THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 52, Issue of December 26, pp. 52873–52880, 2003
Printed in U.S.A.

Cardiolipin Stabilizes Respiratory Chain Supercomplexes*

Received for publication, July 31, 2003, and in revised form, October 9, 2003
Published, JBC Papers in Press, October 15, 2003, DOI 10.1074/jbc.M308366200

Kathy Pfeiffer‡, Vishal Gohil§, Rosemary A. Stuart¶, Carola Hunte‖, Ulrich Brandt‡, Miriam L. Greenberg§, and Hermann Schägger‡**

From the ‡Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, D-60590 Frankfurt, Germany, the §Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202, the ¶Biology Department, Marquette University, Milwaukee, Wisconsin 53051-1881, and the ‖Max-Planck-Institut für Biophysik, D-60439 Frankfurt am Main, Germany

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 c_1 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 ADP-ATP translocator (6–8), for the stability of respiratory complex III (ubiquinol:cytochrome *c* oxidoreductase or cytochrome *c* reductase) (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 F_1F_0-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 c_1, with the head group of cardiolipin residing in close proximity to the internal site of quinone reduction (Q_i) 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 Δ*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 Δ*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 tafazzin 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

* This work was supported by Deutsche Forschungsgemeinschaft Sonderforschungsbereich 472 and the Fond der Chemischen Industrie (to H. S., C. H., and U. B.) and by National Institutes of Health Grant HL62263 (to M. L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, Haus 25B, D-60590 Frankfurt, Germany. Tel.: 49-69-6301-6927; Fax: 49-69-6301-6970; E-mail: schagger@zbc.kgu.de.

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 crista morphology (34). The state of activity of monomeric and dimeric complex V is controversial. Paumard *et al.* (34) showed comparable ATP hydrolysis activities for the monomeric and dimeric forms of yeast complex V, whereas Tomasetig *et al.* (35) reported active monomeric and inactive dimeric forms of bovine complex V.

The availability of a Δ*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: Δ*crd1* (FGY2) and the corresponding parental strain FGY3 (6), wild-type W303-1A (37), Δ*qcr6* (38), Δ*qcr9* (39), Δ*qcr10* (40), Δ*cox12* (41), and Δ*cox13* (42). Δ*cox8* 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)[1] 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.) and enhanced chemiluminescence reagent (ECL, Amersham Biosciences) were used for protein detection.

Phospholipid Determination—Yeast strains were grown in the presence of 10 μCi of ^{32}P/ml of culture at 30 °C to early stationary phase. Cells were then washed and digested by zymolyase 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 [^{32}P]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 ratios of 1, 2, 3, and 4 (g/g) and 0.3, 0.6, 0.9, and 1.2 (g/g), respectively. The samples were used directly without centrifugation.

Enzyme Activities—Ubiquinol:cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) were measured by the antimycin-sensitive reduction and cyanide-sensitive oxidation of cytochrome *c*, respectively (550–540 nm; $\epsilon = 19\ mM^{-1}\ cm^{-1}$) in the presence of 70 μM yeast cytochrome *c*. Decylbenzoquinol (75 μM) was used as 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; $\epsilon = 4.2\ mM^{-1}\ cm^{-1}$) 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 glycine (pH 7.8) with or without addition of 5 μg/ml oligomycin. The buffer was then replaced with 35 mM Tris, 270 mM glycine, 14 mM $MgSO_4$, 0.2% $Pb(NO_3)_2$, 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 (Δ*crd1*) 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. 1*A*). 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 Δ*crd1* 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 in the second dimension, as exemplified in Fig. 2. Quantification of complex III on two-dimensional gels from the Δ*crd1* 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 Δ*crd1* 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 Δ*crd1* 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 Δ*crd1* 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 Δ*crd1* mitochondria were retained following CN-PAGE (Fig. 1*B*). 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

[1] The abbreviations used are: BN, blue native; CN, colorless native.

