Complex I Function Is Defective in Complex IV-deficient Caenorhabditis elegans

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Cytochrome c oxidase (COX) is hypothesized to be an important regulator of oxidative phosphorylation. However, no animal phenotypes have been described due to genetic defects in nuclear-encoded subunits of COX. We knocked down predicted mal phenotypes have been described due to genetic defects in the mitochondrial regulator of oxidative phosphorylation. However, no animal transport may, in fact, harbor a single genetic defect.

The mitochondrial respiratory chain (MRC) consists of five multisubunit complexes termed complexes I through V. The physical organization of these complexes is controversial. Two extreme models of their structure have been proposed. A “liquid-state model” of the mitochondrial respiratory chain depicts the respiratory complexes embedded in the inner mitochondrial membrane as separate entities, functionally connected to each other by the mobile electron carriers, coenzyme Q and cytochrome c. This model postulates that random collision among all respiratory components can account for measured electron transport rates in the inner mitochondrial membrane. Lateral diffusion of each component is regarded as sufficient to generate contact between MRC components (1, 2).

However, data also exist to support a “solid-state” model of respiratory complexes. This model proposes that mitochondrial respiratory complexes are organized into very large supercomplexes (3–8). Stoichiometric architectures of supercomplexes have been identified in multiple organisms, from prokaryotes to humans, and include I–III, I–III–IV, and I–II–III–IV (5, 7, 9–15). Supercomplex architecture suggests a kinetic advantage that increases the respiratory rate by providing substrate channeling between components of the supercomplex as well as stabilizing the complexes (5, 14–16).

There is also compelling evidence to show interdependency among the individual components of supercomplexes. In mammalian cell lines complex III assembly is required to maintain an intact complex I (17). Structural defects in complex III affected the amount of complex I, whereas chemical inhibition did not. Patients with defects in cytochrome b not only lose complex III, but also show decreased amounts of complex I, while maintaining normal enzymatic activity of the complex (15). Conversely, the disruption of complex I function caused by nonsense mutations in NDUF54, a subunit of this large multimeric complex, leads to the partial loss of complex III activity in skin fibroblast cultures obtained from Leigh-like patients (18, 19). However, defects in the complex I subunit ND5 did not cause a loss of complex III in the I–III supercomplex (20).

In most eukaryotes cytochrome c oxidase (COX), or complex IV, contains 10 nuclear and 3 mitochondrial encoded subunits. In cell lines, complex IV stabilizes the assembly of complex I. COX10 knock-out mouse cell lines, which were unable to assemble complex IV, showed decreased amounts of complex I, as detected by Western blot analysis following blue native gel electrophoresis. In human cell lines, high COX1 mutation levels can lead to destabilization of complex I (11). In addition, inhibition of COX IV expression caused impairment in complex I assembly in mouse cell lines (21).
Mitochondrial Complex IV Modulates Complex I Function

Although evidence supports interdependence among MRC complexes, the mechanism regulating this phenomenon remains unclear. We hypothesized that supercomplexes exist in the nematode Caenorhabditis elegans. We also hypothesized that decreasing amounts of complex IV subunits would inhibit the assembly of supercomplexes that include complex IV. We expected that, ultimately, reduced levels of complex IV subunits would in turn reduce amounts of associated components of any supercomplex that includes complex IV. These defects could lead to whole animal phenotypes characteristic of mitochondrial dysfunction. Here we use RNA interference (RNAi) to knock down two different predicted subunits of COX.

COX IV and Va are two nuclear-encoded subunits of complex IV. Each controls mitochondrial energy metabolism (22, 23). COX IV does so through an allosteric mechanism when the extramitochondrial ATP/ADP ratio is high. Binding of ATP to the cytosolic domain of COX IV leads to an increase of the $K_m$ of cytochrome $c$ (24), which slows mitochondrial respiration. This mechanism can be abolished by the binding of 3,5-diiodothyronine to COX Va (25).

No animals with nuclear-encoded genetic defects in subunits of complex IV have been reported. Genes predicted to encode the worm homologues of COX IV and COX Va were each subjected to RNAi knockdown. After knockdown, whole animal phenotypes were recorded, as well as function and structure of the MRC. Lifespan and fecundity of the animals were reduced, consistent with mitochondrial dysfunction. Complex IV activity was reduced, commensurate with decreased amounts of complex IV. Surprisingly, we found that the rate of electron transport within complex I was also decreased by knockdown of either complex IV transcript. Despite the decreased enzymatic activity of complex I in the complex IV knockdown animals, the total amount of complex I remained normal. In diagnosis of patients, our results caution that primary complex IV defects could be misinterpreted to be primary complex I-complex IV defects.

