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Evidence that Ubiquinone Is a Required Intermediate for Rhodoquinone Biosynthesis in *Rhodospirillum rubrum*▽

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Rhodoquinone (RQ) is an important cofactor used in the anaerobic energy metabolism of *Rhodospirillum rubrum*. RQ is structurally similar to ubiquinone (coenzyme Q or Q), a polyprenylated benzoquinone used in the aerobic respiratory chain. RQ is also found in several eukaryotic species that utilize a fumarate reductase pathway for anaerobic respiration, an important example being the parasitic helminths. RQ is not found in humans or other mammals, and therefore inhibition of its biosynthesis may provide a parasite-specific drug target. In this report, we describe several *in vivo* feeding experiments with *R. rubrum* used for the identification of RQ biosynthetic intermediates. Cultures of *R. rubrum* were grown in the presence of synthetic analogs of ubiquinone and the known Q biosynthetic precursors demethylubiquinone, demethoxyubiquinone, and demethyldemethoxyubiquinone, and assays were monitored for the formation of RQ_3. Data from time course experiments and *S*-adenosyl-L-methionine-dependent *O*-methyltransferase inhibition studies are discussed. Based on the results presented, we have demonstrated that Q is a required intermediate for the biosynthesis of RQ in *R. rubrum*.

Rhodospirillum rubrum is a well-characterized and metabolically diverse member of the family of purple nonsulfur bacteria (29, 61). *R. rubrum* is typically found in aquatic environments and can adapt to a variety of growth conditions by using photosynthesis, respiration, or fermentation pathways (28, 70). In the light, *R. rubrum* exhibits photoheterotrophic growth using organic substrates or photoautotrophic growth using CO_2 and H_2 (15, 70). In the dark, *R. rubrum* can utilize either aerobic respiration (70, 73) or anaerobic respiration with a fumarate reduction pathway or with nonfermentable substrates in the presence of oxidants such as dimethyl sulfoxide (DMSO) or trimethylamine oxide (15, 58, 73). *R. rubrum* can also grow anaerobically in the dark by fermentation of sugars in the presence of bicarbonate (58). The focus of this work was the biosynthesis of quinones used by *R. rubrum* for aerobic and anaerobic respiration.

Rhodoquinone (RQ; compound 1 in Fig. 1) is an aminoquinone structurally similar to ubiquinone (coenzyme Q or Q [compound 2]) (44); however, the two differ considerably in redox potential (that of RQ is −63 mV, and that of Q is +100 mV) (2). Both RQ and Q have a fully substituted benzoquinone ring and a polyisoprenoid side chain that varies in length (depending on the species; see Fig. 1 for examples). The only difference between the structures is that RQ has an amino substituent (NH_2) instead of a methoxy substituent (OCH_3) on the quinone ring. While Q is a ubiquitous lipid component involved in aerobic respiratory electron transport (9, 36, 60), RQ functions in anaerobic respiration in *R. rubrum* (19) and in several other phototrophic purple bacteria (21, 22, 41) and is also present in a few aerobic chemotrophic bacteria, including *Brachymonas denitrificans* and *Zoogloea ramigera* (23). In these varied species of bacteria, RQ has been proposed to function in fumarate reduction to maintain NAD^+/NADH redox balance, either during photosynthetic anaerobic metabolism (12, 15–18, 64) or in chemotrophic metabolism when the availability of oxygen as a terminal oxidant is limiting (23). Another recent finding is that RQH_2 is capable of inducing Q-cycle bypass reactions in the cytochrome bc_1 complex in *Saccharomyces cerevisiae*, resulting in superoxide formation (7). If RQ/RQH_2 coexists in the cytoplasmic membrane with Q/QH_2 in *R. rubrum*, it might serve as both a substrate for and an inhibitor of the bc_1 complex (47).

RQ is also found in the mitochondrial membrane of eukaryotic species capable of fumarate reduction, such as the flagellate *Euglena gracilis* (25, 53), the free-living nematode *Caenorhabditis elegans* (62), and the parasitic helminths (65, 66, 68, 72). Similar to *R. rubrum*, these species can adapt their metabolism to both aerobic and anaerobic conditions throughout

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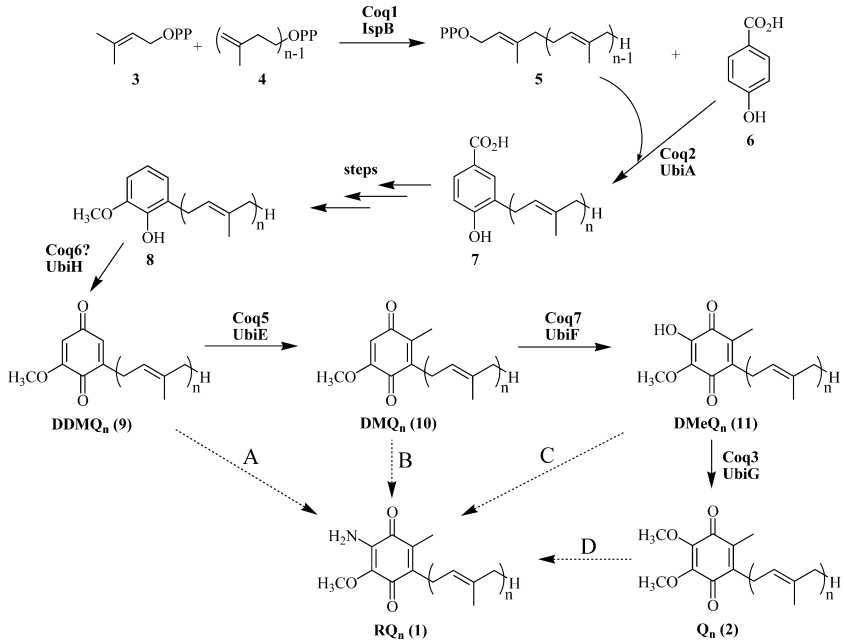

