Cardiolipin prevents rate-dependent uncoupling and provides osmotic stability in yeast mitochondria

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The role of cardiolipin in mitochondrial function was studied by comparing the energy-transforming and osmotic properties of mitochondria isolated from the *Saccharomyces cerevisiae* cardiolipin synthase-null mutant *crd1Δ*, which has no cardiolipin, and the isogenic wild type. The results indicated that the importance of cardiolipin for energetic coupling strongly depends on the rate of oxidative phosphorylation, which was set by using NADH (maximal rate limited by coupling mechanism) or ethanol (moderate rate limited by electron supply) as a respiratory substrate, or by modulating the steady-state rate of NADH supply. The absence of cardiolipin resulted in only a small effect on oxidative phosphorylation proceeding at a moderate rate, but led to significant uncoupling (decreased ADP/O and increased state 4 respiration) at the maximal rate of respiration. This indicates that cardiolipin prevents rate-dependent uncoupling in the

energy-transforming apparatus. This role of cardiolipin may derive from its strong interaction with, and modulation of the function of, respiratory complexes, and from its effects on the physical properties of the membrane. The importance of cardiolipin for mitochondrial osmotic properties was determined by comparing oxidative phosphorylation, release of matrix enzyme, shrinking ability and volume dynamics upon hypotonically induced swelling in *crd1Δ* and wild-type mitochondria. Opening of the yeast mitochondrial unspecific channel (YMUC) in the wild-type and mutant mitochondria was also tested. It was found that the lack of cardiolipin strongly undermines the osmotic stability of the mitochondrial membrane.

Key words: mitochondrial swelling, oxidative phosphorylation, phospholipid.

INTRODUCTION

The unique dimeric structure of cardiolipin and its nearly exclusive location in mitochondrial energy-transducing membranes suggest that this lipid plays a crucial role in oxidative phosphorylation. Indeed, numerous studies *in vitro* revealed that cardiolipin binds tightly to many mitochondrial enzymes and is necessary for their full activity [1–3]. The more recent identification of the gene encoding cardiolipin synthase (*CRD1*) and construction of a *crd1Δ* null mutant lacking cardiolipin [4–6] opened the door to experiments *in vivo* that address the significance of cardiolipin in the overall process of oxidative phosphorylation. Initial studies of cardiolipin-lacking mitochondria suggested that cardiolipin may be important for oxidative phosphorylation. However, the extent to which respiration was affected in the *crd1Δ* was not clear, as a moderate decrease in efficiency of energy transformation [7] or an almost complete shut-down of ATP production [8] in *crd1Δ* mutant mitochondria were reported. The present work was undertaken to unify these seemingly diverse results, and to obtain a greater understanding of the role of cardiolipin in mitochondrial function.

The crucial functions of the inner mitochondrial membrane are energy transformation and regulation of mitochondrial volume. There is a tight interaction between mitochondrial metabolism and volume, since the volume is affected by changes in respiratory state (low-amplitude swelling cycles) [9], by membrane permeability and in response to the composition of the medium. In turn, changes in mitochondrial volume influence the activities of many mitochondrial enzymes and other metabolic parameters [10,11]. Whereas the mechanism of this influence

is not quite clear, altered membrane fluidity is one possible explanation [10]. Thus membrane composition, and the presence of cardiolipin in particular, may be an important factor governing relations between volume and function in mitochondria.

Mitochondria from *crd1Δ* mutant cells lack cardiolipin. However, when grown in non-fermentable carbon sources, they accumulate the precursor lipid phosphatidylglycerol [5,8]. Therefore, a comparative study of wild-type and mutant mitochondria would enable us to determine the functional importance of the unique dimeric structure of cardiolipin (diphosphatidylglycerol) as opposed to phosphatidylglycerol. In this study, we compared cardiolipin-deficient and wild-type mitochondria with respect to energetic coupling at different rates of oxidative phosphorylation, and to osmotic properties. We determined that the functional importance of cardiolipin for energy transformation strongly depends on the respiratory rate. Cardiolipin provides significant improvement of energetic coupling at the maximal rate of oxidative phosphorylation, but has little effect at moderate rates limited by the electron supply. In addition, cardiolipin appears to be crucial for osmotic stability of the yeast mitochondria.