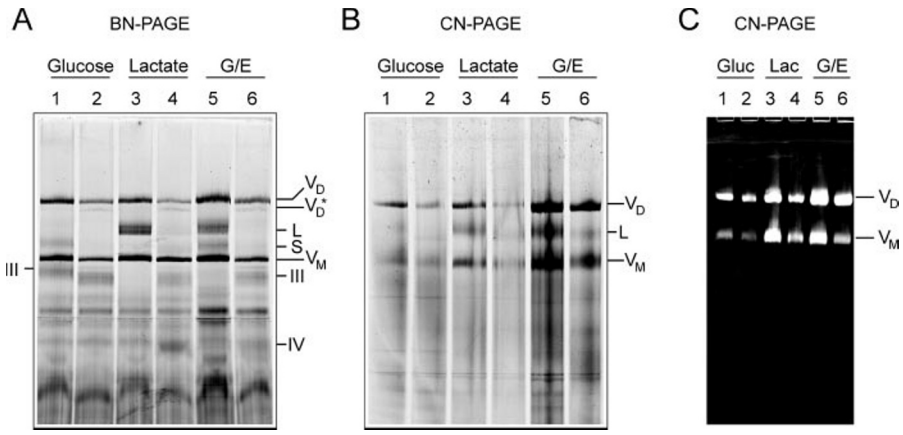

FIG. 1. **Cardiolipin-deficient respiratory chain supercomplexes largely dissociate under BN-PAGE conditions, but not under CN-PAGE conditions.** V_M and V_D, monomeric and dimeric complex V, respectively; V_D*, a subcomplex of dimeric complex V that was observed when cardiolipin was lacking; *S* and *L*, small and large respiratory chain supercomplexes comprising dimeric complex III and one or two copies of complex IV, respectively; *III*, complex III; *IV*, complex IV. The parental strain (*lanes 1*, *3*, and *5*) and the cardiolipin-deficient Δ*crd1* strain (*lanes 2*, *4*, and *6*) were grown on glucose (*Gluc*), lactate (*Lac*), and glycerol/ethanol (*G*/*E*), respectively. *A*, separation of digitonin-solubilized complexes by BN-PAGE. The amounts and ratios of large and small respiratory chain supercomplexes varied with the growth conditions in the parental strains. In the cardiolipin-deficient strain, supercomplexes were hardly detectable following BN-PAGE (two-dimensional resolution was required for detection), although individual complexes III and IV (especially in *lane 4*) were found. The apparent mass of individual cardiolipin-deficient complex III (*lane 2*) was smaller than that of wild-type complex III (*lane 1*). *B*, separation by CN-PAGE. *C*, in-gel lead phosphate precipitation was used as a measure for ATP hydrolysis activity of monomeric and dimeric complex V following CN-PAGE.

