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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

mutants (Fig. 1, Table 1). The steady-state level of the mtDNA-encoded COXI subunit of complex IV also showed a moderate reduction. No differences were observed in the steady-state levels of the complex V subunit ATP-2p. Thus, the *nuo-1* mutations differentially affect the steady state levels of individual complex I subunits and affect the levels of at least one subunit in complex IV.

We investigated the effects of riboflavin supplementation on the steady state levels of the complex I and complex IV subunits in LB25 and LB27, the strains carrying the two most severe alleles. LB26 was not investigated because the mutant phenotype is mild and the effects of riboflavin supplementation are not large. Mitochondria from the riboflavin-supplemented strains showed significant increases in the relative abundance of NUO-1 protein, increasing to 74% and 72% of wild type, respectively (Fig. 1, Table 2). Surprisingly, COXI levels were also elevated by riboflavin, increasing to 85% and 83% of wild type, respectively (Fig. 1, Table 2). The steady-state levels of the 30-kDa and 23-kDa complex I subunits and of the ATP-2p subunit were not affected by riboflavin. Riboflavin differentially affects the steady state levels of individual complex I subunits. In addition, riboflavin modulates the levels of COXI, a subunit of complex IV, an enzyme without a flavin cofactor.

3.2. nuo-1 mutations impair MRC complex assembly

To determine how the reduced steady state levels of MRC subunits affect the amounts of fully assembled complexes, we resolved mitochondrial proteins by blue native gel electrophoresis (BN-PAGE). The gels were stained for NADH dehydrogenase activity, an activity present in the complex I holoenzyme and in some sub-complexes [38]. A species with high levels of NADH dehydrogenase activity was detected in wild type mitochondria migrating with an apparent molecular weight of ~950-kDa; it most likely corresponds to complex I holoenzyme (Fig. 2A). Much lower levels of NADH dehydrogenase activity are associated with this 950-kDa species in mitochondria from LB25, LB26 or LB27. In addition, LB25 and LB26 mitochondria contain complexes with significant NADH dehydrogenase activity migrating at ~640-kDa and ~600-kDa, respectively. These smaller species are absent from wild type mitochondria. LB27 mitochondria do not contain detectable NADH-dehydrogenase activity in the 600–640 kDa range. In mitochondria from riboflavin-supplemented LB25 and LB27, there is a significant increase in the amount of NADH dehydrogenase activity at ~640 kDa and of another species at ~670 kDa (Fig. 2A). However, riboflavin supplementation does not increase the activity associated with the ~950-kDa species.

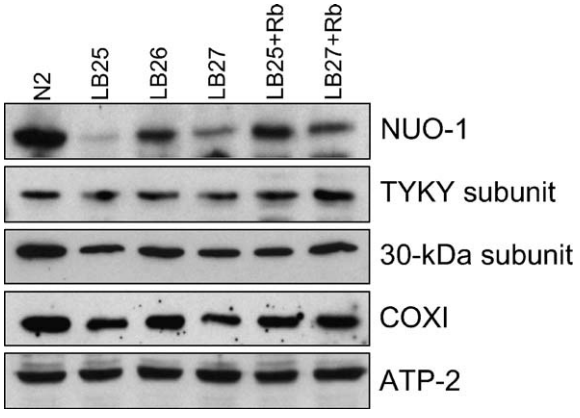

Fig. 1. Steady-state levels of MRC proteins are diminished in mutant mitochondria. 50 μg of isolated mitochondria from the wild type (N2) or the *nuo-1* mutants LB25–LB27 were loaded into each lane and specific subunits of complexes I, IV and V were detected by Western blot analysis. Rb, mitochondria were isolated from strains cultured in the presence of 1 μg/ml riboflavin.

Table 1
Relative steady-state levels of MRC proteins

Protein	N2	LB25	LB26	LB27
NUO-1	1.00	0.29±0.05*	0.75±0.07*	0.45±0.04*
23-kDa	1.00	0.97±0.03	0.99±0.02	0.97±0.02
30-kDa	1.00	0.71±0.02*	0.84±0.02*	0.72±0.05*
COXI	1.00	0.58±0.03*	0.86±0.04*	0.66±0.03*
ATP-2	1.00	1.00±0.02	1.01±0.02	1.00±0.03

Reported values are normalized to levels in the wild type N2. Values are means±S.D. of a minimum of three experiments.
* $P<0.05$ using two-sample *t*-test, compared to N2 wild type value.

