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Coenzyme Q is irreplaceable by demethoxy-coenzyme Q in plasma membrane of *Caenorhabditis elegans*

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Abstract A procedure was developed to isolate fractions enriched in plasma membrane from *Caenorhabditis elegans*. Coenzyme Q_9 (Q_9) was found in plasma membrane isolated from either wild-type or long-lived *qm30* and *qm51 clk-1* mutant strains of *Caenorhabditis elegans*, along with dietary coenzyme Q_8 (Q_8) and the biosynthetic intermediate demethoxy-Q_9 (DMQ_9). NADH was able to reduce both Q_8 and Q_9, but not DMQ_9. Our results indicate that DMQ_9 cannot achieve the same redox role of Q_9 in plasma membrane, suggesting that proportion of all these Q isoforms in plasma membrane must be an important factor in establishing the *clk-1* mutant phenotype.

Keywords: Coenzyme Q; Demethoxy-coenzyme Q; Plasma membrane; *clk-1* mutant phenotype; *Caenorhabditis elegans*

1. Introduction

Ubiquinone (coenzyme Q, Q) is a lipophilic redox molecule that has central function in the mitochondrial respiratory chain, as well as it regulates transition pore in mitochondria [1]. Q also regulates an inducible ceramide-dependent signaling pathway at the plasma membrane of mammals [2]. Q is a highly conserved redox molecule and at least 10 genes are necessary for its biosynthesis [3–6]. Knockout mutation of genes involved in Q biosynthesis, *COQ-7*/*CLK-1* in mice [7] and *coq-3* in *Caenorhabditis elegans* [8] induce arrest of development. There are a multitude of cellular processes that depend on Q [9], including respiration and redox functions at non-mitochondrial membranes that lead to guarantee the success of development and aging [8–11].

Long-lived *C. elegans clk-1* mutant strains accumulate demethoxy-coenzyme Q_9 (DMQ_9), an intermediate of Q_9 biosynthetic pathway [10,12]. DMQ_9 supports partially respiration in these mutants but cannot substitute all functions mediated by Q_9 in non-mitochondrial sites [8,13,14]. Plasma membrane of all eukaryotic cells tested contains an antioxidant system where Q acts as electron carrier, and maintains reduced other antioxidants such as α-tocopherol and ascorbate [15]. Both the impairment of the reduction of Q in this system [16] and the semiquinone produced by NADH-dependent Q reduction [17] are an important source of reactive oxygen species (ROS). The content of Q isoforms and their redox state in the plasma membrane are important factors to determine the cellular physiology, and probably can contribute to the phenotype of these nematodes.

The aim of this work is to study the function of Q isoforms in plasma membrane-enriched fractions of *C. elegans* strains. *C. elegans* N2 strain showed both Q_9 and DMQ_9, and also diet-derived Q_8, and Q_8-less fed N2 nematodes contained solely Q_9 in the plasma membrane. Surprisingly, Q_9 was detected in plasma membrane fractions isolated from both *qm30* and *qm51 clk-1* mutant nematodes along with Q_8 and DMQ_9. Both Q_8 and Q_9 were reduced by NADH in these membranes but DMQ_9 was not significantly reduced. ROS production was lower in plasma membrane of *qm51 clk-1* nematodes. These results can be interpreted in terms of the inability of DMQ_9 to replace Q_9 as substrate of trans-plasma membrane redox reactions, and that does not contribute to ROS production in this membrane.

2. Materials and methods

2.1. Growth culture conditions

N2 (Bristol) wild-type and *qm30* and *qm51* (two Q_9-deficient *clk-1* mutant) strains of *C. elegans* were grown using standard procedures [18]. Worm strains were kindly provided by Dr. T. Stiernagle from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis). Two different strains of *Escherichia coli*, the wild-type OP50 strain, and the Q_8-less GD1 strain, were used separately to feed N2 nematodes, but *clk-1* mutant nematodes were only fed with the wild-type OP50 *E. coli* strain. In this way, worm cultures were supplemented or not with Q_8 (the natural occurring Q homologue in *E. coli*) through the corresponding bacteria strain.