EXPERIMENTAL

Isolation of mitochondria and oxidative phosphorylation assay

The strains used in the present study were FGY3 (wild type) and FGY2 (*crd1Δ*), as described previously [8]. Yeast cultures were grown as described previously [4] in the presence of a non-fermentable carbon source (3% glycerol plus 1% ethanol). Isolation of mitochondria was performed essentially as described in [12], with minor modifications. The medium used for isolation

Abbreviations used: BSA, bovine serum albumin; YMUC, yeast mitochondrial unspecific channel.

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was 0.6 M mannitol, 20 mM Hepes, 1 mM EGTA and 0.2 % (w/v) bovine serum albumin (BSA), pH 6.8. The final mitochondrial pellet was suspended in the isolation medium at approx. 10 mg of protein/ml. The basic reaction medium was 0.6 M mannitol, 20 mM Hepes (pH 6.8), 2 mM MgCl_2 , 1 mM EGTA and 0.1 % BSA. For oxidative phosphorylation assays, this was supplemented with respiratory substrate (see Figure and Table legends) and 10 mM P_i . Respiration rates were measured with the Clark-type electrode at 25 °C, and phosphorylation was estimated from ADP-stimulated respiration based on the total oxygen consumption [13]. To modulate the rate of NADH-driven respiration, the reaction medium was supplemented with an NADH-regenerating system; 2 mM NAD, 4 mM glucose 6-phosphate and NAD-dependent glucose-6-phosphate dehydrogenase [14]. The steady-state rate of NADH supply was set by varying the amount of NADH-producing enzyme. ATP synthesis in this case was determined enzymically in the samples fixed with perchloric acid [15].

Mitochondrial volumes and swelling assay

Mitochondrial volumes were measured by distribution of $^3\text{H}_2\text{O}$ and [^{14}C]mannitol ($^3\text{H}_2\text{O}$ and [^{14}C]inulin or [^{14}C]dextran with permeabilized mitochondria) [16,17], and mitochondrial swelling and shrinkage were monitored by following the changes of apparent absorbance at 540 nm [18]. In experiments investigating the reversibility of swelling, restoration of the medium tonicity was performed with sucrose, which is easily added from a concentrated stock solution. In order to test the fragility of the mitochondrial inner membrane, mitochondria were submitted to hypotonic conditions (29 and 180 mM mannitol) for 2 min, at which time a concentrated sucrose solution was added (up to 0.18 M) to partially restore medium tonicity [19]. Mitochondria were pelleted, and malate dehydrogenase released from the matrix was assayed in the supernatant by oxaloacetate-induced oxidation of NADH in the presence of antimycin A [20].

Permeability-transition monitoring

Mitochondria were incubated in the basic medium supplemented with 0.5 mM P_i . These conditions (isotonic medium containing Mg^{2+} , EGTA and low P_i) ensured stable state 4 respiration, as well as stable levels of membrane potential and mitochondrial volume. Addition of ATP to mitochondria oxidizing NADH caused membrane permeabilization, manifested as mitochondrial swelling, collapse of the membrane potential and acceleration of respiration [21,22], which were monitored in parallel assays. A nearly maximal effect was achieved with 2 mM ATP.

RESULTS AND DISCUSSION

Effect of cardiolipin on energetic coupling

We showed previously that yeast mitochondria lacking cardiolipin exhibit only a moderate decrease in efficiency of oxidative phosphorylation driven by oxidation of ethanol [7]. In the present work, the functional importance of cardiolipin for the overall process of oxidative phosphorylation was studied in a wide range of respiration rates and medium tonicities.

The rate of oxidative phosphorylation was modulated by using NADH and ethanol as respiratory substrates. In yeast mitochondria, unlike mammalian mitochondria, only the external respiratory substrate NADH has been shown to provide the maximal capacity of energy transformation, due to the higher activity of NADH dehydrogenase than other substrate dehydrogenases [23,24]. In yeast mitochondria, both NADH and ethanol

Table 1 Respiration rates in NADH- and ethanol-oxidizing mitochondria from wild-type and *crd1Δ* yeast cells

Nine wild-type and ten *crd1Δ* mitochondrial batches were tested. V_3 and V_4 indicate state 3 and 4 respiration, respectively.

