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Conserved in situ arrangement of complex I and III₂ in mitochondrial respiratory chain supercomplexes of mammals, yeast, and plants

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We used electron cryo-tomography and subtomogram averaging to investigate the structure of complex I and its supramolecular assemblies in the inner mitochondrial membrane of mammals, fungi, and plants. Tomographic volumes containing complex I were averaged at ~4 nm resolution. Principal component analysis indicated that ~60% of complex I formed a supercomplex with dimeric complex III, while ~40% were not associated with other respiratory chain complexes. The mutual arrangement of complex I and III₂ was essentially conserved in all supercomplexes investigated. In addition, up to two copies of monomeric complex IV were associated with the complex I,III₂ assembly in bovine heart and the yeast Yarrowia lipolytica, but their positions varied. No complex IV was detected in the respiratory supercomplex of the plant Asparagus officinalis. Instead, an ~4.5-nm globular protein density was observed on the matrix side of the complex I membrane arm, which we assign to γ-carbonic anhydrase. Our results demonstrate that respiratory chain supercomplexes in situ have a conserved core of complex I and III₂, but otherwise their stoichiometry and structure varies. The conserved features of supercomplex assemblies indicate an important role in respiratory electron transfer.

Significance

Oxygenic phosphorylation entails the transfer of electrons from organic substrates to molecular oxygen by four large protein complexes in the mitochondrial inner membrane. Loss of electrons during this process can produce toxic side products and must be prevented. Three of the electron-transfer complexes form supercomplexes, which are thought to be instrumental in channeling electrons from the substrate to the acceptor. We used electron cryo-tomography and subtomogram averaging to determine the in situ structure and organization of the respiratory chain supercomplexes in three different eukaryotic lineages. We discovered that the mutual arrangement of the two largest components—complex I and complex III—is essentially the same in all supercomplexes, indicating that this arrangement is important for electron transfer.

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The authors declare no conflict of interest.

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relevance. To address these questions, we applied cryo-ET and subtomogram averaging to mitochondrial membranes of three different eukaryotic lineages. We show that, in all three cases, complex I forms a conserved assembly with dimeric complex III. In mammals and fungi, up to two copies of monomeric complex IV are attached to the I,II2 core, but there appears to be no complex IV in the plant supercomplex. Principal component analysis indicated that a variety of supercomplexes can coexist in the same membrane. Our results demonstrate that supercomplexes are prevalent in the inner mitochondrial membrane, but their structure and composition varies between species and even within one species. Their conserved structural features signify an important role in respiratory electron transfer.

Results
Complex I Is Visible in Tomographic Volumes. Respiratory chain supercomplexes were visualized in situ by cryo-ET of mitochondrial membranes from bovine heart, the yeast Y. lipolytica, and the plant Asparagus officinalis. To improve contrast, matrix proteins of bovine and yeast mitochondria were removed by osmotic shock, while asparagus mitochondria underwent spontaneous disruption. Membrane fragments were resuspended in buffer before cryo-EM grid preparation.

Lipid bilayer membranes were clearly visible in the tomographic volumes, and individual ATP synthase and complex I molecules in the inner membrane were easily identified by their shape (Fig. 1 and Fig. S1). When viewed parallel to the membrane, ATP synthase had the characteristic lollipop appearance of a 10-nm spherical head connected to the membrane by a thin 5-nm stalk (29–31), whereas the matrix arm of complex I had an angular, rhomboid profile (Fig. SL4) (31). Parallel slices at a distance of 10 nm above the membrane surface indicated a near-hexagonal shape for the ATP synthase F1 head and a bar-shaped density for the complex I matrix arm (Fig. S1R), as expected from the cross-sections and density envelopes of ATP synthase and complex I structures determined by subtomogram averaging or single-particle cryo-EM, Fourier-filtered to ~25 Å resolution (Fig. S1 C–F). In tomographic slices at this level, only densities that were consistent with either the ATP synthase F1 head or the complex I matrix arm were observed. Mitochondrial ATP synthases form double rows in the inner membrane (Fig. 1 A–C) (30, 31), whereas the densities assigned to complex I are randomly distributed in flat membrane regions (Fig. 1 D–F). Complex I and ATP synthase can thus be distinguished unambiguously in electron cryo-tomograms of inner mitochondrial membranes.

Detergent-Solubilized Mammalian Supercomplex.