EXPERIMENTAL PROCEDURES

Nematodes and Bacteria

Wild type C. elegans (N2) and the Escherichia coli strain HT115 were obtained from the Caenorhabditis Genetics Center. HT115 bacteria containing plasmids used for RNAi knockdown were obtained from GeneService, United Kingdom. Each culture of worms grown for analysis of any sort either had a degree of knockdown measured by qRT-PCR (see below), or lack of complex IV function confirmed by measuring rates of oxygen consumption by mitochondria using TMPD/ascorbate as an electron donor (see below).

Worm Culture and Mitochondrial Isolation

N2 was grown on either W09C5.8 or Y37D8A.14, the 2 clones of E. coli containing the plasmids that express RNAi corresponding to the target genes, COX IV and COX Va, respectively. The control worms were fed HT115 on Nematode Growth Medium plates (3 days) and transferred to liquid culture (3 additional days) when most animals were young adults. Worm preparation and mitochondrial isolation were performed as previously described (26, 27).

RNAi

The knockdown worms required 4 and 6 days on the plates and in liquid culture, respectively. In this manner two generations of worms were exposed to RNAi or control bacteria. 5 mM isopropyl β-D-1-thiogalactopyranoside was used to induce RNAi synthesis from the bacteria. Temperature was controlled at 20 °C throughout and the cultures were never allowed to clear. Gene knockdown was checked by qPCR. RNA isolation and quantitative reverse transcriptase-PCR were done by standard techniques.

Life Span, Growth Rate, Fecundity, and Anesthetic Response

Life Span—Nematodes were grown for two generations on the RNAi strain being studied or the control strain, OP50. Adult nematodes were allowed to lay eggs for 4–6 h on plates containing lawns of the control or RNAi producing bacteria. The adults were removed and the eggs were allowed to hatch. The day of egg laying for this F1 generation was defined as day 0 of life. Animals were moved to new plates on day 3 of life and plated at a density of 20–22 animals per 35-mm plate. The animals were then moved every 2 days. Death, defined as failure to respond to a light touch, was scored each day. Each experiment consisted of 100–150 animals and was repeated in triplicate. The total number of animals for the three experiments was recorded.

Growth Rate—In the experiments described for lifespan, the first day of adulthood was recorded by scoring animals for the presence of eggs.

Fecundity—Animals were grown on a bacterial lawn of either HT115 or one of the RNAi clones for two generations. Single larvae were then transferred to new plates with the appropriate lawn. These animals were moved every 2 days and their offspring were counted on the plates from which they were transferred. Offspring from 15 such animals were used to determine average number of offspring.

Behavior in Anesthetic—Freshly washed young adult worms from liquid cultures of each strain were transferred to agar plates and exposed to varying concentrations of the volatile anesthetic, halothane to determine the $EC_{50}$ values (effective concentration at which 50% of the animals are immobilized). Dose-response analysis is performed as previously described (28).

Mitochondrial Function

Polarography Assay—The polarographic measurement of oxygen consumption of intact mitochondria by a Clark electrode was performed as previously described (29). Malate, succinate, and TMPD/ascorbate were used as the electron donors to complex I-, complex II- and complex IV-dependent respiration, respectively. The complex IV rate was measured in the presence of high ADP and 2,4-dinitrophenol to maximize respiratory capacity of complex IV.

Electron Transport Chain (ETC) Assay—Enzyme activities were measured at 30 °C, exclusively using cholate-treated mitochondria (1 mg of mitochondrial protein solubilized in 0.1 M
Mitochondrial Complex IV Modulates Complex I Function

25 μg of mitochondrial protein were separated by 2D SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in phosphate-buffered saline/Tween 20 (0.1%) and then incubated with either anti-NDUFS3 (MS112, Mitosciences, Eugene, OR) or anti-adenosine nucleotide transporter (MSA02, Mitosciences). Secondary antibodies were from Santa Cruz Biotechnology. Chemiluminescence substrate (SuperSignal® West Pico, Pierce) was used to develop the reactions. ImageQuant (TL version 2005) software from Amersham Biosciences was used to quantify levels of expression. The expression levels of protein reacting with anti-NDUFS3 were normalized to that of anti-adenosine nucleotide transporter.

Mass Spectrometric Analysis of the Polypeptides in BNGs

BNGs were analyzed following the standard mass spectrometry procedure (36). In brief, the in-gel tryptic digests of the bands cut from BNGs were analyzed using a capillary column LC-tandem mass spectrometer. An LCQ-Deca ion trap mass spectrometer system (ThermoFinnigan, San Jose, CA) was equipped with a microelectrospray ionization source (Protana, Odense, Denmark), which was operated under microspray conditions at a flow rate of 0.2 μl/min. The digests were analyzed using the data-dependent mode, recording a mass spectrum and three collision-induced dissociation spectra of ions ranging in abundance over several orders or magnitude. The entire sets of collision-induced dissociation spectra in each digest were subjected to data base search against the NCBI data base using the C. elegans taxonomy filter.

