Significance of Respirasomes for the Assembly/Stability of Human Respiratory Chain Complex I*

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Hermann Schägger§, René de Coo§, Matthias F. Bauer||, Sabine Hofmann**, Catherine Godinot‡‡, and Ulrich Brandt‡

From the §Institut für Biochemie I, Zentrum der Biologischen Chemie, Fachbereich Medizin, Universität Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany, the †Department of Neurology, Erasmus University Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, the Institute of Clinical Chemistry, Molecular Diagnostics and Mitochondrial Genetics, Academic Hospital Munich-Schwabing, 80804 Munich, Germany, the ¶Diabetes Research Institute, Academic Hospital Munich-Schwabing, 80804 Munich, Germany, and the ‡Center of Molecular and Cell Genetics, Unit 5534 of the CNRS and of the University Lyon-1, 69622 Villeurbanne, France

We showed that the human respiratory chain is organized in supramolecular assemblies of respiratory chain complexes, the respirasomes. The mitochondrial complexes I (NADH dehydrogenase) and III (cytochrome c reductase) form a stable core respirasome to which complex IV (cytochrome c oxidase) can also bind. An analysis of the state of respirasomes in patients with an isolated deficiency of single complexes provided evidence that the formation of respirasomes is essential for the assembly/stability of complex I, the major entry point of respiratory chain substrates. Genetic alterations leading to a loss of complex III prevented respirasome formation and led to the secondary loss of complex I. Therefore, primary complex III assembly deficiencies presented as combined complex III/IV defects. This dependence of complex I assembly/stability on respirasome formation has important implications for the diagnosis of mitochondrial respiratory chain disorders.

The mammalian system of oxidative phosphorylation comprises four mitochondrial respiratory chain complexes, NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV). These enzyme complexes couple the oxidation of NADH and FADH₂ to vectorial proton transport across the mitochondrial membrane. F₂F₅-ATP synthase (complex V) then uses the resulting electrochemical potential to synthesize ATP from ADP and inorganic phosphate. Oxidative phosphorylation complexes from bovine mitochondria are functionally active when isolated as individual complexes. This had been one of the major reasons why a “liquid state” model of the respiratory chain allowing free complexes to diffuse laterally and independently of one another was generally favored (reviewed in Ref. 1). Another major reason was the pioneering work of Hackenbrock and co-workers (2, 3) who demonstrated that the concentration of free-diffusing components in the inner mitochondrial membrane and the collision rate between them is enough to explain the observed respiratory rates in mitochondria.

However, recent data favor a “solid state” model with permanently associated complexes. Cruciat et al. (4) and Schägger and Pfeiffer (5) isolated a stoichiometrically assembled respiratory chain supercomplexes from yeast and mammalian mitochondria with high yield. In bovine mitochondria almost all complex I was found associated with dimeric complex III. Additional association of complex IV with this core structure seemed to be more detergent-sensitive, eventually leading to the isolation of different supercomplexes with a variable copy number of complex IV. The largest of these respirasomes containing four copies of complex IV has been proposed to constitute a complete respirasome (5).

The assembly into respirasomes has been suggested to have a number of potential advantages compared with individual complexes, e.g. substrate channeling of quinones and/or cytochrome c, sequestration of reactive intermediates like ubisemiquinone, and stabilization of individual complexes by supramolecular assembly (5). However, experimental evidence confirming or dismissing specific functions is just beginning to emerge.

Substrate channeling directs the mobile electron carriers ubiquinone and cytochrome c directly to the next enzyme rather than allowing competition with other enzymes or exchange with substrate pools and is required for electron flow at low substrate reduction levels. Recently, substrate channeling of ubiquinone between complexes I and III has been experimentally verified for bovine mitochondria by determining flux control ratios (6). Sequestration of the reactive intermediate ubisemiquinone is essential, because ubisemiquinone can react with oxygen to generate superoxide anion radical, which is involved in the pathogenicity of mitochondrial disorders (7), but a potential requirement of stable respirasomes for sequestration of ubisemiquinone has not been verified experimentally so far. Recently we have shown structural stabilization of a bacterial complex I by assembly in a complete respirasome in Paracoccus denitrificans (8). A similar role of respirasomes for the stability of individual complexes in mammals would have considerable implications for human respiratory chain disorders, especially for the etiology of combined deficiencies of multiple complexes in mitochondrial encephalomyopathies.

Toward a molecular understanding of the structural and functional roles of respirasome formation and the etiology of mitochondrial neuromuscular disorders, the focus of the present work was to analyze the structural integrity of human respirasomes in patients with defined defects of individual respiratory chain complexes. Here, we present evidence for an...
human respiratory chain supercomplexes.

