In the inner mitochondrial membrane, the respiratory chain complexes generate an electrochemical proton gradient, which is utilized to synthesize most of the cellular ATP. According to an increasing number of biochemical studies, these complexes are assembled into supercomplexes. However, little is known about the architecture of the proposed multicomplex assemblies. Here, we report on the electron microscopic characterization of the two respiratory chain supercomplexes I,II,III and I,II,III,IV in bovine heart mitochondria, which are also two major supercomplexes in human mitochondria. After purification and demonstration of enzymatic activity, their structures in projection were determined by single particle image analysis. A difference map between the supercomplexes I,II,III and I,II,III,IV closely fits the x-ray structure of monomeric complex IV and shows its location in the assembly. By comparing different views of supercomplex I,II,III,IV, the location and mutual arrangement of complex I and the complex III dimer are discussed. Detailed knowledge of the architecture of the active supercomplexes is a prerequisite for a deeper understanding of energy conversion by mitochondria in mammals.

All living organisms use a series of integral membrane protein complexes for energy conversion and ATP synthesis. In eukaryotes, electrons are transported by the respiratory chain, starting from NADH via complex I (NADH:ubiquinone oxidoreductase) or from succinate via complex II (succinate:ubiquinone oxidoreductase), the membrane integral electron carrier ubiquinol, complex III (ubiquinol:cytochrome c oxidoreductase), the peripheral electron carrier cytochrome c, and complex IV (cytochrome c oxidase) to the terminal acceptor molecular oxygen (1). The electron transport chain generates a proton gradient across the inner mitochondrial membrane, which is used by complex V (F_{0}F_{1}-ATP synthase) to synthesize ATP. In the last decade, structures of the individual respiratory chain complexes from various organisms have been determined. Atomic models exist for bovine heart mitochondrial complex III (2) and IV (3). A high resolution structure of complex I is not yet available, but electron microscopy indicates that it is L-shaped in all organisms investigated, and a 2.2-nm resolution map from cryo-electron microscopy exists for the bovine heart complex I (4).

Two alternative models for the arrangement of the respiratory chain complexes in the membrane have been proposed. According to the currently favored random collision model (5), all components of the respiratory chain diffuse individually in the membrane, and electron transfer depends on the random, transient encounter of the individual protein complexes and the smaller electron carriers. In the solid state model (6) proposed 50 years ago, the substrate is channeled directly from one enzyme to the next. Recently isolated stoichiometric assemblies, so-called supercomplexes, support this model. Respiratory supercomplexes of different compositions have been described in bacteria (e.g. Paracoccus denitrificans (7)) and in mitochondria from Saccharomyces cerevisiae (8, 9), other fungi (10), higher plants (11–13), and mammals (8, 14–16) by means of blue native polyacrylamide gel electrophoresis (BN-PAGE), gel filtration and immunoprecipitation. In these studies, supercomplexes of various stoichiometries have been detected, such as assemblies of monomeric complex I (1), dimeric complex III (11), and complex IV in various copy numbers (IV_{n}). A crucial function of these respiratory supercomplexes may be the stabilization of the individual complexes (7, 10, 15, 17, 18). Kinetic evidence by inhibitor-titration studies in bovine heart mitochondria is consistent with both models for the arrangement of the respiratory chain complexes and suggests their coexistence (19).

Recently, an electron projection map of a plant supercomplex consisting of only complex I and dimeric complex III (11) has been presented for Arabidopsis thaliana (20). But so far, the supramolecular architecture of a stoichiometrically defined supercomplex comprising all three complexes (I, III, and IV) has not been determined. By BN-PAGE analysis, the most abundant supercomplexes in bovine heart mitochondria are a supercomplex consisting of complex I and dimeric III and another supercomplex containing complexes I and dimeric III and IV with molecular masses of ~1,500 kDa and 1,700 kDa, respectively (8, 14). By electron microscopy, we could image both supercomplexes from bovine heart mitochondria and localized complex IV unambiguously in the larger assembly.

