Cardiolipin Stabilizes Respiratory Chain Supercomplexes*

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Cardiolipin stabilized supercomplexes of Saccharomyces cerevisiae respiratory chain complexes III and IV (ubiquinol:cytochrome c oxidoreductase and cytochrome c oxidase, respectively), but was not essential for their formation in the inner mitochondrial membrane because they were found also in a cardiolipin-deficient strain. Reconstitution with cardiolipin largely restored wild-type stability. The putative interface of complexes III and IV comprises transmembrane helices of cytochromes b and c1 and tightly bound cardiolipin. Subunits Rip1p, Qcr6p, Qcr9p, Qcr10p, Cox8p, Cox12p, and Cox13p and cytochrome c were not essential for the assembly of supercomplexes; and in the absence of Qcr6p, the formation of supercomplexes was even promoted. An additional marked effect of cardiolipin concerns cytochrome c oxidase. We show that a cardiolipin-deficient strain harbored almost inactive resting cytochrome c oxidase in the membrane. Transition to the fully active pulsed state occurred on a minute time scale.

Cardiolipin is an anionic phospholipid consisting of two phosphatidyl residues that are linked by a glycerol moiety (1). In non-photosynthetic eukaryotic cells, it is almost exclusively found in mitochondrial membranes (2), where it serves specific roles in mitochondrial structure and function that can be grouped into two categories: stabilization of physical properties of membranes and specific interactions with proteins and modulation of their functions (2, 3). Cardiolipin deficiency leads to alteration in the fluidity and stability of mitochondrial membranes. Osmotic stability of mitochondrial membranes is impaired, as apparent from inefficient energy transformation by oxidative phosphorylation, swelling of mitochondria, decreased ATP/oxygen ratio concomitant with increased state 4 respiration, and reduced membrane potential (4, 5).

Specific effects of cardiolipin on oxidative phosphorylation enzymes have been primarily studied using bovine mitochondrial proteins. Cardiolipin binding is essential for the function of the ATP synthase (6–8), for the stability of respiratory complex IV (cytochrome c oxidase) (9, 12), and for the stability of respiratory complex IV (cytochrome c oxidase) (13, 14). Coupled to the electron transport reactions of complexes III and IV, these respiratory chain complexes generate an electrochemical proton gradient across the mitochondrial inner membrane, which is then used to synthesize ATP by complex V, the mitochondrial F1F0-ATP synthase (15). Cardiolipin binds tightly to bovine complex V, which seems to influence considerably the catalytic and structural properties of this multiprotein complex (16). Cardiolipin appears also to be involved in the membrane attachment of cytochrome c (2, 17). Decreased cardiolipin synthesis corresponds to cytochrome c release in palmitate-induced cardiomyocyte apoptosis (18).

Some integral membrane proteins have been crystallized, and the binding sites of tightly bound lipids have been localized (19–21). In the structure of yeast complex III, a total of six lipid molecules including one cardiolipin have been detected (22, 23). This cardiolipin molecule is bound tightly in a cavity formed by transmembrane helices of cytochromes b and c1, with the head group of cardiolipin residing in close proximity to the internal site of quinone reduction (Q) and to the entrance of the cardiolipin/lysine (CL/K) proton conduction pathway. It was suggested that this cardiolipin may have a specific function for the activity of complex III (22).

In eukaryotic mitochondria, cardiolipin is synthesized from the precursors phosphatidylglycerol and phosphatidyl-CMP by the cardiolipin synthase Crd1p (24–26). The Saccharomyces cerevisiae null mutant crd1 does not contain cardiolipin. The level of the precursor phosphatidylglycerol is elevated when cells are grown on non-fermentable carbon sources, but only a slight increase is evident on glucose (6, 25). Using the crd1 null mutant and the isogenic wild-type strain under appropriate growth conditions therefore allows discrimination between specific cardiolipin, phosphatidylglycerol, and general lipid effects (6). Cardiolipin-deficient strains are viable under fermenting and non-fermenting conditions, except at elevated temperature, indicating that cardiolipin is nonessential for oxidative phosphorylation. However, almost complete uncoupling and shut down of oxidative phosphorylation at 40 °C have been described (27). In humans, reduced cardiolipin and defective cardiolipin remodeling due to mutations in the taflazin gene cause Barth syndrome, which is characterized by dilated cardiomyopathy, neutropenia, skeletal myopathy, and abnormal mitochondria (28).