EXPERIMENTAL PROCEDURES

Isolation of mitochondria from human skeletal muscle, solubilization of mitochondrial membranes by detergents, blue-native (BN) PAGE, two-dimensional SDS–PAGE, staining, and densitometric quantification of individual oxidative phosphorylation complexes in two-dimensional gels was performed essentially as described (10) with minor modifications. Briefly, 10 mg of skeletal muscle (wet weight, stored at −80 °C) was homogenized in 0.5 ml of sucrose buffer (250 mM sucrose, 20 mM sodium-phosphate, pH 7.2) using a tightly fitting homogenizer. Following a 10-min centrifugation at 25,000 × g the mitochondria-containing sediment was suspended with 20 μl of low salt buffer (50 mM NaCl, 50 mM imidazole, pH 7.0) and solubilized with 7.5 μl of dodecylmaltoside (10%) for solubilization of individual respiratory chain complexes or with 7.5 μl of digitonin (20%) for solubilization of respiratory chain supercomplexes. Water-soluble digitonin from Fluka (catalog number 37006, >50% purity) was used directly without recrystallization. Following a 15-min centrifugation at 100,000 × g, Coomassie G250 dye was added to the supernatant (7.5 μl from a 5% stock in 750 mM 6-aminoheptanoic acid), and the total volume was applied to 0.5 × 0.15 cm sample wells for BN-PAGE.

Low digitonin/protein ratios (~0.1 g/g) have been used to prepare mitoplasts (11, 12), whereas the solubilization of oxidative phosphorylation complexes from bovine mitochondria starts with a 10-fold higher ratio (~1 g/g). The solubilization and recovery of all complexes after BN-PAGE is optimal and near quantitative with 3–8 g digitonin/g of protein (5, 13). For convenience, solubilization of crude human mitochondrial sediments was optimized for the wet weight of the skeletal muscle specimens without using protein-determination methods. To make solubilization largely independent of experimental variation, we added digitonin in large excess. Thus standard digitonin addition, as described above, is far above the quantities required for near quantitative solubilization of oxidative phosphorylation complexes. Reducing the digitonin amounts to 50 or 25% of the standard addition led to a comparable recovery of all complexes after BN-PAGE except that the amount of dimeric complex V increased from hardly detectable to considerable quantities (~15% of monomeric complex V; data not shown).

RESULTS AND DISCUSSION

Conventional BN-PAGE using dodecylmaltoside for solubilization of mitochondria resolves individual respiratory chain complexes (10). SDS-PAGE in a second dimension then separates the subunits as shown for control skeletal muscle in Fig. 1A. To elucidate the structural organization of the human respiratory chain from skeletal muscle of control individuals and patients with defined defects of individual complexes we modified the protocol for BN-PAGE using the mild detergent digitonin for membrane protein solubilization, because this detergent has been shown to retain respiratory chain supercomplexes from yeast and bovine mitochondria (4, 5).

In the skeletal muscle of control individuals two supercomplexes were present, a smaller complex (Fig. 1B, S) with an apparent mass of ~1.5 MDa and a larger one (L) with estimated 1.7 MDa. These supercomplexes differ in their composition. Both contained monomeric complex I and dimeric complex III (immunological identification not shown). Complex L contained one copy of complex IV in addition. Respiratory chain complexes II, III, and IV, and the F1F0-ATP synthase (complex V) were also found as individual complexes under the conditions of BN-PAGE. All human complex I was exclusively assembled into supercomplexes.

We first analyzed the structural and functional consequences of the loss of complex IV on the composition and integrity of the supercomplexes L and S. Patient P1 harbored a genetic alteration in the SURF1 gene (homoyzogous 758del2bp, patient 12 in Ref. 14) associated with classical Leigh syndrome and severe complex IV deficiency. This patient showed normal amounts of individual complexes except for complex IV that was selectively reduced to 10% (Table I). A considerable fraction of this 10% residual complex IV was stably assembled into supercomplex L (Fig. 2A), but the ratio of complexes L and S was altered compared with the control (Fig. 1B). In the control, the staining intensity of complex S was less than that of complex L, whereas the intensities were inverted for the patient. This indicated that the loss of complex IV reduced the amounts of supercomplex L and correspondingly increased stable supercomplex S. However, it did not influence the steady-state levels of complexes I and III as quantified in two-dimensional gels using dodecylmaltoside for solubilization (Table I). Thus, complexes I and III form the stable core of respirasomes to which complex IV can bind also.

Next, we investigated the fate of the individual respiratory chain complexes in patients with a primary defect located in complex I or complex III. It can be assumed that loss of either complex I or complex III leads to the absence of the stable core structure. In a patient (P2) carrying an isolated deficiency of complex I the supercomplexes S and L and individual complex I were absent. Complexes III, IV, and V were found in normal amounts as individual complexes (Fig. 2B and Table I). Thus, the presence of fully assembled complex I is not essential for the assembly and stability of complexes III, IV, and V.