**EXPERIMENTAL PROCEDURES**

**Bovine Heart Mitochondria Preparation and Supercomplex Isolation**—Bovine heart mitochondria were isolated (16, 21), frozen in liquid nitrogen, and stored at ~80 °C. Solubilization with 1% (w/v) digitonin (Calbiochem, high purity) was done according to Refs. 8, 10, 13, and 16 in buffer containing 150 mM KOAc, 30 mM HEPES, 10% (w/v) glycerol, 0.5 mM Pefabloc \textsuperscript{TM} SC, pH 7.4, at 4 °C but at a ratio of 28 g of detergent/1 g of protein. To the digitonin extract, sample buffer was added containing 5% (w/v) Coomassie G-250, 500 mM e-aminoacaproic acid, 50 mM bis-tris, pH 7.0, to obtain a detergent-to-dye ratio of 4 (w/w).

BN-PAGE was performed with linear gradient gels of a polyacrylamide concentration (T) gradient of 3–13 or 3–5%, overlaid with 3%
stacking gels using buffers with 25 mM imidazole, 500 mM e-
aminocaproic acid, 0–20% (w/v) glycerol, pH 7.0, based on Ref. 22. Gel
electrophoresis was performed in buffer containing 7.5 mM imidazole, 50
mM tricine, 0.02–0.002% (w/v) Coomassie G-250, pH 7.0 (cathode), and
25 mM imidazole, pH 7.0 (anode).

Protein bands were cut out of the gel and electroeluted in buffer (25 mM
tricine, 7.5 mM bis-tris, 25 mM e-aminocaproic acid, pH 7.0) at 4 °C, 75 V,
and 2 mA for at least 12 h according to a modified version of Refs. 23 and 24.
Afterward, 0.1% (w/v) digitonin was added to prevent protein aggregation.

Matrix-assisted Laser Desorption Ionization-Mass Spectrometry
(MALDI-MS)—For MALDI-MS analysis, proteins were separated by
SDS-PAGE on a 14% polyacrylamide gel at room temperature and sil-
ver-stained. Protein bands were cut out, subjected to in-gel trypsiniza-
tion, and identified by MALDI time-of-flight mass spectrometry
(Applied Biosystems Voyager DE PRO) as described previously (25).

Enzymatic Analysis—NADH dehydrogenase activity of complex I
was determined by in-gel formazan precipitation in 100 mM Tris, 768
mM glycine, 0.04% (w/v) 4-nitro blue tetrazolium chloride, 100 μM
β-NADH, pH 7.4, according to a modified version of Refs. 10 and 26.
The cytochrome c oxidase activity of complex IV was visualized by
precipitation of 3,3′-diaminobenzidine oxides and indamine polymers
in test buffer containing 50 mM sodium phosphate, 0.5 mg/ml 3,3′-
diaminobenzidine-tetrahydrochloride, 0.5 mg/ml cytochrome c (horse
heart), 20 units/ml catalase, and 75 mg/ml sucrose, pH 7.4 (10, 26, 27,
28). Spectrophotometric activity assays of complexes I and III
were performed by a procedure modified from Ref. 8 at 20 °C in 150 mM
NaCl, 75 mM imidazole, 200 μM β-NADH, 750 μM decylubiquinone, pH
7.4, but with 0.1% (w/v) digitonin. NADH-ubiquinol reductase activity
of complex I was measured by the rotenone (50 μM)-sensitive oxidation
of NADH (200 μM; ε 6.2 mM⁻¹ cm⁻¹) at a wavelength of 340 nm. The
cytochrome c reductase activity of complex III was determined by the
antimycin (5 μM)-sensitive cytochrome c reduction (70 μM; ε 21.1
mM⁻¹ cm⁻¹) at a wavelength of 550 nm. 10 mM KCN was added to
inhibit cytochrome c oxidation by complex IV. To eliminate buffer-de-
dendent effects during the measurements, the test buffer was incubated
without protein, and the absorptions were observed. For the actual tests,
the buffers with supercomplexes and substrates were observed at the
given wavelengths (340 or 550 nm) for 1–3 min. The absorptions
decreased linearly, and the decrease/time unit was determined. After-
ward, the inhibitors rotenone or antimycin, respectively, were added.
The decrease in absorption with the inhibitor was less than that without
and was subtracted from the absorption before inhibition to determine
the protein-dependent absorption change. All tests were performed
three times, and the S.D. was determined.