Statistical Analysis

Analysis of variance was used to analyze groups of data for significant differences. Unpaired Student’s t test was employed to calculate statistical significance of specific pairs if a difference was noted with analysis of variance. Standard deviations (S.D.) and n listed under supplemental data are to enable the reader to determine significance. Error bars in the figures represent mean ± S.E. for each experiment.

RESULTS

RNAi—The average percentages of COX IV and COX Va knockdown determined by qPCR from 4 independent cultures were 53.1 ± 7 and 27.4 ± 4%, respectively (see below, and Fig. 1).

Phenotypes—Nematodes treated with RNAi for either sub-unit COX IV (W09C5.8) or COX Va (Y37D8A.14) required an additional day to reach adulthood compared with N2. Lifespan was significantly shortened by 3–5 days, and fecundity was less than half of normal (Table 1). No change in anesthetic sensitivity was seen.

Polarography—MRC function of intact mitochondria isolated from the knockdown worms and N2 was determined by rates of oxygen consumption. As expected, complex IV respiration was decreased by both RNAi knockdowns (Fig. 1). In addition, the state 3 rates of both complex I- and complex II-dependent oxidative phosphorylation were significantly lower in COX IV knockdown worms than in N2. COX Va knockdown significantly decreased complex I-dependent rates but no significant change was seen in the complex II-dependent rates. The defects in MRC function in both RNAi worms were consistent with complex IV deficiencies. The amount of knockdown of the target gene correlated with TMPD/ascorbate rates; respiratory rates were lowest in those preparations with the greatest RNAi effect (Fig. 1B).

Electron Transport Chain—KCN-sensitive cytochrome c oxidase activities in COX IV and COX Va knockdown worms
complex I in the COX IV and COX Va worms were 59 and 48% lower than in the wild type, respectively (Fig. 2B). NADH-ferricyanide reductase (NFR), a measure of proximal complex I function, was not decreased (Fig. 2C). However, rotenone-sensitive NADH-cytochrome c oxidoreductase (CI–III), a measure of electron flow through complex I to III was decreased in COX Va knockdowns (Fig. 2D). When analyzed by analysis of variance, complex II–III rates in the knockdowns (Fig. 2E, CI–III) were statistically unchanged compared with control. Antimycin A-sensitive decylubiquinol-cytochrome c oxidoreductase activities (complex III) in the knockdown worms were also not statistically different from wild type (Fig. 2F). Because II–III and III activity were unchanged, we concluded that complex II is unaffected by knockdown of complex IV. Complex I and complex IV activity were specifically decreased in the mutants; we therefore asked whether the amounts of these complexes were also changed.

**Supercomplex Organization in C. elegans Mitochondria**—BNGs were used to determine the amount of MRC complexes in the worms with and without RNAi treatment. To optimize the BNG conditions, we first tested dodecyl maltoside (MS), Triton X-100 (TX), and digitonin (DT), as detergents in our isolation (Fig. 3 and supplementary data Fig. S1). We also varied the protein to detergent mass ratio in multiple attempts to optimally isolate supercomplexes as discreet entities. Digitonin at a detergent/protein mass ratio of 6/1 best preserved most MRC supercomplexes (Fig. 3 and supplementary data). Mass spectrometry was used to identify the polypeptides in each band appearing in digitonin (6/1) BNGs. Of the MRC proteins, 26 complex I subunits, 5 complex III subunits, 6 complex IV subunits, and 12 complex V subunits were identified (Table 2). Mitochondrial matrix proteins were found in the same bands as MRC supercomplexes, although the ratio of non-MRC/MRC peptides was less than 1/4, and most commonly seen in isolated complex III. The presence of specific subunits led to the identification of the complexes contained in each band.

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**FIGURE 1.** A, the effect of RNAi knockdown of COX IV and COX Va. The integrated mitochondrial functions of RNAi worms were determined by the OXPHOS assay, using malate, succinate, and TMPD/ascorbate as the electron donors to drive complex I-, complex II-, and complex IV-dependent respirations, respectively. The RNAi effect on the target genes (COX IV and COX Va) was measured by quantitative reverse transcriptase-PCR. Complex IV-dependent respiratory rates in both knockdown worms were significantly lower than N2. Similarly, the state 3 rates of complex I- and complex II-dependent respiration were significantly lower in the knockdown worms. Error bars represent mean ± S.E. from four independent isolations. * and ** indicate statistical significance as p < 0.05 and <0.005, respectively. B, the correlation between the target gene expression level and complex IV-dependent respiratory rate. Respiratory rates were measured as the rate of disappearance of oxygen in nanoatom of O/min/mg of mitochondrial protein. The extents of RNAi knockdown were determined by relative quantification of the level of mRNA expression of the target genes to the housekeeping gene and then normalized to that of control worms (N2).