Yeast	Respiration rate (nmol of O_2 /min per mg of protein)					
	NADH			Ethanol		
	V_4	V_3	$V_{3\text{uncoupled}}$	V_4	V_3	$V_{3\text{uncoupled}}$
Wild type	88 ± 14	237 ± 49	420 ± 104	75 ± 15	164 ± 41	155 ± 43
<i>crd1Δ</i>	131 ± 35*	228 ± 51	333 ± 63	73 ± 15	135 ± 25	148 ± 31

* $P < 0.01$ compared with wild-type respiration with NADH and *crd1Δ* respiration with ethanol.

Table 2 Coupling parameters in NADH- and ethanol-oxidizing mitochondria from wild-type and *crd1Δ* yeast cells

RCR, respiratory control coefficient.

Yeast	Substrate ...	NADH		Ethanol	
		ADP/O	RCR	ADP/O	RCR
Wild type		1.58 ± 0.1	2.53 ± 0.14	1.64 ± 0.08	2.13 ± 0.21
<i>crd1Δ</i>		1.21 ± 0.15*	1.75 ± 0.38†	1.55 ± 0.17	1.86 ± 0.21

* $P < 0.01$ compared with wild-type respiration with NADH and *crd1Δ* respiration with ethanol.

† $P < 0.01$ compared with wild-type respiration with NADH.

feed electrons to coupling sites 2 and 3 of the respiratory chain (coupling site 1 is absent from yeast) [23]. NADH- and ethanol-driven respiration in mutant and wild-type mitochondria are compared in Tables 1 and 2. Respiration rates vary with the substrate in two respects, as shown in Table 1. First, the rate of state 3 respiration is about 1.5-fold faster with NADH than with ethanol, reflecting the different activities of the substrate dehydrogenases. Secondly, with NADH, the uncoupled respiration significantly exceeds the phosphorylating respiration which, therefore, is limited by the coupling mechanism. In contrast, with ethanol, uncoupled respiration did not differ from phosphorylating respiration, apparently due to the higher degree of control exerted upon respiration by alcohol dehydrogenase upstream of the respiratory chain.

Data presented in Tables 1 and 2 show that NADH-driven oxidative phosphorylation is significantly more sensitive to the lack of cardiolipin. They demonstrate a considerably increased state 4 respiration rate, and decreased respiratory control and ADP/O coupling coefficients in NADH-oxidizing mutant mitochondria. At the same time, ethanol-driven oxidative phosphorylation exhibited only moderate uncoupling in mitochondria lacking cardiolipin, the degree of which was even less pronounced in this set of experiments than we observed previously [7]. We attribute this difference primarily to improvement of the mitochondrial isolation procedure and assay conditions.

Viewed from a different perspective, acceleration of the electron flow caused by changing the respiratory substrate from ethanol to NADH did not appreciably damage coupling in wild-type mitochondria (respiratory control even increased), but caused a clear uncoupling effect in mutant organelles.

In order to exclude possible effects of the different substrate dehydrogenases, NADH-driven respiration was modulated by

Figure 1 Effect of limiting NADH supply on energetic coupling in wild-type (●) and *crd1Δ* (○) yeast mitochondria

Mitochondria (0.15 mg of protein/ml) were incubated in the basic medium supplemented with 10 mM P_i , 2 mM NAD, 4 mM glucose 6-phosphate and various amounts (0.1, 0.2 and 1 units/ml) of glucose-6-phosphate dehydrogenase.

variation of the steady-state NADH supply in an NADH-regenerating system [14]. In this case, acceleration of NADH oxidation also caused lowering of the ADP/O coupling coefficient in cardiolipin-lacking mitochondria (Figure 1).

Antioxidant butylated hydroxytoluene did not improve coupling parameters of cardiolipin-lacking mitochondria (results not shown), suggesting against participation of reactive oxygen species in mitochondrial deterioration.

