The kynurenine pathway is essential for rhodoquinone biosynthesis in *Caenorhabditis elegans*

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A key metabolic adaptation of some species that face hypoxia as part of their life cycle involves an alternative electron transport chain in which rhodoquinone (RQ) is required for fumarate reduction and ATP production. RQ biosynthesis in bacteria and protists requires ubiquinone (Q) as a precursor. In contrast, Q is not a precursor for RQ biosynthesis in animals such as parasitic helminths, and most details of this pathway have remained elusive. Here, we used *Caenorhabditis elegans* as a model animal to elucidate key steps in RQ biosynthesis. Using RNAi and a series of *C. elegans* mutants, we found that arylamine metabolites from the kynurenine pathway are essential precursors for RQ biosynthesis *de novo*. Deletion of *kynu-1*, encoding a kynureninase that converts L-kynurenine (KYN) to anthranilic acid (AA) and 3-hydroxykynurenine (3HKYN) to 3-hydroxyanthranilic acid (3HAA), completely abolished RQ biosynthesis but did not affect Q levels. Deletion of *kmo-1*, which encodes a kynurenine 3-monooxygenase that converts KYN to 3HKYN, drastically reduced RQ but not Q levels. Knockdown of the Q biosynthetic genes *coq-5* and *coq-6* affected both Q and RQ levels, indicating that both biosynthetic pathways share common enzymes. Our study reveals that two pathways for RQ biosynthesis have independently evolved. Unlike in bacteria, where amination is the last step in RQ biosynthesis, in worms the pathway begins with the arylamine precursor AA or 3HAA. Because RQ is absent in mammalian hosts of helminths, inhibition of RQ biosynthesis may have potential utility for targeting parasitic infections that cause important neglected tropical diseases.

Adaptation to hypoxia is crucial for survival in several animal lineages (1). Such is the case with helminths (parasitic nematodes and platyhelminths), which are facultative anaerobes and live part of their life cycle under low-oxygen tension in the gastrointestinal tract of their vertebrate hosts. One of the key adaptations of these lineages is the use of an alternative electron transport chain (ETC)[7] that allows them to harvest energy under hypoxic conditions (2–4). In the absence of oxygen, complex II of this alternative ETC functions in the opposite direction to the conventional ETC. To facilitate this reversal, fumarate is used as the final electron acceptor, and rhodoquinone (RQ) functions as the electron transporter. RQ differs from ubiquinone (Q), the electron transporter in the conventional ETC, by one substituent on the benzoquinone ring: RQ has a 2-amino substituent, whereas Q has a methoxy group in this position (Fig. 1*A*). RQ has a lower redox potential than Q (−63 mV *versus* 110 mV, respectively) (5, 6), enabling RQ to receive electrons from NADH through complex I and donate them to fumarate though complex II (Fig. 1*B*) (3, 7). In contrast to other fermentative pathways, the alternative ETC allows proton pumping and ATP synthesis through complex V, leading to higher efficiency in harvesting energy. This pathway, in which RQ is the signature metabolite, is also found in some bacteria and protists (1).

The biosynthetic pathway of RQ has been extensively studied in *Rhodospirillum rubrum*. In this organism, RQ biosynthesis requires Q as a precursor (8). Subsequently, it was discovered that the *R. rubrum rquA* gene is essential for RQ but not for Q biosynthesis (9). Despite a comprehensive study of *R. rubrum* knockout mutants, using aerobic *versus* anaerobic transcrip-

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This article contains Figs. S1 and S2 and Tables S1–S7.

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[7] The abbreviations used are: ETC, electron transport chain; AA, anthranilic acid; 3HAA, 3-hydroxyanthranilic acid; 3HKYN, 3-hydroxykynurenine; KYN, kynurenine; KYNA, kynurenic acid; Q, ubiquinone; RQ, rhodoquinone; EV, empty vector; 4HB, 4-hydroxybenzoic acid; pABA, *para*-aminobenzoic acid.

tome data and comparative genomic analysis, no other genes besides *rquA* were identified to be essential for RQ biosynthesis (10). In parallel, it was shown that unicellular eukaryotes also possess a homolog of the *rquA* gene, most likely acquired by horizontal gene transfer (11). These studies indicate that *rquA* is the gene signature for RQ biosynthesis in bacteria and protists. More recently, the heterologous expression of *R. rubrum rquA* in two non-RQ–producing species, *Escherichia coli* and *Saccharomyces cerevisiae*, resulted in the *in vivo* conversion of native Q to synthetic RQ (12). Despite these advances, the biosynthesis of RQ in animals has not been elucidated, and the key genes involved have remained elusive.