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**TABLE 1**
Phenotypical study of COX IV and COX Va knockdown worms

| Phenotype     | N2       | COX IV  | COX Va |
|---------------|----------|---------|--------|
| Mean lifespan (days) | 17 ± 1.1 | 14 ± 9  | 12 ± 1.5 |
| Fecundity (eggs/worm)    | 254 ± 22 | 124 ± 14 | 107 ± 17 |
| Days to adulthood (days) | 3 ± 0.1  | 4 ± 0.3  | 4 ± 0.4  |
| EC50 (halothane)         | 3.2 ± 0.2 | 2.9 ± 0.2 | 2.8 ± 0.2 |

The phenotypes of worms after two generations of exposure to RNAi.
In the digitonin gel, multiple bands of greater than 1,236 kDa contained subunits of supercomplex I:III:IV (Fig. 3A, DT) (Table 2). Two other bands at 1,048 and 1,000 kDa were also identified as supercomplex I:III:IV. The 950-kDa band contained only supercomplex I:III. Three bands at 880, 800, and 720 kDa were identified as complex V. Molecular mass analysis predicts that the 880-kDa band represents dimeric complex V, whereas the 720-kDa band is the monomer. Each band contained peptides from all complex V subunits. Relative amounts of these three bands to each other varied slightly in digitonin-based gels, but the sum of their staining was relatively constant (data not shown). The band at 600 kDa corresponds to dimeric

FIGURE 2. The effects of COX knockdown on electron transport chain assays. A, enzymatic activities in absorbance units/min/mg of protein of KCN-sensitive cytochrome c oxidase (CIV). B, enzymatic activity of rotenone-sensitive NADH-decyldiquinone oxidoreductase (CI). C, enzymatic activity of NADH-ferricyanide reductase (NFR). D, enzymatic activity of rotenone-sensitive NADH-cytochrome c oxidoreductase (CI–III). E, enzymatic activity of antimycin A-sensitive decylubiquinol-cytochrome c oxidoreductase (CIII) were spectrophotometrically measured in N2 as wild type and in both complex IV knockdown worms. Error bars represent S.E. from three to five independent worm cultures. * and ** indicate statistical significance as \( p < 0.05 \) and \( 0.01 \), respectively.
complex III. Complex IV is located at 420 kDa, predicted by molecular mass to be dimeric complex IV. A band at 500 kDa, which is positive for CIV-IGA, was not analyzed by mass spectrometry. However, its molecular mass matches the summation of complex III and complex IV (300 and 200 kDa, respectively). Although complex III is usually found only in dimeric form, we cannot rule out that a monomer may form with complex IV. Thus, the band at 500 kDa may represent a supercomplex III:IV but with the monomeric form of each. We also identified bands from the Triton X-100-treated lane (supplementarydata).

FIGURE 3. The organization of respiratory complexes in C. elegans. Mitochondrial proteins from N2 were solubilized by digitonin (DT), Triton X-100 (TX), or maltoside (MS) and were electrophoresed in 3.5–11% gradient acrylamide gels. The gels were subsequently further stained with Coomassie Blue (A) or used to perform IGA assays for complex IV (CIV-IGA) or complex I (CI-IGA) as shown B and C, respectively. The numbers in the left panel indicate the approximate molecular masses (kDa) of the corresponding protein bands.

Mitochondrial Complex IV Modulates Complex I Function

decreased in both knockdowns, compared with the wild type, whereas supercomplex I:III was increased. RNAi treatment did not affect amounts of isolated complex III or complex V.

CIV-IGA verified loss of complex IV in the knockdown animals (Fig. 4B). Compared with wild type activity, we observed a decrease in COX activity at 420 kDa, the dimeric complex IV, in both RNAi worm strains. The activity of the complex IV component in supercomplex I:II-I:IV was also decreased in the RNAi-treated worms. CI-IGA was decreased in supercomplex I:III:IV after RNAi treatment in the knockdown worms, consistent with the decrease in supercomplex I:III:IV formation (Fig. 4C). However, CII-IGA of supercomplex I:III increased. The ratio of CI-IGA activity in I:III:IV to the total CI-IGA, averaged over 4 independent gels, was significantly decreased in the knock-out animals compared with N2 (Fig. 5A).

Complex I Formation Was Unchanged in COX IV and COX Va Worms—Because ETC activity of complex I was decreased with COX subunit knockdown, we attempted to quantify complex I more precisely. In the BNGs solubilized with digitonin (as in Fig. 4), total CI-IGA, normalized for protein loading, was the same in N2 and RNAi knockdown strains (Fig. 5B). Triton X-100 solubilization isolates complex I most completely (Fig. 3 and supplemental data). At a detergent to protein mass ratio of 5/1, Triton X-100-based BNG revealed one individual complex I band and two supercomplex I:III bands (Fig. 5C) as determined by mass spectrometry (data not shown) and corroborated by CI-IGA (Fig. 5D). Both individual complex I and supercomplex I:III were not significantly decreased from N2 in COX IV or COX Va knockdown animals. The total complex I in each of the complex IV knockdown worms was the same as in the wild type animals. Western blot analysis of mitochondrial protein probed with an antibody to a complex I subunit (orthologue of NUO-2, data not shown) showed no differences in levels of expression between the knockdown strains and N2 (supplementary data Fig. S1).

