Minireview

Quinones in long-lived *clk-1* mutants of *Caenorhabditis elegans*

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Abstract Ubiquinone (UQ) (coenzyme Q) is a lipophilic redox-active molecule that functions as an electron carrier in the mitochondrial electron transport chain. Electron transfer via UQ involves the formation of semiubiquinone radicals, which causes the generation of superoxide radicals upon reaction with oxygen. In the reduced form, UQ functions as a lipid-soluble antioxidant, and protects cells from lipid peroxidation. Thus, UQ is also important as a lipophilic regulator of oxidative stress. Recently, a study on long-lived *clk-1* mutants of *Caenorhabditis elegans* demonstrated that biosynthesis of UQ is dramatically altered in mutant mitochondria. Demethoxy ubiquinone (DMQ), that accumulates in *clk-1* mutants in place of UQ, may contribute to the extension of life span. Here we elucidate the possible mechanisms of life span extension in *clk-1* mutants, with particular emphasis on the electrochemical property of DMQ. Recent findings on the biochemical function of CLK-1 are also discussed. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ubiquinone; Demethoxy ubiquinone; *clk-1*; Ageing; Reactive oxygen species; Longevity

1. Introduction

Caenorhabditis elegans is one of the most extensively used model organisms in elucidating the mechanisms of life span regulation [1]. Some of the biological pathways implicated in ageing have been identified from the study of various long- and short-lived mutant strains [2,3]. Studies of a group of long-lived mutants that includes mutants in the *daf-2*, *age-1*, and *daf-16* genes revealed that life span is affected by an insulin-receptor-like signaling cascade that also affects the formation of dormant dauer larvae [4,5]. The mechanism of life span extension by this signaling pathway is under intense study [1]. Another group of long-lived mutants include *eat* mutants, which are deficient in food uptake [6]. Life span extension in *eat* mutants possibly results from restricted caloric intake [7], as observed in other organisms [8]. It has been suggested that these phenomena are linked to the generation of reactive oxygen species (ROS), which is believed to be one of the causes of ageing [8].

clk-1 mutants were identified as long-lived strains, which also exhibit a slow-down in developmental and behavioral rates, including the cell cycle, development, and rhythmic adult behaviors [9]. The biological pathway altered in *clk-1* mutants appears to be distinct from that of the insulin-receptor-like signaling cascade, since the life span of *daf-2*, *clk-1* double mutants is several times longer than that of either *clk-1* or *daf-2* mutants [7]. The observation that *eat-2*, *clk-1* double mutants do not further increase life span [7], raised the possibility that the biological pathways that are altered by *clk-1* and *eat-2* mutations may overlap. However, until recently, neither the biochemical alteration in *clk-1* mutants nor the exact function of the CLK-1 protein were known (reviewed in [2,10–13]). Recently, we [14], and others [15] demonstrated that *clk-1* mutants do not contain ubiquinone (UQ). We showed instead that the mutants accumulate a biosynthesis intermediate, demethoxy ubiquinone (DMQ) [14] (Fig. 1). These findings indicate that quinones have a critical role in the process that regulates life span. In this minireview, we consider the possible mechanism of life span extension in *clk-1* mutants. Particular focus is on the electrochemical property of the UQ biosynthesis intermediate, DMQ, as this may reveal the property of quinones that is critical in the process of ageing.

2. Quinone composition in long-lived *clk-1* mutant

UQ is a lipophilic, redox-active molecule (Fig. 1), and functions as an electron carrier in the mitochondrial respiratory chain. UQ is reduced at complex I (NADH-UQ reductase) and complex II (succinate-UQ reductase). Reduced UQ (UQ_{red}) then transfers electrons to complex III (ubiquinol-cytochrome *c* reductase), which regenerates oxidized UQ (UQ_{ox}). Electron transfer via UQ is an essential step in oxidative phosphorylation by the respiratory chain. Not surpris-

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Abbreviations: ROS, reactive oxygen species; UQ, ubiquinone (2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquinone); DMQ, demethoxy ubiquinone (2-methoxy-5-methyl-6-polyprenyl-1,4-benzoquinone); complex I, NADH-ubiquinone reductase; complex II, succinate-ubiquinone reductase; Q_{red}, reduced quinone; complex III, ubiquinol-cytochrome *c* reductase; Q_{ox}, oxidized quinone; 3-hydroxy UQ_2, 2-methoxy-3-hydroxy-5-methyl-6-polyprenyl-1,4-benzoquinone; Q_{sem}, semiubiquinone radical; AOX, alternative oxidase