RQ has been found in all helminths where it has been examined (7, 13). Importantly, RQ is also synthesized by the free-living nematode *Caenorhabditis elegans* (14), which faces hypoxia during development or as an environmental challenge. Studies in *C. elegans* have shown that Q is not a required precursor of RQ. Indeed, a KO strain in *coq-7* (also known as *clk-1*) abolishes Q biosynthesis without affecting RQ biosynthesis (15, 16). Although helminths are not easily approachable, *C. elegans* is a formidable experimental organism (17) and has been used as a model for parasitic nematodes (18). In this study, we elucidate key steps in the RQ biosynthesis pathway using *C. elegans*. We demonstrate that the kynureninase KYNU-1 is essential for RQ biosynthesis, and based on RNAi experiments, we propose that RQ and Q have parallel pathways starting from different precursors. Because RQ is not synthesized or used by mammalian hosts, but is required for parasite survival, the RQ biosynthetic pathway is a unique target for antihelminthic drug design.

Results

KYNU-1 is essential for RQ biosynthesis in C. elegans

Because of the differences in RQ biosynthesis in *R. rubrum* and *C. elegans*, we reasoned that, in the case of animals, the amino group at position 2 of the benzoquinone ring (Fig. 1*A*) may be added at the beginning of RQ biosynthesis, rather than at the end. Because *kynu-1* encodes a kynureninase that catalyzes the synthesis of two arylamines, anthranilic acid (AA) and 3-hydroxyanthranilic acid (3HAA), from L-kynurenine (KYN) and 3-hydroxy-L-kynurenine (3HKYN), respectively (Fig. 2*A*), we examined RQ biosynthesis in a *kynu-1* KO strain. No trace of RQ was observed in the KO animals (Fig. 2*B*). In contrast, Q levels were not reduced in the KO. RNAi-mediated knockdown of *kynu-1* in the *C. elegans* strain *rrf-3*(*pk1426*), which is hypersensitive to RNAi (19), exhibited a significant decrease in RQ levels ($p < 0.001$), with no decrease in Q, as compared with the empty vector (EV) control (Fig. 2*B*). The expression of the WT *kynu-1* allele in the *kynu-1* strain under the control of its own promoter rescued RQ biosynthesis (Fig. 2*B*). These results allow us to conclusively demonstrate that *kynu-1* is essential for RQ biosynthesis and strongly suggest that AA or 3HAA is a key precursor for RQ biosynthesis. Intriguingly, supplementation experiments with AA or 3HAA did not restore RQ biosynthesis in the *kynu-1* strain (Fig. 2*B*). These results indicate that RQ is synthesized *de novo* in *C. elegans*.