The in Situ Structure of the Bovine Respirasome Closely Resembles the Detergent-Solubilized Mammalian Supercomplex. Four different assemblies of respiratory chain complexes were observed in the inner membrane of bovine heart mitochondria (Fig. 2). About 44% of the subvolumes contained a single copy of complex I (I1); 16% contained one complex I and one complex III dimer (I1,III); 30% contained one complex I, one complex III, and one complex IV (I1,III,IV); and 10% contained one complex I, one complex III, and two separate complex IV monomers (I1,III,IVa,b). The I1,III,IV supercomplex is thus the most common respiratory chain supercomplex in the mammalian inner mitochondrial membrane under our conditions. The in situ structure of component complexes in the I1,III,IV supercomplex is very similar to the detergent-solubilized mammalian I1,III,IV supercomplex (Fig. S4) determined by single-particle cryo-EM (24–28). The single-particle structures indicate some variation in the positions of complex III and complex IV relative to complex I. This is most notable for complex IV, which has
been suggested to adopt a tight conformation in which complex IV is more closely appressed to complex III<sub>2</sub>, or a loose conformation, in which complex IV is up to 12 Å further removed from the I<sub>1</sub>III<sub>2</sub> (25). Overlays with the single-particle maps suggest that the in situ assembly is most similar to the loose conformation (Fig. S4).

**Yeast and Mammalian Supercomplexes Have the Same Stoichiometry but Different Structures.** Since the widely studied yeast Saccharomyces cerevisiae lacks a conventional complex I (33), we investigated the membranes of the obligate aerobic yeast Y. lipolytica. The structure of the Y. lipolytica complex I has been determined by X-ray crystallography (9, 13) and single-particle cryo-EM (11).

As with bovine mitochondria, several different arrangements of respiratory chain complexes were found in Y. lipolytica (Fig. 3). These included (i) complex I by itself (I<sub>1</sub>, 40%); (ii) complex I plus a complex III dimer (I<sub>1</sub>III<sub>2</sub>, 13%); (iii) complex I with complex III<sub>2</sub> and one complex IV monomer (I<sub>1</sub>III<sub>2</sub>IV<sub>c</sub>, 14%); and (iv) complex I, complex III<sub>2</sub>, and two separate complex IV monomers (I<sub>1</sub>III<sub>2</sub>IV<sub>c</sub>cd, 30%). The latter form is thus the most common supercomplex in Y. lipolytica. A fifth group containing complex I, complex III<sub>2</sub>, and the complex IV monomer (I<sub>1</sub>III<sub>2</sub>IV<sub>c</sub>) was detected with low frequency (<3%).

The location of complex III<sub>2</sub> relative to complex I was the same as in bovine mitochondria, but the position of the complex IV monomers varied. In particular, no complex IV was found in the position most commonly observed in the bovine heart supercomplexes (Fig. S4). This position (IV<sub>c</sub>) coincides with the second complex-IV–binding site (IV<sub>c</sub>) in the bovine I<sub>1</sub>III<sub>2</sub>IV<sub>c</sub> supercomplex (Fig. 2D).

In the supercomplexes, complex IV can interact with complex I or complex III<sub>2</sub>. The fit of component complexes to the subtomogram average volumes shows which subunits interact in each case. Interactions with complex I always appeared to involve subunit 7A of complex IV and either ND5 or the 39-kDa subunit of complex I. The complex III<sub>2</sub>–interactions appeared to involve subunits cox7A and 8B of complex IV and regions of subunits QCR9, QCR8, or UCR1 of complex III<sub>2</sub> (Fig. 5). Distances between component complexes were larger than would be expected for direct protein contacts (C<sub>B</sub>-C<sub>P</sub> of 10–20 Å), suggesting that some interactions are mediated by membrane lipid. None of the complex-IV–containing respirasomes contained a complex IV dimer as in the crystal structures of bovine complex IV (7, 16, 17).

**Plant Respirasomes Do Not Contain Complex IV.** PCA classification of subvolumes from the inner mitochondrial membrane of the plant A. officinalis indicated that 42% contained a single copy of complex I, whereas the remaining 58% also contained a complex III<sub>2</sub> dimer. Complex III<sub>2</sub> occupied essentially the same position as in the bovine and Y. lipolytica supercomplexes but was rotated away from the complex I matrix arm by about 10°. No density resembling complex IV was observed in any of the plant subvolumes.

