Ubiquinone (UQ) is a lipid co-factor that is involved in numerous enzymatic processes and is present in most cellular membranes. In particular, UQ is a crucial electron carrier in the mitochondrial respiratory chain. Recently, it was shown that clk-1 mutants of the nematode worm Caenorhabditis elegans do not synthesize UQ9, but instead accumulate demethoxyubiquinone (DMQ9), a biosynthetic precursor of UQ9 (the subscript refers to the length of the isoprenoid side chain). DMQ9 is capable of carrying out the function of UQ9 in the respiratory chain, as demonstrated by the functional competence of mitochondria isolated from clk-1 mutants, and the ability of DMQ9 to act as a co-factor for respiratory enzymes in vitro. However, despite the presence of functional mitochondria, clk-1 mutant worms fail to complete development even on bacteria that do not produce UQ9. Here we show that clk-1 mutants cannot grow on bacteria producing only DMQ8, and that worm coq-3 mutants, which produce neither UQ9 nor DMQ9, arrest development even on bacteria producing UQ9. These results indicate that UQ is required for nematode development at mitochondrial and non-mitochondrial sites and that DMQ cannot functionally replace UQ at those non-mitochondrial sites.

Ubiquinone (UQ) is a prenylated benzoquinone that is an essential co-factor in the mitochondrial respiratory chain, where its function is best characterized. UQ is also found in many other locations in the cell, such as the lysosome and Golgi membranes, as well as in nuclear and plasma membranes (1). The exact role of UQ at these extramitochondrial sites is being actively explored (e.g. Refs. 2 and 3).

The gene clk-1 of the nematode Caenorhabditis elegans affects many physiological rates, including embryonic and post-embryonic development, rhythmic behaviors, reproduction, and life span (4). clk-1 encodes a 187-amino acid protein that is localized in mitochondria (5) and that is homologous to the yeast protein C0p7p, which has been shown to be required for UQ biosynthesis (6). clk-1 has also been shown to be necessary for UQ biosynthesis in worms (7, 8) and in the mouse (9). Indeed, UQ9 is entirely absent from mitochondria purified from worm and mouse clk-1 mutants (8, 9) (the subscript refers to the length of the isoprenoid side chain). Instead, these mitochondrial accumulate demethoxyubiquinone (DMQ9), which is an intermediate in the synthesis of UQ9 (8, 9). Consistently, recent evidence suggests that clk-1 encodes a DMQ hydroxylase (10), which converts DMQ to ubiquinol. In Escherichia coli, DMQ9 is able to sustain respiration in isolated membranes although at a lower rate than Q8 (11). Similarly, DMQ9 is capable of sustaining electron transport in eukaryotic mitochondria, as the function of purified mitochondria (5), and mitochondrial enzymes (8), from clk-1 worm mutants appears to be almost intact compared with the wild type. In addition, synthetic DMQ9 has been shown to function in vitro as a co-factor for electron transport from worm complex I and, albeit more poorly, from complex II (8). Finally, it was found that in the absence of any exogenous UQ the oxygen consumption of mouse embryonic stem cells with a deleted mclk1 gene is only reduced by 5% (9).

Recently, it has been found that clk-1 mutants are unable to grow on a UQ-deficient bacterial strain despite the presence and the activity of DMQ9 (7). Although, dietary UQ is generally not capable of reaching mitochondria (reviewed in Ref. 1), this has been interpreted to suggest that DMQ9 is insufficient for normal mitochondrial function and that dietary bacterial UQ9 can reach the mitochondria and function there in trace amounts (7).

To resolve these issues, we have generated a strain carrying a knockout mutation in the nematode gene coq-3, which encodes a methyltransferase required for UQ synthesis, and whose inactivation does not lead to DMQ accumulation in yeast (12). We find that coq-3 worms are not able to complete development even on bacteria that contain UQ9. These results indicate that 1) dietary UQ cannot complement a UQ deficiency in the absence of DMQ, 2) the growth impairment of clk-1 mutants without a dietary supply of UQ is due to a non-mitochondrial requirement for UQ, and 3) DMQ cannot functionally replace UQ at the non-mitochondrial sites.