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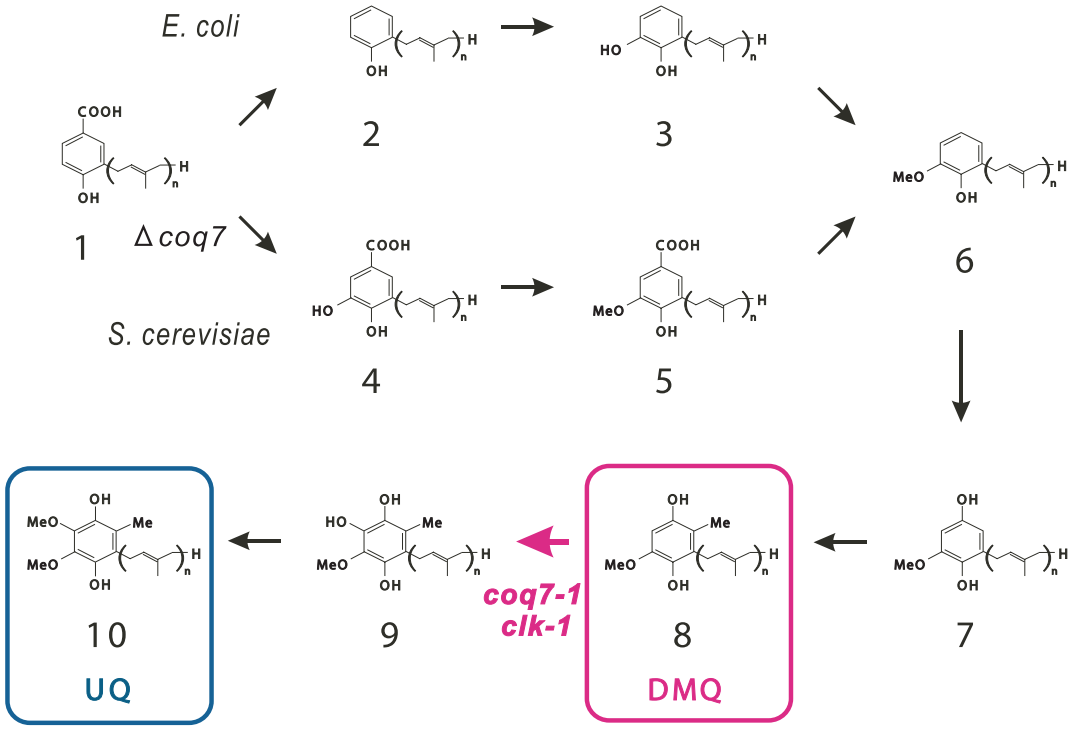

Fig. 1. UQ biosynthesis pathway proposed from the genetic studies of *E. coli* [18] and *S. cerevisiae* [19]. Compounds indicated in this figure are: 1, 4-hydroxy-3-polyprenylbenzoic acid; 2, 2-polyprenylphenol; 3, 2-hydroxy-6-polyprenylphenol; 4, 3,4-dihydroxy-5-polyprenylbenzoic acid; 5, 4-hydroxy-3-methoxy-5-polyprenylbenzoic acid; 6, 2-methoxy-6-polyprenylphenol; 7, 2-methoxy-6-polyprenyl-1,4-benzoquinol; 8, DMQ; 9, 3-hydroxy UQ; 10, UQ. *C. elegans clk-1* mutants accumulate DMQ_9 [14]. Yeast *coq7* deletion mutant accumulates compound 1, and *coq7* missense mutant (*coq7-1*) contains DMQ_6 [60].

ingly, UQ deficiency in humans has been found to be associated with severe mitochondrial encephalomyopathy [16,17].

The biosynthesis pathway of UQ has been extensively analyzed in *Escherichia coli* [18] and *Saccharomyces cerevisiae* [19]. Fig. 1 shows the steps involved in the aromatic ring formation in the UQ biosynthesis pathway. Part of the biosynthesis steps from 4-hydroxy-3-polyprenylbenzoic acid (compound 1 in Fig. 1) to 2-polyprenyl-6-methoxyphenol (compound 6 in Fig. 1) is considered to be different between *E. coli* and yeast [18,19]. It has been proposed that, in mammals, the order of these steps is the same as in *E. coli* [20,21]. However, none of the enzymes catalyzing the reactions in Fig. 1 have yet been purified from any organism, and little is known about their enzymatic properties. The factors regulating biosynthesis and the localization of UQ to various cellular membranes also remain to be uncovered.

