylation [1,2], it is not absolutely necessary *in vivo* for mitochondrial energy transformation. Cardiolipin-lacking mitochondria are able to create a membrane potential, and to utilize it for ATP synthesis. This is reflected in the normal viability of the *crd1Δ* mutant cells in non-fermentable carbon sources [3]. However, cardiolipin provides significant stabilization and improved efficiency of the mitochondrial energy-transforming system, and it is absolutely necessary for energy coupling at elevated temperatures.

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