EXPERIMENTAL PROCEDURES

Nematode and Bacterial Strains—We used the wild type N2 (Bristol) strain. Mutant strains analyzed include daf-2(e1370), eat-2(ad465), dpy-9(e12), and mau-2(qm160). None of these strains had any difficulty in growing on bacteria that do not produce UQ. A detailed analysis was performed using clk-1(qm30), clk-1(e2519), and clk-1(qm51). Standard procedures were used for bacterial and worm cultures (13), except that the nematode growth medium plates contained 0.5% glucose, to minimize the reversion of UQ-deficient strains. The genotypes of the bacterial strains used are described in Table I.

Production of the coq-3 Knockout Mutation—A null mutation in the C. elegans gene coq-3 was generated according to the protocol previously established by Molder and Barstead (snmc01.omrf.uokhsc.edu/revgen/RevGen.html). A detailed description of the manipulations performed, including the primer sequences used for PCR, can be obtained upon request. See Fig. 2 for a schematic representation of the coq-3 gene (which is part of an operon), the extent of the deletion in coq-3 (qm188).
Ubiquinone at Mitochondrial and Non-mitochondrial Sites

RESULTS

Ubiquinone Is Necessary for C. elegans Development and Fertility—It was recently reported that clk-1 mutants are incapable of completing development when fed on a ubiG E. coli mutant strain (7). The ubiG gene product is required at two steps of the UQ biosynthesis pathway, and ubiG mutants do not produce any UQ (16). We tested whether this growth phenotype resulted from a specific toxicity of the ubiG gene product to E. coli for clk-1 mutants or from the absence of UQ. For this purpose, we systematically analyzed the growth of clk-1 mutant worms on a variety of E. coli strains. We produced strains that are defective for UQ biosynthesis (ubi mutants). Nine E. coli enzymes have been described as participating in UQ biosynthesis (see Fig. 1) (16). They are all membrane-bound, except the first one, ubiC, which is a soluble enzyme. The ubiC enzyme is involved in the pathway at the prenyltransferase ubiA step that attaches the isoprenoid side chain to the quinone ring (eight subunits in E. coli) (18). The other enzymes are grouped in three categories: decarboxylases (ubiD and ubiX), monooxygenases (ubiB, ubiH, ubiF), and methyltransferases (ubiG, ubiE) (19–24). We examined the growth of the three clk-1 mutant strains on strains of bacteria mutant for each of these genes, except ubiC (Table I). The three clk-1 mutant alleles are qm30 and qm51, which are putative nulls, and e2518, a point mutation that results in a milder phenotype (14, 25).

We find that on all the bacterial ubi (mutant) strains tested, L1 larvae from the wild type strain N2 are capable of completing development to adulthood and that these adults have a brood size of around 320, which is similar to their brood size on ubi+ bacteria (OP50) (Table II). This indicates that endogenously synthesized Q is sufficient to maintain a wild type phenotype, without a requirement for dietary UQ. We have also examined a number of worm mutants that are not known to be involved in UQ synthesis (dp-9, eat-2, mou-2), including long lived mutants (daf-2) and a number of strains that show a clk-1-like phenotype that have not yet been fully characterized. In no case was the growth of the mutants impaired on ubi+ bacteria. In contrast, all three clk-1 mutants behave identically on most ubi+ bacterial strains tested: they develop very slowly, or not at all, and produce no progeny (Table II). However, the clk-1 mutants can develop and produce some progeny on ubiD, ubiX, and ubiH mutant strains, which are point mutants that produce residual amounts of ubiquinone (around 15% of the wild type) (16). It appears therefore that relatively low levels of bacterial UQs are sufficient to allow for the growth of clk-1 mutants.