FIG. 1. Proposed pathways for RQ biosynthesis. The number of isoprene units (*n*) varies by species (in *S. cerevisiae*, $n = 6$; in *E. coli*, $n = 8$; in *C. elegans*, $n = 9$; in helminth parasites, $n = 9$ or 10; in *R. rubrum*, $n = 10$; in humans, $n = 10$). RQ is not found in *S. cerevisiae*, *E. coli*, or humans. Known Coq (from *S. cerevisiae*) and Ubi (from *E. coli*) gene products required for the biosynthesis of ubiquinone (Q, compound 2) are labeled. A polyisoprenyl diphosphate (compound 5) is assembled from dimethylallyl disphosphate (compound 3) and isopentyl diphosphate (compound 4). Coupling of compound 5 with *p*-hydroxybenzoic acid (compound 6) yields 3-polyprenyl-4-hydroxybenzoic acid (compound 7). The next three steps differ between *S. cerevisiae* and *E. coli*. However, they merge at the common intermediate (compound 8), which is oxidized to demethyldemethoxyubiquinone ($DDMQ_n$, compound 9). RQ (compound 1) has been proposed to arise from compound 9, demethoxyubiquinone (DMQ_n; compound 10), demethylubiquinone ($DMeQ_n$; compound 11), or compound 2 (by pathway A, B, C, or D). Results presented in this work support pathway D as the favored route for RQ biosynthesis in *R. rubrum*.

their life cycle. For example, most adult parasitic species (e.g., *Ascaris suum*, *Fasciola hepatica*, and *Haemonchus contortus*) rely heavily on fumarate reduction for their energy generation while inside a host organism, where the oxygen tension is very low (30, 65, 72). Under these conditions, the biosynthesis of RQ is upregulated; however, during free-living stages of their life cycle, the helminth parasites use primarily aerobic respiration, which requires Q (30, 65, 72). The anaerobic energy metabolism of the helminthes has been reviewed (63, 67). Humans and other mammalian hosts use Q for aerobic energy metabolism but do not produce or require RQ; therefore, selective inhibition of RQ biosynthesis may lead to highly specific antihelminthic drugs that do not have a toxic effect on the host (35, 48).

R. rubrum is an excellent facultative model system for the study of RQ biosynthesis. The complete genome of *R. rubrum* has recently been sequenced by the Department of Energy Joint Genome Institute, finished by the Los Alamos Finishing Group, and further validated by optical mapping (57). The 16S rRNA sequence of *R. rubrum* is highly homologous to cognate eukaryotic mitochondrial sequences (46). Due to the similarities in structure, the biosynthetic pathways of RQ and Q have been proposed to diverge from a common precursor (67). Proposed pathways for RQ biosynthesis (A to D), in conjunction with the known steps in Q biosynthesis, are outlined in Fig. 1 (31, 34, 60). Parson and Rudney previously showed that when *R. rubrum* was grown anaerobically in the light in the presence of [U-^{14}C]*p*-hydroxybenzoate, ^{14}C was incorporated into both Q_{10} and RQ_{10} (50). In their growth experiments, the specific activity of Q_{10} was measured at its maximal value 15 h after inoculation and then began to decrease. However, the specific activity of RQ_{10} continued to increase for 40 h before declining. These results suggested that Q_{10} was a biosynthetic precursor of RQ_{10}, although this was not directly demonstrated using radiolabeled Q_{10}; hence, the possibility remained that the labeled RQ_{10} was derived from another radiolabeled lipid species. We have done this feeding experiment with a synthetic analog of Q where $n = 3$ (Q_3) and monitored for the production of RQ_3. The synthesis and use of farnesylated quinone and aromatic intermediates for characterization of the Q biosynthetic pathway in *S. cerevisiae* and *Escherichia coli* has been well documented (4, 5, 38, 52, 59). The other proposed precursors of RQ shown in Fig. 1 were also fed to *R. rubrum*, and the lipid extracts from these assays were analyzed for the presence of RQ_3, i.e., demethyldemethoxyubiquinone-3 ($DDMQ_3$; compound 9), demethoxyubiquinone-3 (DMQ_3; compound 10), and demethylubiquinone-3 ($DMeQ_3$; compound 11).