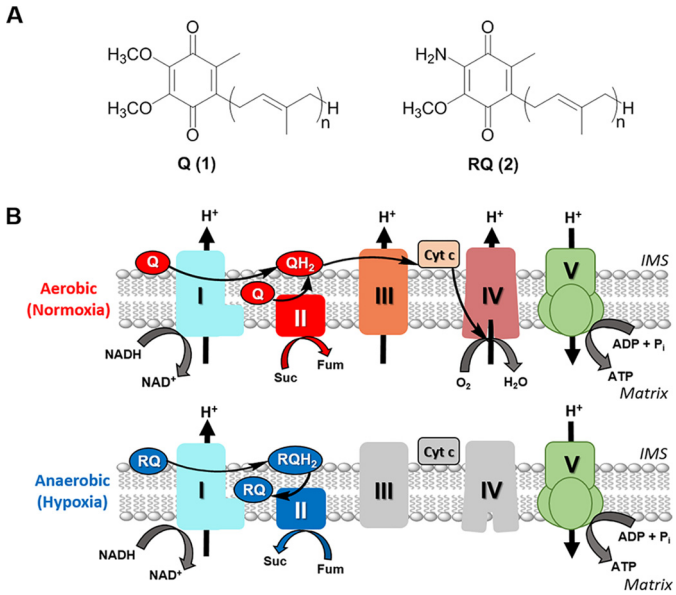

Figure 1. Structure and function of RQ and Q in the mitochondria of helminths. *A,* structures of ubiquinone (UQ or Q, compound 1) and RQ (compound 2), where *n* varies from 6 to 10 depending on species. *B,* Q and RQ are part of the mitochondrial ETC in normoxia and hypoxia, respectively. In normoxia, electrons from NADH and succinate are transferred to Q through complexes I and II, then from QH_2 to cyt *c* through complex III, and finally from cyt c to O_2 through complex IV. In hypoxia, the ETC functions with complexes I and II only. Electrons are transferred from NADH to RQ through complex I and then from RQH_2 to fumarate through complex II. In both ETCs, a proton gradient across the inner membrane is generated and used to produce ATP through complex V. *Suc*, succinate; *Fum*, fumarate.

Because KYN can be converted to 3HKYN by kynurenine 3-monooxygenase, KMO-1, we analyzed a *kmo-1* mutant strain to discriminate whether AA or 3HAA is the RQ precursor. The *kmo-1* strain had significantly reduced RQ levels compared with N2 ($p < 0.001$), but RQ biosynthesis was not completely abolished (Fig. 2*C*). The result is consistent with the fact that a hydroxyl substituent can be introduced at position 3 of the aromatic ring by *kmo-1*–dependent and *kmo-1*–independent routes (20, 21). Because KYNU-1 is required for the biosynthesis of both metabolites, this would explain the absolute requirement of this gene. Because both *kynu-1* and *kmo-1* belong to the kynurenine pathway (Fig. 2*A*), we analyzed a KO mutant in *afmd-1*, the gene that precedes *kynu-1* in the pathway. *afmd-1* encodes the kynurenine formamidase that converts L-formyl kynurenine to KYN. Surprisingly, the *afmd-1* strain only reduced RQ levels to about half that of N2 ($p < 0.001$). We also analyzed a mutant strain in *haao-1*, which encodes a 3-hydroxyanthranilic acid oxygenase (HAAO-1) downstream of KYNU-1 in the pathway (Fig. 2*A*). This strain did not affect RQ biosynthesis (Fig. 2*C*). Thus, 2-amino-3-carboxymuconic semialdehyde is unlikely to be a precursor for RQ biosynthesis. Instead, RQ biosynthesis most likely branches from the AA or 3HAA precursors in the kynurenine pathway.

kynu-1 expression is restricted to the hypodermis of the worm

To assess the expression pattern of *kynu-1* during the *C. elegans* life cycle, we generated and analyzed a translational reporter strain expressing GFP under the control of the *kynu-1* promoter (IH25 strain). We detected expression of *kynu-1* in the embryo early in the E lineage and epidermis. This pattern