DMQ is one of the biosynthesis intermediates of UQ (Fig. 1). In *clk-1* mutant mitochondria, DMQ_9 (numbers indicate the isoprenoid side chain length) is accumulated to the same level as UQ_9 in wild-type mitochondria [14]. Interestingly, DMQ_9 supports respiration of *clk-1* mutant mitochondria to a considerable degree [14]. This finding is consistent with the observation that oxygen consumption is not severely deteriorated in *clk-1* mutants [22], and indicates that DMQ is a redox-active molecule. While DMQ_2 accepts electrons from complex I at the same rate as UQ_2, electron transfer from complex II to DMQ_2 was slightly reduced [14]. A similar observation in *E. coli ubiF* mutant [23] and in mouse *mclk1* knockout mutants [24], which also contain DMQ instead of UQ, indicates that the structure and the mid-point potential of DMQ might be more favorable to function as an electron acceptor at complex I than at complex II. On the other hand, 3-hydroxy UQ_2 (2-methoxy-3-hydroxy-5-methyl-6-geranyl-1,4-benzoquinone; compound 9 in Fig. 1) does not support the electron transfer neither from complex I nor from complex II [14]. From these findings it is clear that not all the UQ biosynthesis intermediates can function as electron carriers in the respiratory chain.

3. Role of quinones as pro- and antioxidant

One of the functions of UQ is to transfer electrons in the respiratory chain, which generates the proton motive force across the inner mitochondrial membrane for oxidative phosphorylation. However, electron transfer in the respiratory chain is also accompanied by the generation of ROS [25,26]. This event is most likely to occur via the formation of a by-product of UQ_{red} oxidation, semiubiquinone radicals (UQ_{sem}) at complex III [25], in which UQ_{red} undergoes single electron oxidation/reduction by the proton motive Q-cycle [27]. UQ_{sem} is highly reactive to molecular oxygen and generates superoxide radicals [25,26], which are toxic to proteins, especially to those containing iron–sulfur clusters [28]. Furthermore, the formation of superoxide results in the generation of its dismutated product, hydrogen peroxide, from which hydroxyl radical can be formed [26]. These ROS cause extensive oxidative damage to biological macromolecules, a process that likely is an important factor in aging [8,29,30].

Contrary to this prooxidant activity of UQ_{sem}, UQ_{red} has been regarded as a lipid-soluble antioxidant [31,32]. This notion is supported by the fact that yeast strains deficient in UQ biosynthesis show increased sensitivity to the products of au-

tooxidized polyunsaturated fatty acids [33]. The maintenance of UQ_{red} level is possibly achieved partly by DT-diaphorase and cytosolic NADPH-quinone reductase [34,35], in addition to the reduction by the respiratory chain. It has been shown that UQ_{red} is capable of reducing fatty acid radicals either directly [36,37], or indirectly via regeneration of α-tocopherol [31,38–40]. This antioxidative action of UQ_{red} exerts beneficial effects for the treatment of cardiomyopathy and the recovery from ischemia, as well as the protection of neuronal cells [41–43]. In fact, the serum UQ_{red}/UQ_{ox} ratio can be used as a reliable index to estimate the level of oxidative stress [44]. However, it has been also known that UQ_{sem}, which is produced as the result of radical scavenging action of UQ_{red}, is prooxidative, and causes the production of superoxide and lipid peroxidation [45]. It is presently unproven how the radical chain reaction derived from UQ_{sem} is quenched, to maintain the net antioxidant action of UQ_{red}.

Considering these properties of quinones, and the fact that the primal cause of the ROS generation is the autooxidation of Q_{sem} by the interaction with oxygen [46], the important parameter in evaluating the pro-/antioxidant action of quinones would be the reactivity of various quinones toward oxygen. We have therefore examined whether these parameters are distinct between UQ_{sem} and DMQ_{sem}, to get insight into the possible mechanism of life span extension in *clk-1* mutants.

4. Electrochemical properties of DMQ

The redox and catalytic properties of quinones for oxygen reduction can be evaluated from cyclic voltammetry of quinone-modified electrodes. UQ_2 and DMQ_2 adsorb on a glassy carbon electrode to form a monolayer. The UQ_2- and DMQ_2-modified electrodes gave a couple of reduction and oxidation wave under anaerobic conditions. The electrode reaction is written by [47]:

$$Q_{ox} + \overset{e^-}{\rightleftarrows} Q_{sem} \overset{e^-(+2H^+)}{\rightleftarrows} Q_{red} \quad (1)$$

The formal redox potential ($E^{\circ\prime}$) was evaluated as 85 mV and 68 mV vs. SHE (standard hydrogen electrode) for UQ_2 and DMQ_2, respectively, at pH 7.0 (Kano, unpublished data). The observed lower $E^{\circ\prime}$ of DMQ_2 compared with UQ_2 partly explains why DMQ_2 accepts electrons more efficiently from complex I (NADH/NAD^+; −320 mV) than from complex II (succinate/fumarate; +30 mV).

