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Riboflavin enhances the assembly of mitochondrial cytochrome *c* oxidase in *C. elegans* NADH-ubiquinone oxidoreductase mutants

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Abstract

Mitochondrial respiratory chain dysfunction is responsible for a large variety of early and late-onset diseases. NADH-ubiquinone oxidoreductase (complex I) defects constitute the most commonly observed mitochondrial disorders. We have generated *Caenorhabditis elegans* strains with mutations in the 51 kDa active site subunit of complex I. These strains exhibit decreased NADH-dependent respiration and lactic acidosis, hallmark features of complex I deficiency. Surprisingly, the mutants display a significant decrease in the amount and activity of cytochrome *c* oxidase (complex IV). The metabolic and reproductive fitness of the mutants is markedly improved by riboflavin. In this study, we have examined how the assembly and activity of complexes I and IV are affected by riboflavin. Our results reveal that the mutations result in variable steady-state levels of different complex I subunits and in a significant reduction in the amount of COXI subunit. Using native gel electrophoresis, we detected assembly intermediates for both complexes I and IV. Riboflavin promotes the assembly of both complexes, resulting in increased catalytic activities. We propose that one primary pathogenic mechanism of some complex I mutations is to destabilize complex IV. Enhancing complex I assembly with riboflavin results in the added benefit of partially reversing the complex IV deficit.

Keywords: Mitochondria; Complex I; Complex IV; *C. elegans*

1. Introduction

The best-known function of mitochondria is to provide cellular energy via the process of oxidative phosphorylation (OXPHOS). OXPHOS is mediated by the mitochondrial respiratory chain (MRC), which is composed of four membrane-bound electron-transporting protein complexes (I–IV) that generate a proton gradient across the mitochondrial inner membrane and the ATP synthase (complex V) that utilizes the proton gradient for ATP generation. Defects in one or more MRC complex impair OXPHOS and can result in mild to severe disease or even lethality. Deficiency in complex I, the NADH-ubiquinone oxidoreductase, is the most prevalent form of MRC disorder [1–4]. Mammalian complex I is the largest respiratory chain complex, composed of at least 46 subunits, seven of which are encoded in the mitochondrial DNA (mtDNA) [5,6]. A flavin mononucleotide (FMN) co-factor serves as the entry point for electrons from NADH oxidation, while up to eight iron–sulfur clusters facilitate electron transfer through the enzyme [7–10]. Electron transfer from NADH to ubiquinone is coupled to vectorial proton movement across the inner membrane [11]. Complex I dysfunction is linked to cardiomyopathies, encephalomyopathies and neurodegenerative disorders such as Parkinson's disease and Leigh syndrome [3,4,12].

The exact pathophysiological mechanisms involved in most mitochondrial diseases remain poorly understood. To better describe the bioenergetic and biochemical consequences of MRC dysfunction, we developed a *Caenorhabditis elegans* model of complex I deficiency [13,14]. *C. elegans* complex I is composed of at least 40 subunits, many of which share very high sequence identity to their human homologues [15,16]. Our nematode strains express missense mutations in the nuclear-encoded *nuo-1* gene, the nematode orthologue of the human *NDUFV1* gene [13]. The *nuo-1* and the *NDUFV1* genes encode the 51-kDa subunit of complex I, which carries the NADH-binding site, the FMN cofactor and an iron–sulfur cluster. Patients with *NDUFV1* mutations present with a myriad of symptoms including hypotonia, myoclonic epilepsy, brain atrophy, macrocystic leukodystrophy, acute metabolic acidosis

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and Leigh syndrome [17–19]. We created nematode strains expressing the A352V, T434M or the A443F amino-acid substitutions in the NUO-1 protein. These mutants demonstrate hallmark features of complex I dysfunction such as lactic acidosis and decreased NADH-dependent respiration. In addition, we noted specific catalytic deficiencies in complexes I and IV, particularly in the more severe A352V and A443F mutants [13].

A variety of pharmacological agents are used to treat MRC dysfunction, although there is little solid evidence supporting their use [20,21]. Riboflavin (vitamin B_2) produces significant increases in fertility in all three *nuo-1* mutants [13]. Interestingly, riboflavin has previously been associated with successful treatments for complex I deficiency [22–25]. Riboflavin is a precursor to the flavin cofactors FMN and FAD (flavin adenine dinucleotide), which serve as coenzymes for numerous reactions involving one and two-electron oxidation-reduction reactions. Complex I utilizes FMN as the initial electron acceptor during the oxidation of NADH. Another important riboflavin-dependent metabolic reaction is catalyzed by the pyruvate dehydrogenase complex (PDHC), an FAD-containing enzyme that oxidizes pyruvate and releases acetyl-coenzyme A. This reaction is the major source of substrate for the Krebs cycle. If PDHC activity is low, pyruvate generated by glycolysis can accumulate and be converted to lactate, resulting in lactic acidosis. Riboflavin supplementation of complex I-deficient worms may also stimulate PDHC activity and the Krebs cycle; this is consistent with the marked attenuation of lactic acidosis we noted in the A443F mutant by riboflavin [13].