In *S. cerevisiae* and *E. coli*, the last O-methylation step in Q biosynthesis is catalyzed by the *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases Coq3 and UbiG, respectively (26, 52); this final methylation step converts DMeQ to Q. Using the NCBI Basic Local Alignment Search Tool, an *O*-methyltransferase (GeneID no. 3834724 Rru_A0742) that had 41% and 59% sequence identity with Coq3 and UbiG, respectively, was identified in *R. rubrum*. *S*-Adenosyl-L-homocysteine (SAH) is a well-known inhibitor of SAM-dependent methyl-

transferases (13, 24). Because SAH is the transmethylation by-product of SAM-dependent methyltransferases, it is not readily taken up by cells and must be generated *in vivo* (24). SAH can be produced *in vivo* from *S*-adenosine and L-homocysteine thiolactone by endogenous SAH hydrolase (SAHH) (37, 71). A search of the *R. rubrum* genome also confirmed the presence of a gene encoding SAHH (GeneID no. 3836896 Rru_A3444). It was proposed that if DMeQ is the immediate precursor of RQ, then SAH inhibition of the methyltransferase required for Q biosynthesis should have little effect on RQ production. Conversely, if Q is required for RQ synthesis, then inhibition of Q biosynthesis should have a significant effect on RQ production. Assays were designed to quantify the levels of RQ_3 produced from $DMeQ_3$ and Q_3 in *R. rubrum* cultures at various concentrations of SAH.

MATERIALS AND METHODS

Bacterial growth. Wild-type *R. rubrum* (ATCC 11170) was cultivated under lighted anaerobic conditions at 32°C in an Innova 4430 incubator shaker (New Brunswick Scientific, Edison, NJ) equipped with a full-spectrum fluorescence lamp with a light output of 365 lx (Verilux Full Spectrum F20T12VLX; Veriflux, Inc., Waitsfield, VT). Cultures were grown in screw-cap medium bottles (600 ml) filled to capacity for 4 to 6 days. The medium recipe was adapted from reference 49 and contained NH_4Cl (37 mM), DL-malic acid (30 mM), yeast extract (2 g/liter; total nitrogen, 9.8%; amino nitrogen, 5.1%, amino N/total N ratio, 0.52; sodium chloride, 0.3%; Research Products International Corp.), morpholinepropanesulfonic acid buffer (0.5 g/liter), $MgSO_4$ (1.4 mM), $CaCl_2$ (0.7 mM), H_3BO_3 (0.5 mM), $Na_2EDTA \cdot 2H_2O$ (54 μM), biotin (1 μg/liter), and the trace elements $FeSO_4 \cdot 7H_2O$ (16 μM), $Na_2MoO_4 \cdot 2H_2O$ (4.5 μM), $MnCl_2$ (1.5 μM), $ZnSO_4 \cdot 7H_2O$ (0.5 μM), $NiSO_4 \cdot 6H_2O$ (0.1 μM), and $CoCl_2 \cdot 6H_2O$ (0.1 μM) solubilized with HCl (60 μM). The final pH was adjusted to 7 with NaOH, and prior to inoculation, KH_2PO_4 buffer (pH 7, 0.15 mM) was added. All of the values in parentheses are final concentrations.

All *in vivo* assays were prepared in 60-ml screw-cap glass centrifuge tubes in a Herasafe biological safety cabinet (Thermo Fisher Scientific, Waltham, MA) under aerobic conditions. Upon the addition of substrate and other assay components, tubes were immediately filled to capacity (with culture or medium) and sealed. Separate experiments using a Traceable Digital Oxygen Meter (accurate to ±0.4 mg/liter; Control Company, Friendswood, TX) showed that approximately 1.5 h was required for cultures of the same optical density (OD) to become anaerobic (<0.2 mg/liter oxygen) after sealing.

Time course assays. To quantify the biosynthesis of RQ_3 over time, *R. rubrum* assays were prepared in duplicate using the substrates $DMeQ_3$ and Q_3, which were synthesized as previously reported (38, 52). Stock solutions of each substrate were prepared in absolute ethanol (Pharmco-Aaper, Brookfield, CT), and concentrations were determined using a UV/Vis spectrophotometer (Agilent 8453; Agilent Technologies, Foster City, CA) at 275 nm with molar extinction coefficients of 15,900 and 15,100 M^{-1} cm^{-1}, respectively (36). Each substrate was added at a 1 μM final concentration to separate 650-ml *R. rubrum* cultures (each with a starting OD at 660 nm [OD_{660}] of 1.7). Each 650-ml culture was divided into 10 60-ml tubes and filled to capacity (final volume per tube, 63 ml). The tubes were incubated at 32°C under tungsten illumination and harvested at 0, 4, 8, 12, and 24 h.

Control assay mixtures were prepared as described above, with RQ_3 (1 μM) as the substrate, and incubated for 0, 4, 8, 12, and 24 h. RQ_3 was synthesized as previously reported (7), and concentrations were determined in ethanol using a molar extinction coefficient of 10,300 M^{-1} cm^{-1} (36). Additional control assay mixtures containing a 1 μM final concentration of $DMeQ_3$, Q_3, or RQ_3 were prepared with dead *R. rubrum* cells or no cells and incubated in the light at 32°C for 0 or 24 h. Dead cells were produced by heating for 50 min at 131°C in an autoclave (Tomy ES-315; Alfa Medical, Westbury, NY). Assay mixtures containing no substrate were also prepared using live *R. rubrum* culture and dead cells and incubated in the light at 32°C for 0 or 24 h.