Under O_2-saturated conditions, the voltammogram exhibited the catalytic reduction current around $E^{\circ\prime}$. The predominant reaction is the catalytic reduction of O_2 by Q_{sem} to generate $O_2^{\bullet-}$, as written by [48]:

$$Q_{sem} + O_2 \rightarrow Q_{ox} + O_2^{\bullet-} \quad (2)$$

$$Q_{ox} + e^- \rightarrow Q_{sem} \quad (1')$$

At potentials more negative than $E^{\circ\prime}$ of the quinones where Q_{red} is the predominant species, no significant catalytic effect was observed. The result clearly evidences that Q_{sem} is much more susceptible to the autooxidation than Q_{red}. The electricity in the background-subtracted catalytic cathodic wave (Q_{cat}) is reasonably attributable to the reduction of O_2 as well as the adsorbed quinone, while that of the cathodic wave under anaerobic conditions (Q_Q) is attributable to the reduction of the adsorbed quinone alone. The Q_{cat}/Q_Q value of UQ_2 was 1.43, and is larger than that of DMQ_2 (=1.16) (Kano, unpublished data). This means that UQ_2 is a stronger catalyst than DMQ_2 in terms of the O_2 reduction into $O_2^{\bullet-}$. In other words, UQ_2 is more toxic than DMQ_2 during the reduction under aerobic conditions.

Basically, the catalytic ability of quinones in the O_2 reduction is governed by $E^{\circ\prime}$ of quinones and the thermodynamic stability of the semiquinone intermediate, as expressed by the semiquinone formation constant K ($=[Q_{sem}]^2/[Q_{ox}][Q_{red}]$); the more negative in $E^{\circ\prime}$ and/or the larger in K, the more strongly the Q_{red} reduces O_2 [48]. Interestingly, $E^{\circ\prime}$ of DMQ_2 is 17 mV more negative than that of UQ_2, in spite of lower catalytic activity of DMQ_2. Therefore, it might be expected that DMQ_2 is smaller in K than UQ_2.

5. Mechanism of life span extension in *clk-1* mutants

The data in the previous section clearly indicate that, although DMQ_2 retains lower redox potential compared to UQ_2, the prooxidant action of DMQ_{sem} would be weaker than UQ_{sem}. This property of DMQ_{sem} might lead to the reduced production of ROS in *clk-1* mutants, and consequently, to the slow ageing phenotype.

In addition to the weaker prooxidative activity of DMQ_{sem}, the slightly reduced respiration in *clk-1* mutant mitochondria may also contribute to the reduced ROS production. Although the respiration of *clk-1* mutant mitochondria measured in vitro was nearly at the wild-type level at complex I, the respiration was slightly retarded when succinate was used as substrate [14,49]. Since complex II is also an enzyme in TCA cycle, the slower rate of electron transfer at complex II in vivo may result in the reduction of the NADH/NAD^+ ratio, leading to the slower respiration from complex I. Supporting this possibility is the slightly reduced oxygen consumption in *clk-1* mutants [22], as well as the lower metabolic rate of *daf-2*, *clk-1* double mutants compared to *daf-2* mutant [50]. Slower respiration may lead to a decreased transmembrane electric potential difference ($\Delta\psi$), which critically affects the rate of ROS generation via the generation of UQ_{sem} [51]. Taken together, it is likely that ROS generation in *clk-1* mutants might be reduced by the synergistic effect of the less prooxidative property of DMQ, and the reduced rate of respiration due to the change in structure and mid-point potential of quinones.

Despite the above considerations, some recent works provide an alternative point of view. For example, the analysis of point mutations in the redox-active subunits of complex III has suggested that lowering the rate of respiration and ROS generation could not increase the life span of *daf-2* mutants that already have high ROS resistance [30]. This is in contrast to what is observed with *clk-1* mutations, which can more than double the life span of *daf-2* mutants [7]. We have also recently shown that DMQ cannot efficiently replace UQ at an unidentified cellular site, distinct from the electron transport chain [24,52]. It is possible therefore that the increased life span of *clk-1* mutants is due to the physiological consequences of the replacement of UQ by DMQ at other cellular sites in addition to the respiratory chain (see below).