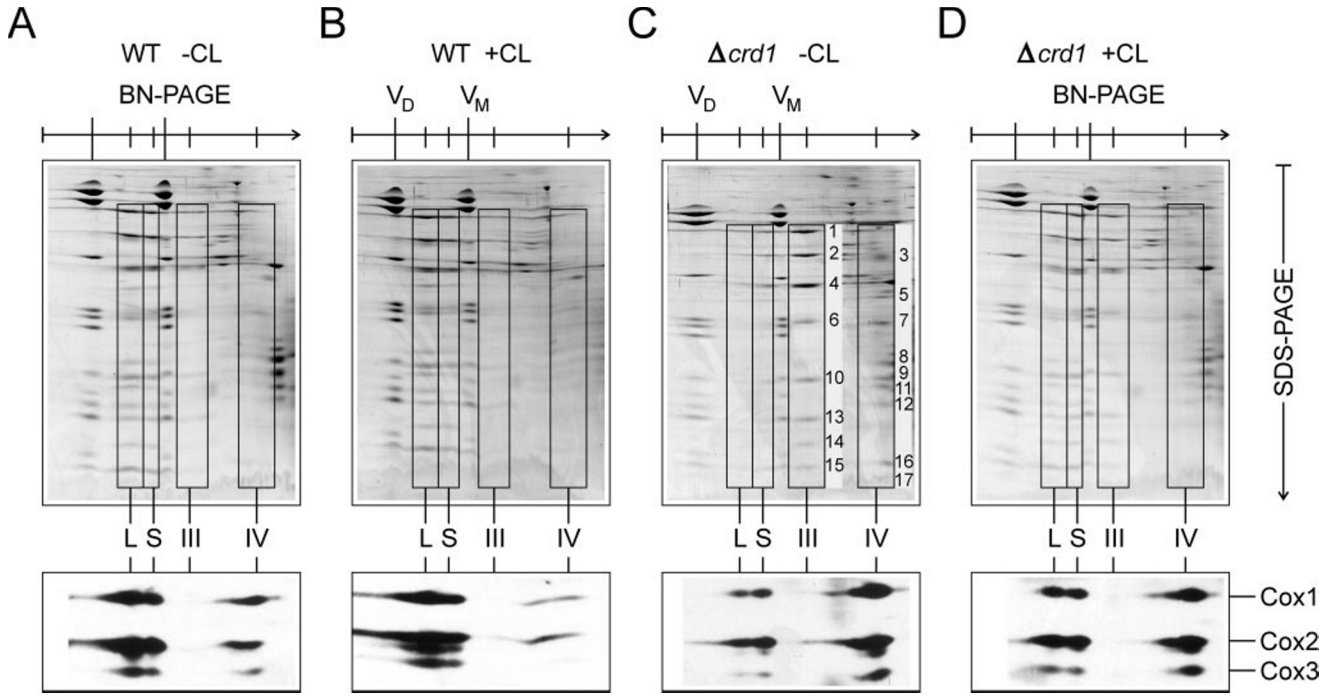

FIG. 2. **Stabilization of respiratory chain supercomplexes by addition of cardiolipin.** Mitochondria from parental wild-type (*WT*) and Δ*crd1* strains grown on glycerol/ethanol were used directly (−*CL*) or after supplementation with cardiolipin (+*CL*) for BN-PAGE and two-dimensional SDS-PAGE. *Boxes* indicate the expected locations of the large (*L*) and small (*S*) supercomplexes, individual complex III (*III*), and individual complex IV (*IV*) on the Coomassie Blue-stained two-dimensional gels (*upper panels*). *A*, mitochondria from the control parental strain (*WT* −*CL*); *B*, mitochondria from the wild-type strain supplemented with additional cardiolipin (*WT* +*CL*); *C*, mitochondria from the cardiolipin-deficient Δ*crd1* strain (Δ*crd1* −*CL*); *D*, mitochondria from the Δ*crd1* strain reconstituted with cardiolipin (Δ*crd1* +*CL*). Supplementation with cardiolipin reduced the amounts of individual complexes III and IV and correspondingly increased the amounts of large and small supercomplexes (see Table III for quantification). The assignment of subunits of complexes III and IV is as follows: *band 1*, Qcr1p; *band 2*, Qcr2p; *band 3*, Cox1p; *band 4*, cytochrome *b* + Cyc1p; *band 5*, Cox2p; *band 6*, Rip1p; *band 7*, Cox3p; *band 8*, Cox4p; *band 9*, Cox5Ap; *band 10*, Qcr7p; *band 11*, Cox13p; *band 12*, Cox6p; *band 13*, Qcr8p; *band 14*, Qcr10p; *band 15*, Qcr9p; *band 16*, Cox7p + Cox9p; *band 17*, Cox8p. Antibodies against cytochrome *c* oxidase subunits 1–3 (*Cox1*, *Cox2*, and *Cox3*, respectively) were used for visualization of complex IV in the corresponding Western blots (*Lower panels*). V_M and V_D, monomeric and dimeric complex V, respectively.

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. 1*A*).

Individual complex III from the Δ*crd1* strain seemed slightly smaller than free complex III from the parental strain (Fig. 1*A*, *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 Δ*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

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

Complex III was densitometrically quantified on Coomassie Blue-stained two-dimensional gels (data not shown) using *lanes 1–6* from Fig. 1 (*A* and *B*). The percentage of total complex III contained in each of the three fractions is indicated. WT, wild-type strain.

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

dimeric ATP synthase was usually observed in mitochondria from the Δ*crd1* strain (Fig. 1*A*). 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. 1*B*). 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 Δ*crd1* strains were similar, as estimated by in-gel lead phosphate precipitation, which correlated with ATP hydrolysis rates (Fig. 1*C*). 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 Δ*crd1* strains was almost completely 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 Δ*crd1* and parental strains grown on glycerol/ethanol were supplemented 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. 2*A*), were found not to be associated with complex III. Upon addition of cardiolipin, this minor amount of individual complex IV was further reduced (Fig. 2*B* and Table III). Addition of cardiolipin to Δ*crd1* mitochondria had a pronounced effect. Using Δ*crd1* mitochondria directly, major amounts of complex IV were found as free individual complex IV (Fig. 2*C*). However, after addition of cardiolipin, a large fraction of this individual complex IV was found assembled with complex III (Fig. 2*D*). 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.

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 (Δ*qcr10*, Δ*cox8*, Δ*cox12*, and Δ*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 (Δ*cox12*) is shown in Fig. 3*A*. Deletion of Qcr9p severely reduced the amounts of supercomplexes; however, small amounts were still detectable (Fig. 3*B*). 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. 3*D*). Δ*qcr6* grown on glucose contained almost exclusively the large supercomplex, whereas the parental strain contained large and small supercomplexes at about equimolar ratios (Fig. 3*C*). 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, 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 c_1 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 Δ*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 Δ*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 Δ*crd1* mitochondria, which were initially rather slow, but increased considerably with time. The nonlinearity is seen best using Δ*crd1* mitochondria without detergent (*trace 4*), but is also apparent

TABLE II

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

	Lactate		Glucose		Glycerol/ethanol	
	WT[a]	Δ*crd1*	WT	Δ*crd1*	WT	Δ*crd1*
	%		%		%	
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	8.73 ± 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	46.43 ± 2.41	41.58 ± 4.63	38.96 ± 2.68