DISCUSSION

Nematode COX IV (encoded by the gene W09C5.8) has 24% identity to human COX IV in amino acid sequence, whereas nematode COX Va (encoded by Y37D8A.14) has 30.5% identity to human COX Va. RNAi treatment of COX IV and COX Va inactivated the expression of their respective genes as shown by qPCR data. Knockdown of either gene impaired respiration and electron transport in a manner consistent with COX deficiency. Therefore, knockdown of these two highly conserved compo-
### TABLE 2

MRC proteins identified by mass spectrometry in each protein band in digitonin-based BNGs

Only MRC proteins are listed. Protein names are obtained from *C. elegans* Genetics Center (CGC) and HUGO Gene Nomenclature Committee (HGNC), respectively. Absence of a subunit does not preclude its presence in the protein band as mass spectrometry may miss individual proteins. These data are used to indicate which complexes are in each band and not to identify all subunits present. Turquoise shading indicates complex I subunits, yellow complex III, pink complex IV, and grey complex V.

| C. elegans peptide (CGC’s name) | Human’s homolog (HGNC’s symbol) | Protein band’s molecular weight (KDa) |
|---------------------------------|---------------------------------|-----------------------------------|
|                                 |                                 | 1,236                             |
|                                 |                                 | 1,048                             |
|                                 |                                 | 1,000                             |
|                                 |                                 | 950                               |
|                                 |                                 | 880                               |
|                                 |                                 | 800                               |
|                                 |                                 | 720                               |
|                                 |                                 | 600                               |
|                                 |                                 | 420                               |
| NUO-1                           | NDUVF1                          | +                                 |
| NUO-2                           | NDUFS3                          | +                                 |
| NUO-4                           | NDUFA10                         | +                                 |
| NUO-5                           | NDUFS1                          | +                                 |
| F53F4.10                        | NDUVF2                          | +                                 |
| C33A12.1                        | NDUFA5                          | +                                 |
| Y54F10AM.5                      | NDUFA8                          | +                                 |
| Y53G8AL.2                       | NDUFA9                          | +                                 |
| Y94H6A.8                        | NDUFA12                         | +                                 |
| C25H3.9a                        | NDUFB5                          | +                                 |
| ZK809.3                         | NDUFB6*                         | +                                 |
| Y51H1A.3b                       | NDUFB8*                         | +                                 |
| C16A3.5                         | NDUFB9                          | +                                 |
| F59C6.5                         | NDUFB10                         | +                                 |
| GAS-1                           | NDUFS2                          | +                                 |
| Y54E10BL.5                      | NDUFS5                          | +                                 |
| W01A8.4                         | NDUFB4                          | +                                 |
| T20H4.5                         | NDUFS8                          | +                                 |
| C18E9.4                         | NDUFB3                          | +                                 |
| F42G8.10a                       | NDUFB11                         | +                                 |
| NUO-3                           | NDUFA6                          | +                                 |
| W10D5.2                         | NDUFS7*                         | +                                 |
| F45H10.3                        | NDUFA7                          | +                                 |
| TAG-99                          | NDUFS2                          | +                                 |
| F44G4.2                         | NDUFB2                          | +                                 |
| D2030.4                         | NDUFB7                          | +                                 |
| UCR-2.1                         | UQRC2C                          | +                                 |
| UCR-2.2                         | UQRC2C                          | +                                 |
| UCR-2.3                         | UQRC2C                          | +                                 |
| T02H6.11                        | UQRCB                           | +                                 |
| F45H10.2                        | UQRCQ                           | +                                 |
| W09C5.8                         | COX4I1                          | +                                 |
| CCO-2                           | COX5A                           | +                                 |
| CCO-1                           | COX5B                           | +                                 |
| TAG-174                         | COX6A                           | +                                 |
| Y71H2AM.5                       | COX6B1                          | +                                 |
| F26E4.6                         | COX7C                           | +                                 |
| H28O16.1d                       | ATP5A1                          | +                                 |
| ATP-2                           | ATP5B                           | +                                 |
| Y69A2AR.18a                     | ATP5C1                          | +                                 |
| ATP-5                           | ATP5H*                          | +                                 |
| ASB-2                           | ATP5F1                          | +                                 |
| ASB-1                           | ATP5F1                          | +                                 |
| ASG-2                           | ATP5L                           | +                                 |
| ASG-1                           | ATP5L                           | +                                 |
| R04F11.2                        | ATP5I                           | +                                 |
| F58F12.1                        | ATP5D                           | +                                 |
| F32D1.2                         | ATP5E*                          | +                                 |
| R05D3.6                         | ATP5E*                          | +                                 |
components of the MRC indicate that they are orthologues of their mammalian counterparts in complex IV of the MRC. This study demonstrated that COX IV and COX Va depleted worms grew slowly, had shortened lifespans, and decreased fecundity, consistent with mitochondrial dysfunction. Lifespans of COX IV and COX Va knockdown worms were 14.1/11006.9 and 12.1/11006.15 days, respectively (compared with 17.1/11006.11 days for N2). This differed from the report of Lee et al. (37), which found that COX IV knockdown worms lived longer than the wild type. However, they used FUDR to limit offspring and allow an RNAi screen for 5,690 genes. We also studied lifespan in the presence of FUDR (data not shown) and found results similar to those reported by Lee et al. (37). We did not use FUDR here to assay conditions under normal life stresses. Lifespan may be directly affected by sterility per se. Our data indicate that disruption of complex IV makes the animals unable to meet the increased stress of oocyte production, leading to a shortened lifespan.