Cardiolipin-lacking mitochondria, in contrast with wild-type organelles, exhibit rate-dependent uncoupling, indicating that an augmented electron supply upstream of the respiratory chain decreases the ADP/O coefficient and increases state 4 respiration. Numerous reports suggest that coupling efficiency may depend on the respiratory flux due to non-ohmic leak or intrinsic slip in respiratory complexes [14,25,26]. The absence of cardiolipin may decrease activity of mitochondrial enzymes since this phospholipid can activate them by direct interaction and by improvement of mitochondrial protein import [1,8]. In yeast mitochondria, the ATP/ADP antiporter was shown to be especially dependent on the presence of cardiolipin [8]. At the same time, the effect of enzymic deficiencies on the overall process of oxidative phosphorylation is characterized by a threshold behaviour, so that a modest decrease in enzyme activity may exert a negligible effect on mitochondrial energy transformation [27,28]. After reaching the threshold level, inhibition of the ATP/ADP antiporter decreases both phosphorylating and respiratory fluxes [29,30]. The increase in state 4 respiration and decrease in ADP/O coefficient (but not state 3 respiration flux) observed in the present experiments suggests that fast electron flow disturbs coupling between respiration and phosphorylation rather than merely revealing a deficiency in the ATP/ADP antiporter in *crd1Δ* mitochondria.

It is important to note that Fitton et al. [26] observed rate-dependent uncoupling in yeast mitochondria upon modulation of electron flow by respiratory substrate, and attributed this to the intrinsic slip in the respiratory chain. In contrast, Ohnishi and Hagihara [23] found nearly constant ADP/O ratios in yeast mitochondria under similar conditions. In our experiments, the

Figure 2 Oxidative phosphorylation in mitochondria from wild-type (WT) and *crd1Δ* yeast cells incubated in isotonic (0.6 M mannitol) and hypotonic (0.3 M mannitol) medium

Mitochondria were added to 0.15 mg of protein/ml (Mit), ADP to 200 μ M (ADP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to 5.9 μ M (FCCP).

inverse relationship between coupling and respiratory rate was observed in cardiolipin-deficient organelles, but not in those of the wild type. These data suggest that rate-dependent uncoupling is a conditional feature of the yeast mitochondria, and one of the factors preventing it is the presence of cardiolipin in the inner membrane.

Effect of cardiolipin on mitochondrial osmotic stability

Mitochondrial function is accompanied by osmotic volume change. High-amplitude swelling is associated with mitochondrial damage, but moderate swelling is a physiological event. For instance, in mammalian mitochondria, swelling of about 30% of the initial volume serves as a mediator between hormonal stimuli and oxidative phosphorylation [10]. Similarly, swelling of liver mitochondria artificially induced by lowering of the medium tonicity (2-fold) does not damage and even somewhat improves energetic coupling [31].

In accordance with these observations, we found that placing mitochondria from wild-type cells into medium containing 0.3 M instead of 0.6 M mannitol does not significantly alter mitochondrial coupling. However, cardiolipin-lacking mitochondria in this slightly hypotonic medium exhibited strong uncoupling (Figure 2). Accelerated respiration in state 4 and lowered ADP/O coefficient suggest that moderate swelling stretches the cardiolipin-lacking mitochondrial membrane and makes it more permeable to ions. Further lowering of the medium tonicity increases permeability of the mitochondrial membrane to large molecules, as shown by increased release of the matrix enzyme malate dehydrogenase, a sensitive marker for detection of damage to the inner mitochondrial membrane [19,20]. Figure 3 demonstrates that, at low tonicity, release of malate dehydrogenase from mutant mitochondria clearly exceeds that from the wild-type organelles.

Additional evidence for the importance of cardiolipin for mitochondrial osmotic behaviour is given by determination of the shrinking ability of strongly swollen mitochondria. Mito-

Figure 3 Release of malate dehydrogenase from wild-type (WT) and *crd1Δ* yeast mitochondria

Mitochondria were incubated in medium in which the tonicity was varied with mannitol. Malate dehydrogenase in the supernatant was assayed and expressed as the percentage of activity released by 0.1% Triton X-100.