[a] 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 Δ*crd1* 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	Δ*crd1*	Δ*crd1* + 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

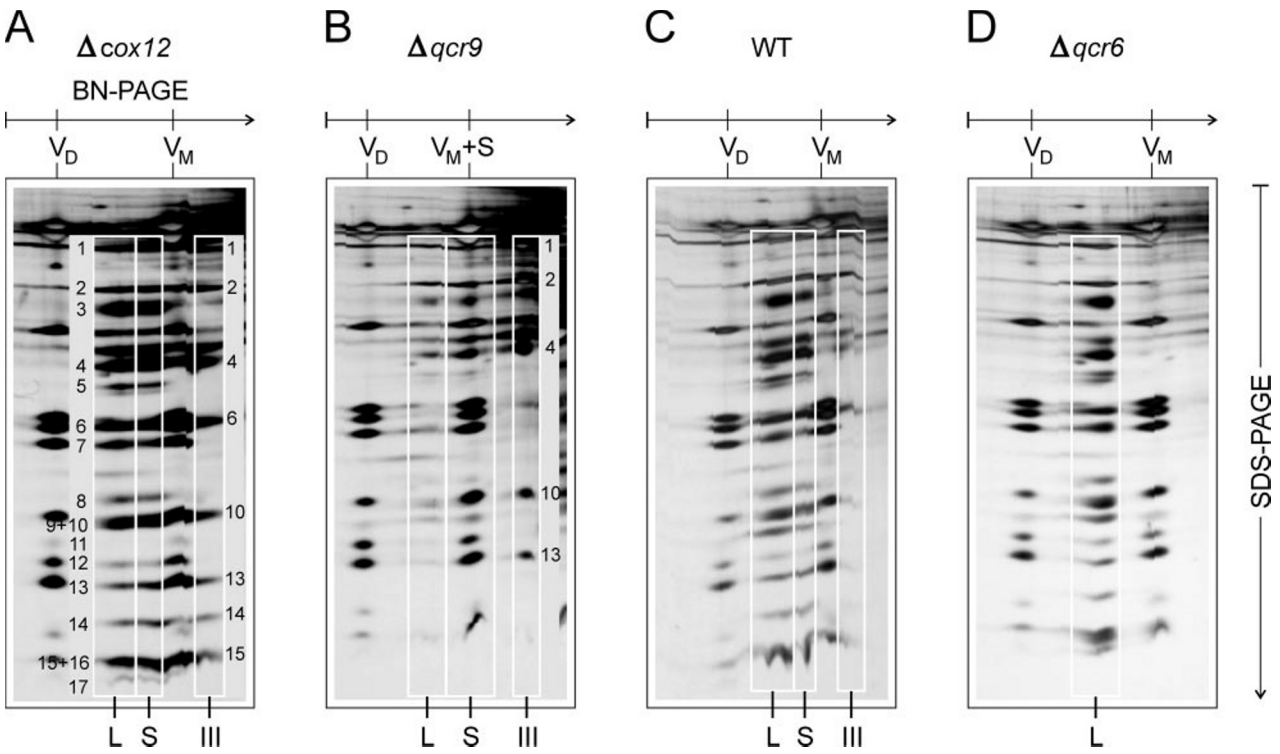

FIG. 3. **Subunits that are not required for assembly of the core structures of complexes III and IV are not essential for supercomplex formation.** Mitochondrial oxidative phosphorylation complexes from *S. cerevisiae* parental and null mutant strains grown on galactose and lactate were solubilized 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_M and V_D, 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*, Δ*cox12* strain. The amounts and ratios of small and large supercomplexes were comparable with those in the parental strain. *B*, Δ*qcr9* 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*, Δ*qcr6* strain. In contrast to the parental strain grown under same conditions, the Δ*qcr6* 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 Δ*crd1* 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 Δ*crd1*