Recently, the oxidative phosphorylation enzymes in the mitochondrial membrane of S. cerevisiae were found to be organized into supramolecular assemblies. Respiratory chain complexes III and IV assemble into small and large supercomplexes (29–31), which comprise dimeric complex III and one or two copies of complex IV, respectively. The major functional
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Role of these supramolecular assemblies or “respirasomes” seems to be substrate channeling of the substrate cytochrome c that enhances the overall quinol oxidase rate (29, 31, 32). Complex V (ATP synthase) was found to exist as a dimer in yeast (33). The dimeric form seems to be essential for the control of biogenesis of the inner mitochondrial membrane and for generating mitochondrial cristae morphology (34). The state of activity of monomeric complex V is controversial. Paumard et al. (34) showed comparable ATP hydrolysis activities for the monomeric and dimeric forms of yeast complex V, whereas Tomasetti et al. (35) reported active monomeric and inactive dimeric forms of bovine complex V. The availability of a crd1 null mutant provided the opportunity to elucidate the specific functions of cardiolipin for the assembly and stability of these oxidative phosphorylation supercomplexes and for the enzymatic properties of the individual complexes. Previously published preliminary results seem to indicate that cardiolipin is essential for the formation of respiratory chain supercomplexes from complexes III and IV (32, 36). The detailed analysis performed in this work reveals a more complex situation and provides insight into the interaction between complexes III and IV: cardiolipin is not essential for the formation of respiratory chain supercomplexes and dimeric ATP synthase in the mitochondrial membrane. However, it stabilizes respiratory chain supercomplexes as well as the individual complexes and is required to prevent formation of the resting state of cytochrome c oxidase in the membrane.

EXPERIMENTAL PROCEDURES

Yeast Strains—The yeast strains used in this study were as follows: Δcrd1 (FGY2) and the corresponding parental strain FGY3 (6), wild-type W303-1A (37), Δqcr1 (38), Δqcr9 (39), Δqcr10 (40), Δcox2 (41), and Δcox13 (42). Δcox8 was constructed by replacing the COX8 open reading frame with the HIS3 gene according to Wach et al. (43).

Electrophoresis and Western Blotting—Mitochondria from S. cerevisiae strains were isolated and processed for first dimension blue native (BN) PAGE as described (33, 44), except that the detergent/protein ratios indicated below were used. Staining, quantification of gels, and electroblotting were performed as described (33, 45). Colorless native (CN) PAGE followed the standard protocol (44), except that acrylamide gradient gels containing no detergent were used. Monoclonal antibodies against S. cerevisiae Cox1p, Cox2p, and Cox3p (Molecular Probes, Inc.) were used for protein detection.

Phospholipid Determination—Yeast strains were grown in the presence of 10 μCi of 32P/ml of culture at 30°C to early stationary phase. Cells were then washed and digested by zymolase to yield spheroplasts. Total (whole cell) phospholipids were extracted (46), separated by one-dimensional thin layer chromatography (47), and analyzed by phosphorimaging. Incorporation of radiolabeled [32P]orthophosphate into individual phospholipids is expressed as a percentage of the total radiolabel incorporated into phospholipids.

Reconstitution with Cardiolipin—Aliquots of sedimented yeast mitochondria (400 μg of protein) were suspended in 40 μl of 50 mM NaCl and 50 mM imidazole HCl (pH 7.0). Cardiolipin (2 μl) from a stock solution (20 mg/ml in 5% digitonin and 50% ethanol) and 1 μl of digitonin (10% in water) were added to set a cardiolipin/protein ratio of 1:10 (g/g) and a digitonin/protein ratio of 0.5 (g/g). Sample preparation for BN-PAGE was as follows. After a 2-h incubation on ice, 10 μl of digitonin (10% stock solution in water) was added to obtain a digitonin/protein ratio of 3 (g/g), which is sufficient for quantitative solubilization. Following centrifugation for 10 min at 100,000 × g, the supernatant was applied to a 1.6 × 10-mm sample well for BN-PAGE. Sample preparation for catalytic activity measurements was similar. After a 2-h incubation on ice, digitonin or dodecyl maltoside was added to obtain detergent/protein ratio of 1:2, 3, and 4 (g/g) and 0.5, 0.6, 0.9, and 1.2 (g/g), respectively. The samples were used directly without centrifugation.