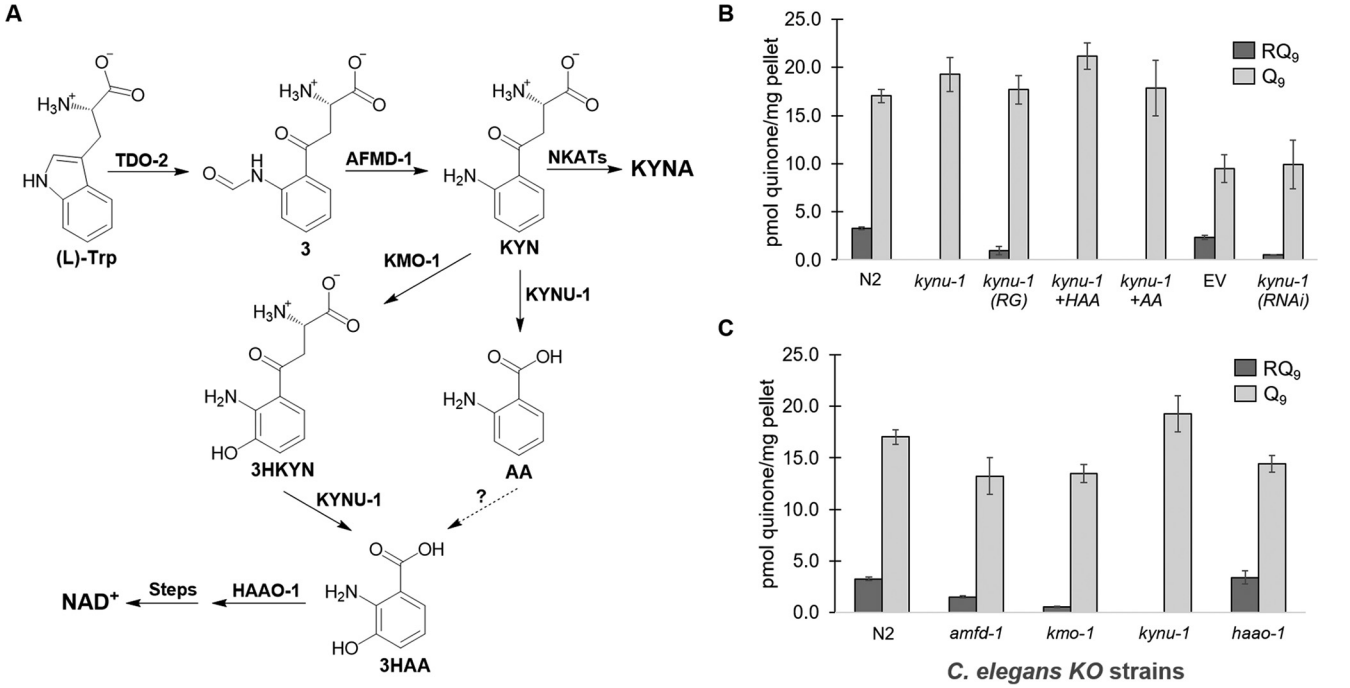

Figure 2. Kynurenine pathway is essential for RQ biosynthesis. *A,* in the kynurenine pathway, L-tryptophan is first converted to L-formyl kynurenine (compound 3) by tryptophan 2,3-dioxygenase (*TDO-2*), which is then converted to KYN by an arylformamidase (*AFMD-1*). Kynurenine (KYN) is a branch point and can be converted to the following: 1) KYNA by kynurenine aminotransferases (*NKATs*); 2) 3HKYN by kynurenine 3-monooxygenase (*KMO-1*); or 3) AA by the kynureninase (*KYNU-1*). KYNU-1 also transforms 3HKYN to 3HAA, which has also been proposed to form from AA. Finally, 3HAA is converted to 2-amino-3-carboxymuconic semialdehyde (not shown) by 3-hydroxyanthranilic acid oxygenase (HAAO-1), which is then converted to NAD^+. *B,* deletion of *kynu-1* from N2 *C. elegans* abolished RQ biosynthesis. Overexpression of *kynu-1* WT allele in the mutant *kynu-1* strain restored RQ biosynthesis (*RG*). Supplementation with 3HAA and AA did not rescue RQ levels. The *kynu-1* RNAi significantly reduced RQ levels compared with the EV control in *rrf-3*(*pk1426*) worms. *C, amfd-1* and *kymo-1* strains significantly reduced RQ levels, compared with N2, whereas the *haao-1* strain had no effect. A full statistical analysis is given in Table S1.

was maintained in the L1 stage, but starting at L2 through adulthood expression was only seen in the epidermis (Fig. 3 and Fig. S1). Because RQ is supposed to play a key role as part of an alternative ETC under hypoxia, we analyzed whether expression of *kynu-1* was affected after exposure to 0.4% oxygen during 24 h in adult worms. We did not observe any obvious increase in GFP fluorescence nor a difference in the spatial expression of the reporter. This suggests that *kynu-1* expression is not regulated under hypoxic conditions.