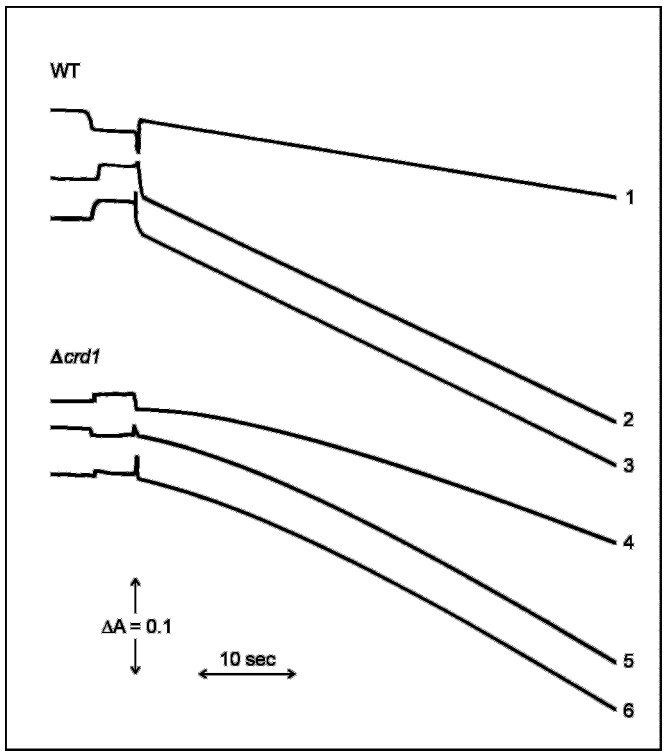

FIG. 4. **Typical cytochrome *c* oxidase traces for mitochondria from the parental and Δ*crd1* strains.** Linear initial rates were found for the parental wild-type (*WT*) strain without using detergent (*trace 1*) and after solubilization by dodecyl maltoside and digitonin (*traces 2* and *3*, respectively). Slow initial rates and activation during the turnover were found for Δ*crd1* 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 Δ*crd1* 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 Δ*crd1* strain).

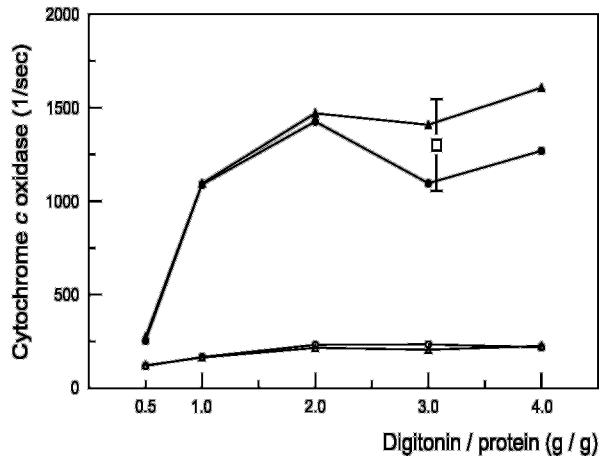

FIG. 5. **Cytochrome *c* oxidase rates in mitochondria from the parental and Δ*crd1* strains.** ●, parental strain; ▲, parental strain supplemented with cardiolipin (digitonin removed accessibility barriers for substrate cytochrome *c* and allowed for maximal cytochrome *c* oxidase rates after complete solubilization (2 g of digitonin/g of protein)); ○, cardiolipin-deficient mitochondria; △, Δ*crd1* mitochondria reconstituted with cardiolipin (supplementation with cardiolipin did not enhance the transition from the resting to pulsed state); □, Δ*crd1* 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 Δ*crd1* mitochondria with cardiolipin had no positive effect on cytochrome *c* oxidase activity, although the same reconstitution protocol that stabilized the supercomplexes structurally was used (Fig. 2). The reduced apparent catalytic activity was found not to be due to an altered K_m for yeast cytochrome *c*, but rather reflected a slow conversion of resting cytochrome *c* oxidase to the pulsed or fast state, a process that seemed not to be complete during the activity measurements. This was deduced from the observation that full activity (1100–1500 s^{-1}) (Fig. 5) and linear initial rates were obtained with digitonin-solubilized cytochrome *c* oxidase from cardiolipin-free Δ*crd1* mitochondria if it was reduced with μM concentrations of reduced cytochrome *c* for at least 1 min. Using pre-reduced cytochrome *c* oxidase, the K_m values for yeast cytochrome *c* were 5.5 ± 1.5 μM for the parental strain and 6 ± 1.0 μM for the Δ*crd1* strain.

Quinol oxidase rates in membranes (~50 s^{-1}; monitoring the combined activities of complexes III and IV) were not adversely affected by the lack of cardiolipin in Δ*crd1* mitochondria. This low overall rate was limited by cytochrome *c* reductase (complex III), and partial activation of resting cytochrome *c* oxidase (complex IV) during turnover was sufficient to approach normal quinol oxidase rates.