2.2. PCR analysis

To check genomic DNA, worms from the three different strains (N2, *qm30* and *qm51*) were subjected to a lysis procedure followed by PCR analysis. Briefly, 3–4 of the corresponding worm strain were picked up from NGM agar plates and transferred to PCR tubes containing 3 μl of lysis buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% Gelatin and 1 mg/ml proteinase K). After pelleting, the worms were frozen in liquid N_2 for 10 min and then lysed by heating them at 65 °C for 60–90 min, followed by proteinase K inactivation at 95 °C for 15 min. These worm lysates were used as DNA templates to run a conventional touch-down PCR. Amplified PCR fragments were subjected to agarose gel electrophoresis, and isolated bands were cut, cleaned up and cloned into pGEM®-T Easy vector (Promega, Madison, WI) to further amplification by transforming competent bacteria. Plasmids bearing the corresponding insert fragments were then retrieved by the Perfectprep® Plasmid Mini kit of Eppendorf AG (Hamburg, Germany) and sent for their sequence analysis (standard sequencing primers for pGEM®-T Easy vector were used) to MGW-Biotech AG (Ebersberg, Germany).

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For primers designing, the sequence with the Accession No. ZC395.2, the unspliced transcript sequence of the CLK-1 gene, along with 600 pb of its 3′-UTR was retrieved from the WormBase server.

The *qm30* checking primers (covering the last exon of the gene) were:
sense primer: (862) 5′-GTTGAAGAACTCATTGGACA-3′ (881).
antisense primer: (1851) 5′-TAATCGCTTATACGGTAGCT-3′ (1832).

The *qm51* checking primers (covering the second intron of the gene) were:
sense primer: (584) 5′-GTTCATCTGTTGGTTCAGTA-3′ (603).
antisense primer: (919) 5′-TTCATTTTGGAATACCTTTC-3′ (900).

For N2 worms, both pairs of checking primers were used in our PCR analysis.

2.3. Membranes isolation

All the process was carried out at 4 °C. Worms were harvested by centrifugation and live nematodes were selected by flotation in 30% sucrose, washed and resuspended in ice-cold lysis buffer (37 mM Tris–maleate, pH 6.4, 0.5 M sucrose, 2 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, and 1% (v/v) Sigma protease inhibitor cocktail). Worm suspension was sonicated for 4×1 min at maximal power in a BANDELIN sonopuls UW 2070 tip sonicator, homogenized in a Teflon-glass potter homogenizer for 10 min and centrifuged at $1000 \times g$ for 20 min. Resulting supernatant was centrifuged at $12000 \times g$ for 10 min, and the resulting supernatant was pelleted at $105000 \times g$ for 1 h. This pellet was resuspended in 5 ml of TE buffer (25 mM Tris–HCl, pH 7.6, 1 mM EDTA) containing 20% (w/v) sucrose (STE20, the number indicates sucrose percentage in w/v) and applied on the top of a discontinuous sucrose gradient made of 3 ml 48% (w/v) sucrose and 4 ml of 30% (w/v) sucrose in TE buffer. The gradient was subjected to centrifugation at $105000 \times g$ for 4 h, and the collected interphases were diluted in membrane buffer (25 mM Tris–HCl, pH 7.6 containing 10% glycerol (v/v), 1 mM EDTA, 1 mM PMSF, and 1% (v/v) Sigma protease inhibitor cocktail), pelleted at $105000 \times g$ for 1 h, resuspended in plasma membrane buffer, aliquoted and stored at −80 °C until use. Plasma membrane enriched fraction partitioned into the interphase between 20% and 30% of the sucrose gradient. The $12000 \times g$ pellet (crude mitochondrial fraction) was resuspended in STED20 buffer (STE20 buffer containing 1 mM DTT). Pure mitochondria fractions were isolated by a sucrose step gradient composed by two STED48 and STED34 layers of 4 ml. Samples already resuspended in STED20 buffer were applied onto STED34 layer and centrifuged $100000 \times g$ for 4 h at 4 °C in a SW40Ti rotor (Beckman, Los Angeles, USA). Pure mitochondria located at the interphase between 34% and 48% of the sucrose gradient were collected, washed and then resuspended in membrane buffer and kept at −80 °C until use. Purity of fractions was checked by enzyme marker analysis: *p*-nitrophenyl phosphatase and 5′-nucleotidase for plasma membrane, succinate:cytochrome *c* reductase for mitochondria, galactosyl transferase for Golgi apparatus, and NADPH:cytochrome *c* reductase for endoplasmic reticulum [19]. Protein concentration was determined by the modified Bradford method [20].

