Altered quinone biosynthesis in the long-lived clk-1 mutants of Caenorhabditis elegans *

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Running title; Altered quinone biosynthesis in the C. elegans clk-1 mutants
Summary

Mutations in the *clk-1* gene of *Caenorhabditis elegans* result in an extended life span and a average slowing down of developmental and behavioral rates. However, up to now, 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 biosynthesis, and show that *clk-1* mutants mitochondria do not contain detectable levels of ubiquinone (UQ$_9$). Instead, the ubiquinone biosynthesis intermediate, demethoxy ubiquinone (DMQ$_9$), is present at high levels. This result demonstrates that CLK-1 is absolutely required for the biosynthesis of UQ$_9$ 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 DMQ$_9$ can function as an electron carrier in the respiratory chain. To test this possibility, the short side chain derivative DMQ$_2$, was chemically synthesized. We find that DMQ$_2$ 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-UQ$_2$, cannot. The accumulation of DMQ$_9$ 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.
Introduction

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 been 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 (UQ)1 in yeast (9, 10). Therefore, yeast coq7/cat5 mutants, which lack UQ6, 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 GFP 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 to 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 analyze the respiratory activities of mutants mitochondria and find that DMQ 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 (CGC).

Mitochondria preparation-Nematodes were grown at 20° C on NGA plates, which contain 3-fold bacto peptone with the supplement of *E. 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 become 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 xg for 5 min. at 4° C (16). The worms were homogenized in 0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA (MSE), containing 1 mM phenylmethylsulfonyl fluoride (PMSF), using glass-glass homogenizer (Iwaki, Tokyo) with the inclusion of glass beads (0.10-0.11 mm, B. Brown Melsungen AG). The degree of breakage was checked under light-microscope. The homogenates were then centrifuged at 1,080 xg for 10 min. at 4° C. The pellet, containing glass beads, was washed with MSE, and was centrifuged at 1,080 xg for 10 min. The supernatants were then centrifuged at 23,500 xg for 10 min., and the pelleted mitochondrial fraction was resuspended in MSE.

Identification of quinones-Quinones were extracted from lyophilized mitochondria (3.0 mg protein). The mitochondria were vortexed in EtOH/n-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 two times.
After drying the pooled extracts under a stream of nitrogen gas, the residue was redissolved in ethanol and analyzed by HPLC. Quinones were applied to a reverse-phase column (Inertsil ODS-3, C-18, 5 µm, 4.6 mm x 250 mm, GL Science, Tokyo), and was eluted in isocratic condition (1 ml/min), with diisopropyl ether/MeOH (1/4, v/v) as described (17). The eluted quinones were identified by comparing their retention times with authentic UQ₉ (Sigma). The spectral characteristics of each quinone were monitored using photodiode array UV-VIS detector (Shimadzu SPD10-A). The concentration of quinones were determined spectrophotometrically using coefficients of $E_{\text{1%1cm}} = 185$ for UQ (18), and $\varepsilon_{\text{max}} = 14.5$ mM$^{-1}$ cm$^{-1}$ at 271 nm for DMQ (19). The mass spectrum of the quinone accumulated in clk-1 (qm51) was analyzed by Hitachi M-8000 LC/MS 3DQ system with atmospheric pressure chemical ionization.

**Enzyme Assays**—NADH-cytochrome c reductase activity and succinate-cytochrome c reductase activity were assayed as described (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 ferricytochrome c. NADH-quinone reductase activity was assayed in 50 mM potassium phosphate buffer (pH 7.7), containing 200 µM NADH, 2 mM KCN, and 90 µM quinone analogues. The oxidation of NADH was monitored at 340 nm, using millimolar extinction coefficient of 6.2 for NADH. Succinate-quinone reductase activities were measured as described (21). DMQ₂ and 3-hydroxy-UQ₂ were synthesized as previously described (22). All the assays were performed at 20° C.
Result 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 minutes from N2 mitochondria is identical to standard UQ9 for both elution time and absorption property (Fig. 1A, B, Fig. 2A). However, a corresponding peak was not observed in clk-1 mutants. The mutant mitochondria instead exhibited a major peak eluting one minute earlier than UQ9 (Fig. 1C-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 were 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).

UQ is known as an essential component of the respiratory electron transfer chain, and is required for
mitochondrial aerobic respiration. In fact, UQ deficiency in humans causes mitochondrial encephalomyopathy (24), and yeast coq7-1 mutant, which contains DMQ$_6$ but not UQ$_9$, is defective in aerobic growth (9). Given this, the apparent absence of UQ$_9$ (Fig. 1) and the normal level of respiration observed in clk-1 mutants (7, 14, 15) raise the question of how electron transfer is carried out in clk-1 mutants. Since a large amount of DMQ$_9$ is accumulated in clk-1 mutants mitochondria, one possibility might be that DMQ$_9$ serves as an electron carrier in mutants mitochondria. To test this possibility, we measured various electron transfer activities in clk-1 mutants mitochondria (Table I). Succinate-cytochrome c reductase activity of mutants mitochondria was measured and found to be only slightly affected, confirming the previous report (14). Similarly, the activity of NADH-cytochrome c reductase in clk-1 mutants was comparable to that in the wild-type strain (Table I). These results indicate the possibility that the electron transfer between complex I and complex III in clk-1 mutants mitochondria might be mediated by endogenous DMQ$_9$, with almost the same efficiency as UQ$_9$.