DISCUSSION

In contrast to previous studies (32, 36), cardiolipin was found not to be essential for the assembly of supercomplexes of complexes III and IV in the inner mitochondrial membrane because respiratory chain supercomplexes could be isolated from a cardiolipin-deficient strain by CN-PAGE and gel filtration. Cardiolipin instead improved the stability of the supercomplexes: although wild-type supercomplexes could be observed under BN-PAGE conditions, supercomplexes from the cardiolipin-deficient strain were prone to dissociation. Supplementation with cardiolipin largely restored wild-type stability. Other phospholipids, including phosphatidylethanolamine, phosphatidylcholine, and phosphatidylglycerol, that are present in the cardiolipin-deficient strain (Table II) could not prevent dissociation of supercomplexes under BN-PAGE conditions.

Because supercomplexes are formed even in the absence of cardiolipin, we searched for direct protein-protein interactions of complexes III and IV. Supercomplexes of respiratory chain complexes III and IV have been identified in eukaryotic mitochondria and also in bacterial membranes, *e.g.* from *P. denitrificans* (49). Bacterial supercomplexes contain homologs to central eukaryotic subunits, but do not contain homologs to supernumerary eukaryotic subunits. This suggests that complexes III and IV may be linked via hydrophobic central proteins also in eukaryotes. We have shown in this work that all subunits that are not essential for assembly of core structures of the individual complexes are not essential for assembly into supercomplexes. The amounts of the large supercomplex were even increased when Qcr6p, an acidic subunit (pI 3.98) containing several stretches of acidic residues, was missing. Because also the Rieske iron-sulfur protein is not essential, only two subunit homologs to bacterial complex III remain: cytochromes *b* and c_1. We therefore suggest that the interface of complexes III and IV comprises hydrophobic domains of cytochromes *b* and c_1. This interface should be close to Qcr6p because removal of acidic Qcr6p promoted supercomplex formation, potentially by relieving electrostatic repulsion.

In the structure of yeast complex III (50), transmembrane helices of cytochromes *b* and c_1 and Qcr8p form a depression in the transmembrane region of the complex, and Qcr6p protrudes like a lid on top of this cavity (Fig. 6). Two phospholipid molecules, *viz.* cardiolipin L5 and phosphatidylethanolamine L4, are tightly bound in this depression (22), suggesting that these two phospholipids may be involved in gluing complexes