Enzymes involved in Q biosynthesis are also involved in RQ biosynthesis

In the case of *S. cerevisiae*, Q can be synthesized from 4-hydroxybenzoic acid (4HB) or 4-aminobenzoic acid (pABA) in parallel pathways using common enzymes in most steps (Fig. 4*A*) (22, 23). Thus, we reasoned that some of the enzymes may also be involved in RQ biosynthesis. We performed RNAi knockdown assays of *coq-3*, *coq-5*, *coq-6*, and *coq-7* genes. We found that *coq-5* and *coq-6* RNAi significantly decreased both Q and RQ levels compared with controls ($p < 0.001$), whereas *coq-3* had a smaller effect on both (Fig. 4*B* and Table S1). As expected, *coq-7* RNAi significantly decreased Q levels ($p = 0.010$) but not RQ. The mRNA levels for the silenced genes indicated efficient interference in all but the *coq-3* RNAi samples (Fig. S2). These results clearly indicate that COQ-5 and COQ-6 are involved in both Q and RQ biosynthesis. Our results support the existence of parallel pathways that use several common enzymes to synthesize Q and RQ from different precursors.

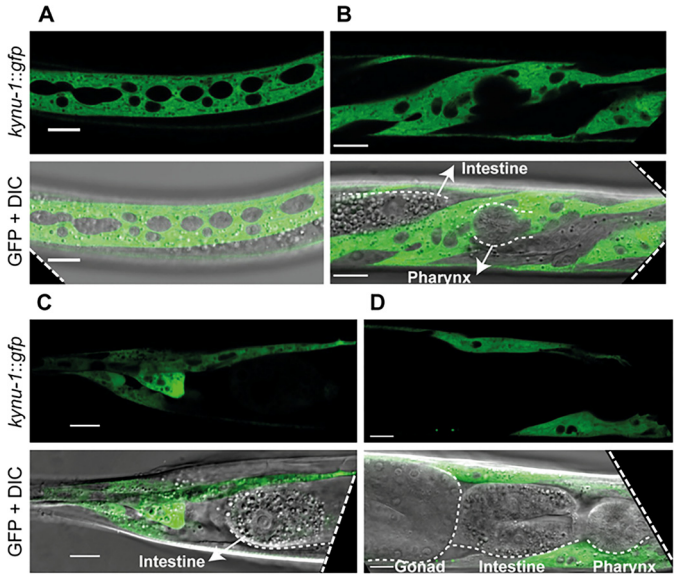

Figure 3. *kynu-1* is expressed in the hypodermis with cytosolic localization. Confocal images of selected planes show head, middle body, and tail regions of P*kynu-1*::*kynu-1*::*gfp* transgenic animal expression. *A,* middle body region. *B,* head of an L3 worm (lateral views). *C,* tail. *D,* head of an adult worm (lateral views). Pharynx, intestine, and gonad are indicated. *Scale bar,* 10 μm.

Discussion

The biosynthesis of RQ in animals has remained a puzzle for decades (24). In bacteria and protists, RQ is derived from Q, and *rquA* is the gene signature for its biosynthesis (9, 11). In contrast, in animals, RQ is not derived from Q, and no RQ-specific