A conspicuous difference to the bovine and yeast respirasomes is a 4.5-nm, roughly spherical density on the matrix side of the plant complex I membrane arm. This density is at the interface of complex I subunits ND2/4 above the supernumerary subunits ESSS, MNLL, B14.5b, and KYFI (bovine heart nomenclature) (Fig. 4). Proteomic analysis suggests that plant complex I contains 49 subunits, of which 40 are homologous to the bovine enzyme (14). Of the nine plant-specific subunits, five are predicted to encode subunits of γ-carbonic anhydrase, an integral and vital part of plant complex I (34, 35). X-ray structures show that γ-carbonic anhydrases are homotrimers measuring roughly 4 × 4.5 nm (36). The atomic model of a γ-carbonic anhydrase fits the spherical 4.5-nm matrix density well. The other plant-specific complex I subunits include two 11 kDa proteins (P1 and P2), a 15–18 kDa translocase-like subunit, and a 68-kDa membrane-anchored l-galactono-1,4-lactone dehydrogenase (GLDH). These are either not associated with mature plant complex I, as is the case for GLDH, or are too small to account for the 4.5-nm density (14, 37). We conclude that the additional matrix density observed in the subtomogram averages of the plant complex I is γ-carbonic anhydrase.

**The In Situ Arrangement of Complexes I and III<sub>2</sub> Is Conserved Across Phyla.** Aligning the various subtomogram averages to complex I indicated that the relative position of I and III<sub>2</sub> in respiratory chain supercomplexes of mammals, fungi, and plants is essentially unchanged (Fig. 5). High-resolution single-particle maps of detergent-solubilized mammalian respirasomes implicate complex I subunits B14.7 and B22 (bovine nomenclature) and complex III subunits QCR9, QCR8, and UCR1 (24, 25, 38) in supercomplex LHII<sub>2</sub> formation and stability. Interactions between these subunits or their fungal and plant equivalents are consistent with our subtomogram averages. When atomic models of the respiratory chain complexes are fitted to the maps, these subunits are next to one another.
Comparison of supercomplexes from three eukaryotic lineages. (A) Subtomogram averages of bovine heart, *Y. lipolytica*, and *A. officinalis* supercomplexes were aligned on complex I volumes. (B) Schematic overlay of complex positions. The relative positions of complexes I and III, in all three species are similar, but III, is rotated clockwise by 12° in *A. officinalis* (A.o.) as seen from the matrix. Supercomplexes of *A. officinalis* contain no visible complex IV but a density consistent with γ-carbonic anhydrase (CA), a known component of plant complex I. Both bovine heart and *Y. lipolytica* supercomplexes contain up to two complex IV monomers. The position of IV, in *Y. lipolytica* coincides with the less frequently observed IV, position in bovine heart. Pink, *B. taurus* (B.t.); blue, *Y. lipolytica* (Y.L.); green, *A. officinalis* (A.o.). (Scale bar: 5 nm.)

(Fig. S6), indicating that the I,III, interactions are conserved throughout the three eukaryotic lineages.

**Discussion**

**Diversity of Respiratory Chain Supercomplexes.** Respiratory chain supercomplexes were first identified by nondenaturing BN-PAGE of mitochondrial membrane extracts (20). Using the mild detergent Triton X-100 and digitonin, Schägger and coworkers (20) observed several high-molecular-weight gel bands, which showed activity for complex I, complex III, and complex IV.

Cryo-ET and subtomogram averaging indicate several types of respiratory chain supercomplexes in the inner mitochondrial membranes of mammals, yeasts, and plants. The bovine subtomogram averages contain clear density for two copies of complex IV with occupancies of 40% for the primary IV, and 10% for the secondary IV, site, proving that supercomplexes I,III, IV, and I,III, IV, exist in the inner membrane. BN-PAGE suggested that respirasomes of bovine heart mitochondria contain up to four complex IV monomers (20). In the gels, the most common supercomplexes were I,III, II, IV, and I,III, IV, as in our subtomogram averages.

Single-particle cryo-EM structures of detergent-solubilized mammalian I,III, IV, supercomplexes have been determined independently by four different groups (24, 25, 27, 28, 38) at medium-to-high resolution. All contain one copy of complex I and one complex III dimer. The relative orientations of complexes I and III in these structures are similar to those in our subtomogram averages (Fig. S4).

The only exception is the recent 17-Å map of a respiratory chain supercomplex from digitonin-solubilized human mitochondria (39), which appears to contain two copies of complex I on either side of a central complex III dimer. Supercomplexes with more than one copy of complex I would be conspicuous in our tomographic volumes, but none were identified.

As in bovine mitochondria, supercomplex I,III, IV, was the largest and most prevalent assembly in blue-native gels of *Y. lipolytica* mitochondria, whereas in plants the only apparent supercomplex was I,III, (40, 41). Small amounts of complex IV have been observed by BN-PAGE, but only in freshly harvested potato tubers (42). All these biochemical results correlate closely with our subtomogram averages, indicating that BN-PAGE is an effective and reliable method for analyzing the supercomplex composition of mitochondrial membranes. The respiratory chain supercomplexes were found mostly in flat regions of the inner mitochondrial membrane (Fig. 1). They appeared to be randomly distributed, with the closest distances ranging from 15 nm up to more than 100 nm (Fig. S7).

