Altered Quinone Biosynthesis in the Long-lived clk-1 Mutants of Caenorhabditis elegans*

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Hiroko Miyadera, Akira Hiraishi, Hikari Taka, Kimie Murayama, Hideto Miyoshi, Kimitoshi Sakamoto, Naoki Ishii, Siegfried Hekimi, and Kiyoshi Kita

From the Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan, the Department of Ecological Engineering, Toyohashi University of Technology, Toyohashi 441-8580, Japan, the Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo 113-8421, Japan, the Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, the Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa 259-1193, Japan, and the Department of Biology, McGill University, Montréal, Québec H3A 1B1, Canada

Mutations in the clk-1 gene of Caenorhabditis elegans result in an extended life span and an average slowing down of developmental and behavioral rates. However, it has not been possible to identify biochemical changes that might underlie the extension of life span observed in clk-1 mutants, and therefore the function of CLK-1 in C. elegans remains unknown. In this report, we analyzed the effect of clk-1 mutation on ubiquinone (UQ) biosynthesis and show that clk-1 mutants mitochondria do not contain detectable levels of UQ9. Instead, the UQ9 biosynthesis intermediate, demethoxyubiquinone (DMQ9), is present at high levels. This result demonstrates that CLK-1 is absolutely required for the biosynthesis of UQ9 in C. elegans. Interestingly, the activity levels of NADH-cytochrome c reductase and succinate-cytochrome c reductase in mutant mitochondria are very similar to those in the wild-type, suggesting that DMQ9 can function as an electron carrier in the respiratory chain. To test this possibility, the short side chain derivative DMQ2 was chemically synthesized. We find that DMQ2 can act as an electron acceptor for both complex I and complex II in clk-1 mutant mitochondria, while another ubiquinone biosynthesis precursor, 3-hydroxy-UQ2, cannot. The accumulation of DMQ9 and its use in mutant mitochondria indicate, for the first time in any organism, a link between the alteration in the quinone species used in respiration and life span.

The understanding of the biological pathways that control life span can be studied in Caenorhabditis elegans through the identification of genes that alter the length of life when mutated (1). For example, mutations in clk-1 are known to cause an extended life span, as well as the slowing of a variety of developmental and physiological events, including the cell cycle, embryogenesis, post-embryonic development, and rhythmic adult behaviors (2, 3). Thus, CLK-1 is expected to play a unique biological role that is necessary to determine the life span and to coordinate these various biological processes. However, the biochemical differences between clk-1 mutants and the wild-type strain, which might indicate the function of CLK-1, have yet to be identified (1, 4–7).

clk-1 encodes a 187-residue polypeptide that is homologous to yeast coq7/cat5 (8). COQ7/CAT5 is located in the inner membrane of yeast mitochondria and is necessary for the biosynthesis of ubiquinone (UQ9) in yeast (9, 10). Therefore, yeast coq7/cat5 mutants, which lack UQ9, are unable to grow on nonfermentable carbon sources (9). Orthologs of clk-1/coq7/cat5 have also been reported from mammals, including human (11–13), and appear to be highly conserved among species.

Recently, a green fluorescent protein fusion to C. elegans CLK-1 was shown to localize to the mitochondria of all the somatic cells of the worm (14). However, in contrast to the situation in yeast, which is defective in respiratory growth, C. elegans clk-1 mutants are able to respire almost normally. In fact, the metabolic capacities and the ATP levels of adult clk-1 mutants are unchanged or even higher than those of the wild-type strain (15), and clk-1 mutants mitochondria exhibit succinate-cytochrome c reductase activity that is comparable with that of wild-type mitochondria (14). These observations suggest that CLK-1 is not exclusively involved in UQ biosynthesis in C. elegans.

In this report, we analyzed the quinone composition of clk-1 mutants mitochondria, to elucidate the effect of clk-1 mutation on the biosynthesis of UQ in C. elegans, and found that UQ biosynthesis is dramatically altered in clk-1 mutants. That is, clk-1 mutants mitochondria do not possess detectable levels of UQ9, and instead contain a UQ biosynthesis intermediate, demethoxy ubiquinone (DMQ9). We further analyzed the respiratory activities of mutants mitochondria and found that DMQ9 can functionally replace UQ to maintain active respiration in clk-1 mutant mitochondria, despite the absence of UQ9.

