CLK-1/Coq7p is a DMQ mono-oxygenase and a new member of the di-iron carboxylate protein family

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Received 10 September 2001; revised 9 October 2001; accepted 16 October 2001

First published online 26 November 2001

Abstract Strains of *Caenorhabditis elegans* mutant for *clk-1* exhibit a 20–40% increase in mean lifespan. *clk-1* encodes a mitochondrial protein thought to be either an enzyme or regulatory molecule acting within the ubiquinone biosynthesis pathway. Here CLK-1 is shown to be related to the ubiquinol oxidase, alternative oxidase, and belong to the functionally diverse di-iron-carboxylate protein family which includes bacterioferritin and methane mono-oxygenase. Construction and analysis of a homology model indicates CLK-1 is a 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone mono-oxygenase as originally predicted. Analysis of known CLK-1/Coq7p mutations also supports this notion. These findings raise the possibility of developing CLK-1-specific inhibitors to test for lifespan extension in higher organisms.

Key words: Aging; Alternative oxidase; Senescence; Gerontogene; Bacterioferritin; *Clk-1*

1. Introduction

Advances in understanding the aging process are being made through the identification and characterization of long-lived genetic mutants in the nematode *Caenorhabditis elegans* (reviewed in [1]). The Clk mutants are a collection of single-gene mutants displaying a common set of phenotypes – namely, maternal rescue, a slowing of rhythmic behaviors such as pharyngeal pumping and defecation, an increase in embryonic development, and an increase in mean lifespan (20–40%) [2]. Of the four known Clk mutations only *clk-1* has been characterized extensively at the protein level [3,4]. Fueled largely by studies in *Saccharomyces cerevisiae*, Coq7p/Cat5p/CLK-1 has been shown to be a mitochondrial protein necessary for ubiquinone (Q) biosynthesis [5,6]. *coq7* mutants are unable to grow on non-fermentable carbon sources in the absence of Q supplementation [6]. In the *coq7-1* point mutant it has been shown that 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone (DMQ) is the most abundantly accumulating Q biosynthetic intermediate [6], originally leading to the suggestion that CLK-1/Coq7p is, or regulates the activity of, a DMQ mono-oxygenase. However, no homology with known mono-oxygenases or their regulatory subunits has to date been identified. Paradoxically, a *coq7* deletion mutant accumulates 3-hexaprenyl-4-hydroxybenzoate (HBB), an earlier Q biosynthetic intermediate [6]. Furthermore, isolated mitochondria from *clk-1* nematodes are able to respire in the presence of succinate [3,7], raising questions as to the true function of CLK-1/Coq7p.

In this study a computational analysis has been employed to reveal the function of CLK-1. CLK-1 orthologues from 18 different species were found to contain 14 absolutely conserved residues distributed in a manner that identifies them as new members of the di-iron-carboxylate family of proteins [8]. Crystallographic data for several di-iron-carboxylate proteins has allowed a molecular model of rat CLK-1 to be established. The structural similarity of CLK-1 to known mono-oxygenases, as well as to another predicted di-iron carboxylate protein, alternative oxidase (AO), led to the finding that the originally proposed substrate of Coq7p/CLK-1, DMQ, could be comfortably accommodated in the model's active site. This suggests CLK-1 is a DMQ mono-oxygenase as predicted. An analysis of known *clk-1*/*coq7* mutations provides further support for this notion. The implications of defective Q biosynthesis and lifespan extension are discussed.

2. Materials and methods

2.1. CLK-1 sequences

The following CLK-1 sequences were obtained from GenBank (accession #): *Rattus norvegicus* (Q63619), *Mus musculus* (P97478), *Homo sapiens* (NP_057222), *C. elegans* (U13642), *Drosophila melanogaster* (AJ252203), *S. cerevisiae* (NP_014768), *Schizosaccharomyces pombe* (T40268), *Rickettsia prowazekii* (AJ235270), *Pseudomonas aeruginosa* (E83564) and *Xylella fastidiosa* (AAF84347). The following CLK-1 sequences were translated from unfinished genomic fragments: *Candida albicans*, *Caulobacter crescentus*, *Pseudomonas putida*, *Pseudomonas syringae*, *Thiobacillus ferrooxidans*, *Legionella pneumophila*, *Bordetella pertussis* and *Bordetella bronchiseptica* (see Acknowledgements).