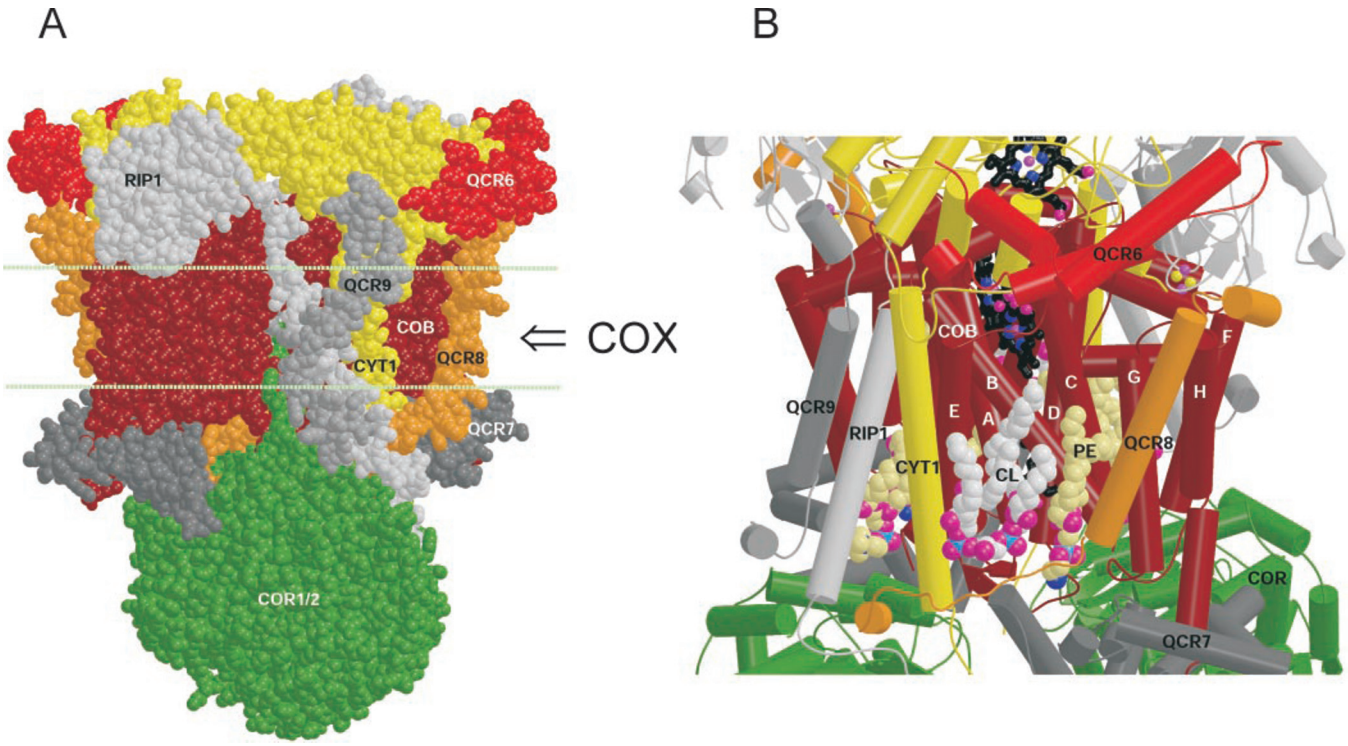

FIG. 6. **Putative interaction site of complexes III and IV.** *A*, the interaction of cytochrome *c* oxidase (*COX*) and complex III is proposed to involve cytochromes *b* and c_1. These two subunits form a depression in the transmembrane region of complex III (50), which is shown in surface presentation with the different subunits labeled and color-coded. Loss of Qcr6p, which protrudes like a lid on top of this cavity, stimulates formation of the large supercomplex. The position of the phospholipid bilayer as deduced from tightly bound phospholipid molecules (22, 23) is indicated with *dotted lines*. *B*, front view on the putative interaction site. Transmembrane helices *A–F* of cytochrome *b* (*COB*) are marked. Cardiolipin L5 (*CL*) and phosphatidylethanolamine L4 (*PE*) are bound in the depression formed by helices of cytochrome *b* and flanked by helices of cytochrome c_1 (*CYT1*) and Qcr8p according to Lange *et al.* (22). Illustrations were prepared using BobScript (53) and Raster3D (54).

III and IV together. The head group of cardiolipin is in close proximity to the internal site of quinone reduction (Q_i) 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 Δ*crd1* 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 cardiolipin. This excludes 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 Δ*crd1* 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.

Acknowledgments—We are grateful to B. L. Trumpower for the kind gift of yeast mutant strains Δ*qcr6*, Δ*qcr9*, Δ*qcr10*, and Δ*cox12*; to B. Kadenbach for the kind gift of Δ*cox13*; and to S. Brunner for generating the Δ*cox8* null mutant.

REFERENCES

1. LeCocq, J., and Ballou, C. E. (1964) *Biochemistry* 3, 976–980
2. Schlame, M., Rua, D., and Greenberg, M. L. (2000) *Prog. Lipid Res.* 39, 257–288
3. Hoch, F. L. (1992) *Biochim. Biophys. Acta* 1113, 71–133
4. Halestrap, A. (1989) *Biochim. Biophys. Acta* 973, 355–382
5. Koshkin, V., and Greenberg, M. L. (2002) *Biochem. J.* 364, 317–322
6. Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Zhiming, G., Klingenberg, M., Pfanner, N., and Greenberg, M. L. (2000) *J. Biol. Chem.* 275, 22387–22394
7. Drees, M., and Beyer, K. (1988) *Biochemistry* 27, 8584–8585
8. Beyer, K., and Nuscher, B. (1996) *Biochemistry* 35, 15784–15790
9. Yu, C. A., and Yu, L. (1980) *Biochemistry* 19, 5715–5720
10. Schägger, H., Hagen, T., Roth, B., Brandt, U., Link, T. A., and von Jagow, G. (1990) *Eur. J. Biochem.* 190, 123–130
11. Hayer-Hartl, M., Schägger, H., von Jagow, G., and Beyer, K. (1992) *Eur. J. Biochem.* 209, 423–430
12. Gomez, B., and Robinson, N. C. (1999) *Biochemistry* 38, 9031–9038
13. Robinson, N. C., Zborowski, J., and Talbert, L. H. (1990) *Biochemistry* 29, 8962–8969
14. Sedlák, E., and Robinson, N. C. (1999) *Biochemistry* 38, 14966–14972
15. Eble, K. S., Coleman, W. B., Hantgan, R. R., and Cunningham, C. C. (1990) *J. Biol. Chem.* 265, 19434–19440
16. Laird, D. M., Parce, J. W., Montgomery, R. I., Spach, P. I., and Cunningham, C. C. (1986) *J. Biol. Chem.* 261, 14851–14856
17. Rytöomaa, M., and Kinnunen, P. K. J. (1994) *J. Biol. Chem.* 269, 1770–1774
18. Ostrander, D. B., Sparagna, G. C., Amoscato, A. A., McMillin, J. B., and Dowhan, W. (2001) *J. Biol. Chem.* 276, 38061–38067
19. Fyfe, P. K., McAuley, K. E., Roszak, A. W., Isaacs, N. W., Cogdell, R. J., and