Endogenous Ubiquinone Is Necessary for C. elegans Development and Fertility—To test whether dietary UQ is sufficient for C. elegans development, we produced a knockout mutation of the worm gene coq-3. coq-3 encodes a methyltransferase whose homologues (Coq3p and UbiG) have been extensively characterized in S. cerevisiae and in E. coli, respectively. The enzyme acts at two different steps of Q synthesis, and neither UQ nor DMQ are produced in the yeast and bacterial mutants (24, 26).
The worm COQ-3 protein is 29% identical to *S. cerevisiae* Coq3p and 28% to *E. coli* UbiG. We used a method of random mutagenesis and PCR-based screening to identify a deletion in *coq-3* (see “Experimental Procedures” and Fig. 2). The deletion *qnm188* removes exons 3 and 4, such that no functional COQ-3 protein can be produced (Fig. 2A). Self-fertilizing *coq-3(qnm188)/+* hermaphrodites produce one-quarter of homozygous *coq-3(qnm188)/coq-3(qnm188)* progeny, as verified by PCR (see “Experimental Procedures” and Fig. 2B). These *coq-3* homozygous develop slowly and appear substantially smaller than wild type worms. Most are sterile (Fig. 2C), but ~25% (*n = 31*) produce some progeny (5–10 eggs) that arrest at the L1 stage and die quickly thereafter. These observations are consistent with a partial maternal effect from the heterozygous mothers of *coq-3* homozygotes, as the phenotype of the first homozygous generation (slow development to adulthood) is less severe than that of the second homozygous generation (arrest at the L1 stage). The maternal effect could be due to UQ provided to the embryo by the mother or to maternal deposits of *coq-3* mRNA or protein. To ascertain whether the observed phenotypes are solely due to the mutation in the *coq-3* gene, we introduced the genomic fragment corresponding to the *coq-3* gene into *coq-3(+)* heterozygotes using the dominant rol-6 (sa1006d) transformation marker by germ line transformation. Homozygous *coq-3* transgenic animals (displaying the marker phenotype, Rol) develop normally and are fertile, indicating that the phenotype we observe is indeed due to the *coq-3* deletion. However, the extrachromosomal array carrying the *coq-3* and *rol-6* sequences is incapable of producing a strong maternal effect. Indeed, homozygous animals without the array (phenotypically non-Rol) issued directly from mothers carrying the array (phenotypically Rol) did not develop beyond the L2 stage. The expression of genes from extrachromosomal arrays is sometimes silenced and is generally poor in the *C. elegans* germline (27). The observation of a maternal effect suggests that the mother deposits an essential product in the oocytes, which here could be UQ or *coq-3* mRNA. In either case, proper expression of *coq-3* in the germ line appears to be necessary for the effect.

We also observed that the brood size of heterozygous *coq-3*/*dpy-4* worms (243 ± 38; *n = 10*) was similar to that of *dpy-4/*+* worms (248 ± 23; *n = 10*) (Fig. 2D), suggesting that the expression level of *coq-3* in the heterozygotes might not be limiting for UQ biosynthesis. The lethal phenotype of *coq-3* mutants on *ubi-1* indicates that dietary UQ is not sufficient for the growth and development of worms. This is consistent with findings in other systems that indicate that dietary UQ cannot reach the mitochondrial compartment or only in extremely small amounts (1). The possibility that dietary UQ could be sufficient for worms was proposed to account for the viable phenotype of clk-1 mutants grown on *ubi-1* bacteria and their lethal phenotype when grown on *ubi-1* mutant bacteria. However, the phenotype of *coq-3* mutants indicates clearly that even in the presence of dietary bacterial UQ9, a total absence of endogenous UQ9 and DMQ9 (as in *coq-3* mutants) is not equivalent to the replacement of endogenous UQ9 by endogenous DMQ9 (as in *clk-1* mutants).

In this context, it is of particular interest that *clk-1* mutants cannot thrive by feeding on *ubiF* mutants (see above). Indeed, UQ biosynthesis in *ubiF* mutants is blocked at the same level as in *clk-1* mutants, and *ubiF* bacteria thus produce DMQ9 (22). As DMQ9 performs efficiently in the mitochondrial respiratory chain (8), our findings indicate that neither endogenous nor dietary DMQ9 can replace UQ at non-mitochondrial sites of UQ requirement.

**Discussion**

Our results suggest that UQ is necessary for *C. elegans* growth and development at different subcellular locations, in particular it appears to be necessary at sites distinct from the mitochondrial respiratory chain (Fig. 3). Indeed, for its respiratory function in the mitochondria, endogenous DMQ9 can functionally replace endogenous UQ9, as indicated by the observation that *clk-1* mutant mitochondria do not appear to contain UQ9 but are functionally competent (8). On the other hand, *coq-3* mutants, in which a failure to manufacture UQ9 and DMQ9 is expected, display a much more severe phenotype than *clk-1* mutants. Thus, at some still unknown site or sites, distinct from the respiratory chain, endogenous DMQ9 or dietary DMQ9 cannot functionally replace endogenous UQ9, while dietary UQ9 can. In fact, *clk-1* mutants, which have functional mitochondria and make DMQ9, cannot develop and grow without dietary UQ9, even in the presence of dietary DMQ9 from *ubiF* bacteria.