EXPERIMENTAL PROCEDURES

Nematode Strains—The wild-type strain used was the N2 (Bristol) strain. Mutant strains used were CB4876 clk-1 (e2519), MQ438 clk-1 (qm51), and MQ50 clk-1 (qm30). MQ50 clk-1 (qm30) was supplied from the Caenorhabditis Genetics Center.

Mitochondria Preparation—Nematodes were grown at 20 °C on NGP plates, which contain 3-fold bacto-peptone with the supplemen of Escherichia coli OP50. Nematodes were collected in M9 buffer and were sedimented in a 200-ml cylinder. The sedimented worms were washed with M9 buffer until the buffer became clear and were applied to Baermann’s Device and left overnight. The nematodes were collected and further purified by centrifugation on 30% (w/v) sucrose, at 750 × g for 5 min at 4 °C (16). The worms were homogenized in 0.21 M mannitol,
Identification of Quinones—Quinones were extracted from lyophilized mitochondria (3.0 mg of protein). The mitochondria were vortexed in EtOH/hexane (2/5, v/v) for 10 min and centrifuged at 15,000 rpm for 5 min at room temperature. The supernatants were pooled, and the extraction of quinones was repeated twice. After drying the pooled extracts under a stream of nitrogen gas, the residue was redisolved in ethanol and analyzed by HPLC. Quinones were applied to a reverse-phase column (Inertsil ODS-3, C-18, 5 μm, 4.6 × 250 mm, GL Science, Tokyo) and was eluted in isocratic condition (1 ml/min), with diisopropyl ether/MeOH (1/4, v/v) as described previously (17). The eluted quinones were identified by comparing their retention times with authentic UQ9 (Sigma). The spectral characteristics of each quinone were monitored at 340 nm, using a millimolar extinction coefficient of 6.2 for NADH, 200 μM potassium phosphate buffer, or 14,500 g at 4 °C. The supernatants were then centrifuged at 1,080 g for 10 min. The supernatants were then centrifuged at 23,500 g for 10 min, and the pellet mitochondrial fraction was resuspended in MSE.

Enzyme Assays—NADH-cytochrome c reductase activity and succinate-cytochrome c reductase activity were assayed as described previously (20) in 50 mM potassium phosphate buffer (pH 7.7), 200 μM NADH, or 10 mM potassium succinate, 2 mM KCN, and 50 μM horse heart ferrocytochrome c. NADH-cytochrome c reductase activity was assayed in 50 mM potassium phosphate buffer (pH 7.7), 200 μM NADH, 2 mM KCN, and 90 μM quinone analogues. The oxidation of NADH was monitored at 340 nm, using a millimolar extinction coefficient of 6.2 for NADH. Succinate-quinone reductase activities were measured as described previously (21). DMQ9, and 3-hydroxy-UQ2, were synthesized as described previously (22). All the assays were performed at 20 °C.

RESULTS AND DISCUSSION

Quinones were extracted from the mitochondria of N2 and clk-1 mutant strains, and the quinone composition was directly analyzed by reverse-phase HPLC (Fig. 1). Three different mutant strains, including a missense mutant (e2519), a deletion mutant (qm30), and a splice acceptor mutant (qm51), were used for the analysis. The major peak at 18.3 min from N2 mitochondria is identical to standard UQ9 for both elution time and absorption property (Fig. 1, A and B, and Fig. 2A). However, a corresponding peak was not observed in clk-1 mutants. The mutant mitochondria instead exhibited a major peak eluting 1 min earlier than UQ9 (Fig. 1, C–E). The slightly polar nature and the absorption property (absorption peak at 270 nm, Fig. 2B) of this compound coincide well with those reported for the ubiquinone biosynthesis intermediate, DMQ9 (see Fig. 3) (19, 23). A mass spectrometry analysis of the accumulated quinone in clk-1 (qm51) mutant detected a molecular ion peak at m/z 765, which corresponds to the molecular mass of DMQ9 (theoretical mass [C53H80O3] = 765.2005) (Fig. 4). For comparison, the mass spectrum of the standard UQ9 (Sigma) showed a molecular ion peak at m/z 795 (theoretical mass [C54H82O4] = 795.2264) (data not shown).