2.2. Computational analyses

CLK-1 proteins were aligned using Clustal W (1.81) (BLOSUM matrix, default settings) [9]. Phylogenetic analysis was performed using WebPHYLIP (V1.3), (PAM 001 distance matrix and FITCH treebuilder) [10]. Regions of sequence conservation within CLK-1 proteins were identified using the Blocks Multiple Alignment Processor [11,40] (Supplementary Fig. 1a (http:/www.elsevier.com/PII/S001457930103099X)). LAMA (local alignment of multiple alignments) Searcher (V28, default settings) [12] was used to screen the Blocks$^+$ and Prints databases (V8.9) for related protein BLOCKS. Of nine originally identified, two (PF01786E and IPB001891F) were selected for further analysis because they each contained invariant

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Abbreviations: AO, alternative oxidase; DMQ, 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone; DMQ_2, 2-diprenyl-3-methyl-6-methoxy-1,4-benzoquinone; FR, fumarate reductase; HBB, 3-hexaprenyl-4-hydroxybenzoate; Q, coenzyme Q or ubiquinone; ROS, reactive oxygen species; RQ, rhodoquinone; TRC, tandem repeat in CLK-1

0014-5793/01/$20.00
PII: S0014-5793(01)03099-X

residues corresponding in position to those of the CLK-1 family block to which they had been aligned (Supplementary Fig. 1b,c).

2.3. Homology modeling of rat CLK-1

The crystal structures of *D. vulgaris* Rubrerythrin (Protein Databank Bank accession # 1B71_A, 1.9 Å resolution), *Escherichia coli* bacterioferritin (1BCF_A, 2.9 Å resolution) and *H. sapiens* H-chain ferritin (2FHA, 1.9 Å resolution) were employed as templates to homology model the predicted four-helical bundle (αα 2–155) of rat clk-1 using Swiss-Model (http://www.expasy.ch/spdbv), and Swiss-PdbViewer (v3.6b3) and programs contained therein [13]. The C-terminal 24 residues of CLK-1 were organized into an amphipathic α-helix, then subjectively placed onto the model. The arrangement chosen buried an exposed hydrophobic face on the modelled four-helical bundle. This arrangement resulted in the juxtapositioning of the only two cysteine residues (Cys 99 and Cys 169), present within the rat clk-1 sequence. Both residues are conserved across all animal and fungal CLK-1 proteins. DMQ was initially orientated into the predicted active site of CLK-1 based on the *Magnaporthe grisea* AO model of Sessions and colleagues [14]. Following refinement, the final position favored hydroxylation at the position 5 quinone ring carbon of DMQ. This placement assigned an immediate function to His 110 of rat CLK-1 and allowed a total of three hydrogen bonds to hold the quinone head group in place. Energy minimization of the final model was performed using the GROMOS96 implementation of Swiss-PDB Viewer. Structural soundness was investigated using Procheck (V3.5.4) [15].

3. Results and discussion

3.1. The CLK-1 family

Database mining was used to identify CLK-1 orthologues from 18 different species (Section 2). Phylogenetic analysis of these proteins reveals CLK-1 likely entered the domain Eucarya along with an ancestral α-proteobacteria, presumably the precursor of present-day mitochondria [16] (Fig. 1). Investigation of the CLK-1 family reveals the following characteristics (refer to Supplementary Fig. 2): (i) 14 residues are strictly conserved and they cluster in either of two, function-unknown, tandem repeat in CLK-1 (TRC) domains [4], (ii) CLK-1 proteins do not tolerate a C-terminal extension, (iii) within each TRC domain insertions and deletions are only tolerated at highly specific sites and this is suggestive of structural conservation (iv) CLK-1 is notably absent from the green plant *Arabidopsis thaliana*.

