Here we present for the first time a three-dimensional cryo-EM map of the *Saccharomyces cerevisiae* respiratory supercomplex composed of dimeric complex III flanked on each side by one monomeric complex IV. A precise fit of the existing atomic x-ray structures of complex III from yeast and complex IV from bovine heart into the cryo-EM map resulted in a pseudo-atomic model of the three-dimensional structure for the supercomplex. The distance between cytochrome c binding sites of complexes III and IV is about 6 nm, which supports proposed channeling of cytochrome c between the individual complexes. The opposing surfaces of complexes III and IV differ considerably from those reported for the bovine heart supercomplex as determined by cryo-EM. A closer association between the individual complex domains at the aqueous membrane interface and larger spaces between the membrane-embedded domains where lipid molecules may reside are also demonstrated. The supercomplex contains about 50 molecules of cardiolipin (CL) with a fatty acid composition identical to that of the inner membrane CL pool, consistent with CL-dependent stabilization of the supercomplex.
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maps obtained by these two approaches. Docking of x-ray crystal structures of individual complexes showed a good agreement using both methods for the interacting surfaces of complexes III and IV and the orientation of complex IV relative to complex III. However, the above organization differs considerably from that for the same supercomplex deduced from a three-dimensional map determined by negative stain EM (23).

*S. cerevisiae* lacks complex I, and the peripheral dehydrogenases are not found associated with respirasome complexes after solubilization from the mitochondrial membrane by detergents (24, 25). The supercomplex, composed of complex III and two complexes IV (III₁IV₂) was previously studied by using negative stain EM (26). Analysis of two-dimensional class averages resulted in a proposed three-dimensional arrangement of the individual complexes in which complex III is flanked on each side by a monomer of complex IV (supercomplex IV₁-III₂-IV₁). The suggested orientation of complexes IV toward complex III in yeast differs considerably from that deduced from the cryo-EM map of the bovine supercomplex (20, 22). Given the differences in the structures of the bovine supercomplex determined by single particle analysis using negative-stain EM versus cryo-EM, a reinvestigation of the yeast supercomplex structure by cryo-EM is warranted. Here we present for the first time a three-dimensional map of a *S. cerevisiae* supercomplex organization obtained by cryo-EM and single particle analysis. Our three-dimensional map determined by cryo-EM reveals more structural details because only a very limited number of views were presented in the negative stain two-dimensional EM class averages (26). In addition, we obtained more data with respect to the transmembrane part of the supercomplex, which was not resolved by negative stain-EM (26). By docking the atomic x-ray structures of complex III from yeast and complex IV from bovine heart into our three-dimensional EM map of the supercomplex, we constructed a pseudo-atomic model of the three-dimensional structure of the supercomplex. Our model in general supports the structure of the yeast supercomplex suggested previously from negative stain data (26). Thus our findings definitely establish that the arrangement of complex IV with respect to complex III in the yeast supercomplex differs significantly from that found in the bovine supercomplex. At the same time our model assumes some specific differences in the interaction of the complexes III and IV compared with atomic model of the yeast supercomplex suggested from negative stain two-dimensional EM class averages. Finally, since the phospholipid CL is required for formation and stabilization of the supercomplex (see above), we also examined the CL species and content in the supercomplex by electrospray ionization mass spectrometry (ESI-MS).

**MATERIALS AND METHODS**

**Yeast Strain and Growth**—*S. cerevisiae* strain USY00b (Mat a, ade2-1, his3-11-15, leu2-3-112, trp1-1, ura3-52, can1R-100, atp2::LEI12, TRP1::ATP2-His₉) was supplied by Dr. David Mueller (Rosalind Franklin University of Medicine and Science, North Chicago, IL) and expresses the F₆,F₈-ATPase β-subunit with a His₉ extension (27). The strain was cultivated in YP-lactate growth medium (pH 5.6) containing 1% yeast extract, 2% peptone, and 3.7% of lactic acid. The cells were harvested (A₆₀₀ of 2.0) by centrifugation and washed with cold TBS buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl).