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 to 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 (Table I, II). This tendency has been also reported for E. coli ubiF mutants, which accumulate
DMQ₈ (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 DMQ₈, are defective in respiratory growth (9). Our results suggest that this is probably due to the relatively small amount of DMQ₈ 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 DMQ₈ to 3-hydroxy-UQ₈. However, there are reasons to believe that CLK-1 may not directly participate in the hydroxylation of DMQ₈, 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-UQ₈ from DMQ₈ (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 DMQ₈ in C. elegans. In addition, Hsu et al. 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 (26). 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 DMQ₈ is one of the major reactions under the control of CLK-1.

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 previously discussed (27). Another observation that suggests that the phenotype of
\textit{clk-1} mutants is not explained solely by the accumulation of DMQ$_9$ 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 \textit{clk-1} alleles. Indeed, we could not find a quantitative difference in the amount of DMQ$_9$ 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 \textit{clk-1}.

One phenotype of \textit{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 DMQ$_9$
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.
Acknowledgements

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References


Footnotes

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1 The abbreviations used are: DMQ₉, demethoxy ubiquinone or 2-methoxy-5-methyl-6-nonaprenyl-1,4-benzoquinone; 3-hydroxy-UQ₉, 2-methoxy-3-hydroxy-5-methyl-6-nonaprenyl-1,4-benzoquinone; UQ₉, 2,3-methoxy-5-methyl-6-nonaprenyl-1,4-benzoquinone; PMSF, phynylmethylsulfonyl fluoride; ROS, reactive oxygen species.
Figure Legends

**Fig. 1**  Elution profiles of quinones from N2 and *clk-1* mutants mitochondria.

Reverse-phase HPLC chromatograms showing the elution of quinones in the isocratic condition (diisopropyl ether/MeOH (1/4, v/v)). The elution of standard UQ9 (A), lipid extracts from N2 (B), *clk-1* missense mutant (*e2519*) (C), deletion mutant (*qm30*)(D), and splice acceptor mutant (*qm51*) (E) mitochondria were monitored at 275 nm. The elution time of the major peak is indicated.

**Fig. 2**  Absorption spectra of quinones in N2 and *clk-1* mutants mitochondria.

The spectra of the quinones from N2 (eluted at 18.31 minutes in Fig. 1B) (A), and *clk-1* mutant (*qm51*) (eluted at 17.11 minutes in Fig. 1E) (B) are monitored by photodiode array UV-VIS detector. Maximum absorption of each compound (λ max) is indicated.

**Fig. 3**  Pathway for biosynthesis of UQ9.

The proposed pathway of UQ biosynthesis in eukaryotes (9). The accumulation of DMQ9 in *clk-1* mutants indicates that CLK-1 is necessary for the step converting DMQ9 to 3-hydroxy-UQ9. The intermediates indicated are (from the top) 3-nonaprenyl-4-hydroxybenzoate; DMQ9, 2-methoxy-5-methyl-6-nonaprenyl-1,4-benzoquinone; 3-hydroxy-UQ9, 2-methoxy-3-hydroxy-5-methyl-6-nonaprenyl-1,4-benzoquinone, and UQ9.
**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. The molecular structure of DMQ₉ is also shown. The peak at m/z 765 corresponds to the molecular ion of DMQ₉.
Table I

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

Quinone content was analyzed using 3.0 mg of mitochondria, as described under “Experimental Procedures”. NADH-cytochrome c reductase and succinate-cytochrome c reductase activities were assayed at pH 7.7 with freshly prepared mitochondria.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Quinone content* (nmol mg⁻¹ (±s.d.))</th>
<th>NADH-cytochrome c reductase† (nmol min⁻¹ mg⁻¹ (±s.d.))</th>
<th>Succinate-cytochrome c reductase† (nmol min⁻¹ mg⁻¹ (±s.d.))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMQ₉</td>
<td>UQ₉</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>n.d.</td>
<td>1.04±0.20</td>
<td>260.0±29.5</td>
</tr>
<tr>
<td>e2519</td>
<td>1.24±0.21</td>
<td>n.d.</td>
<td>236.8±36.4</td>
</tr>
<tr>
<td>qm30</td>
<td>1.16±0.14</td>
<td>n.d.</td>
<td>256.8±27.4</td>
</tr>
<tr>
<td>qm51</td>
<td>1.28±0.13</td>
<td>n.d.</td>
<td>233.6±41.0</td>
</tr>
</tbody>
</table>

*n=3 for N2, e2519, and qm51; n = 4 for qm30.
†n=3 for qm51; n=5 for e2519 and qm30; n=6 for N2.

n.d.; not detectable.
Table II

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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>NADH-quinone reductase (nmol min⁻¹ mg⁻¹ (±s.d.))</th>
<th>Succinate-quinone reductase (nmol min⁻¹ mg⁻¹ (±s.d.))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UQ₂</td>
<td>DMQ₂</td>
</tr>
<tr>
<td>N2 (n=4)</td>
<td>34.4±11.1</td>
<td>24.8±9.0</td>
</tr>
<tr>
<td>e2519 (n=3)</td>
<td>42.3±10.5</td>
<td>28.1±5.6</td>
</tr>
<tr>
<td>qm30 (n=5)</td>
<td>39.6±7.1</td>
<td>26.7±6.1</td>
</tr>
<tr>
<td>qm51 (n=3)</td>
<td>42.2±11.0</td>
<td>32.4±3.9</td>
</tr>
</tbody>
</table>

n.d.; not detectable.
Fig. 1  Miyadera et al.  
(Correspondence; Kita, K.)
Fig. 2    Miyadera et al.
(Correspondence; Kita, K.)
Fig. 3  Miyadera et al.
(Correspondence; Kita, K.)
Fig. 4  Miyadera et al.
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