BNGs of mitochondria revealed that MRC supercomplexes exist in the nematode, much as described in other organisms (4–6, 8–15). We observed decreased amounts of complex IV and decreased complex IV IGA for both complex IV mutants. As COX IV and Va encode core subunits of complex IV, the decrease in functional complex IV, seen in oxidative phosphorylation capacity, ETC and IGA assays, is likely caused by an insufficient amount of this subunit for full complex IV assembly. Our results are similar to the study from Li et al. (38), which found that the inhibition of COX IV expression led to a loss of complex IV assembly in mouse cell lines. Interestingly, we found that CIV-IGA activity was either in a dimeric complex IV (420 kDa) or a supercomplex of complexes I, III, and IV; we did not detect CIV-IGA in a monomeric form. The dimeric form of COX is generally accepted as its functionally active form (39). Our observation is in contrast to the report of Grad and Lemire (35), which found both the monomeric and dimeric forms of cytochrome c oxidase in their CIV-IGA. However, Grad and Lemire (35) did not report the detergent used to solubilize mitochondria. Digitonin may keep more MRC complexes and supercomplexes intact (Fig. 3).

FIGURE 4. Blue native electrophoresis and the IGA. Two representative BNGs (upper and lower) to show the degree of reproducibility in the amount of supercomplexes between isolations. Each gel was loaded in triplicate and cut into thirds for the assays shown in A–C. In each set of three lanes, N2 is the left lane, COX IV is the middle lane, and COX Va is the right lane. A, Coomassie Blue-stained BNGs. Twelve distinct blue protein bands were consistently observed by Coomassie Blue staining. Mass spectrometry identified three bands of 12, between molecular masses 450 and 500 kDa, as containing non-OXPHOS proteins. The other nine bands contained primarily subunits of MRC complexes and were analyzed as follows: supercomplex I:III:IV, 1,236, 1,048, and 1000 kDa; supercomplex I:III, 950 kDa; complex V, 880, 800 and 720 kDa; complex III, 600 kDa; complex IV, 420 kDa. B, CIV-IGA. The dark brown bands indicate the activity of cytochrome c oxidase that reduces diaminobenzidine. Note that activity is found in those bands shown to contain complex IV by mass spectrometry and that activity is decreased in the knockdown strains. C, CI-IGA. The activities of complex I are marked by the deep blue color of formazan crystals that resulted from the reduction of nitro blue tetrazolium by complex I. The positive bands appearing in CI-IGA corresponded to the identification of complex I by mass spectrometry. Note that the amount of complex I staining is relatively unchanged in the knockdown strains.

This study demonstrated that COX IV and COX Va depleted worms grew slowly, had shortened lifespans, and decreased fecundity, consistent with mitochondrial dysfunction. Lifespans of COX IV and COX Va knockdown worms were 14 ± 0.9 and 12 ± 1.5 days, respectively (compared with 17 ± 1.1 days for N2). This differed from the report of Lee et al. (37), which found that COX IV knockdown worms lived longer than the wild type. However, they used FUDR to limit offspring and allow an RNAi screen for 5,690 genes. We also studied lifespan in the presence of FUDR (data not shown) and found results similar to those reported by Lee et al. (37). We did not use FUDR here to assay conditions under normal life stresses. Lifespan may be directly affected by sterility per se. Our data indicate that disruption of complex IV makes the animals unable to meet the increased stress of oocyte production, leading to a shortened lifespan.