**Isolation of Mitochondria and Mitoplasts**—Mitochondria were isolated from spheroplasts of yeast cells as previously described (10) except that 3 mg of Zymolase-20T per g of cells were used, and incubation time was 90 min. Isolated mitochondria were further purified by centrifugation at 30,000 rpm (SW41 Ti) at 4 °C for 90 min through a density gradient containing 60, 32, 23, and 15% layers of sucrose in 15 mM Tris-HCl buffer, pH 7.4 and 20 mM KCl. Mitochondria were either used immediately or flash-frozen with liquid N₂ and stored at −80 °C. Mitoplasts (mitochondria without the outer mitochondrial membrane) for phospholipid analysis were prepared from purified mitochondria according to the protocol described in (10).

**Purification of Mitochondrial Supercomplex III₂IV₂**—Isolated mitochondria (8 mg of protein) were suspended in the 1 ml of lysis buffer containing 2% (w/v) digitonin (Invitrogen), 50 mM potassium acetate, 10% glycerol, 1:50 volume Protease Inhibitor mixture set III (Calbiochem), 1.5 mM phenylmethylsulfonyl fluoride, and 30 mM HEPES-KOH (pH 7.4) for 1 h at 4 °C with gentle shaking. After incubation the lysate was centrifuged at 4 °C for 20 min at 45,000 rpm (TLA55 rotor), and the supernatant was incubated with 1 μl of TBS-equilibrated magnetized Cobalt Beads (Dynabeads TALON catalogue 101.02D, Invitrogen) per 2.2 μl of lysate to remove F₆,F₈-ATPase. Incubation of 1 ml of lysate with magnetic beads was performed for 45 min at 4 °C with constant shaking. After incubation the beads were removed using a magnetic separator. The supernatant (1 ml) was immediately transferred to an 8 ml sucrose gradient (0.75 M to 1.5 M sucrose in 15 mM Tris-HCl (pH7.2), 20 mM KCl, and 0.4% digitonin) and centrifuged at 4 °C for 20 h at 30,000 rpm (TLS-55 swinging bucket rotor). Fractions (80–100 μl) from the gradient were analyzed by Blue Native polyacrylamide gel electrophoresis (BN-PAGE) and Western blotting.

**Analysis of the Supercomplex**—Aliquots of fractions obtained after sucrose gradient centrifugation were subjected to BN-PAGE in a 3–12% acrylamide gradient gel in Bis-Tris buffer (Invitrogen) according to manufacturer’s instructions. After electrophoresis gels were either stained with Bio-Safe Coomassie G-250 (Bio-Rad) or used for Western blotting analysis as we described earlier (28) with primary monoclonal antibody (generous gift of Dr. Bernard L. Trumpower, Dartmouth University, Hanover, NH) to the Qcr7 subunit of complex III and secondary anti-mouse antibody (Stabilized Peroxidase Conjugate, Goat anti-mouse, Thermo Fisher). SuperSignal Western Femto Sensitivity kit was used to develop the signal, which was captured using a Fluro-S Max multi imager (29). For Western blotting with primary antibody against yeast cytochrome c (generous gift of Dr. Alexander Tzagoloff, Columbia University, New York, NY) followed by goat anti-rabbit secondary antibody (Stabilized Peroxidase Conjugate, goat anti-rabbit, Thermo Fisher), the purified supercomplex was separated by NuPAGE in a 4–12% acrylamide gradient gel in Bis-Tris buffer with NuPAGE MES SDS Running buffer (Invitrogen) according to the manufacturer’s instructions. Protein concentrations were
determined using the BCA protein assay kit (Thermo Scientific) according to manufacturer’s instructions.

**Measurements of Supercomplex Enzymatic Activity**—Respiratory activity of the purified supercomplex III$_2$IV$_2$ was measured using a Clark oxygen electrode as KCN-sensitive oxygen consumption with NaBH$_4$-reduced decylubiquinone (Sigma), QH$_2$, as substrate at 30 °C. Rates were determined from the slope of a plot of O$_2$ concentration versus time. Supercomplex purified in the sucrose gradient (about 55 µg of protein) was incubated in 50 mM Tris-HCl buffer, pH 7.0, 100 mM KCl, 0.13 mM sucrose, 0.05% digitonin in a final volume of 3 ml. QH$_2$ (40 µM) was added to start the reaction, and oxygen consumption was stopped by addition of 50 µM KCN.