**Structural Variability of Respirasomes.** Comparison of cryo-EM structures of the mammalian supercomplex I,III, IV, at sub-nanometer resolution has indicated some degree of variability in the relative positions of the three components in the detergent-extracted supercomplex. Complex III can be twisted by 25° relative to complex I (24), and complex IV can form either a tight or loose association with complex III, (25). The more loosely assembled complexes were suggested to be artifacts of purification, as they were not abundant or their abundance increased with time, suggesting gradual dissociation in detergent (24, 25). However, the loose conformation most closely resembles that of respirasomes in the membrane. The slightly tighter packing of component complexes in isolated supercomplexes may reflect the removal of lipids by detergent upon isolation.

**Subunit Contacts.** Detailed comparisons of subtomogram average volumes to single-particle cryo-EM structures reveal which subunits establish contacts between component complexes. Interactions involve subunits B14.7 and B22 of complex I and QCR8 of complex III, Homologs for all three subunits are found in mammals, fungi, and plants (14), consistent with our observation that the I,III, interaction is conserved in the three eukaryotic lineages.

Recent proteomic and mutational analysis suggested that cox7A2L (also known as the supercomplex assembly factor 1, or SCAF1) is essential for the interaction of complexes III, and IV in mammals (18). Homologs of cox7A2L are present in yeasts, but a Blast search (43) did not identify a plant homolog. The absence of cox7A2L in plants would explain why the *A. officinalis* supercomplex does not contain complex IV.

Osmotic shock, as applied to the bovine heart and *Y. lipolytica* membranes that we examined, is unlikely to affect subunit contacts in the supercomplexes, as these interactions are mediated primarily by hydrophobic protein surfaces within the lipid bilayer, or by lipids. Neither would be particularly sensitive to changes in ionic strength or the concentration of osmolytes, which would act primarily on the membrane-exposed, hydrophilic parts of the complex. A well-documented example is the interaction between ATP synthase dimer rows. If the weak interactions between these dimer rows is less strong and specific than the interaction between the membrane is fragmented by osmotic shock (46, 47). The dimer rows persist, irrespective of whether the inner membrane is fragmented by osmotic shock (Fig. 1A and B) or under isotonic conditions (Fig. 1C). Although the rows tend to be shorter (because in intact mitochondria they are longer than the membrane fragments), their structure appears to be unchanged.

The interaction between ATP synthase dimers in the dimer rows is less strong and specific than the interaction between the components of respiratory chain supercomplexes. This is indicated by (i) the variable interdimer distance (30, 31), which suggests weak interactions, and (ii) the defined structure of supercomplexes, which can be isolated by detergent solubilization, whereas this is not the case for ATP synthase dimer rows. If the weak interactions between ATP synthase dimers persist, osmotic shock will not affect the stronger interactions in the supercomplexes.

Our present study shows that the interface between complex I and III, in the supercomplexes from bovine heart, *Yarrowia*, and plants is essentially the same, whether or not the mitochondria were osmotically shocked (Fig. 5). This supports our conclusion that osmotic shock has no effect on the supercomplex structure.

**Complex IV Dimers.** All X-ray structures of bovine complex IV (7, 16, 17) show a dimer, whereas there are no complex IV dimers in any of
our supercomplex volumes. Apparently, dimer formation depends on the presence of subunits CoxA2 and CoxA1 in complex IV. If CoxA1 and CoxA2 (or SCA1) are present instead, this results in the formation of complex-IV-containing supercomplexes (18). As CoxA2 is an isoform of CoxA2, and CoxA1 an isoform of CoxA2, complex IV could contain only one of each. The isoforms thus determine whether complex IV assembles into dimers or supercomplexes. Most likely complex IV dimers and monomers that are not part of a supercomplex coexist in the membrane, but both would be too small to be easily detected in our tomographic volumes.

**Role in Electron Transfer.** The evidence in support of mitochondrial respiratory chain supercomplexes in detergent solution is unambiguous, and we now show that the supercomplexes are ubiquitous in the mitochondrial inner membrane. However, their functional significance has not been clear. For many years, a role in translocation and force generation in mitochondrial ATP synthase. However, there appears to be no need for such a conduit, provided that the quinol-binding sites are close enough. Ubiquinol reduced by complex I can diffuse freely within the lipid bilayer, but the close proximity and mutual orientation of the binding sites would ensure that it is trapped by complex III, as soon as a binding site becomes available. The observed functional asymmetry of isolated bovine respirasomes (24) suggests that, of the two monomers in the complex III dimer, only one is active. In the single-particle structure (24), the active monomer faces the complex I quinol site, enhancing the efficiency of electron transfer in the supercomplex.