To confirm that the high molecular weight NADH dehydrogenase-active species are in fact related to complex I, we used Western blot analysis with an antiserum directed against the bovine 51-kDa subunit, which crossreacts with *C. elegans* NUO-1. In wild type mitochondria, we detected a strong but diffuse signal centered at ~1-MDa, which corresponds closely to the position of the ~950-kDa NADH dehydrogenase-active species (Fig. 2B). This confirms that the *C. elegans* complex I is ~950 kDa in size, similar to mammalian complex I. Immunologically-detectable, high molecular weight species containing NUO-1 are considerably less abundant in LB26 and almost absent in LB25 and LB27. This is in agreement with the steady-state levels of NUO-1 determined by Western blot analysis after denaturing gel electrophoresis (Fig. 1). The diffuse NUO-1-specific signal in LB26 mitochondria is shifted towards smaller molecular weights, being centered at ~800-kDa. This suggests that the ~800-kDa signal either represents a complex I sub-complex or that the holoenzyme is unstable during long electrophoresis required for BN-PAGE. Mitochondria from riboflavin-supplemented LB25 and LB27 demonstrate significant increases in NUO-1-specific signal. The NUO-1-specific signal in these

Table 2
Effects of riboflavin on protein steady-state levels

Protein	LB25	LB25+Rb	LB27	LB27+Rb
NUO-1	0.29±0.05	0.74±0.04*	0.45±0.04	0.72±0.03*
23-kDa	0.97±0.03	0.98±0.02	0.97±0.02	0.99±0.01
30-kDa	0.71±0.02	0.73±0.01	0.72±0.05	0.76±0.04
COXI	0.58±0.03	0.85±0.04*	0.66±0.03	0.83±0.04*
ATP-2	1.00±0.02	1.02±0.05	1.00±0.03	0.99±0.01

Reported values are normalized to levels in the wild type N2. Values are means±S.D. of a minimum of three experiments.
* $P<0.05$ using two-sample *t*-test, compared to the corresponding unsupplemented strain.

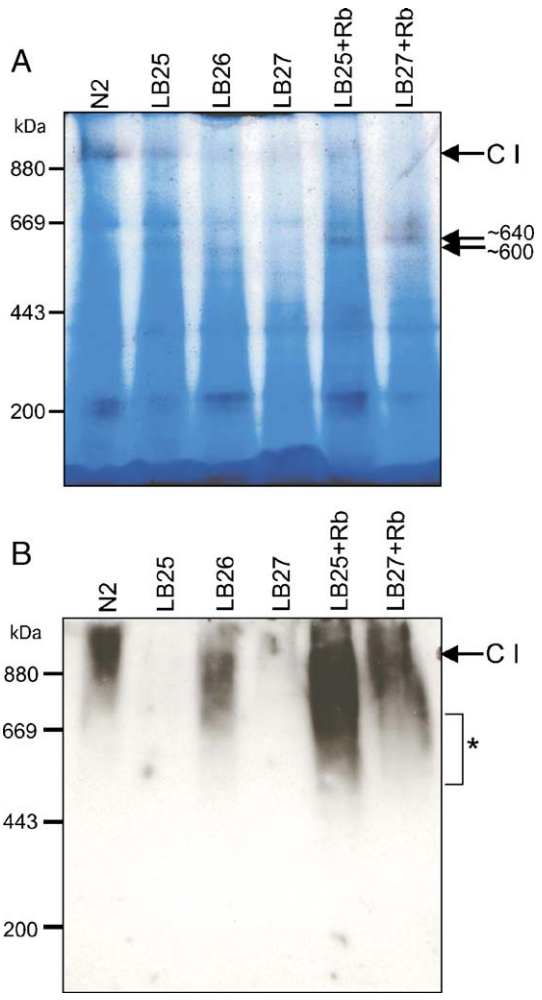

Fig. 2. Complex I assembly is impaired in *nuo-1* mutant mitochondria. (A) Mitochondrial proteins (300 μg in each lane) were resolved by BN-PAGE and the gel stained for NADH dehydrogenase activity. (B) Mitochondrial proteins (300 μg in each lane) were resolved by BN-PAGE and the gel was transferred for Western blot analysis with polyclonal antiserum against the bovine 51-kDa subunit of complex I. Rb, mitochondria were isolated from strains cultured in the presence of 1 μg/ml riboflavin; C I, fully assembled complex I holoenzyme; ~600 and ~640 indicate the sizes of LB26 and LB25-specific assembly intermediates, respectively.

samples is even more diffuse and is found as far down as ~550-kDa (Fig. 2B). The signal by Western blot overlaps with the position of the ~640-kDa species seen by NADH dehydrogenase activity staining, strongly suggesting that this species is a NUO-1-containing assembly intermediate of complex I. The diffuseness of the signal also suggests that NUO-1 is not present in a single discrete species or that this species is unstable under the conditions of electrophoresis.