Figure 4 Shrinkage of hypotonically swollen wild-type (WT) and *crd1Δ* yeast mitochondria

Mitochondria were added to the basic medium containing isotonic (0.6 M) or hypotonic (0.025 M) mannitol, and absorbance at 540 nm, was monitored. Shrinkage of swollen mitochondria was induced by the addition of sucrose to 0.18 M.

chondrial volume kinetics upon hypotonic swelling and partial restoration of medium tonicity were monitored by absorbance (light scattering) of the mitochondrial suspension (Figure 4). In response to restoration of osmotic support (addition of sucrose), wild-type mitochondria exhibited an immediate, fast shrinkage followed by a lower rate of shrinkage that approached the initial level of absorbance. In contrast, cardiolipin-deficient mitochondria showed only a transient shrinkage immediately after the addition of sucrose, and then reverted to continuous swelling.

The difference between the osmotic behaviour of the mutant and wild-type mitochondria is further supported by the isotopically measured hypotonic volume changes. As observed in Figure 5, the volume increase in *crd1Δ* mitochondria was less pronounced than in the wild type with decreasing tonicity. Mitochondrial swelling occurs via unfolding of inner-membrane cristae; when the physical limit of swelling is reached, the mitochondria become leaky as a result of stretching and damage to the membrane [10]. Our observations suggest that cardiolipin-deficient mitochondria are more subject to this damage than wild-type organelles.

Figure 5 Dependence of the matrix volume of wild-type (WT; ●) and *crd1Δ* (○) mitochondria on the tonicity of suspending medium

Medium contained variable concentrations of mannitol, 10 mM Hepes (pH 6.8), 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM P_i and 0.1% BSA.

Swelling is not only a response to altered tonicity, but is also a key component of the permeability transition (opening of the megapore) [32–34], which may play a role in cell death. In mammalian cells, permanent opening of the megapore causes mitochondrial swelling and uncoupling leading to necrosis. Transient opening of the megapore may cause rupture of the outer membrane and cytochrome *c* release, leading to activation of the caspase cascade and apoptosis [33]. Although yeast mitochondria do not demonstrate a permeability transition typical of the mammalian one, they do possess a yeast mitochondrial unspecific channel (YMUC), which has some functional similarity to the mammalian megapore [22,35]. We wished to determine if the altered osmotic properties of cardiolipin-lacking mitochondria affect YMUC opening.

Opening of the YMUC was induced by ATP in isotonic, mannitol-based, low-phosphate medium containing a respiratory substrate. This resulted in acceleration of respiration, collapse of the membrane potential and swelling, detected by the decrease in absorbance (light scattering) of the mitochondrial suspension. Figure 6 demonstrates that the decrease in absorbance was less pronounced in the mutant organelles than in wild-type mitochondria under conditions in which the membrane potential was largely dissipated and respiration completely released, as determined by no further response to the uncoupler. Accordingly, the isotopically measured volume increase upon permeability transition was from 1.62 ± 0.3 to 3.02 ± 0.41 $\mu l/mg$ of protein in the wild-type mitochondria and from 1.7 ± 0.28 to 2.49 ± 0.19 $\mu l/mg$ of protein in the mutant.

We implemented a third method to induce mitochondrial swelling, i.e. permeabilization of the mitochondria with the pore-forming antibiotic alamethicin [22]. In this experiment as well, the osmotic response of mutant mitochondria was 50–60% that of the wild type (results not shown).