6. Function of CLK-1

The identification of DMQ_9 in *clk-1* mutants raised the possibility that CLK-1 is critical in the biosynthesis of UQ_9 from DMQ_9. CLK-1 is localized in mitochondria [49], and its homologs, yeast COQ7 and mouse mCLK1 localize in the matrix side of the inner mitochondrial membrane [53,54]. The high degree of homology of *clk-1*/*COQ7* between organisms [55] implied that the function of CLK-1/Coq7p might be conserved throughout evolution. However, until recently, its biochemical function had been unknown.

A recent report from Stenmark et al. [56] revealed that bacterial *clk-1*/*COQ7* homologs from *Pseudomonas aeruginosa* and *Thiobacillus ferrooxidans*, are able to complement the UQ deficiency of *E. coli ubiF* mutant, indicating that *clk-1*/*COQ7* catalyze the monooxygenation of DMQ. It was also found that *clk-1*/*COQ7* genes contain the conserved -EXXE- motifs of di-iron containing monooxygenase/hydroxylases [56]. Although this needs to be confirmed by further biochemical analyses, these findings, together with the high conservation of the primary structure of CLK-1/Coq7p between bacteria and mitochondria [56], indicate that mitochondrial CLK-1/Coq7p might also function as a hydroxylase. The -EXXE- motif has also been identified in plants and trypanosome quinol-oxidase (known as cyanide-insensitive alternative oxidase, AOX) [57,58], that is also located on the matrix side of the inner mitochondrial membrane [57]. Thus, it would be reasonable to place CLK-1 and AOX within the family of di-iron enzymes, which contain binding sites for both quinones and oxygen, and play an important role in oxygen metabolism.

Interestingly, yeast Coq7p has been shown to play a regulatory or structural role in the UQ biosynthesis pathway, since *COQ7* is absolutely required for the functional expression of Coq3p, which participates in several UQ biosynthesis steps [59]. This possibility is also consistent with the fact that while a yeast *coq7* point mutant contains a small amount of DMQ_6, the *coq7* deletion mutant does not contain DMQ_6, but accumulates 4-hydroxy-3-hexaprenylbenzoic acid (compound 1 in Fig. 1) [60]. The findings that worm and mammalian *clk-1*/*COQ7* complement the respiratory deficiency of yeast *coq7* deletion mutant [55,61] indicate that the regulatory function of Coq7p might be retained in the multicellular CLK-1/Coq7p proteins.

Finally, the multiple developmental and behavioral phenotypes of *clk-1* mutants raise the question of whether mitochondrial CLK-1/Coq7p participates solely in UQ biosynthesis, or whether it has retained additional functions involved in regulating these multiple phenotypes. UQ_9 is equally undetectable in phenotypically weak (*e2519*) and strong (*qm30* and *qm51*) *clk-1* alleles [14]. It suggests that either the UQ_9 deficiency is not responsible for the *clk-1* phenotype, or that very low levels of UQ_9 are made in *e2519* mutants, and that such levels are sufficient for some phenotypic relief. To further elucidate the function of CLK-1 in higher eukaryotes, we have recently created a *mclk1* knockout in mice [24]. We found that the homozygous mutants are UQ deficient, as is the case in *C. elegans*. However, these homozygotes were embryonic lethal, despite the presence of large amounts of DMQ_9 that could partially (65%) support respiration. This finding suggests that UQ_9, and/or mCLK1, may play an essential role in the developmental process of mammals, in addition to its functions in energy metabolism [24].

7. Conclusion

The identification of *clk-1* mutants revealed that a change in quinone species could alter metabolism and life span in eukaryotes, possibly via altering the rate of ROS generation in mitochondria.

Besides its important role in respiration, recent data indicate that UQ is an indispensable component in various biological machineries. For instance, UQ is used as signaling molecule in the bacterial Arc two-component system, in which UQ_{ox} inhibits kinase activity of ArcB [62]. UQ also serves as an electron acceptor in the bacterial Dsb system, which catalyzes thioester bond formation [63]. In eukaryotes, UQ is an activator of uncoupling protein, which regulates the membrane potential and the rate of ATP synthesis in mitochondria [64]. Furthermore, there is evidence now, both in worms [52] and in mice [24], that DMQ cannot functionally replace UQ for at least one of these functions. Given this, it is possible that life span extension in *clk-1* mutants is the result of the alteration of one or more UQ-dependent processes.

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