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To identify the proteins in the bands appearing on BNG as completely as possible, we took advantage of high sensitivity and specificity of mass spectrometry. Supercomplex I:III:IV appeared as a stack of multiple bands at the upper most part of the gel (1,236 kDa and higher). The previous study by D’Aurelio et al. (11) showed that supercomplex I:III:IV in human cybrid cell lines can be assembled into 4 different stoichiometries. Each contained 1 copy of complex I and 2 copies of complex III,
Mitochondrial Complex IV Modulates Complex I Function

We did not observe a band in BNGs that contained only complex I subunits in any gel that used digitonin as a detergent. We interpret these results to indicate that under physiologic conditions virtually all complex I is bound to complex III in C. elegans. Similar observations have been made in plants, where 50–90% of complex I forms part of supercomplex I:III2 (6, 40, 41). In bovine heart mitochondria only 15% of complex I was found in free form (7). All human complex I in skeletal muscle was shown to be exclusively assembled into supercomplexes (15). It may then be a general feature among mitochondria that almost all complex I is associated with supercomplexes. In contrast, we did not find complex II, the smallest of the ETC complexes, in any supercomplex on BNGs, nor did we identify it as an isolated entity.

RNAi inactivation of COX IV and COX Va demonstrated a relationship between supercomplexes I:III and I:III:IV, as well as a linked regulation of complexes I and IV. The amount of supercomplex I:III increased when the amount of complex IV and supercomplex I:III:IV decreased (Fig. 4, A and C). Similar to our finding, Schagger et al. (15) demonstrated that a patient with decreased complex IV caused by a SURF1 mutation had a decreased ratio of supercomplex I:III:IV to supercomplex I:III. They did not report ETCs for this patient. In the report by Schagger et al. (15), amounts of complex I did not decrease, even though the residual complex IV levels were only 10% of normal. However, SURF1 is not a structural subunit of complex IV, but rather a protein involved in its assembly. In studies of patients with missense mutations in COX 10, also a COX assembly factor (42), complex I levels were normal. In a separate study, fibroblast lines from two patients with no measurable complex IV (43) contained normal amounts of complex I. In contrast, Diaz et al. (44) found that a knock-out of COX10 in a mouse fibroblast line drastically reduced both supercomplex I:III and supercomplex I:III:IV. They proposed that the complete absence of complex IV in a COX10 knock-out led to a global decrease of NADH oxidoreductase supercomplexes as determined by BNGs. Li et al. (21) established mouse cell lines (38) that decreased COX IV levels more than 75% and COX activity by more than a half as shown by CIV-IGA (38). These cells exhibited more than a 50% decrease in the levels of two nuclear-encoded complex I subunits, GRIM-19 and NDUFA9 (21); supercomplex formation was not investigated. They concluded that unstable complex assembly, as opposed to decreased protein synthesis, led to a reduction in complex I subunits. Although D’Aurelio et al. (11)

**FIGURE 5.** Complex IV disruption alters the ratio between supercomplex I:III and I:III:IV but not the total amount of complex I. A, the amounts of complex I as present in supercomplexes I:III and I:III:IV were measured from 4 independent digitonin-based CI-IGAs. Quantitative analysis shows significant changes in the amount of CI-IGA in supercomplex I:III to the total amount of CI-IGA in both complex IV-depleted worms compared with N2. B, the total amount of CI-IGA present in both supercomplexes I:III and I:III:IV, after normalization to a total complex V is not perturbed by complex IV disruption. C, Triton X-100-based BNGs also confirm that the total amount of complex I in complex IV-depleted worms is unchanged as shown by Coomassie Blue stain. D, Triton X-100-based BNGs corroborates that the total amount of complex I in complex IV-depleted worms is unchanged as shown by CI-IGA.

i.e. I:III2, as well as different copy numbers of complex IV, varying from 1 to 4 copies. The uppermost bands in our BNGs are likely to be I:III2:IV, I:III2IV3, I:III2:IV2, and I:III2:IV. However, the molecular weight of each band could not be accurately defined by its mobility in the gel. In addition, it would not have been exactly the cumulative molecular weight of all complex components because not every subunit of complexes I, III, and IV has been identified by mass spectrometry in our gels (Table 2). For example, supercomplex I:III:IV was also identified at both 1,048 and 1,000 kDa, but each band was composed of fewer ETC subunits than the higher molecular band at greater than 1,236 kDa. This most probably represents relative binding strength of different subunits to the complexes, resulting in the dissociation of those subunits during the isolation.

The isolation of supercomplexes and their stoichiometries in worms are dependent on the amount and type of detergent used for mitochondrial solubilization. Like other reports, we obtained very different BNGs using digitonin, Triton-X, and maltoside as detergents (supplementary data Fig. S1) (5, 6). Digitonin showed the most preservation of supercomplexes, as well as of IGA of complex IV, both as part of a supercomplex and as a homodimer. It is important to remember that the ETC functional studies (most importantly, those for complex I) were not done under the conditions used for BNGs, but under standard conditions normally used for measuring electron transport chain activities (see “Experimental Procedures”).

### References

(11)
Mitochondrial Complex IV Modulates Complex I Function

have argued that a threshold decrease in complex IV is necessary to decrease complex I, results from patients would indicate that even very low levels of complex IV do not decrease complex I levels.