Mutations leading to complex I deficiency may be localized to either nuclear or mtDNA-encoded complex I genes or in genes encoding proteins that mediate the assembly of subunits and cofactors into the holoenzyme [26–29]. In contrast, clinical deficiency of cytochrome *c* oxidase is almost never a result of mutation in one of its structural subunits but rather is a result of an assembly factor defect [30,31].

To better understand the molecular bases of pathogenic complex I mutations, we have investigated the assembly and catalytic function of complexes I and IV in *C. elegans nuo-1* mutants. *nuo-1* mutations are pathogenic because they destabilize complex I and impair the assembly of complex IV. Here, we also report that the assembly defects for both complexes I and IV are riboflavin-responsive. Complex IV assembly defects are indirectly corrected by riboflavin supplementation because complex IV is not a flavoprotein.

2. Materials and methods

2.1. Strains

Worms were cultured as described [32]. We used the following *C. elegans* strains: N2 (Bristol) wild type; LB25, *nuo-1*(*ua1*) II, *unc-119*(*ed3*) III, *uaEx25* [p016bA352V]; LB26, *nuo-1*(*ua1*) II, *unc-119*(*ed3*) III, *uaIs26* [p016bT434M]; LB27, *nuo-1*(*ua1*) II, *unc-119*(*ed3*) III, *uaEx27* [p016bA443F] [13]. Worms cultured in liquid medium were supplemented with 1 μg/ml riboflavin with additional riboflavin added every second day until harvested.

2.2. Electrophoresis and Western blot analyses

Mitochondria were isolated as previously described [13]. Fifty μg of mitochondrial protein were solubilized in gel-loading buffer and resolved by electrophoresis on 10% or 12% sodium dodecyl sulfate (SDS) polyacrylamide gels [14]. Proteins were transferred electrophoretically to nitrocellulose or polyvinylidene fluoride membranes. Blots were treated with rabbit polyclonal antisera against the bovine 51-kDa subunit (a gift from Dr. M. Yamaguchi, USA), the *N. crassa* TYKY subunit (a gift from Dr. F. Nargang, Canada) or the *Saccharomyces cerevisiae* ATP-2p [33]. Another source of antiserum against the 51-kDa protein gave similar results, indicating the specificity of the bovine serum. Mouse monoclonal antisera against the human NDUFS3 protein (30-kDa complex I subunit) or COXI (MitoSciences, Eugene, Oregon) were also used. For development, blots were treated with peroxidase-labeled goat anti-rabbit or goat anti-mouse secondary antibodies. The Enhanced Chemiluminescence Western Blotting System (Amersham Biosciences, Buckingham, UK) or the Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Inc., Rockford, IL) were used for detection. Signal quantification was performed with the BioRad Gel Doc 1000 Image Analysis System and Molecular Analysts software (BioRad Laboratories, Hercules, CA).

2.3. Native gel electrophoresis and histochemical staining

Blue native gradient gels (4–13% for complex I analysis; 5–15% for complex IV analysis) were loaded with 300 μg mitochondrial protein as described [34]. Following electrophoresis, NADH dehydrogenase activity was detected by incubating gels in 20 ml 50 mM Tris–HCl, pH 7.4 containing 0.5 mM tetranitroblue tetrazolium and 5 mM NADH at 37 °C for 80 min in the dark with gentle rocking. For the detection of cytochrome *c* oxidase activity, gels were incubated in 20 ml 50 mM Tris–HCl, pH 7.4 containing 0.1% 3,3′-diaminobenzidine, 0.1% cytochrome *c*, 0.02% catalase at 37 °C for 90 min in the dark with gentle rocking. Gels used for Western blot analysis were incubated in 20 mM Tris-base, 150 mM glycine, 20% (v/v) methanol, 0.08% (w/v) SDS for 10 min before electrophoretic transfer to Immobilon-P membranes (Millipore Corp., Billerica, MA) [35]. Following transfer, excess stain was removed as described [36].

2.4. MRC assays

Enzymatic activities were measured on an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK) as described [37]. Rotenone-sensitive NADH-decylubiquinone oxidoreductase activity was measured at 340 nm using 65 μM 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone as electron acceptor, 2 μg/ml antimycin A, 0.13 mM NADH. Rotenone was added to 2 μg/ml. Cytochrome *c* oxidase activity was measured at 550 nm using 15 μM cytochrome *c* as electron acceptor in the presence of 0.45 mM lauryl maltoside. Potassium cyanide was added to 1 mM.

3. Results

3.1. Steady-state levels of complexes I and IV are reduced in nuo-1 mutants

We investigated the steady state levels of complexes I and IV in mitochondria isolated from wild type *C. elegans* and from three complex I-deficient strains: LB25, LB26 and LB27. LB25 and LB27 carry extrachromosomal transgenic arrays expressing the A352V and A443F *nuo-1* alleles, respectively. LB26 carries an integrated transgene encoding the T434M *nuo-1* mutation. The relative abundance of three complex I subunits was determined by Western blot analysis. We observed severe reductions in the amounts of NUO-1 protein (51-kDa subunit), moderate reductions in the amounts of 30-kDa subunit but no reduction in the amounts of TYKY (23-kDa) subunit in the