The fact that the mutual arrangement of complexes I and III2 is essentially conserved in organisms as diverse as yeasts, mammals, and plants argues strongly for a role in the conduction of reduced quinol. Thus, the main function of supercomplexes in the mitochondrial inner membrane may be to avoid the loss of reducing power by maintaining the close proximity and favorable orientation of the key respiratory chain components. This would ensure that ubiquinol diffusion does not become rate-limiting in the crowded mitochondrial inner membrane (48, 49).

**Methods**

**Isolation of Mitochondria.** Bovine heart mitochondria were isolated in 0.25 M sucrose and 0.1 M Tris-HCl, pH 7.4 (50, 51). Yeast mitochondria were isolated from Y. lipolytica strain E129 in 0.6 M sorbitol and 0.1 M Tris HCl, pH 7.4 (31). White asparagus shoots were purchased at a local supermarket and ground in a Waring blender in 400 mM mannitol, 25 mM Mops–KOH, pH 7.8, 10 mM EDTA, 10 mM DTT, 1% (wt/vol) PVP-40, and 0.01% (wt/vol) BSA (fatty acid-free), with added protease inhibitors. Mitochondria were isolated, fragmented, and washed in 400 mM mannitol, 10 mM Mops–KOH, pH 7.4, and 1 mM EDTA essentially as described for potato tubers (31), except that a self-forming Percoll density gradient (28%) was used instead of a step gradient.

**Sample Preparation for Cryo-ET.** For bovine heart and Yarrowia, 20 μL of isolated mitochondria at a concentration of 4–5 mg/mL was diluted with 25 vol of osmotic shock buffer (10 mM Tris, pH 7.8). Asparagus mitochondria were diluted with 25 vol of SEM buffer (250 mM sucrose, 10 mM Mops–KOH, pH 7.2, 1 mM EDTA). Membrane fragments were pelleted by centrifugation (13,000 × g at 4 °C for 20 min) and resuspended in 15 μL of buffer. The suspension was mixed 1:1 with Fiducial markers (6- or 10-nm gold particles conjugated to protein A; Aurion) before being applied to glow-discharged quartz grids (R2/2) and plunged-frozen in liquid ethane using a home-made guillotine (52).

**Data Collection.** Tomograms were collected as described (52) using a Krios transmission electron microscope (FEI) operating at 300 kV in corotated mode. Single-axis tilt series (±60°, 1.5° intervals, start angle of 24°) were recorded on a K2 summit detector with postcolumn energy filter (Gatan Quantum 976) at a tilt width of 20 e-°. Nominal magnification in FETEM mode was 33,000 (0.43 nm/pixel) for Y. lipolytica and 44,000 (0.33 nm/pixel) for bovine and asparagus membranes. Exposure times were adjusted automatically during tilt series acquisition to maintain a constant dose of 8 e-°/px. The total dose per tilt series was kept below 80 e-°/Å2. All tilt series were collected at 4° to 6° μm defocus with the automatic data collection program Latitude (Gatan).

**Tomogram Reconstruction and Subtomogram Averaging.** Tilt series were aligned and tomograms were reconstructed using IMOD (53). Contrast was enhanced by nonlinear anisotropic diffusion (54). Subvolumes containing complex I were extracted from the unfiltered tomogram and aligned in PEET (55) using pre-defined angles calculated from the vector describing the position of the peripheral arm in relation to the membrane. Initial alignment was restricted to ±180° about the axis of the peripheral arm, using a single subvolume as initial reference. Subsequent alignments were performed with the degree of rotation relative to the peripheral arm restricted to ±20°. The average from each round of refinement was used as the reference for the next round. All references were masked to contain only one complex I peripheral arm density. For the bovine supercomplex, 324 subvolumes were averaged: 316 for Yarrowia, and 8 for Asparagus. Principal component analysis (PCA) was performed on all volumes using a mask around the expected position of complex III, or complex IV. Resolution was estimated by Fourier shell correlation on two half-sets after alignment. Subtomogram averages were filtered to reduce noise with the median filter (IMOD, ref. 53) or the Ferrini Fourier filter [SPIDER (56)] (Fig. 5B). All molecular fits and figures were produced in Chimera (57).

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