Isolated complex III deficiencies can be caused by mutations in cytochrome b, the only mitochondrial encoded subunit of complex III. A heteroplasmic G290D mutation in the cytochrome b gene of patient P3 (80% mutated mtDNA in muscle) was previously found to be associated with impaired assembly of complex III and progressive exercise intolerance (15–17). In another patient, P4, a 4-base-pair deletion generating truncated cytochrome b (85% mutated mtDNA in muscle) was associated with Parkinsonism/MELAS overlap syndrome (18). In both patients, complex III was severely reduced or even below the detection limit; complexes IV and V were found at normal levels (Fig. 2, C and D and Table I). Dimeric complex V was detected in Fig. 2C, although the dimeric form was not found for other patients. This is explained by the variation of the actual digitonin/protein ratio because of the preparation of mitochondrial sediments in different laboratories. It does not affect the recovery of oxidative phosphorylation complexes (see
complex V (VDIM into supercomplex L. Reasons for the unusual observation of dimeric P2 with an isolated complex I deficiency.

Subunit staining intensities were normalized to the complex V α subunit to normalize for the mitochondria content.

| Patient | Genetic defect | Complex I | Complex III | Complex IV |
|---------|----------------|-----------|-------------|------------|
| P1      | SURF1          | %         | %           | %          |
| P2      | Unknown        | <20       | 126         | 91         |
| P3      | Cytochrome b   | <20       | <15         | 75         |
| P4      | Cytochrome b   | <20       | <15         | 79         |

Controls (n = 5): 100 ± 23; 100 ± 8; 100 ± 10.

Fig. 2. Analysis of mitochondrial complexes from patients muscle with specific genetic alterations. Assignment of complexes I–V, L, S, and individual subunits are as described in the legend to Fig. 1. The 47-kDa band for the core proteins of complex III is assigned also. Digitonin was used for BN-PAGE in A–C, and dodecylmaltoside for D. All gels were silver-stained. A, patient P1 with a mutation in the SURF1 gene associated with isolated complex IV deficiency. B, patient P2 with an isolated complex I deficiency. C, patient P3 with an assembly deficiency of complex III and a parallel deficiency of complex I. Minimal amounts of residual complexes I and III were found assembled into supercomplex L. Reasons for the unusual observation of dimeric complex V (Vdim) with this patient are discussed under “Results and Discussion.” D, patient P4 with an assembly deficiency of complex III. Complex III subunits (arrows) were considerably reduced. Complex I was below the limit of detection.

Fig. 3. Immunological detection of complex I subunits. Dodecylmaltoside was used for the separation of individual mitochondrial complexes by BN-PAGE, followed by two-dimensional SDS-PAGE for resolution of the subunits. The positions of complexes I–V (I–V) in BN-PAGE are indicated. A polyclonal antibody against bovine complex I detected the 75- and 51-kDa subunits of complex I as indicated. A, two-dimensional blot for a control individual (comparable with Fig. 1). The 75-kDa subunit is detected at four locations (marked I–IV), as discussed under “Results and Discussion.” B, two-dimensional blot for patient 4 (duplicate gel of what is shown in Fig. 2) with overall reduced signal intensities.

complex I was used for the detection of complex I subunits on Western blots (Fig. 3) for a control individual (comparable with Fig. 1A) and for patient 4 (duplicate gel of Fig. 2D). On the control blot (Fig. 3A) we found the largest 75-kDa subunit of complex I at four different locations. 1) Highest signal intensity was found at the location of complex I (Fig. 3A, I). 2) A signal close to the size of complex III (Fig. 3A, 2) indicated the presence of a subcomplex of complex I with an apparent mass of ~500 kDa. The presence of a subcomplex, and not an oligomeric form of the 75-kDa subunit, is evident from the additional detection of a weak signal for the 51-kDa subunit. At present it is not known if this subcomplex represents a stable assembly intermediate or is dissociated from complex I under the conditions of BN-PAGE. 3) A potential subcomplex (Fig. 3A, 3) showed an apparent mass of ~400 kDa. 4) A spot close to the running front (Fig. 3A, 4) presumably represented the individual 75-kDa subunit. Overall reduced signal intensities on the blot for the patient (Fig. 3B) indicated reduced amounts of complex I, which is in accordance with the densitometric data given in Table I, and suggested that complex I has not been stably assembled and thus was rapidly degraded.