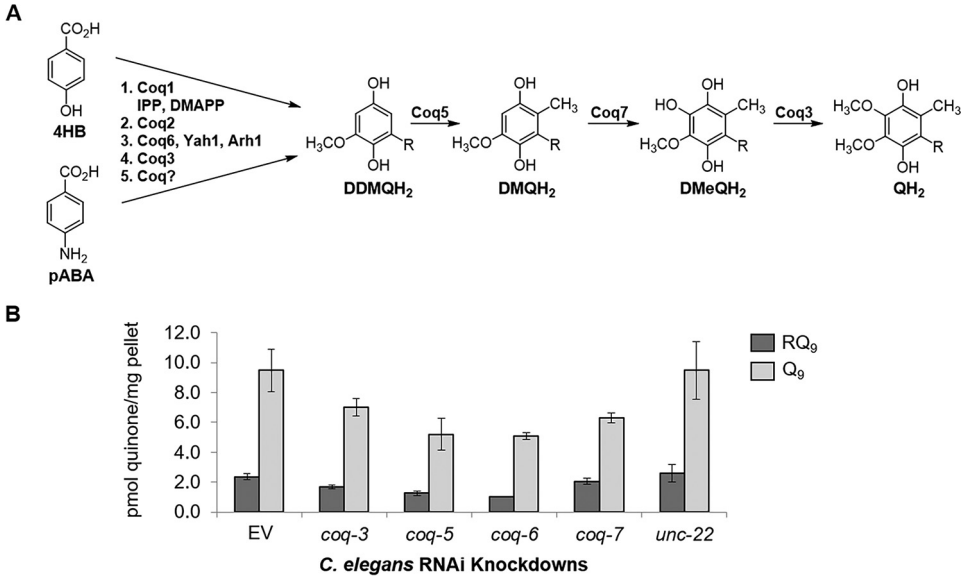

Figure 4. Biosynthesis of RQ shares common enzymes with the Q biosynthetic pathway. *A,* Q biosynthetic pathway in yeast can start from either 4HB or pABA. These pathways share the enzymes Coq1, Coq2, Coq6 (with Yah1 and Arh1), and Coq3. They merge at the common precursor demethyldemethoxyubiquinone ($DDMQH_2$), which is converted to QH_2 in three steps by Coq5, Coq7, and Coq3, respectively. *B,* RNAi strains of *coq-3*, *coq-5*, and *coq-6 C. elegans* show significant reduction of both RQ and Q, as compared with the EV and *unc-22* controls (Table S1). RNAi of *coq-7* significantly reduces Q levels, but RQ biosynthesis is unaffected (Table S1).

gene has been discovered. By analogy to the biosynthetic pathway of Q in yeast from pABA (22), we reasoned that the 2-amino substituent of RQ could be derived from an arylamine precursor. While this manuscript was in preparation, a different group independently reported the essential role of KYNU-1 for RQ biosynthesis, using the *kynu-1* strain CB1003 (25). Our study was performed with the *kynu-1* strain Tm4924. The genetic rescue of Tm4924 and RNAi experiments that we performed confirmed this finding. These results indicate that AA and/or 3HAA are RQ precursors. Consistent with this view, the strain used in this study has been previously reported to show increased levels of KYN and 3HKYN (21). Interestingly, supplementation with AA or 3HAA did not rescue RQ biosynthesis, suggesting the absence of transporters for uptake of these metabolites. The *kmo-1* strain showed significantly reduced levels of RQ. Thus, whether AA or 3HAA or both are precursors of RQ is unclear. The *kynu-1*–dependent, *kmo-1*–independent biosynthesis of 3HAA has been postulated in several studies (20, 21), but to the best of our knowledge, no clear evidence regarding this reaction has been reported. In any case, the drastic decrease of RQ biosynthesis in the *kmo-1* strain would suggest that 3HAA is an RQ precursor. The mutant strain in *afmd-1*, upstream of *kynu-1*, did not completely abolish RQ biosynthesis. This result would be explained if the *afmd-1* strain is not a null-mutant or if KYN can be acquired from *E. coli.*

KYNU-1 expression was mostly restricted to the hypodermis, suggesting that the precursor of RQ is transported to other tissues. Two genes of the kynurenine pathway, *tdo-2* and *kmo-1*, have also been found to be expressed almost exclusively in the worm hypodermis (26). KYN, AA, and 3HAA transport to other worm tissues is likely to be highly relevant because they are also precursors for other key metabolites, such as quinolinic acid and kynurenic acid. Interestingly, enigmatic deposits of fluorescent AA glycosyl esters are found in gut granules in dying worms (20). We found that the expression of *kynu-1* was not up-regulated under hypoxic conditions. KYNU-1 may be constitutively expressed because it is also essential for *de novo* synthesis of NAD^+ (27). An important conclusion of our study is that the kynurenine pathway is a complex metabolic hub, and that AA or 3HAA is a likely branch point for RQ biosynthesis.