2.4. Western blot analysis

Crude mitochondrial fraction (25 μg of protein) of the three different nematode strains (wild-type N2, *qm30* and *qm51 clk-1* mutants) were denatured overnight at room temperature in SDS–PAGE loading buffer containing an extra 4% SDS. Samples were then subjected to a SDS–PAGE, with the stacking gel containing 7 M Urea and the separating gel at 12.5% acrylamide, and transferred onto a PVDF membrane for Western blot analysis. Primary antibodies were used at 1:500 dilutions. Anti-Coq7p primary antibody was raised in chicken by AgriSera (Vännäs, Sweden) by using a common peptide of both human and *C. elegans* Coq7 protein as antigen. Anti-*S. cerevisiae* $F_1\beta_0$ ATPase primary antibody was raised in rabbit and was a gift of Dr. R. Serrano from the "Instituto de Biología Molecular y Celular de Plantas" (Valencia, Spain). This anti-yeast primary antibody was used as a cross-reacting antibody to control the loading of protein in the SDS–PAGE. HRP-conjugated secondary antibodies (anti-chicken IgG and anti-rabbit IgG, respectively) were used at 1:5000 dilutions. The Immun-Star™ HRP substrate kit from Bio-Rad (Hercules, CA) was used to develop the PVDF membrane.

2.5. Quantification and redox state of Q isoforms in the plasma membrane of C. elegans

Two methods were used for the extraction of Q isoforms from plasma membrane of *C. elegans*: (i) a standard hexane extraction of ethanolic SDS-treated plasma membranes for quantification of total content of Q isoforms (recovered in their oxidized state) [4], and (ii) a rapid extraction procedure for the evaluation of the reduced/oxidized ratio of Q isoforms [21]. The redox state of Q isoforms in plasma membrane of *C. elegans* was studied after its incubation (50 μg/0.1 ml of 50 mM Tris–HCl, pH 7.4) in the absence and in the presence of 0.1 mM NADH for 30 min at 30 °C. β-Mercaptoethanol was added to the incubation mixture to a final concentration of 0.5 mM to prevent reoxidation of the reduced Q forms during the extraction process. Incubation was stopped by addition of 330 μl *n*-propanol and centrifuged for 5 min at $15000 \times g$. The clear supernatant was immediately subjected to HPLC separation as described [21]. Assignment of peaks was done by the criteria described elsewhere [4], and reduced forms were assigned after their chemical reduction with $NaBH_4$.

2.6. Assay for H_2O_2 production at the plasma membrane of C. elegans

Plasma membrane (0.2 mg/ml) was incubated in a 100 μl final volume (50 mM Tris–HCl, pH 7.4) in the absence and in the presence of 10 μM NADH at room temperature in a 96-well plate. Cumulative H_2O_2 produced over 3 h of incubation was measured by the *Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit* (Molecular Probes, Eugene, USA) according to manufacturer instructions. Fluorescence (λ_{ex} = 532 nm, λ_{em} = 580 nm) of *Amplex® Red* was monitored and analyzed in a Typhoon 9410 imager (Amersham Pharmacia Biotech, Uppsala, Sweden). The amount of H_2O_2 was calculated against calibration curves done with commercial H_2O_2 diluted in the same buffer solution as sample assays (Sigma–Aldrich, Madrid, Spain).

2.7. Statistical analysis

Data are expressed as means ± S.E.M. Changes in variables for different assays were analyzed by either Student's *t* test (single comparisons) or one-way analysis of the variance (ANOVA) for multiple comparisons. If ANOVA revealed significant changes between samples, multiple unpaired Student's *t* tests were performed. Differences among means were considered significant when $P < 0.05$. The software package used was SigmaStat v.2.0.3 (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Purity of membrane fractions

The purity of both plasma membrane- and mitochondria-enriched fractions isolated from *C. elegans* was established by the assay of the enzyme markers included in Table 1 [19]. Plasma membrane-enriched fraction showed an increase of about 20-folds of plasma membrane markers and almost the absence of markers of the other endomembranes. Mitochondria-enriched fraction showed an increase of about 14-folds of its specific marker compared to plasma membrane markers that were not detected, and a significant decrease of other non-specific markers.

3.2. Content of Q isoforms in the plasma membrane

The concentration, as well as their redox state, of Q isoforms extracted from plasma membrane fractions from *C. elegans* are shown in Fig. 1. Plasma membrane of wild-type nematodes fed on Q_8-replete bacteria contained both Q_9 and dietary Q_8, and a small amount of DMQ_9, while plasma membrane of Q_8-less fed wild-type nematodes only contained Q_9. Along with these Q isoforms, other non assigned compounds – rhodoquinone-9 is likely to be the peak eluting at ca. 21.5 min, according to previous reports [10] – were also observed in chromatograms (Fig. 1). None of these non-assigned peaks changed after the incubation of plasma membrane samples with NADH. Wild-type yeast strains can also accumulate DMQ_6 [22]. Plasma