These data implied that complex III defects that involve not just catalytic activity but also the assembly of the multiprotein complex will always present as combined deficiencies of complex III and I in humans and should not present as isolated enzyme deficiencies. To confirm this hypothesis, we screened skeletal muscle biopsies of more than 100 patients with neuromuscular disorders by two-dimensional gel analysis for the assembly of the respiratory chain complexes. More than 30 patients with multiple deficiencies of two or more complexes, 9 with isolated complex I deficiencies, 9 with isolated complex IV deficiencies, and 4 with isolated complex V deficiencies were identified. Thus, an individual loss of complex I, or complex IV, or complex V appears not to affect assembly/stability of the other complexes in general. We found two combined deficiencies of complex III/I (patients P3 and P4). However, we could not find any isolated complex III deficiency suggesting that defects in the correct assembly of complex III may always cause combined deficiencies of complexes III and I.

Surprisingly, the parallel severe reduction of the steady-state levels of assembled complexes III and I in patients P3 and P4, as based on the two-dimensional gel analysis (Table I), is not stringently mirrored by enzymatic activities determined in the respective muscle tissues. The catalytic activities of complex III were significantly reduced in patients P3 and P4 (21 and 9% of the control average, respectively; Table II, No. 1 and 2). Thus, these enzymatic measurements are in good accord-
Catalytic activities of the patients and the control range (in parentheses) are expressed as the percent of the average control activity. Complex III, decylbenzoquinon:cyanochrome c reductase activity; Complex II+III and I+III, succinate and NADH:cyanochrome c reductase, respectively; Complex I, NADH:decelbenzoquinone reductase; aa, amino acids.

| No. | Cytochrome b mutation | Refs | Complex III | Complex II+III | Complex I+III | Complex I |
|-----|-----------------------|------|-------------|---------------|--------------|-----------|
| 1   | G290D                 | 15, 17, and this work P3 | 21 (50–150) | 9 (60–140) | 53 (35–165) |           |
| 2   | 4-bp deletion, loss of 330 aa | 18, and this work P4 | 25 (64–136) | 10 (no range) | 30 (no range) |           |
| 3   | Q352X, loss of 29 aa   | 26   | 27 (64–136) | 10 (no range) | 30 (no range) |           |
| 4   | G142X, loss of 238 aa  | 26   | No data    | 14 (67–133) | Normal       |           |
| 5   | G190X, loss of 190 aa  | 27   | No data    | 14 (67–133) | Normal       |           |
| 6   | C353X, loss of 41 aa   | 28   | Depressed polarographic oxidation rates with NADH and flavoprotein-dependent substrates indicating complex III deficiency | Histochemistry and immunonochemical but no enzymatic data |
| 7   | W326X, loss of 55 aa   | 29, patient 5 | No data | 54 (67–153)* | 43 (67–133)* | 76 (81–119)* |
| 8   | G166X, loss of 215 aa  | 30   | No data    | 12 (85–115)* | 7 (83–137)* | 60 (78–122)* |
| 9   | 24-bp deletion, loss of 8 aa | 29, patient 1 | No data | 9 (67–133)* | 5 (67–133)* | 87 (81–119)* |
| 10  | W112X, loss of 287 aa  | 29, patient 4, 31 |           |           |           |           |
| 11  | W141X, loss of 238 aa  | 29, patient 3 |           |           |           |           |

* Mean range given by S.D.

From the large number of cytochrome b mutations we selected nonsense mutations, because missense mutations were rarely analyzed for complex III assembly, and a normally assembled non-functional complex III is not expected to influence assembly/stability of complex I. From nine cases with nonsense mutations in the cytochrome b gene (Table II, Nos. 3–11), two have been classified as combined deficiencies of complexes III and I as detected by enzymatic means (Nos. 3 and 4). With five cases (Table II, Nos. 5–9) that have been classified as isolated complex III deficiencies, a parallel reduction of complex III and complex I activities seems not to be convincingly excluded. A deficiency of complex III was documented using various analytic techniques, but complex I activity was not determined or described as normal without giving data, or complexes III and I were comparably reduced (Table II, No. 9). The data for two cases (Table II, Nos. 10 and 11) seemed not to be in accordance with our proposal that an assembly deficiency of complex III is relevant for this study. Despite a severe reduction at the protein level, complex I activities might appear near normal. This specific aspect has to be considered when diagnosing complex III deficiencies. Isolated complex III deficiency does occur with functional deficiencies of complex III that do not affect assembly of complex III.

Combined complex III/I defects associated with alterations in the NDUFS4 protein of complex I have been observed, and explanations for this combined deficiency have been presented (19). It has been suggested that the truncated NDUFS4 protein may interfere with the formation of supercomplexes thereby leading to a combined deficiency of complexes I and III, or enhanced radical production caused by complex I deficiency may have a secondary influence on complex III activity. Further studies to obtain a definite explanation for these specific complex III/I defects will be required.

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