This model is consistent with the findings by numerous studies on UQ uptake and metabolism in other systems, such as in yeast (28). The *clk-1* mutation results in almost complete loss of UQ9 in the mitochondrial matrix (29). Therefore, the results are consistent with the idea that both UQ9 and DMQ9 are necessary for the mitochondrial respiratory chain.
FIG. 2. Characterization of the coq-3 deletion mutant. A, genomic structure of the coq-3 gene. coq-3 is located on linkage group IV of C. elegans and is part of an operon, comprising the gdi-1 gene and a gene (Y57G11C.12) encoding a subunit of NADH-ubiquinone oxidoreductase. coq-3 contains five predicted exons. The deletion in coq-3 (qm188) mutants removes 2456 bp, eliminating exons 3 and 4, and consequently no functional protein can be produced. Primers (SHP) used for PCR screening of the deletion library and individual worms are indicated. B, PCR analysis of genomic DNA from N2, coq-3/dpy-4, and coq-3/coq-3 worms. To check the presence of a deletion in the coq-3 gene, PCR analyses were carried out using genomic DNA from single worms. Each DNA preparation was simultaneously tested with primers recognizing sequences either outside the coq-3 gene (SHP 1774 and SHP 1775, lanes 2–4) or inside the obtained deletion (SHP 1840 and SHP 1865, lanes 5–7). See A for the primer localization. When using primers amplifying the whole coq-3 gene, a band of 4.3 kb was obtained from a wild type worm (lane 2). In contrast, a mutant band was amplified at 1.8 kb from a coq-3/coq-3 worm. DNA from heterozygotes only amplified the mutant band (lane 2), probably because the PCR conditions are in favor of the shorter amplicon. When using primers annealing in the deletion region, both wild type and heterozygote worms gave a PCR product of 1.1 kb (lanes 5 and 6), while no band was detected from a coq-3/coq-3 worm, which underlies their sterile phenotype. D, brood size analysis. Experiments were performed twice, yielding similar results. The sample size is indicated in parentheses. Growth is scored as the capacity to reach adulthood and be fertile. We analyzed coq-3/dpy-4 heterozygotes, maternally rescued coq-3/coq-3 (m−/z−), coq-3/coq-3 rescued with a transgene carrying the wild type sequence of the coq-3 gene (coq-3/coq-3 rescue), and dpy-4/dpy-4 as well as dpy-4/+ as controls. The results show that coq-3/dpy-4 heterozygotes have a brood size comparable with dpy-4/1+ or dpy-4/dpy-4 animals. coq-3 homozygotes rescued with a wild type version of the coq-3 gene are fertile, but their brood-size is low.
Endogenous UQ is able to reach all of the locations where UQ is necessary, while dietary UQ only accesses non-mitochondrial sites. On the other hand, endogenous DMQ does not function at those sites, but is active in mitochondrial respiration. Mitochondria are depicted in red, and the undetermined non-mitochondrial locations are represented in blue. Arrows point to locations within the cell where a given quinone is functional.

Our studies clarify the roles of endogenous and dietary UQ in the worm’s biology. Also, we demonstrate for the first time the functional importance of UQ at non-mitochondrial locations for an organism’s viability. Action of dietary UQ at non-mitochondrial sites could underlie the beneficial effects of dietary UQ for patients with mitochondrial diseases (1). For example, UQ has been found to participate in reactions that regulate the redox state of the cell at the plasma membrane (28). Disease states that arise from deficient mitochondria are often found to increase cellular oxidative stress, and dietary UQ could stimulate a protective function at the plasma membrane (28). In addition, in bacteria, quinones have recently been found to act as the primary signal of the redox state of the cell (29). In E. coli, UQ negatively modulates the phosphorylation status and function of ArcB, an important global regulator of gene expression. The eventual discovery of additional roles for UQ in eukaryotes, and in particular as a signaling cue, will help to better understand the pleiotropic effects of mutations in genes that affect UQ, including clk-1.

Finally, we note that the coq-3 and clk-1 mutant strains provide genetic models to identify compounds that could selectively replace ubiquinone at the mitochondria and/or at non-mitochondrial sites. The development of such bio-available ubiquinone mimetics could be of great medical interest.

Acknowledgments—We thank Claire Bénard and Robyn Branicky for careful reading of the manuscript and Robert Poole, Georges Javor, Philip Rather, Catherine Clarke, David Clark, Bernard Lemire, and the E. coli stock center at Yale for sharing bacterial clones.

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*J. Biol. Chem.* 2002, 277:2202-2206.
doi: 10.1074/jbc.M109034200 originally published online November 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109034200

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