**Phospholipid Extraction from Supercomplex for Mass Spectrometry**—Phospholipids were extracted from the supercomplex and mitoplasts (~20 µg of protein) with a mixture of methanol:chloroform:0.1 n HCl (containing 11 mM ammonium acetate) in a proportion of 1:1:0.9. Samples were vortexed for 1 min and centrifuged at 3000 × g. The organic layer was transferred to a glass tube and dried under a stream of N$_2$. Dried phospholipid samples were resuspended in 100 µl of hexane-isopropanol (30:40, v/v) (30). For quantification of CL using mass spectrometry, 0.8 nmol of 1,1,2,2-$\text{-tetratetramyristoyl cardiolipin (CL (14:1)$_4$}$(Avanti Polar Lipids, Alabaster, AL) was used as a reference and 0.8 nmol of CL ((14:1)$_4$) as a standard. This ratio, along with the slope and intercept of the standard curve made with reference standard CL as described in (30), was used to establish the standard curve. Quantification of CL was done by deposited on freshly glow discharged (2 min) Quantifoil holey carbon grids (West Chester, PA). Prior to glow discharge, the holey carbon grids were coated with a continuous thin carbon film that was prepared by evaporating carbon on a freshly cleaved mica sheet from a graphite rod using a Denton Vacuum DU-502A Evaporator. Grids were rapidly frozen in liquid ethane using a home made gravity-driven plunger apparatus. Cryo-frozen samples were imaged at liquid nitrogen temperature using a Polara 300 kV electron microscope (FEI Company) using a 4K × 4K CCD camera (TVIPS GmbH, Gauting, Germany). Images were recorded at low dose conditions at 3 to 7 µm underfocus and a ×39,000 nominal magnification.

**Data Processing, Map Visualization, and Analysis**—All image processing steps were done using the SPARX software package (31). From 200 micrographs a total of 23,715 particles were manually selected and windowed using e2boxer.py with a pixel size of 4.58 Å on the specimen scale. Class averages were calculated using the Iterative Stable Alignment and Clustering (ISAC) program, from which 252 averages were produced (32). By visual inspection, we selected from this set a subset of 230 plausible views of the supercomplex III$_2$IV$_2$.

An initial model was calculated with the three-dimensional projection matching method using the selected class averages and starting from a template structure constructed using the electron density map obtained from the atomic model of *S. cerevisiae* complex III (PDB accession number 1KYO) and an oblate spheroid as a reference structure, a three-dimensional reconstruction was generated from averages. The horizontal lines indicate the known location of the membrane (MEM) bilayer relative to complex III. The orientation of complex III with respect to the inter-membrane space (IMS) and mitochondrial matrix (MA) is also shown.

**FIGURE 1. Complex III-oblale spheroid reference model.** Beginning with the electron density map of complex III obtained from the atomic model of *S. cerevisiae* complex III (PDB accession number 1KYO) and an oblate spheroid as a reference structure, a three-dimensional reconstruction was generated from averages. The horizontal lines indicate the known location of the membrane (MEM) bilayer relative to complex III. The orientation of complex III with respect to the inter-membrane space (IMS) and mitochondrial matrix (MA) is also shown.
evenly distributed projection directions and the refinement was continued until we obtained a stable three-dimensional structure at a resolution of 24 Å, as determined by the FSC @ 0.5 test (33). During the refinement the power spectrum of the structure was adjusted to match that of the electron density map calculated from the atomic model of yeast cytochrome bc1 complex and low-pass filtered with frequency cut-off calculated from the FSC curve. The atomic models of S. cerevisiae complex III (PDB accession numbers 1KYO or 3CX5, both giving the same results) and bovine complex IV (PDB accession number 1OCC) were used for modeling the supercomplex structure. For the former case the PDB file was modified by removing the antibody fragment chains and, unless otherwise indicated, the cytochrome c (chain w) structure; subunit Qcr10p is not present in the PDB file since it was lost prior to structural determination. Only a monomer of the dimeric bovine complex IV was used while chains K and M not present in yeast were removed. The initial approximate placement of each x-ray structure in the supercomplex structure was done manually based on distinct structural features and subsequently refined using the UCSF Chimera program by maximization of the local correlation coefficient between the two maps (34).