Jones, M. R. (2001) *Trends Biochem. Sci.* 26, 106–112
20. McAuley, K. E., Fyfe, P. K., Ridge, J. P., Isaacs, N. W., Cogdell, R. J., and Jones, M. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 14706–14711
21. Mizushima, T., Yao, M., Inoue, N., Aoyama, H., Yamashita, E., Yamaguchi, H., Tsukihara, T., Nakashima, R., Shinzawa-Itoh, K., Yaono, R., and Yoshikawa, S. (1999) *Acta Crystallogr. Sect. A* 55, suppl., P06.04.069
22. Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) *EMBO J.* 20, 6591–6600
23. Palsdottir, H., Lojero, G. C., Trumpower, B. L., and Hunte, C. (2003) *J. Biol. Chem.* 278, 31303–31311
24. Jiang, F., Rizavi, H. S., and Greenberg, M. L. (1997) *Mol. Microbiol.* 26, 481–491
25. Tuller, G., Hrastnik, C., Achleitner, G., Schiefthaler, U., Klein, F., and Daum, G. (1998) *FEBS Lett.* 421, 15–18
26. Chang, S.-C., Heacock, P. N., Mileykovskaya, E., Voelker, D., and Dowhan, W. (1998) *J. Biol. Chem.* 273, 14933–14941
27. Koshkin, V., and Greenberg, M. L. (2000) *Biochem. J.* 347, 687–691
28. Vreken, P., Valianpour, F., Nijtmans, L. G., Grivell, L. A., Plecko, B., Wanders, R. J. A., and Barth, P. G. (2000) *Biochem. Biophys. Res. Commun.* 279, 378–382
29. Boumans, H., Grivell, L. A., and Berden, J. A. (1998) *J. Biol. Chem.* 273, 4872–4877
30. Cruciat, C. M., Brunner, S., Baumann, F., Neupert, W., and Stuart, R. A. (2000) *J. Biol. Chem.* 275, 18093–18096
31. Schägger, H., and Pfeiffer, K. (2000) *EMBO J.* 19, 1777–1783
32. Schägger, H. (2002) *Biochim. Biophys. Acta* 1555, 154–159
33. Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schägger, H. (1998) *EMBO J.* 17, 7170–7178
34. Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D. M., Brèthes, D., di Rago, J.-P., and Velours, J. (2002) *EMBO J.* 21, 221–230
35. Tomasetig, L., DiPancrazio, F., Harris, D. A., Mavelli, I., and Lippe, G. (2002) *Biochim. Biophys. Acta* 1556, 133–141
36. Zhang, M., Mileykovskaya, E., and Dowhan, W. (2002) *J. Biol. Chem.* 277, 43553–43556
37. Rothstein, R. J., and Sherman, F. (1980) *Genetics* 94, 871–889
38. Schmitt, M. E., and Trumpower, B. L. (1990) *J. Biol. Chem.* 265, 17005–17011
39. Phillips, J. D., Graham, L. A., and Trumpower, B. L. (1990) *J. Biol. Chem.* 268, 11727–11736
40. Brandt, U., Uribe, S., Schägger, H., and Trumpower, B. L. (1994) *J. Biol. Chem.* 269, 12947–12953
41. LaMarche, A. E., Abate, M. I., Chan, S. H., and Trumpower, B. L. (1992) *J. Biol. Chem.* 267, 22473–22480
42. Follmann, K., Arnold, S., Ferguson-Miller, S., and Kadenbach, B. (1998) *Biochem. Mol. Biol. Int.* 45, 1047–1055
43. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* 13, 1793–1808
44. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* 217, 220–230
45. Schägger, H. (1995) *Electrophoresis* 16, 763–770
46. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* 226, 497–509
47. Leray, C., Pelletier, X., Hemmendinger, S., and Cazenave, J. P. (1987) *J. Chromatogr.* 420, 411–416
48. Zerbetto, E., Vergani, L., and Dabbeni-Sala, F. (1997) *Electrophoresis* 18, 2059–2064
49. Berry, E. A., and Trumpower, B. L. (1985) *J. Biol. Chem.* 260, 2458–2467
50. Hunte, C., Koepke, J., Lange, C., Roßmanith, T., and Michel, H. (2000) *Structure* 8, 660–684
51. Wilson, M. T., Peterson, J., Antonini, E., Brunori, M., Colosimo, A., and Wyman, J. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 7115–7118
52. Moody, A. J. (1996) *Biochim. Biophys. Acta* 1276, 6–20
53. Esnouf, R. M. (1999) *Acta Crystallogr. Sect. D* 55, 938–940
54. Merritt, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. Sect. D* 50, 869–873