SAH inhibition assays. To inhibit the methyltransferase involved in Q_3 biosynthesis from $DMeQ_3$, *R. rubrum* assay mixtures were prepared in duplicate using 60-ml screw-cap glass centrifuge tubes filled with 50 ml of bacterial culture (OD_{660} of 2.2). SAH was generated *in vivo* as follows. *S*-Adenosine (≥99% pure; Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO (99.9% pure; molecular biology grade; Sigma-Aldrich, St. Louis, MO) at concentrations of 0.5, 0.1, and 0.02 M, and 0.6 ml of each was added to assay mixtures to give final concentrations of 5, 1, and 0.2 mM; the same volume of DMSO (0.6 ml) was added to the 0 mM SAH controls. DL-Homocysteine thiolactone hydrochloride (99% pure; Sigma-Aldrich, St. Louis, MO) was dissolved in water at 1.0, 0.2, and 0.04 M concentrations, and 0.6 ml of each was added to assay mixtures to give final concentrations of L-homocysteine thiolactone of 5, 1, and 0.2 mM. Prior to the addition of substrate, tubes were sealed and preincubated under lighted conditions for 10 min at 32°C to allow the generation of SAH. Following preincubation, an ethanolic solution of each substrate was added to give a final concentration of 1 μM $DMeQ_3$ or 1 μM Q_3; the same net volume of ethanol (125 μl) was added to all assay mixtures. Tubes were then filled to capacity with additional medium to ensure anaerobic growth. Controls were also prepared without $DMeQ_3$ and Q_3 at final SAH concentrations of 5 and 0 mM. All assay mixtures were incubated under lighted conditions at 32°C for 4 h.

Lipid extraction of cells. Lipid extractions were performed on all *R. rubrum* assay mixtures (excluding the no-cell assay mixtures) to isolate the quinones for analysis. Once the assay mixture incubation period was complete, cultures were transferred to separate 250-ml plastic centrifuge bottles. Cells were harvested by centrifugation at 2,000 × *g* for 30 min at 4°C (Avanti J-E high-performance centrifuge with a JA-10 rotor; Beckman-Coulter, Fullerton, CA). Cell pellets were resuspended in 5 ml of deionized water, transferred to 10-ml glass centrifuge tubes, and collected by centrifugation at 2,000 × *g* for 30 min at 4°C (Dupont Sorvall RT 600B tabletop centrifuge with a Sorvall H100B rotor; Block Scientific, Bohemia, NY). After pouring off the supernatant, the tubes were flushed briefly with N_2 gas and sealed. The masses of the wet pellets were obtained before they were stored at −85°C. Prior to extraction, the pellets were thawed and Q_2 (0.5 pmol/10 μl injection volume; Sigma Aldrich, St. Louis, MO) was added as an internal standard. The pellets were extracted with 2 ml methanol (Optima grade; Fisher Scientific, Pittsburgh, PA)–2 ml petroleum ether (J. T. Baxter, Phillipsburg, NJ) containing 10 μM 2,6-di-*tert*-butyl-4-methylphenol (BHT; Sigma-Aldrich, St. Louis, MO) and 110 μl deionized water. After thorough vortex mixing, the suspensions were separated into layers by centrifugation at 1,000 × *g* for 5 min at 4°C. The etherial layers were transferred to 5-ml glass centrifuge tubes, and the methanol layers were reextracted with another 2 ml of petroleum ether solution (containing 10 μM BHT). To prevent decomposition, sample tubes were stored on ice during the extraction procedure and kept away from direct light. The combined etherial layers were dried under a steady stream of N_2 gas. The resulting dark blue residue from each assay mixture was immediately resuspended using 20 μl of hexanes (Optima grade; Fisher Scientific, Pittsburgh, PA) and 80 μl of absolute ethanol for liquid chromatography (LC)-mass spectrometry (MS) analysis.

Extraction of no-cell assay mixtures. Due to the absence of a pellet, extractions were performed immediately following incubation using a 125-ml separatory funnel. The assay medium was transferred to the separatory funnel with 10 ml brine and 18 ml petroleum ether (containing 1 μM BHT). The ether layer was transferred to a 100-ml round-bottom flask and concentrated *in vacuo* using a rotary evaporation (Büchi Rotovapor R-205; Büchi Labortechnik, Flawil, Switzerland). A second extraction was performed using 18 ml of petroleum ether solution. The second ether layer was added to the residue in the 100-ml flask and concentrated as before. The combined residue was then resuspended in 4 ml of petroleum ether solution, transferred to a 5-ml glass centrifuge tube, and dried under N_2 gas. The extracts were stored at −85°C and eventually resuspended in 20 μl hexanes and 80 μl ethanol for LC-MS analysis as in the other extractions.

Preparation of standards. LC-MS standards were prepared containing Q_3 (0.05, 0.25, 1, 2.5, 5, or 10 pmol/10-μl injection), RQ_3 (0.015, 0.075, 0.15, 0.75, 1.5, or 3 pmol/10-μl injection), and Q_2 (0.5 pmol/10-μl injection) in absolute ethanol. Lipid extractions were performed on the standards following the same procedure used for the cell pellets.