RESULTS

Purification of the III2IV2 Supercomplex—The approach used previously (35) was modified for purification of the active supercomplex III2IV2. Extraction of the supercomplex by digitonin and purification using sucrose gradient centrifugation resulted in contamination of the supercomplex with the F1F1-ATPase dimer, which has a similar molecular mass. To decrease this contamination, the ratio of digitonin to the protein previously used was 6:1 (wt/wt) to induce dissociation of the F1F1-ATPase dimer to monomers. However, these conditions also increased the level of the III2IV supercomplex trimer, which could not be completely separated from the intact supercomplex in the sucrose gradient (35). Several modifications of the published method were made to eliminate the above contaminants thus improving the quality of material subjected to cryo-EM. Strain USY00b expresses a His6-tagged β-subunit of F1F1-ATPase, which was easily removed by incubation of the digitonin extract with cobalt affinity beads. The strain was grown on lactate-containing media with intense aeration, which resulted in high levels of the III2IV2 supercomplex and greatly reduced levels of the III2IV supercomplex trimer due to higher level of expression of complex IV (24). For extraction of the supercomplex, a ratio of digitonin to protein of 2.5:1 (wt/wt) was used, which also reduced the latter contaminant. Purification of the digitonin extract of mitochondria by sucrose gradient centrifugation after removing F1F1-ATPase with cobalt beads resulted in samples containing highly purified III2IV2 supercomplex (Fig. 2A).

It was previously demonstrated that the purified mammalian respirasome contained bound CoQ and cytochrome c (20, 36) and can perform NADH oxidation without addition of any external cytochrome c (36). According to negative stain EM and single particle analysis, some percentage of the yeast supercomplex population of the sample purified using a sucrose gradient appears to contain attached cytochrome c (26). SDS-PAGE followed by Western blot analysis with antibody to cytochrome c confirmed the presence of cytochrome c in our purified supercomplex samples (8 different purifications). Since III2IV2 represents a part of respirasome lacking dehydrogenases, QH2 was used to donate electrons to complex III (Complex III contains bound CoQ). Oxygen consumption upon oxidation of QH2 by the purified supercomplex (Fig. 2B) was recorded without any addition of the external cytochrome c. The process was completely inhibited by 50 μM of KCN, an inhibitor of the complex IV. Activity of the supercomplex sample calculated from the data presented in Fig. 2B was about 240 nmol of O2 min−1 per mg of protein. Taking into account that the concentration of the supercomplex used in this experiment was about 18 μg per ml (or 18 nm) and that cytochrome c was at sub-saturating levels with respect to the supercomplex, we concluded that the recorded reaction was supported by the channeling of cytochrome c between complexes III and IV in the supercomplex and represents activity of the fraction of supercomplex containing bound cytochrome c. Activity of the supercomplex upon
addition of 50 μM of cytochrome c was about 1 μmol of O₂
min⁻¹ per mg of protein, which is comparable with published
results (24).

EM and Image Processing—A total of 23,715 particle images
of the purified active III₄IV₂ supercomplex were manually
selected from 200 micrographs and a total of 252 class averages
were calculated from which 230 averages were selected; see Fig.
2 for a typical micrograph. An initial model for projection
image-based three-dimensional refinement was determined
using an approach based on calculating a three-dimensional
map from the 230 selected averages, for which the three-di-
imensional projection-matching strategy with enforcement of
C2 symmetry was employed (37). The reference structure for
the procedure was constructed to avoid introducing any bias in
the orientation between complexes III and IV in the recon-
structed map. Toward this end, we obtained the reference
structure by combining the electron density map of complex III
converted from the atomic model of yeast cytochrome bc₁ com-
plex and an oblate spheroid as described in “Materials and
Methods” (Fig. 1). Furthermore, to determine the self-consis-
tency and reproducibility of the three-dimensional maps com-
puted by this approach, we made ten noise-corrupted models of
the oblate spheroid model. Each of the ten noisy models was
used as the initial template for three-dimensional projection
alignment of the set of 230 selected averages. The resulting ten
three-dimensional maps were similar demonstrating that ori-
entation between complexes III and IV was reliably established
using this approach. Using the initial model as a starting struc-
ture and a subset of projection images (see “Materials and
Methods” for details), a three-dimensional map (Fig. 3, A–D)
with a resolution of 24 Å was obtained.