Electron Microscopy and Single Particle Analysis—The super-
complex samples were negatively stained with 1% (w/v) uranyl acetate, pH
4. Negative staining with 2% (w/v) ammonium molybdate, pH 6.9, was
also carried out and showed equivalent results. Electron micrographs
were collected using a Philips CM120 at 120 kV under low dose condi-
tions at a magnification of 45,000× on Kodak SO-163 electron image
film. The negatives were checked by optical diffraction for correct de-
focus and lack of drift and astigmatism and digitized on a PhotoScan
scanner (Z/I Imaging, Aalen, Germany) at a pixel size of 7 μm, corre-
sponding to 1.56 Å on the specimen. Subsequently, adjacent pixels were
averaged to yield a pixel size of 4.67 Å. The images were processed using
Imagic V (Image Science Software GmbH, Berlin, Germany).

RESULTS

To structurally characterize the supercomplexes, bovine heart mito-
chondria were solubilized with 1% (w/v) digitonin at a ratio of 28 g of
digitonin/1 g of protein. Afterward, the solubilizate was separated by
BN-PAGE. The protein pattern of respiratory chain complexes resem-
bles that in previous reports (8, 14). It shows the individual complexes
I–V, as well as two prominent supercomplex bands, namely I₁III₂ (con-
sisting of one complex I and a dimer of complex III; 1,500 kDa) and
I₁III₂IV₁ (with one additional complex IV; 1,700 kDa) and some minor
supercomplex bands of higher molecular masses (Fig. 1, a and b). Solu-
bilization was performed at a detergent-to-protein ratio far above the
2–4 g of digitonin/1 g of protein needed for quantitative extraction of
the respiratory chain complexes (8, 16). This was done to obtain I₁III₂
and I₁III₂IV₁ as the two predominant supercomplex species (Fig. 1, a
and b) while minimizing the amounts of complex V dimers, which
because of their comparable mass (~1,500 kDa) would comigrate with
I₁III₂. In addition, sharper protein bands were obtained. The employed
detergent-to-protein ratio thus facilitated the isolation of the super-
complexes I₁III₂ and I₁III₂IV₁.

For biochemical and structural characterization, supercomplexes
I₁III₂ and I₁III₂IV₁ were excised from the gel and electroeluted. The integrity of the purified supercomplexes was verified by an additional
BN-PAGE (Fig. 1c) to detect supercomplex fragments, which might
arise during electroelution. Supercomplex I₁III₂IV₁ remained intact

FIGURE 1. Isolation and activity assay of supercomplexes I₁III₂ and I₁III₂IV₁. a–d, BN-
PAGE (polyacrylamide concentration gradient T, 3–13%) of solubilized bovine heart
mitochondria. b, BN-PAGE (T, 3–5%) of solubilized bovine heart mitochondria. Both
supercomplex bands were in close proximity in a BN-PAGE (T, 3–13%). The separation
was improved on a BN-PAGE with T at 3–5%, c, BN-PAGE (T, 3–13%) of isolated super-
complexes I₁III₂ and I₁III₂IV₁. Supercomplex I₁III₂IV₁ (lane 2) is intact after electroelution,
but the supercomplex I₁III₂ sample (lane 1) shows two additional very weak bands of I
and III. The BN gels a–c were Coomassie R-250-stained. d, BNH dehydrogenase
activity of complex I. Supercomplexes I₁III₂ and I₁III₂IV₁, as well as complex I of solubilized
bovine heart mitochondria (lane 1), isolated I₁III₂ and I₁III₂IV₁ (lane 2) show in-gel NADH
dehydrogenase activity, e, cytochrome c oxidase activity of complex IV. Supercomplex I₁III₂IV₁
(from solubilized bovine heart mitochondria (lane 1) and isolated I₁III₂IV₁ (lane 3) have in-gel
cytochrome c oxidase activity, whereas I₁III₂ (lane 2) lacking complex IV does not. The
position of I₁III₂ (in lane 2) is indicated by a faint blue band resulting from residual Coom-
assie dye of the BN-PAGE bound to the protein.
after isolation, whereas in the supercomplex I\textsubscript{1}III\textsubscript{2} sample, two minor bands representing complexes I (\(\sim 1,000\) kDa) and III\textsubscript{2} (500 kDa) were present (Fig. 1c). The composition of both supercomplexes was confirmed by peptide mass fingerprinting. In total, 20 subunits of the integrated complexes were identified, \textit{e.g.}, the 49- and 30-kDa subunits from complex I, core protein II from complex III, and subunit IV from complex IV (data not shown).