LC-MS quantitation of quinones. LC-MS analysis was performed at the UCLA Molecular Instrumentation Center in Los Angeles, CA. Authentic standards were prepared in the high-performance liquid chromatography (HPLC) running buffer at 1 to 2 pmol/μl and infused to determine optimum conditions for quantifying transition ions by multiple-reaction monitoring (MRM). For Fig. 2, the compounds were infused at 2 pmol/μl in 80:20, ethanol-hexanes (Optima grade; Fisher Scientific, Pittsburgh, PA) under the conditions described below, except that the collision energy was increased to produce the spectra as shown. The collision energy ranged from 35 to 55 V. Quinone separation was accomplished with an HPLC system (Agilent 1200 Binary Pump SL; Agilent Technologies, Foster City, CA) and an autosampler containing thermostat-controlled tray holders and sample stack (PAL system; LEAP Technologies, Carrboro, NC), which was maintained at 4°C. Chromatography was performed using a pentafluorophenyl propyl column [Luna PFP(2), 50 by 2.00 mm, 3 μm, 100 Å; Phenomenex, Torrance, CA]. Farnesylated and geranylated quinones were eluted between

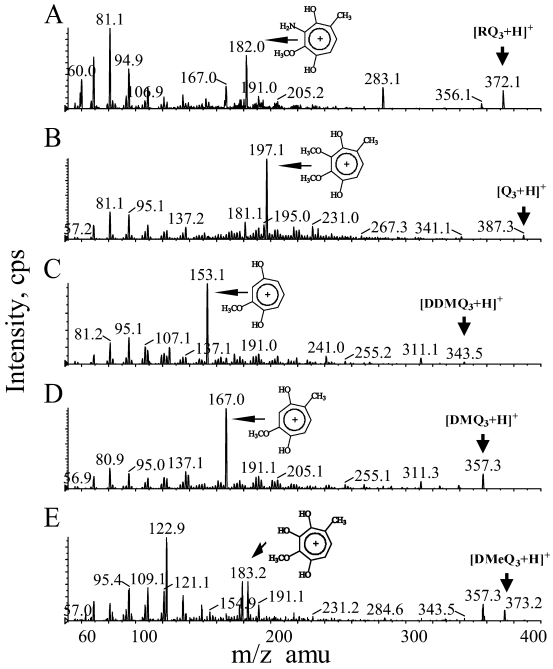

FIG. 2. Mass spectra of farnesylated intermediates. All spectra were obtained using an Applied Biosystems AP 4000 QTrap as specified in Materials and Methods. Intermediates were infused at a concentration of 2 pmol/μl in 80:20 ethanol-hexanes at a flow rate of 20 μl/min. Panel A shows the $[RQ_3 + H]^+$ precursor ion ($C_{23}H_{34}NO_3^+$; exact mass, 372.2) and the RQ tropylium ion ($C_9H_{12}NO_3^+$; exact mass, 182.1). Panel B shows the $[Q_3 + H]^+$ precursor ion ($C_{24}H_{35}O_4^+$; exact mass, 387.2) and the Q tropylium ion ($C_{10}H_{13}O_4^+$; exact mass, 197.1). Panel C shows the $[DDMQ_3 + H]^+$ precursor ion ($C_{22}H_{31}O_3^+$; exact mass, 343.2) and the DDMQ tropylium ion ($C_8H_9O_3^+$; exact mass, 153.1). Panel D shows the $[DMQ_3 + H]^+$ precursor ion ($C_{23}H_{33}O_3^+$; exact mass, 357.2) and the DMQ tropylium ion ($C_9H_{11}O_3^+$; exact mass, 167.2). Panel E shows the $[DMeQ_3 + H]^+$ precursor ion ($C_{23}H_{33}O_4^+$; exact mass, 373.2) and the DMeQ tropylium ion ($C_9H_{11}O_4^+$; exact mass, 183.1; the base peak for this compound is highly collision energy dependent).

1 and 3 min using an isocratic mobile phase of acetonitrile-water (7:3, vol/vol) containing formic acid (0.1%, vol/vol). The acetonitrile used was Optima LC-MS grade (Fisher Scientific, Pittsburgh, PA), the formic acid was >99% pure and packaged in sealed 1-ml ampoules (Thermo-Scientific Pierce Protein Research Products, Rockford, IL), and the water was doubly distilled. All HPLC runs used a flow rate of 0.5 ml/min and an injection volume of 10 μl. All injections were performed in duplicate. Quantitation was accomplished using a triple-quadrupole mass spectrometer with a Turbo electrospray ionization source in positive mode (AP 4000 QTrap; Applied Biosystems, Foster City, CA) with MRM of singly charged ions. Q1 and Q3 were operated at single-unit resolution. Analyst 1.4.1 software was used for data acquisition and processing. Linear slopes were calculated using peak areas with a bunching parameter of 3 and three smoothing functions. Standard error was determined at a 95% confidence interval with a value of $n = 4$. The following global conditions were used for MS/MS analysis of all compounds: dwell time, 100 ms; entrance potential, 10.00 V; curtain gas pressure, 20 lb/in^2; nebulizer gas pressure, 50 lb/in^2; turbo gas pressure, 60 lb/in^2; collision gas, medium; nebulizer voltage, 20 V; temperature, 450°C. Nitrogen gas was used for all applications and was obtained from the boiloff from a bulk liquid nitrogen storage tank. Additional quinone-specific parameters are listed in Table 1.