A pseudo-atomic model of the three-dimensional structure of
the supercomplex was constructed by superimposing the
x-ray structures of complex III (S. cerevisiae, PDB 3CX5) and
complex IV (bovine, PDB 1OCC, monomer) (Fig. 4). The pro-
truding densities at the top center of the supercomplex corre-
spond to the position of Cor1 and Cor2 subunits in complex III
(Figs. 4A and 5A). Our three-dimensional map (Fig. 4) and the
derived pseudo-atomic model (Fig. 5) confirm the previously
suggested overall organization of the S. cerevisiae supercom-
plex, which was based on an arrangement inferred from two-
dimensional class averages obtained by processing of negative
stain data (26). The main finding was that each of the two indivi-
dual complexes IV interacts with complex III through convex
surface of IV, which is opposite to the interaction surface
between the cytochrome c oxidase monomers in the dimer of
complex IV present in the crystal structure (38). However, the
three-dimensional structure obtained by cryo-EM suggests
some differences in orientation of IV relative to III (Fig. 5) com-
pared with conclusions based on the two-dimensional work
(26). The distance between cytochrome c binding sites on cyto-
chrome c₁ subunit of complex III and subunit II of complex IV
is about 6 nm (Fig. 5) versus 4.0 nm as previously reported. This
larger distance would still enable channeling of cytochrome c
between these sites in the supercomplex. Complexes III and IV
are closer to each other at the aqueous-membrane interface
exposed to the mitochondrial inter-membrane space, while
larger spacing between III and IV that is seen in the hydropho-
bic lipid bilayer is consistent with what is observed in the bovine
supercomplex (20, 22). This “gap” (spacing) is sufficiently spa-
cious for insertion of lipids between the individual complexes in
the supercomplex.

Determination and Quantification of CL in the Super-
complex—The phospholipid CL is required for formation of
the stable III₄IV₂ supercomplex (10, 14, 15) and therefore
should be associated with the supercomplex. We determined
the number of CL molecules and their fatty acid species distri-
bution in the supercomplex. CL associated with the II₄IV₂
supercomplex showed the same proportion between the five
major CL species (with values of singly ionized CL of m/z 1,344,
1,372, 1,400, 1,428, and 1,456) as CL found in mitoplasts (rep-
resenting the inner mitochondrial membrane) (Fig. 6). These
numbers correspond to CL species with acyl chains (16:1)_{4}, (16:1)_{3}, (18:1)_{1}, (16:1)_{2} (18:1)_{2}, and (18:1)_{4}, respectively. Thus the major CL species contain only monounsaturated 16:1 and 18:1 acyl chains, which corresponds to published results for yeast mitochondria (39). Total CL was determined as the sum of these five prevalent species. By using CL (14:0)_{4} as an internal standard and CL (18:1)_{4} as a reference standard (see “Materials and Methods”) 40–60 mol of CL per mol of the yeast supercomplex were determined in two independent experiments.

DISCUSSION

Analysis of the yeast supercomplex reported here began with more highly purified samples in which the supercomplex trimer lacking one complex IV and the FoF1-ATPase dimer, significantly present in the earlier report from yeast (26), were minimized. This improvement allowed us to collect enough particle images in different orientations to reconstruct a three-dimensional cryo-EM map of the supercomplex. The final three-dimensional structure shows that the orientation of complex IV relative to complex III is similar but not identical to that inferred from the analysis of the two-dimensional negative stain EM averages of yeast data (26). One complex IV flanks each side of complex III, and the convex side opposite to the complex IV dimer interface found in the x-ray structure opposes complex III. However, we established reliably that there are major differences between the yeast and bovine supercomplex in the orientation of complex III relative to complex IV.

The differences between the organization of the bovine and yeast respirasomes most likely stem from the necessity in mammals of complex III to interact with both complexes I and IV. In yeast where no complex I exists, two complex IV monomers flank complex III. Absence in yeast of a subunit homologous to VIIIa, which is located at the interface between complexes III and IV in the bovine supercomplex, is probably relevant to the differences between yeast and bovine in the mutual orientation of the individual complexes. Within the yeast supercomplex the closest distance between the individual complexes is between complex III subunit Qcr6p and a domain of complex IV subunit IV (corresponds to Cox5ap in yeast), which are exposed to the inter-membrane space (Fig. 5). In the both yeast and bovine there are larger spaces between subunits of complexes III and subunits of complex IV in the hydrophobic bilayer, and these “gaps” are most likely filled with lipids (see below).