The amount of DMQ9 in all the three mutant strains was in the same range as UQ9 content in N2 mitochondria (Table I). In all clk-1 mutants, the peak corresponding to UQ9 was undetectable by UV absorbance, indicating that the levels of UQ9 in clk-1 mutants mitochondria are less than 0.1 nmol/mg. Since clk-1 mutants show normal levels of oxygen consumption (15), and succinate-cytochrome c reductase activity (14), it has been suggested that CLK-1 may not be critically involved in UQ biosynthesis in nematodes (4–7). Our findings, however, clearly demonstrate that clk-1 encodes a protein that is absolutely required for the biosynthesis of UQ in C. elegans (Fig. 3).
To further investigate the activity of DMQ as an electron acceptor, we chemically synthesized a short side chain DMQ$_2$ and measured the activities of NADH-quinone reductase and succinate-quinone reductase. As shown in Table II, DMQ$_2$ was able to accept reducing equivalent from complex I with a rate comparable with UQ$_2$. DMQ$_2$ was also capable of serving as electron acceptor of complex II, although the activity was lower than that with UQ$_2$. In contrast to DMQ$_2$, 3-hydroxy-UQ$_2$, which is a direct precursor of UQ (see Fig. 3), was unable to serve as efficient electron acceptor neither at complex I nor at complex II (Table II), indicating that not all the quinone biosynthesis intermediates are recognized as functional substrates by respiratory complexes. Interestingly, DMQ appears to be a more efficient substrate for complex I than for complex II (Tables I and II). This tendency has been also reported for *E. coli* ubiF mutants, which accumulate DMQ$_6$ (25), suggesting that the structure and the redox potential of DMQ might be more favorable for reduction by complex I than by complex II. The active respiration in *clk-1* mutants mitochondria raises the question as to why yeast *coq7–1* mutants, which also contain DMQ$_6$, are defective in respiratory growth (9). Our results suggest that this is probably due to the relatively small amount of DMQ$_6$ accumulated in *coq7–1* mutants (9) and the inherent lack of complex I in the *S. cerevisiae* respiratory chain.

The finding of an altered quinone composition in *clk-1* mutants is the first indication of a biochemical difference between *clk-1* mutants and wild-type strains and shows that CLK-1 is absolutely required for the step converting DMQ$_9$ to 3-hydroxy-UQ$_9$. However, there are reasons to believe that CLK-1 may not directly participate in the hydroxylation of DMQ$_9$, since *clk-1* and its homologues do not possess any monooxygenase/hydroxygenase motifs in their primary structure (8, 9, 11–13), in contrast to *E. coli ubiF* gene, which has been recently identified to be responsible for the synthesis of 3-hydroxy-UQ$_9$ from DMQ$_9$ (23). The fact that a gene homologous to *E. coli ubiF* does exist in the genome of *C. elegans* (GenBank™ accession number O01884) suggests that it, rather than CLK-1, catalyzes the hydroxylation of DMQ$_9$ in *C. elegans*. In addition, Hsu *et al.* (26) recently reported that yeast CLK-1 homologue, COQ7/CAT5, is necessary for the stable expression of Coq3p, which participates in the O-methylation steps of the UQ biosynthesis pathway. These observations suggest that CLK-1/COQ7/CAT5 may participate in a fundamental regulatory mechanism in the UQ biosynthesis of eukaryotes and that the hydroxylation of DMQ$_9$ is one of the major reactions under the control of CLK-1.

**Fig. 2. Absorption spectra of quinones in N2 and clk-1 mutants mitochondria.** The spectra of the quinones from N2 (eluted at 18.31 min in Fig. 1B) (A) and clk-1 mutant (qm51) (eluted at 17.11 min in Fig. 1E) (B) are monitored by photodiode array UV-visible detector. Maximum absorption of each compound ($\lambda_{\text{max}}$) is indicated.

**Fig. 3. Pathway for biosynthesis of UQ$_9$.** The proposed pathway of UQ biosynthesis in eukaryotes (9) is shown. The accumulation of DMQ$_9$ in *clk-1* mutants indicates that CLK-1 is necessary for the step converting DMQ$_9$ to 3-hydroxy-UQ$_9$. The intermediates indicated are (from the top) 3-nonaprenyl-4-hydroxybenzoate, DMQ$_9$, 3-hydroxy-UQ$_9$, and UQ$_9$.