In a recent review (45), it has been concluded that the role of complex IV in complex I assembly is unclear. It is interesting that in all the data from intact animals, even animals as far apart in the animal kingdom as nematodes and humans, defects in complex IV do not decrease amounts of complex I. However, clearly, in the nematode, low levels of complex IV shift the ratio of complex I from supercomplex I:III:IV to I:III. Even, however, with normal amounts of complex I, this shift in supercomplex formation results in a striking loss of enzymatic activity of within complex I itself (see below).

Complex I enzymatic function, as assayed by standard ETC assays, is decreased in our complex IV knockdowns. ETC assays are not done on complexes isolated from BNGs, but rather from mitochondria treated with cholate that allows entry of substrates specific for the complexes studied (e.g. NADH for complex I). It is possible that defects in complex IV destabilize complex I such that cholate treatment negatively affects complex I function. In either case, complex IV has an effect on complex I function that is measured as per standard protocols for patient diagnosis (30–33).

Two assays revealed defects in complex I function: flow of electrons through complex I to the artificial electron acceptor decylubiquinone, and electron transport through complex I, coenzyme Q, and complex III, to cytochrome c (for COX Va alone). This decreased enzymatic activity was seen despite the fact that the overall amount of complex I, as assayed by Coomassie Blue staining, by CI-IGA activity, or by measurement of NUO2 levels, was not significantly different in these animals compared with N2. Electron movement within the matrix arm of complex I (NFR) was not affected by knockdown of either complex IV subunit. Although each RNAi experimental culture varied slightly in the amount of measured knockdown, knockdown was always verified either by qPCR or measurements of complex IV-dependent respiration. Therefore it is not possible that lack of change in levels of complex I was due to a lack of knockdown in the particular culture. In addition, at least 4 gels from different cultures were used to assess supercomplex organization in knockdown animals.

The total amount of CI-IGA in supercomplexes is not decreased in the mutants, similar to our finding that NFR of complex I is not decreased in the knockdown animals. It is not known exactly what step of electron transport is measured by IGA of BNGs. Our data indicate that CI-IGA involves a proximal part of complex I rather than the entire complex. However, transport of electrons through the entire complex I, as measured using NADH as an electron donor and decylubiquinone as the electron receptor (Fig. 2B) is significantly decreased in both knockdowns. Further complex I–III activity is significantly decreased in one knockdown and approaches significance in the second knockdown ($p = 0.07$). This indicates then that the I:III:IV supercomplex, which is reduced in both knockdown animals, is crucial for normal rates of electron transport within complex I.

Our results are consistent with the model that supercomplexes represent an advantage in electron transfer, in this case even affecting movement through another member of the associated supercomplex. Schafer et al. (10) showed that NADH:ubiquinone reductase was more active in supercomplex I:III$_2$:IV than in supercomplex I:III$_2$. This is the same step of electron transfer that is measured in our complex I assay. They concluded that complex IV enhanced the function of complex I in supercomplex I:III:IV. Their enzyme assays were done with samples directly electroluted from BNGs. Our results corroborate and extend these findings. We have produced a defective complex IV through knockdown of two nuclear genes that encode different subunits of the complex. Both knockdowns lead to a decreased rate of electron transport through complex I while leaving enzymatic activity within the matrix arm unchanged. This is in keeping with the model in which the membrane portion of complex I, in animals, is in extensive contact with complex IV, whereas the matrix arm is not (13). We attribute the decreased rates to loss of the I:III:IV supercomplexes, with relatively decreased activity of the I:III supercomplex in passing electrons within complex I. It appears that complex IV then plays a role in maintaining maximal rates of electron flow within complex I itself through its contact within the membrane bound arm.

Multiple deficiencies of the ETC have been reported in patients in conditions like peripheral arterial disease (I+III (46)) or Parkinson disease (I+IV+V (47), I+IV (48)), in multiple infantile defects (I+IV, I+V (49)), in children with myopathies (I+IV (50)), and in infants with intractable lactic acidosis (I+IV (51)). A combined I+IV defect is cited as the most common combined deficiency in patients (50). Our study demonstrates that a single primary defect can have wide ranging effects on function of multiple different protein complexes of the ETC. This finding adds to the complexity of interpreting ETC assays when used in isolation to diagnose mitochondrial defects. Patients presenting with puzzling multiple defects of electron transport involving complexes I and IV may, in fact, harbor a single genetic defect affecting complex IV.

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Note Added in Proof—A recent study by Maas et al. (Maas, M. F., Krause, F., Dencher, N. A., and Sainsard-Chanet, A. (2008) J. Mol. Biol. 10.1016/j.jmb.2008.12.025) has shown that in the fungus Podospora anserina lack of both complex III and complex IV does not decrease the amount of complex I.

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