The distance reported here between cytochrome c binding sites on cytochrome c_{1} subunit of complex III and subunit II
(Cox2p) of complex IV, which contains the first electron acceptor (CuA cofactor) of complex IV, is about 6 nm (Figs. 5 and 7A). This is a longer distance than that (about 4 nm) estimated from the pseudo-atomic model of the supercomplex created from negative stain data (26). However, the larger distance is still consistent with the possibility of direct channeling of cytochrome c between its respective binding sites on complexes III and IV. Our measurement of the KCN-dependent oxygen consumption by the supercomplex upon oxidation of QH₂ without addition of any external cytochrome c supports this possibility.

Previously, oxygen consumption without external cytochrome c was demonstrated with isolated respirasomes from mitochondria of mouse fibroblast cells (36), which is also consistent with channeling of cytochrome c. In the bovine supercomplex the distances between the cytochrome c binding site in complex IV and cytochrome c binding sites on each cytochromes c₁ monomers of complex III are estimated to be about 10–11 nm (20, 22) (Fig. 7B). Interestingly, one group considers this relatively long distance as an indication that no channeling of cytochrome c takes place (22), while another group, taking into account the diameter of cytochrome c of about 3.4 nm, proposes direct channeling of cytochrome c between complexes III and IV is still possible (20).
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FIGURE 8. Hypothetical channeling of cytochrome c between complexes III and IV in the yeast supercomplex. Part of the density map of the supercomplex showing the protrusion (indicated by black arrow) to the right of cytochrome c (red) (chain w of complex III, PDB 3CX5). Complex III is in light yellow except for Qcr6p in light blue. Complex IV is in magenta except with subunit II (Cox2p in yeast) in dark blue, subunit IV (Cox5ap in yeast) in bright yellow. Cu3, the first electron acceptor in complex IV, is indicated. The inset shows the same protrusion (black arrow) from a different angle.

In the discussion of the structure of S. cerevisiae supercomplex obtained by negative stain, the authors suggested that some particles contain bound cytochrome c and that its “fuzzy” appearance in the two-dimensional class average might be due to flexibility upon binding resulting in an intermediate position of cytochrome c between its respective binding sites on complex III and complex IV. They also suggested that in order to mediate electron transfer, cytochrome c would have to rotate and move between these complexes (26). Subunits like the hinge protein (Qcr6p) of complex III, which was found to be essential for a binding of cytochrome c to complex III, can be important for transfer of cytochrome c from complex III to complex IV (26). Our structural findings and activity measurements are in a good agreement with these suggestions. Interestingly, our three-dimensional density map has a protrusion on the surface of the supercomplex facing the inter-membrane space at the position between cytochrome c binding sites on complexes III and IV in the close contact with Qcr6p (Fig. 8). The dimensions of the protrusion are close to the dimensions of cytochrome c. Further experiments are required to establish whether the indicated protrusion represents cytochrome c attached to the supercomplex and how cytochrome c channeling occurs in the supercomplex.

We found about 50 molecules of CL per molecule of the supercomplex. Our results are consistent with the finding that CL is required for formation and stabilization of the S. cerevisiae III2IV2 supercomplex (10, 14, 15). Tightly bound CL in the crystal structure of the yeast complex III (CL denoted as CN3 in PDB 3CX5 and shown in Fig. 5) was demonstrated to be important for supercomplex stability (16). Although this tightly bound CL is necessary for supercomplex formation, it is probably not sufficient for stable supercomplex formation. There are “gaps” between complex III and complex IV in the supercomplex residing in the hydrophobic bilayer of the membrane, which are most likely filled with lipid including CL. Reconstituation of the supercomplex from purified complex III (which contains only tightly bound CL) and complex IV requires the addition of CL.6 The bovine respirasome contains 200–400 molecules of CL (20). The space between complexes III and IV in yeast is smaller than inter-complex space in the bovine respirasome and the large complex I might contain many CL molecules (20, 22). Thus the lower numbers for CL molecules in the yeast complex is not surprising. CL accumulated in the space between complexes in the supercomplex might play a role in triggering supercomplex dissociation under conditions where CL levels are reduced by either growth conditions or peroxidation. The definite elucidation of CL-dependent dynamics of the supercomplex organization will require further studies.