We also investigated the assembly of complex IV in the *nuo-1* mutants. Mitochondrial proteins were separated by BN-PAGE and the gels stained for cytochrome *c* oxidase activity. Wild type mitochondria contain five distinct cytochrome *c* oxidase-active species (Fig. 3). A major species with an apparent molecular weight of 210-kDa, likely representing monomeric complex IV, appears first during the staining reaction. Minor species of 130, 165, 270 and 420 kDa, the latter likely representing dimeric complex IV, appear later during the in-gel staining reaction. Mitochondria from the LB25, LB26 and LB27 mutants all produce different staining patterns. The 130-, 210- and 270-kDa species are present in all three mutants, although their staining intensities are variably reduced compared to wild type. Complex IV dimer (420-kDa species) is only weakly detected in LB25 and LB27 mitochondria and appears absent in LB26. The 165-kDa species is absent in all three mutants; instead, an alternate species with an apparent molecular weight of ~160-kDa is present. Finally, a smaller additional species at ~120-kDa is present in the mutants but not in wild type.

We further examined the effects of riboflavin on the assembly of complex IV because the mutant strains showed riboflavin-responsive increases in the steady-state level of COXI (Fig. 3). The most obvious riboflavin-dependent effect is the disappearance of the 120-kDa species from the mutants, making the cytochrome *c* oxidase staining patterns more closely resemble that of wild type. However, the 160-kDa species remains intact, as opposed to the 165-kDa species seen in the wild type. The overall cytochrome *c* oxidase staining intensities of the mutant mitochondria are consistently increased by riboflavin, suggesting complex IV assembly and/or stability is enhanced by the vitamin. Our analysis has revealed the presence of aberrant cytochrome *c* oxidase complexes in *nuo-1* mutants and that the abundance of some of these is responsive to riboflavin supplementation.

3.3. Riboflavin increases the activities of complexes I and IV

The in-gel activity staining suggests that riboflavin increases the amounts of assembled, active complexes I and IV. However, the in-gel assays are only semi-quantitative and require that the complexes be stable during electrophoresis for detection. We measured the rotenone-sensitive NADH-decylubiquinone

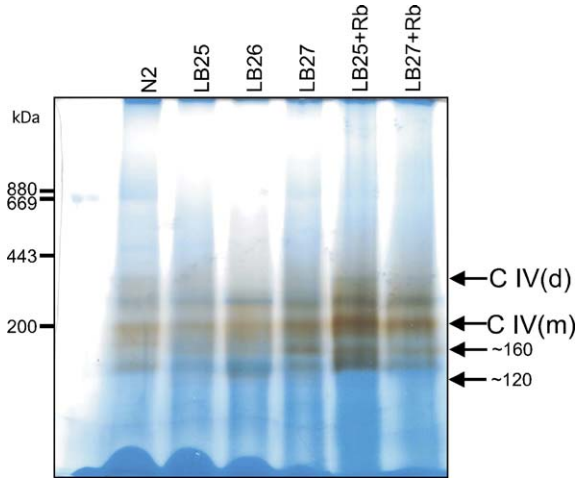

Fig. 3. Complex IV assembly is impaired in *nuo-1* mutant mitochondria. Mitochondrial proteins (300 μg) were resolved by BN-PAGE and the gel stained for cytochrome *c* oxidase activity. Rb, mitochondria were isolated from strains cultured in the presence of 1 μg/ml riboflavin; C IV(m), monomeric complex IV; C IV(d), dimeric complex IV; ~160 and ~120 indicate the sizes of mutant-specific assembly intermediates.

reductase activities in LB25 and LB27 mitochondria; as reported previously, these are approximately 30% of the wild type (Fig. 4A) [13]. Mitochondria isolated from riboflavin-supplemented cultures contain more than twice the amount of complex I activity (Fig. 4A). Likewise, the cyanide-sensitive cytochrome *c* oxidase activities of LB25 and LB27 mitochondria are ~50% of wild type levels (Fig. 4B). The activities also increase substantially when the strains are cultured in the presence of riboflavin.