3.2. CLK-1 is a new member of the di-iron-carboxylate protein family

Conventional sequence analysis has previously been unable to provide clues as to the function of CLK-1. To circumvent this limitation the BLOCKS suite of programs [11] was used to first locate regions of highly conserved residues within the CLK-1 family (Supplementary Fig. 1a). These blocks represent structurally and functionally important regions of the CLK-1 proteins that have withstood evolutionary pressure to change across two phylogenetic domains. A collection of 3011 distinct protein family BLOCKS was then screened to identify a region of CLK-1 which exhibited significant similarity to part of the predicted iron-binding active-site of AO [17] (Supplementary Fig. 1b). A second region corresponding to the crystallographically determined NAD-binding site of Malic enzymes was also located [18] (Supplementary Fig. 1c). AOs are ubiquinol (Q) oxidases found in the mitochondria of plants as well as some fungi and protists [19]. They form an alternative branch in the mitochondrial electron transport chain, diverting electrons away from complex III and IV to catalyze the cyanide-resistant reduction of oxygen to water. This reduction is not directly coupled to proton translocation across the inner mitochondrial membrane. AOs have been associated with heat generation, metabolic

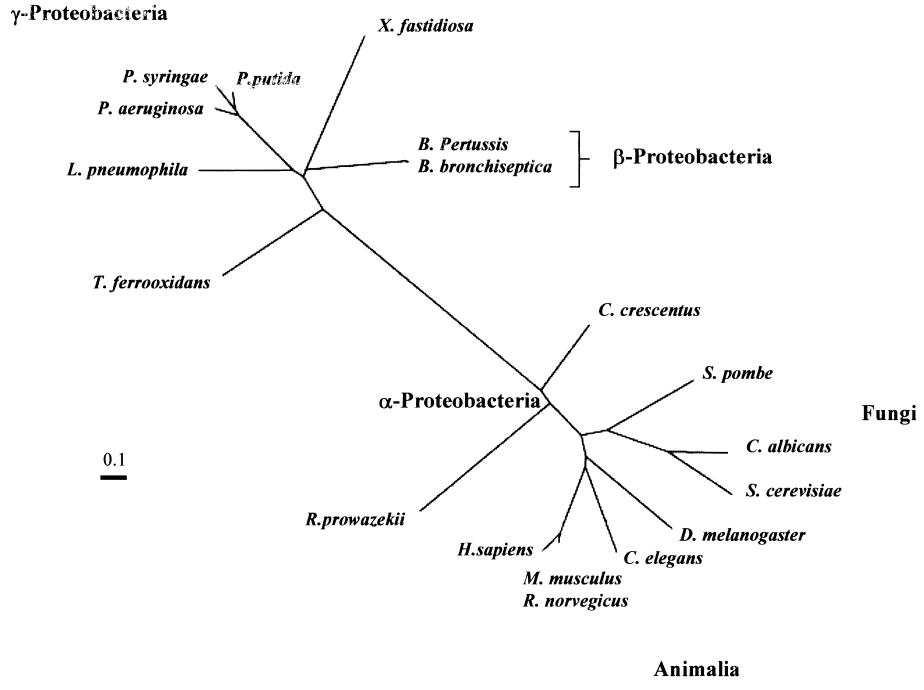

Fig. 1. Unrooted CLK-1 phylogenetic tree. Evolutionary distance (branch length) is scaled according to the expected number of (underlying) amino acid substitutions per site (scale bar = 0.1. A similarly branching tree was obtained when the seed alignment was restricted to both TRC domains, except *C. crescentus* was placed to the right of *R. prowazekii*. Bootstrapping ($N = 100$), maintained the latter distribution 71% of the time).

flexibility, and a decrease in reactive oxygen species (ROS) in respiring mitochondria [14]. Recent studies have assigned the AOs to the di-iron-carboxylate family of proteins [17,20].