Enzyme Activities—Ubihydroquinone:cytochrome c oxidoreductase (complex I) and cytochrome c oxidase (complex IV) were measured by the antimycin-sensitive reduction and cyanide-sensitive oxidation of cytochrome c, respectively (550–540 nm; ε = 19 mm−1 cm−1) in the presence of 70 μM yeast cytochrome c. Decylbenzoquinol (75 μM) was used as a substrate for complex III. Yeast mitochondrial membranes were suspended in 150 mM NaCl and 75 mM imidazole HCl (pH 7.0) at 2.8 mg/ml protein and solubilized by dodecyl maltoside (1 g/g of protein) or digitonin (3 g/g of protein). Quinol oxidase (coupled activities of complexes III and IV) was determined by the cyanide-sensitive oxidation of 75 μM decylbenzoquinol (280–290 nm; ε = 4.2 mM−1 cm−1) in the presence of 1 μM yeast cytochrome c. The ATP hydrolysis activity of complex V was probed by in-gel lead phosphate precipitation. The original protocol (48) was modified to obtain fast and inhibitor-sensitive ATP hydrolysis rates. After CN-PAGE, gels were incubated for 3 h at room temperature in 35 mM Tris and 270 mM glycerol (pH 7.8) with or without addition of 5 μg/ml oligomycin. The buffer was then replaced with 35 mM Tris, 270 mM glycerol (pH 7.8) and 14 mM MgSO4, 0.2% Pb(NO3)2, and 8 mM ATP (pH 7.8). Lead phosphate precipitation was usually detected after ~10–30 min and continued for 1–3 h. ATP hydrolysis on CN gels was ~10 times faster than on BN gels and was oligomycin-sensitive in contrast to BN gels.

RESULTS

Cardiolipin Effects on Respiratory Chain Supercomplexes and Dimeric ATP Synthase—The cardiolipin-deficient (Δcrd1) and parental strains were grown on glucose, lactate, and glycerol/ethanol. Mitochondria were solubilized by digitonin, and the sizes of solubilized complexes were compared after separation by BN-PAGE (Fig. 1A). In the parental strain, complexes III and IV were found assembled into small and large supercomplexes, which contained dimeric complex III and one or two copies of complex IV, respectively. The ratio of the two supercomplexes varied with the growth conditions as described previously (31). Upon analysis of the Δcrd1 strain by BN-PAGE, we could detect individual complexes III and IV, but almost no supercomplexes under all growth conditions. Detection of minor amounts of supercomplexes required resolution by SDS-PAGE, but the second dimension as exemplified in Fig. 2. Quantification of complex III on two-dimensional gels from the Δcrd1 mutant revealed that individual complex III was the predominant form and that minor amounts of complex III assembled with complex IV to form supercomplexes. This either could reflect the situation that formation of supercomplexes in the inner membrane of Δcrd1 mitochondria was reduced or may merely indicate that assembled supercomplexes were less stable compared with the parental strain and therefore prone to dissociation under BN-PAGE conditions. For protein solubilization in BN-PAGE, a neutral detergent and the negatively charged dye Coomassie Blue are used. This combination can mimic some properties of an anionic detergent and may lead to dissociation of proteins or complexes. Therefore, we used two additional experimental approaches to look for supercomplexes in solubilized Δcrd1 mitochondria. During gel filtration in the presence of digitonin, major amounts of complexes III and IV (500 and 200 kDa, respectively, for the individual complexes) coeluted with dimeric complex V (1250 kDa) (data not shown). This seemed to indicate that complexes III and IV were still assembled in supercomplexes and that digitonin (in the absence of the anionic dye Coomassie Blue) did not dissociate the supercomplexes of Δcrd1 mitochondria. Similar results were obtained using CN-PAGE. This method follows the protocol for BN-PAGE, except that Coomassie Blue is omitted from the sample and cathode buffers. In contrast to the results obtained with BN-PAGE, significant amounts of respiratory chain supercomplexes from Δcrd1 mitochondria were retained following CN-PAGE (Fig. 1B). SDS-PAGE in the second dimension (data not shown) indicated that major amounts of complex III were assembled in supercomplexes, and minor amounts were found as individual complex III (Table I). We concluded that mitochondrial membranes lacking cardiolipin still contained supercomplexes, but that these were significantly less stable than supercomplexes in the parental strain. Other phospholipids
that are increased in the mutant, including phosphatidylethanolamine and phosphatidylglycerol (Table II), could not substitute for cardiolipin and could not prevent dissociation of supercomplexes under BN-PAGE conditions (Fig. 1A).