Why does the absence of cardiolipin affect mitochondrial osmotic properties? Cardiolipin is a polymorphic lipid that is able to adopt both bilayer and non-bilayer structures [36]. These lipids can play a role in determining membrane curvature and intermembrane interactions [37–39]. It is conceivable that proper folding of the inner mitochondrial membrane and/or interactions

Figure 6 Permeability transition in wild-type (WT) and *crd1Δ* yeast mitochondria

Kinetics of respiration (top curves), membrane depolarization (middle curves) and absorbance at 540 nm, (bottom curves) in the course of permeability transition induced by 2 mM ATP. The basic reaction medium was supplemented with 0.5 mM P_i and the following additions indicated by arrows: 0.15 (respiration and fluorescence experiments) or 0.45 (absorbance experiments) mg of protein/ml of mitochondria (Mit), 2 mM NADH and 2 mM ATP. Membrane depolarization estimated by 3,3'-dipropylthiobarbiturate iodide [$diSC_3(5)$] fluorescence was as described earlier [7]. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

between the cristae and outer membrane depend on the presence of cardiolipin. Aberrant folding of the membrane and inner–outer membrane interactions in the absence of cardiolipin may thus explain the weakened osmotic stability of cardiolipin-lacking mitochondria.

An interesting point is possible compensation for the lack of cardiolipin provided by its precursor (and nearly monomeric analogue) phosphatidylglycerol. In mutant cells grown on a non-fermentable carbon source, phosphatidylglycerol accumulates to about the same level as cardiolipin is present in wild-type cells (with respect to phospholipid phosphorus) [8]. These cells exhibit less-pronounced mitochondrial defects than cells lacking both cardiolipin and phosphatidylglycerol [8]. At the same time, phosphatidylglycerol cannot replace cardiolipin in its strong interaction with major mitochondrial enzyme complexes [40–43]. Furthermore, results of the present and other works [7,8] show that phosphatidylglycerol cannot fully compensate for the lack of cardiolipin for oxidative phosphorylation. Therefore, phosphatidylglycerol can substitute for cardiolipin in some, but not all, functions. The unique functions of cardiolipin may be related to its distinctive physical properties, such as its dimeric structure and ability to form non-bilayer membrane structures [36].

In conclusion, our results demonstrate that cardiolipin prevents rate-dependent uncoupling of oxidative phosphorylation and ensures osmotic stability of yeast mitochondria. Our findings that cardiolipin-lacking mitochondria are extremely sensitive to variation in respiratory rate and medium tonicity help to explain the apparent discrepancy in previous studies. In the present study, we observed good coupling of *crd1Δ* mitochondria oxidizing ethanol in isotonic medium. In [8], oxidative phosphorylation was studied with ethanol + α -glycerophosphate as respiratory substrates in medium containing 0.25 M sucrose, which is slightly hypotonic for yeast mitochondria [44]. Comparing these conditions, we observed that the addition of α -glycero-

phosphate to ethanol-oxidizing *crd1Δ* mitochondria accelerated respiration by 50 % and simultaneously decreased ADP/O by 20 % (results not shown). Substitution of 0.25 M sucrose for 0.6 M mannitol leads to a drop of ADP/O by 46 %. In contrast to the mutant, wild-type mitochondria remained well coupled under these conditions. This suggests that the greater uncoupling of *crd1Δ* mitochondria in [8] compared with the present results is due to the relatively low medium tonicity and fast respiration rate in the previous study.

In summary, cardiolipin-lacking mitochondria remain satisfactorily coupled only under a limited range of conditions, i.e. at submaximal respiration rate (Tables 1 and 2, and Figure 1), strict isotonicity (Figures 2–6) and optimal temperature [7]. Deviation from optimal conditions by increasing the rate of respiration, decreasing the tonicity or increasing the temperature were tolerable for wild-type mitochondria, but dramatically damaged coupling in *crd1Δ* mitochondria. Thus from a physiological point of view, cardiolipin acts as a stabilizer of mitochondrial function.

Our data indicate that the essential component of mitochondrial damage caused by the lack of cardiolipin is acceleration of state 4 respiration at the high electron supply. It was reported recently that some drugs used in the treatment of chronic peripheral ischaemic and venous diseases (e.g. aescine, Ginkor Fort, naftidrofuryl and naphthoquinone) normalize mitochondrial energetics by lowering state 4 respiration [45,46]. In light of the results reported here, substances exerting such effects may be useful for treatment of pathologies in which intramembrane interactions involving cardiolipin are disturbed, such as adriamycin cancer therapy or production of anti-cardiolipin antibodies [3].

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