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Silencing of FAD synthase gene in *Caenorhabditis elegans* upsets protein homeostasis and impacts on complex behavioral patterns

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ABSTRACT

Background: FAD synthase is a ubiquitous enzyme that catalyses the last step of FAD biosynthesis, allowing for the biogenesis of several flavoproteins. In humans different isoforms are generated by alternative splicing, isoform 1 being localized in mitochondria. Homology searching in *Caenorabditis elegans* leads to the identification of two human FAD synthase homologues, coded by the single copy gene *R53.1*.
Methods: The *C. elegans R53.1* gene was silenced by feeding. The expression level of transcripts was established by semi-quantitative RT-PCR. Overall protein composition was evaluated by two-dimensional electrophoresis. Enzymatic activities were measured by spectrophotometry and oxygen consumption by polarography on isolated mitochondria.
Results: From *R53.1* two transcripts are generated by *trans*-splicing. Reducing by 50% the transcription efficiency of *R53.1* by RNAi results in a 50% reduction in total flavin with decrease in ATP content and increase in ROS level. Significant phenotypical changes are noticed in knock-down nematodes. Among them, a significant impairment in locomotion behaviour possibly due to altered cholinergic transmission. At biochemical level, impairment of flavoenzyme activities and of some KCN-insensitive oxygen-consuming enzymes is detected. At proteomic level, at least 15 abundant proteins are affected by *R53.1* gene silencing, among which superoxide dismutases.
Conclusion and General Significance: For the first time we addressed the existence of different isoforms of FAD-metabolizing enzymes in nematodes. A correlation between FAD synthase silencing and flavoenzyme derangement, energy shortage and redox balance impairment is apparent. In this aspect *R53.1*-interfered nematodes could provide an animal model system for studying human pathologies with alteration in flavin homeostasis/flavoenzyme biogenesis.

1. Introduction

The relevance of riboflavin (vitamin B_2, Rf) in cell metabolism rests on its role as precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the redox cofactors of a large number of dehydrogenases, reductases and oxidases [1,2]. Rf to FAD conversion occurs via the sequential actions of riboflavin kinase, or ATP:riboflavin 5'-phosphotransferase (RFK, EC 2.7.1.26), and of FAD synthase, or ATP: FMN adenylyl transferase (FADS or FMNAT, EC 2.7.7.2).

The sub-cellular localization of FADS in eukaryotes has been matter of a long-lasting controversy. De Luca and Kaplan first demonstrated that the enzyme responsible for FAD production is located in the cytosol of rat liver [3]. Since then, FADS has been purified from rat liver cytosolic fractions only as a protein with molecular mass of about 53 kDa by SDS–PAGE [4,5]; a cytosolic localization has also been demonstrated for yeast Fad1p [6]. Therefore, for many years it had been assumed that in eukaryotes FAD biosynthesis occurred only in the cytosol. However, using cell fractionation and activity measurements, the presence of FADS activity was then detected in mitochondria from rat liver [7,8], *Saccharomyces cerevisiae* [9,10] and *Nicotiana tabacum* Yellow Bright-2 [11].

Abbreviations: ALS, amyotrophic lateral sclerosis; FAD, flavin adenine dinucleotide; FMN, flavin adenine mono-nucleotide; FADS, FAD synthase; FCCP, carbonyl cyanide *p*(trifluoromethoxy)-phenyl-hydrazone; FUM, fumarase; GR, gluthatione reductase; IPTG, isopropyl β-D-1-thiogalactopyranoside; NGM, Nematode Growth Medium; OXPHOS, oxidative phosphorylation; Rf, riboflavin; RFK, riboflavin kinase; RR-MADD, Riboflavin Responsive Multiple Acyl-CoA Dehydrogenase Deficiency; RNAi, RNA interference; SDH, succinate dehydrogenase; SOD, superoxide dismutase; TCA cycle, tricarboxylic acid cycle

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The hypothesis that different isoforms with compartment-specific functions may exist in eukaryotes has been recently supported by the cloning and functional characterization of two products of the human FADS gene, *FLAD1*. *FLAD1* encodes different transcript variants, among which are transcript variants 1 and 2. The two corresponding protein products differ for an extra-sequence of 97 amino acids at the N-terminus, present only in isoform 1 (hFADS1), which contains a putative mitochondrion-targeting peptide predicted by bioinformatics [12]. Accordingly, a recent paper conclusively demonstrates that, in humans, hFADS1 is mitochondrial whereas hFADS2 is cytosolic [13].

A reduction of FADS activity has been suspected in human adults with hypothyroidism since the level of erythrocyte glutathione reductase (GR, EC 1.8.1.7), a FAD-containing enzyme, is significantly reduced [14]. A FADS alteration was also suggested in a patient with a neuromuscular metabolic disease (Riboflavin Responsive Multiple Acyl-CoA Dehydrogenase Deficiency, RR-MADD), in whom FAD level was half that of control samples and a number of mitochondrial FAD-containing enzymes were reduced but could be restored by Rf treatment [15]. Recently, a significant reduction in FADS was observed at the protein level in serum from patients with nasopharyngeal carcinoma [16] and at the transcriptional level in whole blood from patients with amyotrophic lateral sclerosis (ALS) [17]. Moreover, FADS was the antigen target of monoclonal IgA in a sporadic ALS patient, who also had an IgA gammopathy [17].