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**Fig. 4. Mass spectrum of the quinone biosynthesis intermediate from *clk-1* (qm51) mutant mitochondria.** The mass spectrum of the quinone accumulated in *clk-1* (qm51) mitochondria, which corresponds to the peak eluting at 17.1 min in Fig. 1E, is shown. The molecular structure of DMQ$_9$ is also shown. The peak at m/z 765 corresponds to the molecular ion of DMQ$_9$. 

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Altered Quinone Biosynthesis in the C. elegans clk-1 Mutants

Quinone content and enzymatic activities of N2 and clk-1 mutant mitochondria

Assays were performed at pH 7.7, using 90 μM short side chain quinone analogues as electron acceptor, as described under “Experimental Procedures.”

### Table I

| Strain | Quinone content | NADH-quinone reductase | Succinate-quinone reductase |
|--------|-----------------|------------------------|-----------------------------|
|        | UQ9 | DMQ9 | NADH min⁻¹ mg⁻¹ (±S.D.) | Succinate min⁻¹ mg⁻¹ (±S.D.) |
| N2     | ND  | 1.04 ± 0.20 | ND  | 260.0 ± 29.5 |
| e2519  | 1.24 ± 0.21 | ND  | 236.8 ± 36.4 |
| qm30   | 1.16 ± 0.14 | ND  | 258.6 ± 27.4 |
| qm51   | 1.28 ± 0.13 | ND  | 233.6 ± 41.0 |

* a = 3 for N2, e2519, and qm51; b = 4 for qm30.

### Table II

Enzymatic activities of clk-1 mutant mitochondria using synthetic UQ analogues as electron acceptor

| Strain | UQ9 | DMQ9 | 3-Hydroxy-3 | NADH min⁻¹ mg⁻¹ (±S.D.) | Succinate min⁻¹ mg⁻¹ (±S.D.) |
|--------|-----|------|-------------|------------------------|-----------------------------|
| N2 (n = 4) | 34.4 ± 11.1 | 24.8 ± 9.0 | 6.1 ± 1.5 | 24.8 ± 5.2 |
| e2519 (n = 3) | 42.5 ± 10.5 | 28.1 ± 5.6 | 7.1 ± 1.3 | 36.0 ± 10.0 |
| qm30 (n = 5) | 39.6 ± 7.1 | 26.7 ± 6.1 | 6.7 ± 2.1 | 28.5 ± 8.6 |
| qm51 (n = 3) | 42.2 ± 11.0 | 32.4 ± 3.9 | 6.5 ± 1.4 | 29.6 ± 14.0 |

* ND, not detectable.

What is the relation between UQ biosynthesis and the overall phenotype of clk-1 mutants? The normal rate of respiration observed in clk-1 mutants by distinct methodologies (present study and Ref. 15) strongly implies that the phenotype of clk-1 mutants is not the direct consequence of decreased energy metabolism, as has been discussed previously (27). Another observation that suggests that the phenotype of clk-1 mutants is not explained solely by the accumulation of DMQ9 in the adult mitochondria is the absence of correlation between the severity of the overall mutant phenotype and the severity of the biochemical phenotype in the three clk-1 alleles. Indeed, we could not find a quantitative difference in the amount of DMQ9 between the weaker missense mutant (e2519) and the more severe mutants (qm30 and qm51) (Table I). These observations suggest that UQ biosynthesis might be only one of the processes that is regulated by clk-1.

One phenotype of clk-1 mutants that is not very different in the different alleles is the increase in life span (2, 3). The alteration of the content of different quinones, which is similar in all alleles, might thus contribute to slower aging. Reactive oxygen species (ROS) produced as a by-product of electron transport are widely believed to be an important determinant of aging (28–30). An important source of ROS is the ubisemiquinone radical, which is a reaction intermediate during the reduction and the oxidation of UQ in complex I and complex III (31). Possibly, the chemical properties of the semiquinone produced from DMQ9 allow for a lesser level of ROS production, and thus to a slower rate of oxidative damage accumulation, which in turn could promote a long life span.

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