4. Discussion

We have modeled pathogenic human complex I mutations in *C. elegans* in an effort to elucidate the biochemical and bioenergetic mechanisms of MRC. Our previous study suggested that mutations in complex I cause pathology in at least three ways: (1) A reduced ability to oxidize NADH perturbs the cellular redox balance and impairs other metabolic pathways. (2) The rate of oxygen free-radical generation is accelerated, leading to oxidative stress. (3) Impaired OXPHOS results in an energy deficit. In this work, we have extended our understanding of these complex I mutations by investigating their effects on the assembly and activity of complexes I and IV. In addition, we have revealed an unexpected effect of riboflavin on the assembly of the cytochrome *c* oxidase complex.

A key result of our investigation is the clear demonstration of reduced levels of complex I holoenzyme in *nuo-1* mutants. Similar observations have been reported for human complex I mutations affecting a variety of nuclear or mtDNA-encoded subunits [27,28,38–41]. Of the complex I subunits we investigated, the NUO-1 protein itself showed the greatest reduction in steady-state levels. The A352, T434 and A443 mutations we introduced into the *nuo-1* gene affect conserved residues. We believe these residues are important for protein folding and that newly-synthesized mutant NUO-1 protein is more susceptible to degradation and/or is inefficiently assembled into holoenzyme. Similarly, when the equivalent T434 mutation was modeled in *Neurospora crassa*, the mutant protein was less efficiently assembled than the wild type and resulted in approximately half the wild type activity [42]. In contrast to our results, the equivalent A352 mutant did not assemble into complex I and the authors suggested that the subunit was degraded [42]. In agreement with our conclusions, the *Neurospora* data suggest that the decreased levels of complex I activity in the membrane are mainly due to diminished assembly or stability, rather than to reduced activity of the mutated enzymes [42].

The A352, T434 and A443 residues are not located in known cofactor-binding motifs but we do not have sufficiently high resolution structural information to eliminate a role for them in cofactor binding [7]. Recently, the organization of the iron-sulfur centers of the *Thermus thermophilus* complex I was determined by X-ray crystallography [43]. The authors suggested that the FMN cofactor of the 51-kDa subunit may be located between iron–sulfur centers N1a and N3, judging from possible access channels for NADH [43].

Unlike bovine or human complex I, there is no information on the localization of specific subunits in the *C. elegans* complex I holoenzyme. There is, however, mounting evidence that mammalian complex I is assembled from a series of modules or sub-complexes [44]. Both the 30-kDa and the 23-kDa subunits are located within the peripheral arm of complex I and more specifically with the Iλ fraction, the same sub-complex that contains the 51-kDa subunit [5,45]. The mutations in *C. elegans nuo-1* affect the steady-state level of the 30-kDa subunit but not of the 23-kDa subunit. This suggests that the stability or assembly of certain sub-complexes is compromised in the mutants, leading to the loss of specific subunits. Very little is known about the 30-kDa subunit, as it contains no redox cofactors, while the 23-kDa subunit is believed to house two iron–sulfur clusters [7]. Models for the assembly of mammalian complex I have been proposed but these are incomplete or insufficiently detailed to allow us to suggest why the levels of 30-kDa and 23-kDa subunits are differentially affected by the

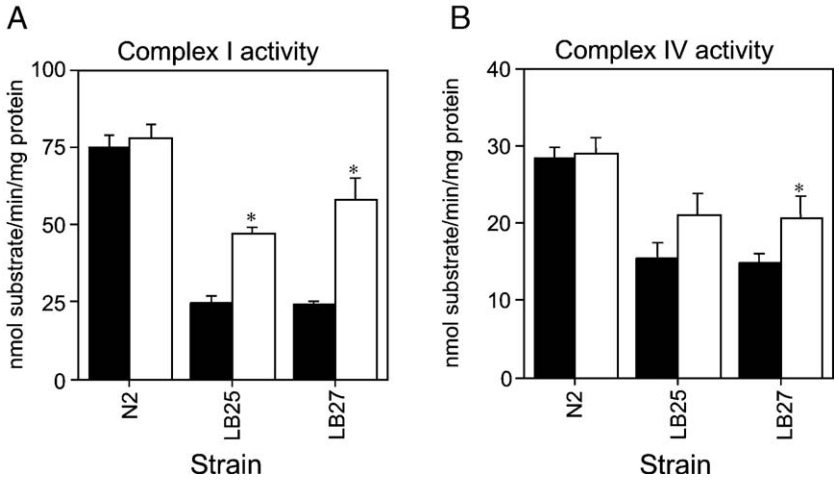

Fig. 4. Riboflavin increases the enzyme activities of complexes I and IV in *nuo-1* mutants. (A) Complex I activity was measured as the rotenone-sensitive NADH-decylubiquinone oxidoreductase activity. (B) Complex IV was measured as the cyanide-sensitive oxidation of reduced cytochrome *c*. Reported values are means of four independent trials. Black bars represent activities in mitochondria of strains grown in the absence of riboflavin; white bars represent activities of strains cultured in the presence of 1 μg/ml riboflavin. *$P<0.05$ compared to corresponding unsupplemented sample using a two-sample *t*-test.