Our study reveals that from the kynurenine pathway branch point, Q and RQ biosynthesis in *C. elegans* make use of common enzymes, because *coq-5* and *coq-6* RNAi led to a significant decrease of both quinones. The fact that the enzymes involved in Q biosynthesis do not have strict substrate specificity is highlighted by the parallel pathways of Q biosynthesis in yeast that start from different precursors (23). In addition, neofunctionalization of Coq enzymes has been described for the COQ-5 bacterial ortholog (UbiE/MenG) in the biosynthesis of menaquinone (28). A scheme depicting a possible pathway for RQ biosynthesis in *C. elegans* is shown in Fig. 5, which utilizes COQ-2, COQ-3, COQ-5, and COQ-6. The order of these proposed steps will need to be determined.

Our results highlight the existence of two independent evolutionary pathways for RQ biosynthesis. Interestingly, *R. rubrum*, and protists that synthesize RQ, lack the kynurenine pathway and use Q and RquA for the biosynthesis of RQ. In contrast, KYNU-1 is present in all helminths and in bivalves, suggesting that the kynurenine pathway has been co-opted for RQ biosynthesis. Our findings have practical applications for the identification of potential targets in the RQ biosynthetic pathway for antihelminthic drug development. Parasitic helminth infections have become a global health epidemic, and in the face of emerging drug resistance, new treatments are necessary to combat them (29). A key issue regarding future studies is to understand why mammals, and other animals that possess the kynurenine pathway, do not synthesize RQ. The discovery of key enzymatic steps that discriminate between Q and RQ

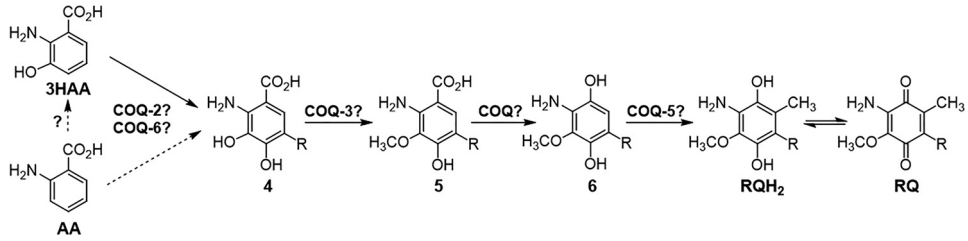

Figure 5. Proposed pathway for RQ biosynthesis in *C. elegans*. Either 3HAA or AA are proposed to be arylamine precursors to RQ. The Q biosynthetic enzymes, COQ-2 and COQ-6, may be used to form the common precursor, 2-amino-3,4-dihydroxy-5-nonaprenylbenzoic acid (compound **4**). *O*-Methylation of compound **4** can be achieved using a *S*-adenosylmethionine–dependent methyltransferase, most likely COQ-3. The resulting compound **5** must be decarboxylated and hydroxylated, respectively, using the COQ enzyme(s), which would be analogous to those used in the Q biosynthetic pathway, to form the final 1,4-hydroquinone precursor to RQH_2 (compound **6**). The COQ-5 *C*-methyltransferase is proposed to catalyze the final methylation step to form RQH_2, which can be oxidized to RQ. In *C. elegans*, *R* represents a tail with nine isoprenoid units ($n = 9$).

precursors will be highly relevant to target a metabolic pathway that is essential for helminth survival within the mammalian host under hypoxic conditions, such as those found in the intestine.

Experimental procedures

C. elegans strains and culture conditions

The *C. elegans* strains used in this study are listed in Table S2. Transgenic lines were obtained according to Ref. 30. The pRF4 plasmid containing the injection marker *rol-6(su1006)* was co-injected with constructs containing *Pkynu-1*::*kynu-1*::*gfp* cloned into the pPD95.77 plasmid and injected into *kynu-1*(*tm4924*) animals. Independent transgenic lines were isolated and observed. The general methods used for culturing and maintenance of *C. elegans* are described in Ref. 31. All chemical reagents were purchased from Sigma. Chemical supplementation was carried out adding 10 mM AA or 10 mM 3HAA to NGM agar plates.