Recent reports indicate that in addition to CL two related proteins, Rcf1 and Rcf2, are also important for supercomplex formation and stability (42–44). These are small proteins of about 20 kDa containing two transmembrane domains. Rcf1 has a predicted long coiled coil extramembrane domain, which may not contribute significant electron density. On the other hand, we cannot exclude the possibility that several Rcf1 proteins can provide a connection between individual complexes through an interaction of their coiled coil domains exposed to the inter-membrane space. Since digitonin extracted supercomplex contains these proteins (42–44), they must be present in our purified supercomplex and for example may be responsible for the protrusion exposed to the inter-membrane space. Further structural studies are required to identify the location of the Rcf proteins in the supercomplex structure.

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REFERENCES
1. Schägger, H. (2001) Respiratory chain supercomplexes. IUBMB Life 52, 119–128
2. Stuart, R. A. (2008) Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria. J. Bioenerg. Biomembr. 40, 411–417
3. Wittig, I., and Schägger, H. (2009) Supramolecular organization of ATP synthase and respiratory chain in mitochondrial membranes. Biochim. Biophys. Acta 1787, 672–680
4. Lenaz, G., and Genova, M. L. (2010) Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. Antioxid. Redox Signal. 12, 961–1008
5. Dudkina, N. V., Kouril, R., Peters, K., Braun, H. P., and Boekema, E. J. (2010) Structure and function of mitochondrial supercomplexes. Biochim. Biophys. Acta 1797, 664–670
6. Yamashita, T., Nakamaru-Ogiso, E., Miyoshi, H., Matsumo-Yagi, A., and Yagi, T. (2007) Roles of bound quinone in the single subunit NADH-quinone oxidoreductase (Ndi1) from Saccharomyces cerevisiae. J. Biol.

6 S. Bazan, E. Milejkovskaya, and W. Dowhan, personnel communication.
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Chem. 282, 6012–6020

7. Piccoli, C., Scriba, R., Boffoli, D., and Capitanio, N. (2006) Control by cytochrome c oxidase of the cellular oxidative phosphorylation system depends on the mitochondrial energy state. *Biochem. J.* 396, 573–583

8. Lenaz, G., and Genova, M. L. (2009) Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. *Int J Biochem. Cell Biol.* 41, 1750–1772

9. Boumans, H., Grivell, L. A., and Berden, J. A. (1998) The respiratory chain in yeast behaves as a single functional unit. *J. Biol. Chem.* 273, 4872–4877

10. Zhang, M., Mileykovskaya, E., and Dowhan, W. (2005) Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. *J. Biol. Chem.* 280, 29403–29408

11. Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) Specific roles of protein-phospholipid interactions in the yeast cytochrome bc$_1$ complex structure. *EMBO J.* 20, 6591–6600

12. Paldottir, H., and Hunte, C. (2004) Lipids in membrane protein structures. *Biochim. Biophys. Acta* 1666, 2–18

13. Shinzawa-Itoh, K., Aoyama, H., Muramoto, K., Terada, H., Kurauchi, T., Inoue, N., Uchida, N., and Yoshikawa, S. (2012) Mitochondrial respiratory chain supercomplexes are destabilized in Barth Syndrome patients. *Proc. Natl. Acad. Sci. U.S.A.* 109, 283, 1363–1373

14. Vukotic, M., Oeljeklaus, S., Wiese, S., Vögtle, F. N., Meisinger, C., Meyer, A. H. E., Zieseniss, A., Katschinski, D. M., Jans, D. C., Jakobs, S., Warscheid, B., Rehling, P., and Deckers, M. (2012) Rcf1 mediates cytochrome oxidase supercomplex assembly and respirasome formation, revealing heterogeneity of the enzyme complex. *Cell Metab.* 15, 336–347

15. Chen, Y. C., Taylor, E. B., Dephoure, N., Heo, J. M., Tonhato, A., Pandrourdou, I., Nath, N., Denko, N. C., Gygi, S. P., and Rutter, J. (2012) Identification of a protein mediating respiratory supercomplex stability. *Cell Metab.* 15, 348–360

27. Mueller, D. M., Puri, N., Kabaleeswaran, V., Terry, C., Leslie, A. G., and Walker, J. E. (2004) Ni-chelate-affinity purification and crystallization of the yeast mitochondrial F1-ATPase. *Protein Expr. Purif.* 37, 479–485