The enzymatic activity of each supercomplex component was tested to confirm their functional integrity after isolation. NADH dehydrogenase activity of complex I and cytochrome c oxidase activity of complex IV, respectively, were visualized by in-gel activity staining. NADH dehydrogenase activity of complex I was displayed by purple bands resulting from in-gel formazan precipitation (10, 26). The cytochrome c oxidase activity of complex IV was determined by the cytochrome c-dependent oxidation of 3,3′-diaminobenzidine to brown oxide and indamine precipitates (10, 27, 28). Thus, complex I activity for supercomplexes I\textsubscript{1}III\textsubscript{2} and I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1}, as well as complex IV activity for supercomplex I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1} in the isolated samples, were demonstrated (Fig. 1, a and c). Spectroscopic activity assays using specific inhibitors were employed to assess NADH:ubiquinol reductase activity of complex I and cytochrome c reductase activity of complex III in the electroeluted samples. For the latter activity, no in-gel assay exists (28). Under the conditions used, complex I in supercomplex I\textsubscript{1}III\textsubscript{2} displayed about half the activity of that in supercomplex I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1} (Table 1). Complex III was active in supercomplex I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1}, but supercomplex I\textsubscript{1}II\textsubscript{3} showed only minor cytochrome c reductase activity (Table 1). In conclusion, both isolated supercomplexes displayed activity, but supercomplex I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1} was significantly more active.

For electron microscopy, the purified supercomplexes I\textsubscript{1}III\textsubscript{2} and I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1} were negatively stained with 1% uranyl acetate (Fig. 2). Electron micrographs of both supercomplex samples showed predominantly triangular particles measuring 30–33 nm on the longest side (Fig. 2). In addition, smaller particles were seen in the I\textsubscript{1}III\textsubscript{2} sample (Fig. 2a). As there are no other proteins present in the sample, the smaller particles might be individual complex III dimer arising from partial disintegration of the supercomplex. This observation is consistent with the minor supercomplex I\textsubscript{1}III\textsubscript{2} dissociation seen on BN-PAGE (Fig. 1c). Another less frequent view on the micrographs is an L-shaped particle (Fig. 2). It can be concluded that the two views represent the two preferential orientations of the supercomplexes on the carbon support film with opposite sides attached to the film. Apparently, one orientation is preferred over the other.

Another typical view was observed less frequently than the triangular views (Fig. 2). This was characterized by a bright stain-excluding line of
complex I, III2, nor IV has a noticeable domain exposed to the intermembrane space (3), which cannot be identified in the present side view. Neither dimer (2) (Figs. 4a–c) nor mirror images (average of 66 particles). c, difference map of I,III2,IV1 minus I,III2, d, top view (from the matrix space) of I,III2,IV1. e, side view of supercomplex I,III2,IV1 (average of 70 particles). To get the correct side view from a top view (a), the carbon film would be on the bottom of the top view (red line). To derive the two top views (a and d) from the side view (e), the film would be on the bottom (red line) or on the top (green line) of the side view, respectively. Because the red and the green lines are not parallel in e, the top views in a and d are not mirror images. I, top view of the bovine complex IV x-ray structure as seen from the intermembrane space (green, Protein Data Bank (PDB) accession code 1OCC). The scale bar represents 10 nm.

Because the asymmetric nature of the supercomplex, the two top views are not exactly 180° rotated relative to each other and thus do not form mirror images. By comparing the top and side views of supercomplex I,III2,IV1, the matrix arm of complex I can be identified in the top views as a bright stain-excluding density at the sharpest corner of the triangle (Fig. 4, a and d). In the side view, the distal domains of the complex I matrix arm extend beyond the membrane arm (Fig. 4e). This is visible in the top views as a diffuse extension (Fig. 4; a, b, and d).