Individual complex III from the \(\Delta crd1\) strain seemed slightly smaller than free complex III from the parental strain (Fig. 1A, lanes 1 and 2). We assign this mass difference to the absence of bound cardiolipin because the subunit composition of complex III was found to be identical in the parental and \(\Delta crd1\) strains.

In contrast, the lack of cardiolipin had no detectable effect on the size of monomeric and dimeric ATP synthases following BN-PAGE. However, an additional faint band just below the...
dimeric ATP synthase was usually observed in mitochondria from the \( \Delta crd1 \) strain (Fig. 1A). This additional band contained subunits of ATP synthase as identified by two-dimensional SDS-PAGE (data not shown), but not all subunits could be detected due to the small protein amounts. This additional band was not detected by CN-PAGE (Fig. 1B). We concluded that the lack of cardiolipin had little effect on the stability of dimeric ATP synthase compared with the significant effect on the stability of respiratory chain supercomplexes.

The ATP hydrolysis activities of monomeric and dimeric ATP synthases from the wild-type and \( \Delta crd1 \) strains were similar, as estimated by in-gel lead phosphate precipitation, which correlated with ATP hydrolysis rates (Fig. 1C). This indicates that dimeric ATP synthase is not an inactive form of complex V, as was recently described for the bovine enzyme (35). An ~10–30-min incubation with ATP was sufficient to observe strong lead phosphate precipitation on gels following CN-PAGE. ATP hydrolysis by monomeric and dimeric complex V from the wild-type and \( \Delta crd1 \) strains was almost completely inhibited by oligomycin-sensitive (data not shown). Following BN-PAGE, a comparable precipitation intensity required ~10 h of incubation and could not be inhibited by oligomycin.

Stabilization of Respiratory Chain Supercomplexes by Addition of Cardiolipin—Mitochondria from \( \Delta crd1 \) and parental strains grown on glycerol/ethanol were solubilized with cardiolipin and analyzed by BN-PAGE and two-dimensional electrophoresis (Fig. 2). In mitochondria from the parental strain, only small amounts of free individual complex IV, which required immunological techniques for detection (Fig. 2A), were found not to be associated with complex III. Upon addition of cardiolipin, this minor amount of individual complex IV was further reduced (Fig. 2B and Table III). Addition of cardiolipin to \( \Delta crd1 \) mitochondria had a pronounced effect. Using \( \Delta crd1 \) mitochondria directly, major amounts of complex IV were found as free individual complex IV (Fig. 2C). However, after addition of cardiolipin, a large fraction of this individual complex IV was found assembled with complex III (Fig. 2D). The band of individual complex III was significantly reduced, and the amounts of both supercomplexes containing one and two copies of complex IV, respectively, were significantly increased (Table III). Cardiolipin addition did not affect the ratio of dimeric and monomeric ATP synthases.

### Table I

Partitioning of total complex III to large and small supercomplexes and individual complex III following BN-PAGE and CN-PAGE

|                     | Large complex | Small complex | Individual complex III |
|---------------------|--------------|---------------|------------------------|
| WT, glucose         | 39           | 46            | 15                     |
| BN-PAGE             | 36           | 44            | 20                     |
| \( \Delta crd1 \), glucose | 0            | 15            | 85                     |
| BN-PAGE             | 27           | 40            | 33                     |
| WT, lactate         | 89           | 11            | 0                      |
| BN-PAGE             | 87           | 13            | 0                      |
| \( \Delta crd1 \), lactate | 22         | 28            | 50                     |
| BN-PAGE             | 71           | 29            | 0                      |
| WT, glycerol/ethanol| 47           | 36            | 17                     |
| BN-PAGE             | 52           | 36            | 12                     |
| \( \Delta crd1 \), glycerol/ethanol | 12       | 23            | 65                     |
| BN-PAGE             | 31           | 45            | 24                     |