Di-iron-carboxylate proteins are non-heme, di-iron-oxo cluster-containing proteins which participate in a range of functions including mono-oxygenation, iron regulation and reactive radical production (reviewed in [8]). Their name derives from the use of several carboxylates and histidines to coordinate a pair of iron atoms. Other members include bacterioferritin, ferritin, methane mono-oxygenase, ribonucleotide reductase R2 and stearoyl-acyl-Δ^9 desaturase. Class I and II di-iron-carboxylate proteins are characterized by the presence of a duplicated motif comprised of a pair of consecutive helices. An iron-coordinating E (single letter code) or D is located in the first helix, and an EXXH motif in the second (Fig. 2a). Outside of these conserved residues there is no obvious homology (cf. Supplementary Fig. 3). As illustrated in Fig. 2, a strong case can be put forward that CLK-1 proteins are bona fide members of the ferritin-like di-iron-carboxylate proteins. Indeed, seven strictly conserved residues can immediately be accounted for as Fe-interacting. The unusual structural similarity between class I and II proteins has allowed a molecular model of rat CLK-1 to be generated (Fig. 3a). Several features are immediately evident from the proposed model. Foremost is the bipolar distribution of charged and uncharged residues, consistent with the observation CLK-1 is a peripheral membrane-associated protein [21], as well as the presence of a large hydrophobic cavity leading to the di-iron-containing active site (see Supplementary Fig. 4 for details).

3.3. CLK-1 is a DMQ mono-oxygenase

Two studies have rekindled the possibility that CLK-1 is a DMQ mono-oxygenase [7,22]. Both studies show *clk-1* nematodes are unable to synthesize Q and instead accumulate DMQ. This phenotype was found to be allele independent; interesting since *clk-1 (qm51)* is a splice acceptor mutant and purportedly null for Clk-1 mRNA [3]. On the basis of the *coq7* deletion mutant, one might have predicted these worms would accumulate HBB [6]. Also of interest were two additional findings, namely, that 2-diprenyl-3-methyl-6-methoxy-1,4-benzoquinone (DMQ_2) could act as an electron carrier from complex I but only poorly from complex II in isolated *clk-1* mitochondria [7], and that full respiratory activity in *clk-1* worms appeared to require dietary-obtained Q [22]. These observations strongly suggest that the primary defect in *clk-1* worms relates to their inability to synthesize Q. Very simply it suggests CLK-1 is a DMQ mono-oxygenase (Clarke and colleagues have suggested Coq7p/CLK-1 is a component of a multi-enzyme complex specializing in the production of Q [6]. Recent studies using *coq3* support this notion [23]. The absence of HBB in *clk-1(qm51)* may indicate it is not a true null mutation or, instead, that the proposed complex exhibits differing stabilities in yeast and worms following the loss of CLK-1).

It is noteworthy that the substrate of AO is Q. Both Q and DMQ are large substrates differing simply by the presence of a methoxy group on the position 5 ring carbon of the quinone headgroup. Furthermore, it is of particular interest that class I and II di-iron-carboxylate proteins are characterized by the