To localize complex IV in the I,III2,IV1 projections, the smaller supercomplex I,III2 was compared with the larger one. ~1,400 images were selected and analyzed. The great majority showed triangular views, similar to the I,III2,IV1 top views but with two sides of approximately the same length (Fig. 4b). A difference map of I,III2,IV1 minus I,III2 displays a density of ~13 × 7 nm (Fig. 4c) at the side opposite the complex I matrix arm. This density matches closely the projection of a complex IV monomer viewed from the intermembrane space toward the matrix (Figs. 4f and 5, d, g, and h). It is worth noting that the complex IV monomer interacts with the other components of the supercomplex (Fig. 5) through the interface, which is the dimer interface in the x-ray structure (3).

In the top views, complex IV and the matrix arm of complex I could be assigned unambiguously. Two densities remain that each would fit the membrane arm of complexes I or III2 (Fig. 5, g and h). The top view two-dimensional projections were calculated from the published three-dimensional data (2) of complex III dimer and filtered to 3.0 nm. They were cross-correlated with the two densities in the supercomplex where III2 might be located. The cross-correlation coefficients for the upper density in the supercomplex and III2 were ~0.55–0.6, whereas the ones for the lower density with III2 were ~0.7. Thus complex III2 viewed from the intermembrane side has a higher similarity with the lower density in the supercomplex top view. However, as the cross-correlation coefficients for the two locations differ only by 10–15%, neither location of I and III2 in the top view can be ruled out at present (Fig. 5, g and h).

**DISCUSSION**

The two respiratory chain supercomplexes I,III2 and I,III2IV1 from bovine heart mitochondria were isolated in an active state and were...
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structurally characterized by transmission electron microscopy. Our results show that the two digitonin-solubilized supercomplexes I,III$_2$ and I,III$_2$IV$_1$ are structurally related to one another, I,III$_2$ being a building block of the larger supercomplex I,III$_2$IV$_1$. The structures we found for the two supercomplexes studied confirm their stoichiometries of I,III$_2$ and I,III$_2$IV$_1$, as deduced from BN-PAGE (8, 14, 16). Mitochondrial complex III forms a dimer in the crystal structures of the bovine (2), chicken (31), and yeast (32) enzymes. The crystal structures suggest that the complex is functional as a dimer, which is supported by data showing that the two monomers differ in their cytochrome $c$ binding properties and their ubiquinone reduction sites (33) and that electrons are transferred between equivalent hemes in the two monomers (34). The fact that a complex III dimer is seen in the supercomplexes of both bovine heart (this work) and plant mitochondria (20) strongly supports the existence of a functional dimer for complex III. In addition, our finding of well defined structures for the supercomplexes supports biochemical data showing that complex III dimer is essential for assembly/stability of complex I (7, 10, 15, 17, 18).

Bovine heart complex IV is also dimeric in the crystal structure (3) but is clearly monomeric in the supercomplex (Fig. 4, c and f). Complex IV is associated with the rest of the supercomplex through the concave face, which is the dimer interface in the x-ray structure. No functional role of complex IV dimerization has been suggested, and our finding of a single copy in the supercomplex suggests a structural rather than a functional role of dimer formation. The crystal dimer contact in the 13-subit complex IV is not very extensive and is formed by both copies of subunit VIb interacting with each other on the intermembrane space side and by the N terminus of subunit VIa interacting with subunit III of the other monomer near the matrix interface. Between these contact sites, the two monomers are separated by 8–10 Å, enough space to accommodate lipids. It is conceivable that dimer formation competes with supercomplex formation using the same contact interface. The existence of larger supercomplexes in bovine heart mitochondria, which differ only in the copy number of complex IV from 1 to 4 (8, 14), suggests that the association of complex IV is rather promiscuous. However, the abundance and high stability of I,III$_2$IV$_1$ indicates that the association of complex IV dimerization has been suggested, and our finding of both approaches are under way.

Our data confirm the stoichiometries of I,III$_2$ and I,III$_2$IV$_1$ that were proposed based on biochemical data (8, 14, 16). In addition these data suggest that the supercomplex I,III$_2$IV$_1$ is a major physiological module of the respiratory chain in mammalian mitochondria.

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