Potential Interaction Site of Complexes III and IV—Complexes III and IV from *S. cerevisiae* contain subunits that are not essential for assembly of a core structure of the individual complexes. We asked whether deletion of the genes for these subunits, viz. Qcr6p, Qcr9p, and Qcr10p of complex III and Cox8p, Cox12p, and Cox13p of complex IV, can adversely affect the formation of small and large supercomplexes containing dimeric complex III and one or two copies of complex IV, respectively. Mitochondria of the individual null mutant strains were solubilized by digitonin and screened for the presence of supercomplexes by two-dimensional electrophoresis (BN-PAGE/SDS-PAGE) (Fig. 3). Most deletions (\( \Delta qcr10 \), \( \Delta cox8 \), \( \Delta cox12 \), and \( \Delta cox13 \)) had no significant effect on the amounts of supercomplexes, except that the supercomplexes were somewhat smaller due to the absence of the given subunit. A two-dimensional gel for one representative of this group (\( \Delta cox12 \)) is shown in Fig. 3A. Deletion of Qcr9p severely reduced the amounts of supercomplexes; however, small amounts were still detectable (Fig. 3B). These supercomplexes had significantly lower masses, because not only Qcr9p, but also the Rieske iron-sulfur protein Rip1p and Qcr10p were missing. Cruciat et al. (30) have shown previously that a similar significant effect on the amount and size of supercomplexes occurs upon deletion of the Rieske iron-sulfur protein. In contrast to all deletion strains analyzed so far, deletion of Qcr6p stimulated the formation of the large supercomplex (Fig. 3D). \( \Delta qcr6 \) grown on glucose contained almost exclusively the large supercomplex, whereas the parental strain contained large and small supercomplexes at about equimolar ratios (Fig. 3C). Cytochrome c was excluded as a candidate protein linking complexes III and IV together because cytochrome c was removed from the supercomplexes during BN-PAGE, as determined by immunodetection (data not shown). We concluded that Rip1p, Qcr6p, Qcr9p, Qcr10p, Cox8p, Cox12p, and Cox13p, and cytochrome c were not critical for formation of respiratory chain supercomplexes and that lack of Qcr6p even enhanced the assembly. Of the group of remaining subunits of complexes III and IV, the most likely candidates for a direct link of complexes III and IV seem to be the central subunits: cytochromes b and c₁ of complex III and Cox1p, Cox2p, and Cox3p of complex IV. In fact, assemblies of complexes III and IV have been identified also in *Paracoccus denitrificans*, a bacterium not containing the “supernumerary” eukaryotic subunits (49). Moreover, many mild detergents dissociate the supercomplexes at low ionic strength, suggesting that the interaction of the two complexes primarily involves hydrophobic protein surfaces.

Catalytic Properties of Complexes III and IV—Ubiquinol: cytochrome c oxidoreductase (complex III) activity in the supercomplexes after membrane solubilization by digitonin (3 g/g of protein) was compared with individual complex III activity after dissociation by dodecyl maltoside (1 g/g of protein). Turnover numbers were essentially the same for the parental and \( \Delta crd1 \) mitochondria and were independent of the detergent used (70–90 s⁻¹). The \( K_m \) for yeast cytochrome c was unchanged (3.5 ± 0.4 μM for the parental strain and 3.6 ± 0.6 μM for the \( \Delta crd1 \) strain).