In order to estimate the effects of lower FAD levels in an easily amenable model we studied the transcription regulation of the FADS gene using *Caenorhabditis elegans*. This nematode is one of the best model organisms in biological research, since an enormous amount of information has been accumulated with respect to anatomy, development and genetics. It is easy and inexpensive to maintain in the laboratory on agar plates or in liquid medium with *Escherichia coli* as food source. Its life cycle from egg to adult takes about 3 days at 22 °C. The first-stage larva (L1) hatches from the egg and proceeds through three additional stages of larval development - L2, L3, and L4 - punctuated by moults, before reaching reproductive maturity as an adult [18]. *C. elegans* is endowed with many of the basic organs and systems common to higher animals and its behavioural responses to chemotaxis and thermotaxis are also observed in higher organisms [19]. Finally, *C. elegans* was the first multicellular organism for which the genome was completely sequenced [20]. This achievement has boosted genome-wide research projects devoted to *e.g.* RNAi, ORFeome and interactome [21].

C. elegans has been used as a model of several aspects of neurodegenerative diseases, including those in which aggregation and toxicity of misfolded disease-related proteins have been demonstrated [22,23]. Among these, Rf and FAD have proven to rescue the phenotype of frataxin deficiency [24] and of mitochondrial complex I and IV dysfunction [25].

In this paper we describe structure and expression of the FADS gene of *C. elegans* as well as provide an overview of the effects of partially silencing the gene.

2. Materials and methods

2.1. Materials

All reagents and enzymes for biochemical analysis were from Sigma-Aldrich (USA). Reagents for RNA extraction, purification and retro-transcription (Trizol Reagent, DNAseI Amp Grade, SuperScript III First-Strand System for RT-RNA), chemically competent *E. coli* cells (Shot TOP10F') and TOPO TA Cloning Kit were purchased from Invitrogen (USA). 5'/3'UTR RACE kit, 2nd generation, was purchased from Roche (Switzerland). Reagents used for nematode cultures were from Oxoid (UK). The dye reagent for protein assay was from Bio-Rad (USA). Solvents and salts used for HPLC were from J. T. Baker (USA). Immobiline II monomers and carrier ampholytes were from GE Healthcare (UK). Other chemicals for electrophoresis were from either Merck (Germany) or Bio-Rad (USA).

2.2. C. elegans culture conditions

Wild-type *C. elegans* N2 was maintained on Nematode Growth Medium (NGM) agar plates (51.72 mM NaCl, 1.7% bacto-agar, 0.25% bacto-peptone, 0.0005% cholesterol, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM potassium phosphate pH 6) seeded with *E. coli*, OP50 strain. The *rrf-3* strain was provided by the *Caenorhabditis* Genetic Center. The nematodes were incubated at 22 °C and observed through a stereomicroscope ZEISS Stemi DV4. The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. The end of each larval stage is marked with a moult where a new, stage-specific cuticle is synthesized and the old one is shed. Each stage is easily identified by the relative size of larvae [26]. We analysed number of progeny, developmental timing and behavioural states.

2.3. RT-PCR and semi-quantitative RT-PCR

Nematodes were collected from plates with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 83 mM NaCl, 0.1 mM $MgSO_4$), and lysed by thermal shock. Total RNA was extracted using Trizol Reagent (Invitrogen) and treated with DNAse (deoxyribonuclease I, amplification grade, Invitrogen). RNA concentration was measured at 260 nm. Total cDNA was prepared from 700 ng DNase-treated RNA using SuperScript III First-Strand System for RT-PCR (Invitrogen). Random hexamers were used as primers for the overall cDNA synthesis. When appropriate, oligod(T) were used as primers for the amplification of poly(A)-containing RNA. PCR was performed using 5 PRIME Taq DNA Polymerase (Eppendorf) according to the manufacturer's instructions using gene-specific primers. RT-PCR products were separated through electrophoresis on ethidium bromide-containing 1.2% agarose gel or, when higher resolution was necessary, on ethidium bromide-containing 12% polyacrylamide gel. Gene expression level was analysed through a semi-quantitative reverse transcription-PCR technique, using *ama-1* for expression level normalization of the target genes. Images and quantification were obtained with a Molecular Imager ChemiDoc XRS System (BioRad). Primers were designed on the basis of sequences retrieved from *C. elegans* database (Wormbase, http://www.wormbase.org) and are reported in Supplementary Material_Table 1.

2.4. Cloning

The amplification product was excised from agarose gel, purified using Nucleo Spin Extract II (Macherey-Nagel) and ligated into the pCR 2.1 TOPO cloning vector (TOPO TA cloning kit, Invitrogen). The ligated product was transformed into One Shot TOP10F′ Chemically Competent *E. coli* (Invitrogen). Cells were then plated onto LB-agar plates containing ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, and incubated overnight at 37 °C. Positive colonies were selected, grown overnight in LB broth containing ampicillin, and plasmids were purified using the QIAprep® Spin Miniprep Kit (Qiagen GmbH). Approximately 25 μl of purified plasmid was sent to a commercial Sequence Service (Primm srl, Milano, Italy) for sequencing with M13 reverse and M13 forward primers.

2.5. 3' RACE

3' RACE experiments were performed using 5'/3' RACE 2nd generation kit (Roche) following manufacturer's instructions. Briefly, the first strand cDNA synthesis was initiated at the poly(A)$^+$ tail using