nuo-1 mutations [28,45,46]. Due to the small number of available antibodies against *C. elegans* complex I subunits, more detailed investigations into the assembly and localization of NUO-1 are currently severely restricted.

With respect to complex I subunits, the beneficial effects of riboflavin are limited to the NUO-1 protein. This is perhaps not surprising since it is the NUO-1 subunit itself that carries the FMN cofactor and it is well known that ligands can promote protein folding and stabilization. Supplementation with riboflavin likely results in increased FMN availability, leading to enhanced rates of NUO-1 folding to a more stable cofactor-bound form and to assembly into stable sub-complexes. The appearance of lower molecular weight assembly intermediates and the continued low abundance of 30-kDa subunit in riboflavin-supplemented mutant mitochondria indicate that stabilizing and assembling mutant species of NUO-1 are not sufficient to entirely correct the assembly defects. We did not detect significant increases in the activity of the 950-kDa species by BN-PAGE and histochemical analysis in the riboflavin-treated mutants (Fig. 2A), yet we detected more NUO-1 protein by Western blotting in the 600–1,000 kDa range (Fig. 2B). We suggest that the mutant complexes may not be fully stable during the extended electrophoresis conditions required for BN-PAGE. The diffuseness of the NUO-1 signal detected by Western blotting after BN-PAGE suggests that mutant NUO-1 protein can be assembled into a high molecular weight species but that only a fraction of this species survives BN-PAGE; most of the NUO-1 protein is found in faster migrating species that are probably unstable and have little or no activity. Our results emphasize the importance of studying the assembly pathways of MRC enzymes for diagnosing and treating mitochondrial disease.

We have identified aberrant assembly of complex IV as contributing to the *nuo-1* mutant phenotypes. Patients with mutations in the NDUFS2 and NDUFS4 subunits of complex I presented with a decreased level of complex III dimer [38]. These observations suggest that a physical interaction between complexes I and III may exist. This is supported by genetic evidence; a mutation in cytochrome *b* of complex III produces a combined deficiency in complexes I and III [47]. A supercomplex comprising complexes I and III and up to four copies of complex IV has been detected in mammalian mitochondria [48]. In fact, almost all the complex I was found associated with complex III. Our previous investigations did not identify a consistent effect of the *nuo-1* mutations on complex III activity [13]. Under certain detergent conditions, a direct interaction between complexes I and IV without complex III is detected [48].

Combined deficiencies in complexes I and IV have been reported but the precise molecular lesions are unknown [22,49–51]. Complex IV deficiency has been presumed to be secondary to complex I deficiency [51]. Complex I deficiency may destabilize complex IV by preventing or diminishing the formation of one or more of the supercomplexes seen in mammalian mitochondria. Alternatively, complex I dysfunction may indirectly affect assembly of complex IV through altered expression of complex IV subunits, including isoforms, or of complex IV assembly factors. These altered expression patterns may account for the relatively small size differences in the complex IV species seen by BN-PAGE and activity staining (Fig. 3). Finally, complex I deficiency may result in a lower membrane potential across the mitochondrial inner membrane, which is necessary for complex IV assembly [52]. Riboflavin may thus exert its beneficial effects on complex IV either through enhancing complex I assembly and stability or indirectly through enhancing complex I activity and energy production.

In conclusion, we have shown that the catalytic deficiencies of complexes I and IV observed in *C. elegans nuo-1* mutants are due to their impaired assembly or stability. NUO-I mutants have reduced levels of fully assembled complex I and lower molecular weight assembly intermediates. Riboflavin supplementation stabilizes the steady-state levels of NUO-1, thereby increasing the abundance of fully assembled complex I and of specific assembly intermediates. NUO-1 mutants also demonstrate aberrant complex IV assembly patterns, which are partially corrected by riboflavin. We suggest that the effects of riboflavin are profound because this vitamin improves the activities of both complexes I and IV, which in turn leads to reduced oxidative stress and lactic acidosis and increased metabolic function.

Acknowledgements

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