Initial cytochrome c oxidase traces in standard assays for complex IV of wild-type mitochondria were linear in the absence of detergent (Fig. 4, trace 1) or in the presence of dodecyl maltoside (trace 2) or digitonin (trace 3) for solubilization. This is in contrast to the cytochrome c oxidase rates in \( \Delta crd1 \) mitochondria, which were initially rather slow, but increased considerably with time. The nonlinearity is seen best using \( \Delta crd1 \) mitochondria without detergent (trace 4), but is also apparent...
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**Table II**

Phospholipid composition of the Δcrd1 and isogenic parental strains grown in different media expressed as a percentage of total radiolabeled 32P incorporated into phospholipids (n ≥ 4)

|          | Lactate | WT | Δcrd1 | Glucose | WT | Δcrd1 | Glycerol/ethanol | WT | Δcrd1 |
|----------|---------|----|-------|---------|----|-------|-----------------|----|-------|
| CL       | 7.13 ± 0.64 | ND | 5.92 ± 1.25 | ND | 7.42 ± 1.61 | ND |
| PA       | 8.59 ± 1.84 | 8.22 ± 0.72 | 4.88 ± 0.51 | 4.96 ± 0.45 | 7.55 ± 1.95 | 5.64 ± 0.43 |
| PE       | 6.73 ± 1.25 | 16.19 ± 1.06 | 13.60 ± 1.77 | 16.59 ± 1.30 | 14.26 ± 0.77 | 22.99 ± 2.86 |
| PG       | 0.31 ± 0.12 | 6.70 ± 0.44 | ND | 3.29 ± 0.16 | 0.19 ± 0.08 | 7.27 ± 0.35 |
| PS       | 1.88 ± 0.59 | 1.77 ± 0.41 | 4.94 ± 1.26 | 6.35 ± 0.61 | 3.14 ± 0.37 | 4.05 ± 0.46 |
| PI       | 20.46 ± 1.47 | 17.19 ± 1.77 | 16.81 ± 4.79 | 16.60 ± 4.25 | 22.61 ± 1.86 | 17.20 ± 1.03 |
| PC       | 44.02 ± 3.83 | 42.72 ± 1.99 | 48.05 ± 2.42 | 48.43 ± 2.41 | 41.58 ± 4.63 | 38.96 ± 2.68 |

*WT, wild-type strain; CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; ND, not detectable.

**Table III**

Cardiolipin-dependent partitioning of total complex III to supercomplexes and individual complex III after BN-PAGE

The parental wild-type (WT) strain and Δcrd1 mutant were grown on glycerol/ethanol. Complex III was densitometrically quantified on Coomassie Blue-stained two-dimensional gels (BN-PAGE/SDS-PAGE) as shown in Fig. 2 (a–d) (n = 3). CL, cardiolipin.

| Complex III | WT | WT + CL | Δcrd1 | Δcrd1 + CL |
|-------------|----|---------|-------|-----------|
| In large supercomplex | 47 ± 1 | 52 ± 1 | 12 ± 3 | 33 ± 5 |
| In small supercomplex | 36 ± 1 | 34 ± 2 | 23 ± 4 | 39 ± 5 |
| As individual complex III | 17 ± 1 | 14 ± 1 | 65 ± 7 | 28 ± 3 |

**Fig. 3.** Subunits that are not required for assembly of the core structures of complexes III and IV are not essential for supercomplex formation. Mitochondrial oxidative phosphorylation complexes from S. cerevisiae parental and null mutant strains grown on galactose and lactate were solubilized by digitonin, and the native complexes were separated by BN-PAGE. The subunits were then resolved by SDS-PAGE in the second dimension and silver-stained. V_v and V_m monomeric and dimeric complex V, respectively; L and S, large and small respiratory chain supercomplexes, respectively; III, individual complex III. The assignment of subunits of complexes III and IV is as listed in the legend to Fig. 2 A, Δcox12 strain. The amounts and ratios of small and large supercomplexes were comparable with those in the parental strain. B, Δqcr9 strain. The amounts of supercomplexes were found to be considerably reduced. The small supercomplex showed a considerably reduced size compared with the parental wild-type strain and comigrated with monomeric complex V(V_m + S). The Rieske iron-sulfur protein, Qcr9p, and Qcr10p were missing in individual complex III. C, parental wild-type (WT) strain W303-1A. D, Δqcr6 strain. In contrast to the parental strain grown under same conditions, the Δqcr6 strain contained almost exclusively the large supercomplex.