RESULTS

Time course assays. Figure 2 shows the mass spectra of all five of the farnesylated quinone substrates used in these experiments with their major tropylium product ions highlighted.

TABLE 1. LC-MS parameters for each quinone

MS parameter	Q_2	$DMeQ_3$	Q_3	RQ_3
DP[a] (V)	71.00	71.00	76.00	71.00
CE[b] (V)	27.00	23.00	25.00	31.00
CXP[c] (V)	16.00	16.00	16.00	14.00
Precursor mass $[M+H]^+$ (*m/z*)	319.06	373.21	387.19	372.21
Ion product mass $[M]^+$ (*m/z*)	197.10	183.20	197.10	182.00
LC retention time (min)	1.31	1.64	2.72	1.96

[a] DP, declustering potential.
[b] CE, collision energy.
[c] CXP, collision cell exit potential.

All MRM analyses monitored for the mass transition from each quinone precursor ion ($[M+H]^+$) to its respective tropylium product ion.

The $DMeQ_3$ feeding assays revealed that *R. rubrum* is capable of converting $DMeQ_3$ to both Q_3 and RQ_3 *in vivo* (Fig. 3A), with the amount of Q_3 produced being between 100 and 400 times greater than that of RQ_3 from 24 to 4 h, respectively. A small amount of Q_3 (0.07 ± 0.02 pmol/mg wet pellet weight)

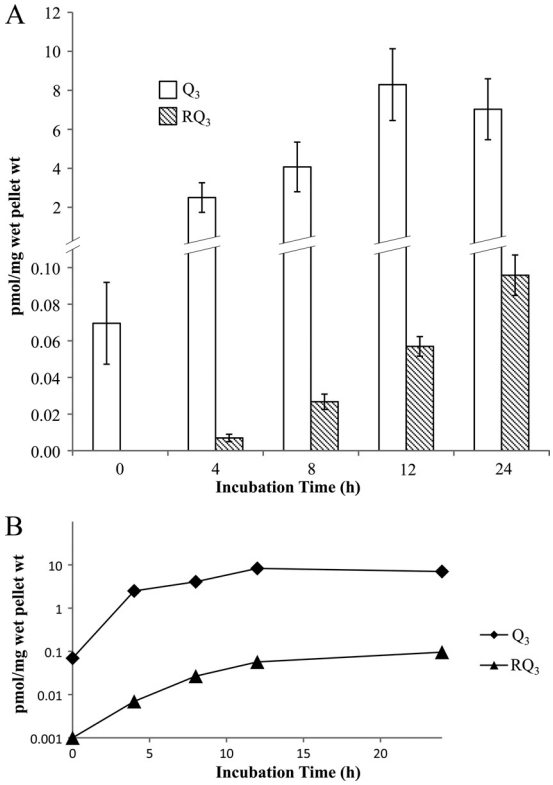

FIG. 3. *R. rubrum* time course assays with $DMeQ_3$. $DMeQ_3$ was added at a 1 μM final concentration to a 650-ml *R. rubrum* culture (with a starting OD_{660} of 1.7). The culture was divided into 10 tubes filled to capacity (63-ml total volume per tube). Tubes were incubated at 32°C under tungsten illumination and harvested at 0, 4, 8, 12, and 24 h. Quinones were extracted and quantified using LC-MS with MRM analysis as described in Materials and Methods. Panel A shows the amounts of Q_3 and RQ_3 (pmol/mg wet pellet weight) produced from $DMeQ_3$ in 24 h. The white bars indicate quantities of Q_3, and the striped bars represent quantities of RQ_3. Panel B shows a logarithmic comparison of the formation of Q_3 and RQ_3 from $DMeQ_3$.

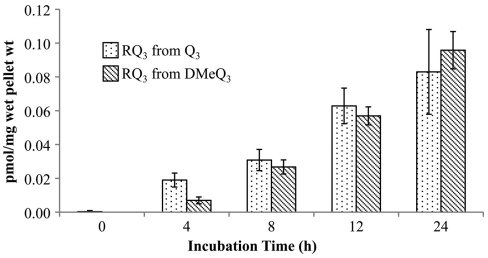

FIG. 4. Comparison of RQ_3 produced from $DMeQ_3$ and that produced from Q_3. $DMeQ_3$ or Q_3 was added at a 1 μM final concentration to *R. rubrum* cultures (with a starting OD_{660} of 1.7) as described in the legend to Fig. 3. The dotted bars correspond to RQ_3 from Q_3, and the striped bars indicate amounts of RQ_3 produced from $DMeQ_3$ (in pmol/mg pellet wet weight).