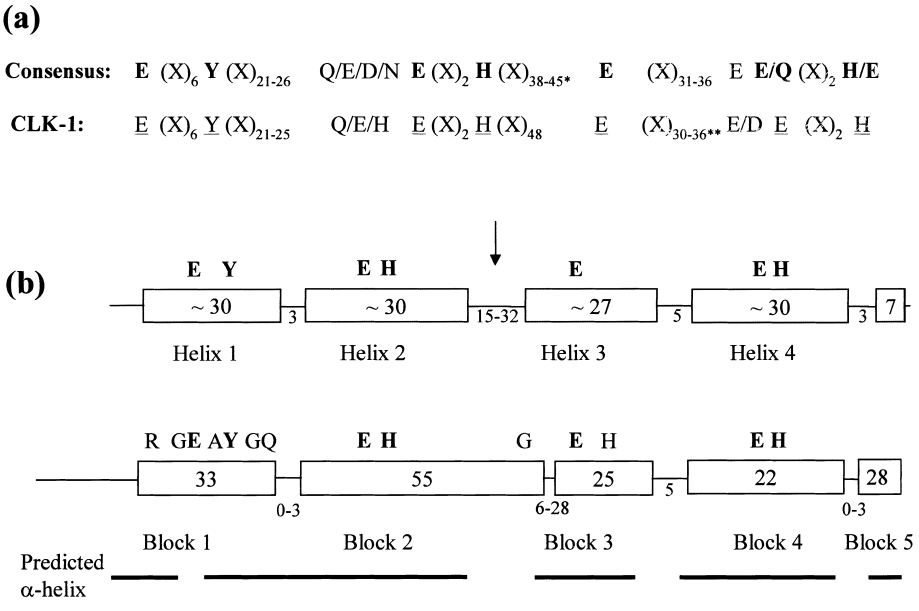

Fig. 2. CLK-1 proteins contain 14 invariant residues forming part of a di-iron-binding motif. a: Seven of 14 invariant CLK-1 residues (bottom sequence, underlined) are predicted to be part of a redox-active di-iron-binding motif. The di-iron-binding consensus sequence (top) of the bacterioferritin–ferritin–rubrerythrin class II di-iron-carboxylate proteins is shown aligned against the proposed corresponding region of CLK-1. Iron-binding residues in the consensus are indicated by bold type. Note Y is a second sphere Fe-coordinating ligand (*52 αα in cyanelle rubrerythrin, **45 and 58 αα in *S. cerevisiae and C. albicans*). b: (Top) Schematic representation of the position of Fe-binding residues (bold) within the four-helix bundle of the bacterioferritin–ferritin–rubrerythrin family of proteins. Approximate lengths of each helix and their intervening sequences are shown (data derived from Fig. 6 of Andrews [37]). Arrow specifies the loop 2–3 dimerization interface of both bacterioferritin and ferritin. (Bottom) Schematic representation of rat CLK-1 showing the position, length and predicted α-helical content of the five highly conserved blocks of residues common to the *clk-1* family (Section 2). Proposed Fe-binding residues (bold) and other invariant residues are marked. CLK-1 is predicted to be an all-helix protein. Secondary structure was identified (75% accuracy) using Predator [38] and the Clustal W multiple sequence alignment shown in Supplementary Fig. 2.