After solubilization by dodecyl maltoside (trace 5) or digitonin (trace 6). About 8-fold higher concentrations of complex IV from Δcrd1 mitochondria had to be used to obtain rates comparable with those in wild-type mitochondria. Assuming that activation was complete during turnover, we used the “linear” parts of the traces and determined the digitonin-dependent cytochrome c oxidase rates (Fig. 5). Wild-type cytochrome c oxidase rates increased considerably after addition of digitonin (1 g/g of protein). This seems to reflect an improved accessibility of the substrate cytochrome c due to partial solubilization by detergent. Quantitative solubilization was achieved at a digitonin/protein ratio of ~2 g/g. Cytochrome c oxidase rates in Δcrd1
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**Fig. 4. Typical cytochrome c oxidase traces for mitochondria from the parental and Δcrd1 strains.** Linear initial rates were found for the parental wild-type (WT) strain without using detergent (trace 1) and after solubilization by dodecyl maltoside and digitonin (traces 2 and 3, respectively). Slow initial rates and activation during the turnover were found for Δcrd1 mitochondria without detergent (trace 4) and also after solubilization by dodecyl maltoside and digitonin (traces 5 and 6, respectively). To obtain comparable cytochrome c oxidation rates for the parental and Δcrd1 strains, greatly different complex IV concentrations had to be used in the test (1.085 nM for the parental strain and 8.7 nM for the Δcrd1 strain).

**Fig. 5. Cytochrome c oxidase rates in mitochondria from the parental and Δcrd1 strains.** ● parental strain; ▲, parental strain supplemented with cardiolipin (digitonin removed accessibility barriers for substrate cytochrome c and allowed for maximal cytochrome c oxidase rates after complete solubilization (2 g of digitonin/g of protein)); ○, cardiolipin-deficient mitochondria; △, Δcrd1 mitochondria reconstituted with cardiolipin (supplementation with cardiolipin did not enhance the transition from the resting to pulsed state); ◼, Δcrd1 mitochondria without addition of cardiolipin after pre-reducing cytochrome c oxidase for at least 1 min. Full transition to the pulsed state and wild-type rates (1100–1500 s⁻¹; error bars) were obtained.

mitochondria showed a similar dependence on digitonin. However, the maximal rates were ~250 s⁻¹ compared with ~1500 s⁻¹ in wild-type mitochondria. Reconstitution of Δcrd1 mito-
III and IV together. The head group of cardiolipin is in close proximity to the internal site of quinone reduction (Q) and to the entrance of the cardiolipin/lysine (CL/K) proton conduction pathway, suggesting that this cardiolipin may have, in addition to its structural role, a potential role in proton conduction (22). In this work, we have compared the decylubiquinol:cytochrome c oxidoreductase activities of the parental and Δclr1 strains and found that the turnover numbers were comparable (70–90 s⁻¹) and also that the $K_m$ values for cytochrome c were independent of the presence of the cardiolipin. This cardiolipin specifies a specific function of cardiolipin for the electron transfer activity of complex III.

Cytochrome c oxidase exists in (at least) two functionally distinct forms, which have been distinguished because they catalyze the overall oxidation of cytochrome c by molecular oxygen at different rates (51, 52). These states of the enzyme have been called the “resting” and “pulsed” states. The former term has been used to refer to the fully oxidized enzyme as isolated, whereas the latter term refers to the enzyme activated by undergoing a redox cycle in which cytochrome $a_3$ is reduced and subsequently reoxidized by molecular oxygen. It was suggested that a dynamic distribution of oxidase between the two states may have regulatory importance in vivo (51). However, the resting state has been observed so far only with isolated complex IV, but not in membranes. Here, we have shown that a cardiolipin-deficient strain harbored largely inactive resting complex IV in the membranes that exhibited wild-type rate constants and $K_m$ values for cytochrome c following pre-reduction. The full activation suggested that the catalytic cycle of complex IV was not critically affected. However, we found that full activation of the cardiolipin-deficient enzyme required >1 min of pre-reduction by reduced cytochrome c, whereas the wild-type enzyme was already in a fully active state or activation was too fast to be detected in standard enzymatic assays. The resting cytochrome c oxidase as found in Δclr1 mitochondria seemed to indicate that a stabilizing effect of cardiolipin for the physiological fast form was missing, and transition to the resting form could occur, which otherwise has been observed only after use of delipidating isolation procedures. The primary role of cardiolipin seems to be stabilization of supercomplexes and individual complexes.

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