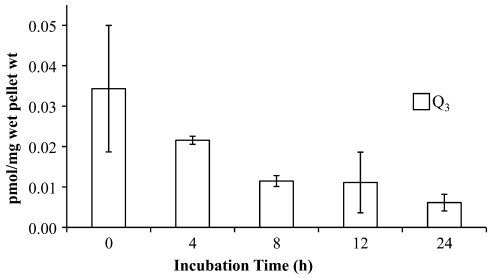

FIG. 5. Q_3 produced from RQ_3 over time. RQ_3 was added at a 1 μM final concentration to *R. rubrum* cultures (with a starting OD_{660} of 1.3) as described in the legend to Fig. 3. Q_3 production (in pmol/mg pellet wet weight) is shown as white bars.

was already formed from $DMeQ_3$ at the 0-h time point, since about 5 min of processing time was required for the transfer of cultures to centrifuge bottles; however, no RQ_3 was detected. RQ_3 was first detected after 4 h, when the amount of Q_3 present had increased by about 36 times (2.5 ± 0.8 pmol/mg wet pellet weight). The amount of Q_3 formed from $DMeQ_3$ reached a maximal level after 12 h (8.3 ± 1.8 pmol/mg wet pellet weight), while the amount of RQ_3 continued to increase throughout the 24-h growth period, reaching a maximum of 0.10 ± 0.01 pmol/mg wet pellet weight. Figure 3B shows a logarithmic comparison of the accumulation of Q_3 versus RQ_3 produced from $DMeQ_3$ in 24 h.

Results from the Q_3 feeding assays also showed the formation of RQ_3 over time. As shown in Fig. 4, there was a very small amount of RQ_3 detected from Q_3 at the 0-h time point (<1 fmol/mg wet pellet weight). The amount of Q_3 recovered at the 0-h time point was about 2 orders of magnitude greater than the amount of Q_3 that had formed from $DMeQ_3$ at the same time point (data not shown). After 4 h, the amount of RQ_3 increased to about twice that produced from the $DMeQ_3$ assay in the same time period. However, by the 12-h time point, the amounts of RQ_3 produced from $DMeQ_3$ and Q_3 were equivalent (within the standard error). The amount of Q_3 recovered from the Q_3 feeding assay after 12 h was roughly equal to the amount of Q_3 formed from the $DMeQ_3$ assay (~10 pmol/mg wet pellet weight; data not shown). Other, unknown, species with the same mass transition as $DMeQ_3$ but with longer retention times were also detected in the Q_3 assays, and the concentration of these species appeared to decrease over time (data not shown).

To test the reversibility of the conversions of $DMeQ_3$ and Q_3 to RQ_3, a control experiment was performed with RQ_3 as the substrate, and lipid extracts were analyzed for Q_3 and $DMeQ_3$ after 0, 4, 8, 12, and 24 h. As shown in Fig. 5, a small amount of Q_3 (0.03 ± 0.01 pmol/mg wet pellet weight) was present at the 0-h time point; however, the amount decreased over time. Another unknown species with the same mass transition as Q_3 but with a shorter retention time was also detected (data not shown); however, no $DMeQ_3$ was detected in these assays. Control experiments were also performed under the same growth conditions and with the same concentrations of $DMeQ_3$, Q_3, and RQ_3 with dead *R. rubrum* cells and with growth medium (no cells) to monitor for degradation or nonenzymatic conversion to RQ_3, Q_3, or $DMeQ_3$. Assays were performed for 0 and 24 h, and lipid extracts were analyzed for the presence of farnesylated products. No RQ_3 was detected from $DMeQ_3$ or Q_3 in the control assays (data not shown). However, there was partial degradation of Q_3 to $DMeQ_3$ after exposure to light for 24 h in the no-cell assays; this degradation was not observed in the dead-cell assays (due to the absence of a pellet, the data are not directly comparable to those obtained from assays with live cultures). A small amount of Q_3 was detected from the $DMeQ_3$ and RQ_3 no-cell assays; however, Q_3 was not detected in the dead-cell assays (again, due to the absence of a pellet, data are not directly comparable to those obtained from assays with live cultures). No farnesylated products were observed in *R. rubrum* control assays in the absence of synthetic substrates.

Feeding assays were also performed using a 5 μM starting concentration of each of the proposed precursors $DDMQ_3$ (compound 9) and DMQ_3 (compound 10). No RQ_3 was detected from either assay after 4 days of growth (data not shown). However, a small amount of Q_3 was detected in both assays (~0.02 pmol/mg wet pellet weight), and DMQ_3 was also formed from $DDMQ_3$ (0.2 pmol/mg wet pellet weight). Since the starting concentrations of $DDMQ_3$ and DMQ_3 in these experiments were five times greater than in the reported $DMeQ_3$ and Q_3 assays, the data are not directly comparable.

SAH inhibition assays. The results presented here suggest that *R. rubrum* can synthesize RQ_3 when provided either Q_3 or $DMeQ_3$. To investigate whether RQ_3 could be produced directly from $DMeQ_3$ in the absence of Q_3, *S*-adenosine and L-homocysteine thiolactone were added to incubation mixtures in order to generate SAH, a competitive inhibitor of the *O*-methyltransferase-mediated conversion of $DMeQ_3$ to Q_3 (Fig. 1). Experiments were first performed to optimize the range of concentrations of SAH necessary for partial and complete inhibition of the O-methylation reaction of $DMeQ_3$ to form Q_3 (0.2 to 5 mM SAH). The average yield of Q_3 from $DMeQ_3$ was reduced by 85% with 0.2 mM SAH compared to the control (0 mM SAH), and *O*-methyltransferase inhibition was greater than 99.9% with 1 and 5 mM SAH (Fig. 6). A similar trend was observed with the amount of RQ_3 formed from $DMeQ_3$; the average yield of RQ_3 was reduced by 89% with 0.2 mM SAH, and no RQ_3 was observed with either 1 or 5 mM SAH (Fig. 6).