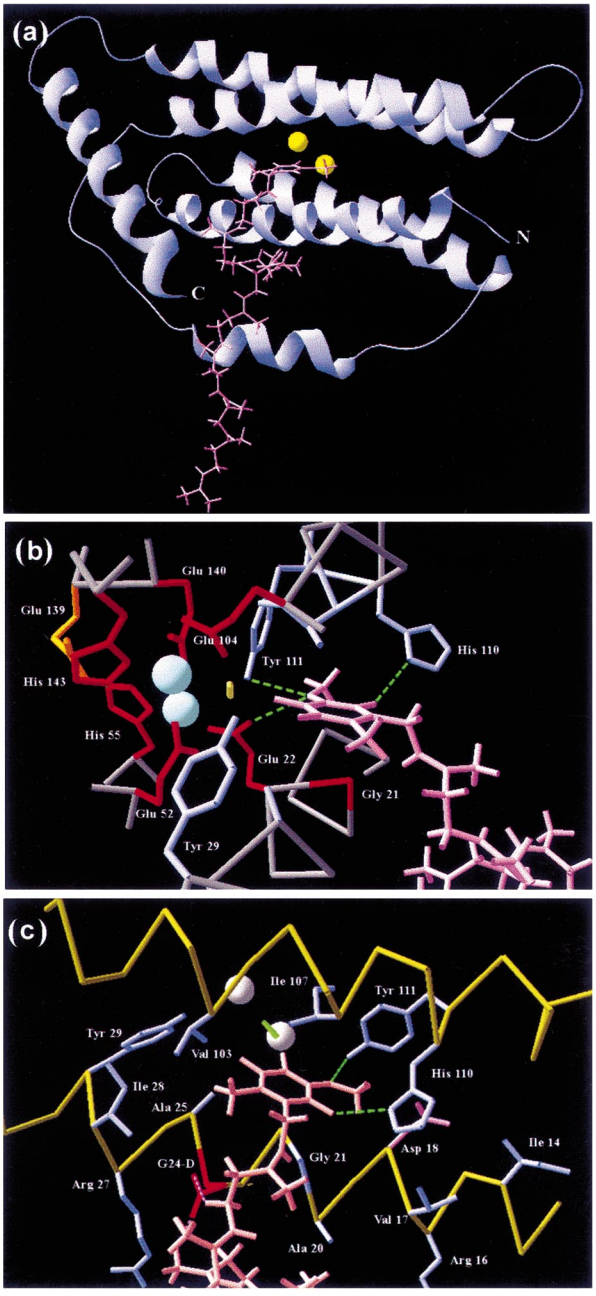

Fig. 3. Homology model of Rat CLK-1. a: The structural co-ordinates of bacterioferritin, human H-ferritin and rubrerythrin were used to homology model the four-helix bundle (αα 8–155) of rat CLK-1. The C-terminal 24 residues were appended as described (Section 2). Yellow balls: Fe atoms; N and C: amino and carboxy termini, respectively. DMQ is inserted into the active site (pink) (see also Supplementary Fig. 3a). b: Proposed active site of rat CLK-1 with DMQ (pink) inserted. The strictly conserved residue His 110 is positioned to hydrogen-bond (green dotted line) the position one ring oxygen of DMQ. Tyr 29 is in a structurally equivalent position as Tyr 34 of human H-chain ferritin. The latter is implicated in transient radical formation [39] and is also conserved in all bacterioferritin–ferritin–rubrerythrin proteins. In CLK-1 proteins, Tyr 29 might participate in the reduction of an Fe intermediate before catalysis. Gln 32 (and indirectly Gly 31) may also participate in this process given its proximity to Tyr 29. Fe atoms, blue balls; complexed O_2, yellow bar; Cα backbone atoms, gray; sidechains are labelled as follows: red, iron coordinating; purple, DMQ headgroup pocket-forming residues; orange, secondary shell Fe-stabilizing residues (also Tyr 29). c: Proposed CLK-1 residues forming the DMQ quinone headgroup binding pocket. His 110, Tyr 111 and Glu 22 (latter not shown) are within H-bonding distance of DMQ ring oxygens. Fe atoms, white; hydrophobic sidechains, purple; acidic sidechains, pink; H-bonds, green dotted lines; Cα backbone, gold; DMQ, orange. The equivalent point mutation (rat CLK-1 G_{24}-D) forming the loss of function *coq7-1 (G_{104}-D)* allele in *S. cerevisiae* [6] is shown in red. The model predicts this point mutation results in loss-of-function because it hinders the entrance of DMQ into the active site.
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fact that they catalyze dioxygen-dependent oxidation–hydroxylation reactions. On the premise that CLK-1 is a DMQ mono-oxygenase, DMQ was tested for its ability to fit into the active site of the model shown in Fig. 3a. As evident in Fig. 3b,c, DMQ not only fits very comfortably into the hydrophobic pocket leading to the di-iron-containing active site, but the quinone headgroup can be positioned in a manner that is predicted to permit the position 5 ring carbon to be hydroxylated by di-iron-bound dioxygen. The strictly conserved residue His 110 also now assumes a function in H-bonding the substrate. Examination of known *clk-1* mutations provides further support for the notion that CLK-1 is a DMQ mono-oxygenase. The *coq7-1* (G_{104}-D) lesion is predicted to place a negative charge at the entrance of the hydrophobic cavity leading to the active site. The *clk-1 (e2519)* point mutation converts the EXXH iron-binding motif of TRC-2 into KXXH, while *clk-1 (qm30)* results in a deletion immediately C-terminal of it. Both the latter mutation presumably destabilize the Fe-binding core.

3.4. Clk-1 and aging

If, as predicted, CLK-1 is a DMQ mono-oxygenase, why do *clk-1* animals have an extended lifespan? One answer might be that in respiring mitochondria, DMQ acts as a surrogate electron carrier and, unlike Q [24], it sustains a smaller degree of uncontrolled electron loss to dioxygen. ROS-related damage might therefore be minimized and, in accordance with the free radical hypothesis of aging [25], aging retarded either directly of indirectly [26]. In *S. cerevisiae*, complex I is absent and an alternative NADH:Q oxidoreductases acts to direct electrons into the electron transport chain without pumping protons [27]. Since it was shown in nematodes that DMQ_2 is a poor substrate for complex II [7], then assuming the latter extends to yeast, this would explain why *coq7-1* is not viable on non-fermentable carbon sources [6]. Paradoxically, each of the three known *clk-1* mutations (*e2519, qm30* and *qm51)* differ in the severity of their phenotypes, including lifespan extension, in a manner corresponding to the severity of their predicted lesion [2]. Despite this each mutant apparently accumulates the same amount of DMQ [7,22]. Also, although the absence of dietary Q was shown to influence the ability of each *clk-1* strain to progress to fertile adults, animals are able to reach the L2 stage before becoming growth arrested [22]. Furthermore, two studies have shown that isolated *clk-1* mitochondria appear to respire normally when provided with the complex II substrate succinate [3,7]. This latter finding directly contradicts the observation that DMQ_2 is a poor complex II electron acceptor [7].