Reporter construct for expression and localization analysis

The expression pattern of *kynu-1* was determined using GFP as a reporter. The translational constructs *Pkynu-1*::*kynu-1*::*gfp* included promoter (1.4 kb), exons, and introns of *kynu-1* in-frame with the *gfp* coding sequence. Sequences were amplified by PCR using appropriate primers (Table S3) from N2 genomic DNA. The PCR products were cloned into the pPD95.77 vector that provides the *unc-54* 3′UTR. For the study of the *kynu-1* expression pattern under hypoxic conditions, adult worms of the transgenic lines expressing the construct *Pkynu-1*::*kynu-1*::*gfp* were grown at 0.42% oxygen, 20 °C during 20 h in a C-Chamber incubator with a ProOx 110 oxymeter (Biospherix, Parish, NY). Worms were immediately mounted for visualization under the microscope. Animals were visualized under a confocal microscope Zeiss LSM 880 and images captured with the Zen black 2.3 software and processed with Fiji (32). Embryos were obtained by a transverse cut in a gravid adult (early stages) or picked directly from the plate (late embryonic stages).

RNAi assay

The expressions of the *C. elegans kynu-1*, *coq-3*, *coq-5*, *coq-6,* and *coq-7 (clk-1)* genes were interfered with by *E. coli* strain HT115 containing the plasmid pL4440 encoding the gene of interest (Table S4). Plasmids without an insert DNA (EV) or encoding *unc-22* were used as controls. *E. coli* strains were grown overnight at 37 °C in LB plus ampicillin (50 μg/ml) and carbenicillin (30 μg/ml), followed by a 2-h outgrowth to obtain a cell density of 0.4–0.6 OD_{600} units. Each strain was seeded onto 20 NGM agar plates (150 μl per plate) plus ampicillin, carbenicillin, and 1 mM isopropyl 1-thio-β-D-galactopyranoside (to induce expression of dsRNA) and incubated for 48 h at 37 °C. RNAi was carried out by plating *C. elegans rrf-3*(*pk1426*), which were age-synchronized to the L1 stage, onto the seeded *E. coli* plates at 22 °C and grown for 7 days. Worms were washed from plates with M9 buffer, divided into aliquots for pelleting, and frozen at −80 °C until use.

Lipid extraction

For lipid extractions of *C. elegans* N2 or mutant strains, 4,000 synchronized L1s were grown on NGM plates at 20 °C to adulthood. For each experiment, ~10,000 adult worms were harvested and washed several times with 18 megohm water to obtain pellets for extraction (~100 mg). Lipid extraction of *rrf-3* strains from RNAi assays was also performed using pellets containing ~100 mg of worms, prepared from feeding plates as described above. Prior to extraction, 1000 pmol of Q_3 internal standards was added to pellets, and then lipids were extracted using hexanes and ethanol as described previously (12).

RNA isolation and RT-quantitative PCR

RNA was extracted from ~100-mg worm pellets using TRIzol reagent and the Zymo Quick-RNA MiniPrep kit and further purified using the Zymo RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). cDNA was prepared using the high-capacity RNA to cDNA kit (Applied Biosystems, Waltham, MA), and TaqMan gene expression assays (Table S4) were optimized and performed for each RNAi strain as described previously (10) using the endogenous control assay, *cdc-42* (Ce02435138_g1).

LC-MS quantitation

LC-MS samples were prepared as described in Ref. 12. Standards were prepared and extracted at the following concentrations: Q_3 (10 pmol/10 μl injection) and RQ_9 (0.75, 1.5, 3.0, 4.5, or 6.0 pmol/10 μl injection). The RQ_9 standard was isolated from *Ascaris suum* lipid extracts at Gonzaga University. In the absence of a standard, the quantity of Q_9 was determined using a picomole conversion from the RQ_9 standard curve and applying a RQ/Q response correction factor of 2.45 determined from RQ_{10}/Q_{10} and

RQ_8/Q_8 standard curves (12). Additional quinone-specific parameters are listed in Table S5. Samples were analyzed in triplicate and the picomole of quinone was determined from the standard curve and corrected for recovery of internal standard. Samples were normalized by milligrams of pellet mass.

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