One explanation that would account for each of these apparent inconsistencies is that the absence of Q might also invoke the assembly of a rhodoquinone (RQ) oxidase-fuma-

rate reductase (FR) into complex II to replace succinate dehydrogenase [28]. In this sense two different electron carriers, namely DMQ and RQ, may be operating at once and both might be predicted to have a lower capacity to lose electrons to dioxygen; and indirectly leading to lifespan extension. Two FR isoforms are readily identifiable in the *C. elegans* genome and elevated RQ levels have already been observed in *clk-1* worms [22]. In the parasitic helminth *Fasciola hepatica*, Q and RQ are likely to share all but their final biosynthetic enzymes [29]. It is reasonable to suggest, then, that the differential ability of each *clk-1* strain to disassemble mutant CLK-1 from its Q biosynthetic complex, so as to allow the incorporation of new RQ biosynthetic enzymes, may be the cause of the phenotypic differences observed between each line. In addition, under appropriate conditions some FRs can also operate in reverse [30]. This then may be the reason why isolated *clk-1* mitochondria appeared to respire normally in the presence of succinate. With regards to L2 arrest, it is noteworthy that a shift from a glyoxylate cycle-based metabolism to one utilizing the tricarboxylic acid cycle occurs around the L1–L2 boundary [31]. Although DMQ levels may not be elevated enough, or indeed sufficient, to support complex I-dependent respiration at this time, it remains formally possible that 2-polyprenyl-6-hydroxyphenol, the Q intermediate which accumulates in *ubiG* bacteria [32], and/or bacterially produced menaquinone (used instead of RQ), could interfere in a dominant negative fashion with DMQ/RQ redox cycling, and this is in fact the cause of the L2 arrest.

3.5. CLK-1 and AO

In light of the above findings, it becomes interesting that AO and CLK-1 are both matrix-facing peripheral-mitochondrial membrane proteins, share very similar predicted structures, have almost identical substrates, and exhibit somewhat complementary expression patterns in the Eucarya kingdoms where they have been identified [33]. Given that *clk-1* appears to be is absent from the green plant *A. thaliana*, it is tempting to speculate that this gene may have given rise to the AOs. A plastid-derived mono-oxygenase activity may have provided the catalyst for loss of CLK-1. The appearance of AOs in some fungi indicates that a *clk-1* gene duplication event may have first occurred shortly after the Animalia separation in Eucarya [34]. This notion is consistent with the clear phylogenetic distinction between plant and fungal AOs [14]. If CLK-1 was free to evolve a ubiquinol oxidase function in plants, then the ability of the AOs to reduce ROS in respiring mitochondria [35], coupled with the fact that AOs are a multigene, differentially regulated family of proteins [36] could provide insight into how some plants are able to maintain long-living tissue.

3.6. Summary

Mutational inactivation of *clk-1* in nematodes leads to the loss of Q and a subsequent increase in lifespan. While the precise role of FR in the lifespan extension of *clk-1* nematodes remains to be determined, the loss of Q, coupled with the notion that CLK-1 is now an enzyme of known active site structure, elicits the simple but far reaching conclusion that the development of lipophilic compounds to modulate the activity of CLK-1 might also provide a means for controlling Q levels in higher organisms to extend their lifespan.

Acknowledgements: I thank Drs. Richard Sessions, Thomas Johnson, Judith Campisi, Chang-Su Lim, and Milena Girotti, as well as Antonio Ubach for their respective help. Unfinished sequence data was generated at the Stanford DNA Sequencing and Technology Center with the support of the NIDR and the Burroughs Wellcome Fund, The Institute for Genomic Research, The Legionella Genome Project, Columbia Genome Centre, and the *B. pertussis* and *B. bronchiseptica* Sequencing Groups at the Sanger Centre, UK.

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