Since SAH was expected to inhibit other SAM-dependent methyltransferases that may indirectly have an effect on RQ_3 biosynthesis, another control experiment was performed. Q_3 was used as the substrate, and the production of RQ_3 was monitored at the same three concentrations of SAH. Under 0.2

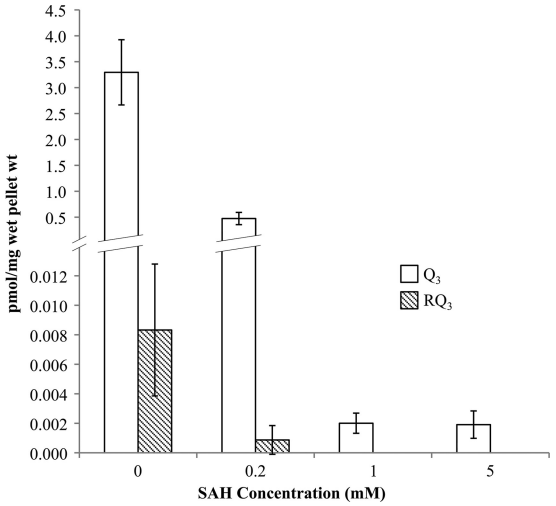

FIG. 6. SAH effect on Q_3 and RQ_3 synthesis from $DMeQ_3$. *R. rubrum* cultures (starting OD_{660} of 2.2) were preincubated with SAH (0, 0.2, 1, or 5 mM) for 10 min prior to the addition of $DMeQ_3$ (1 μM final concentration). SAH was generated *in situ* from *S*-adenosine and DL-homocysteine thiolactone as described in Materials and Methods. Assays were allowed to proceed for 4 h at 32°C under tungsten illumination. The concentrations of Q_3 and RQ_3 were determined from lipid extracts using LC-MS as previously described. Amounts of Q_3 and RQ_3 (in pmol/mg wet pellet weight) produced from $DMeQ_3$ under each assay condition are shown. The white bars indicate quantities of Q_3, and the striped bars represent quantities of RQ_3.

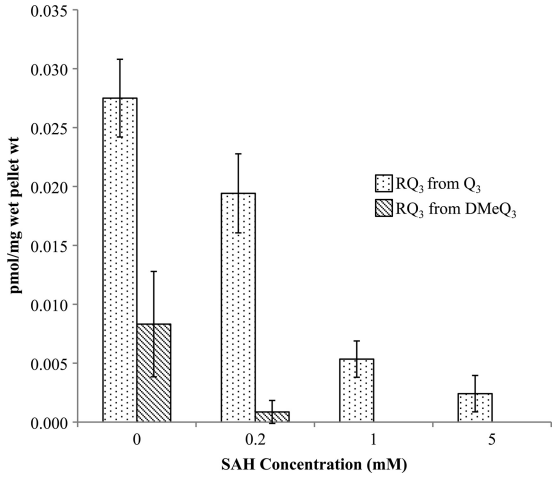

FIG. 7. Comparison of the effects of SAH on RQ_3 synthesis from $DMeQ_3$ versus Q_3. Assays were prepared using the procedure described in the legend to Fig. 6 for the Q_3 control (1 μM final concentration). Shown is a comparison of the amounts of RQ_3 produced from $DMeQ_3$ versus the Q_3 control under each assay condition. The dotted bars correspond to RQ_3 from Q_3, and the striped bars indicate amounts of RQ_3 produced from $DMeQ_3$.

mM SAH inhibition conditions, the amount of RQ_3 produced from Q_3 was reduced by 31% compared to that produced by the 0 mM SAH control. The average yield of RQ_3 was reduced by 81% at 1 mM SAH and by 91% at 5 mM SAH. A comparison of the levels of RQ_3 produced from $DMeQ_3$ versus Q_3 at various concentrations of SAH is shown in Fig. 7. Even though the yields were reduced, RQ_3 was produced under all of the assay conditions with Q_3 as the substrate.

DISCUSSION

The time course studies presented here show that $DMeQ_3$ is a precursor of both Q_3 and RQ_3 in *R. rubrum*. However, formation of RQ_3 was not detected in assays from the other proposed Q biosynthetic intermediates (compounds 9 and 10). The results from the SAH methyltransferase inhibition assays eliminated $DMeQ_3$ as the immediate precursor of RQ_3. These assays indicated that no RQ_3 is produced from $DMeQ_3$ in the absence of Q_3. This observation further supports the results from the time course assay where Q_3 from $DMeQ_3$ was detected prior to that from RQ_3. Our results favor pathway D for the biosynthesis of RQ (Fig. 1); however, this transformation may occur in more than one step.

The synthetic transformation of Q_{10} to RQ_{10} (and iso-RQ_{10}) has been observed by treatment of Q_{10} with NH_4OH in diethyl ether and ethanol (11, 42). It is therefore conceivable that this conjugate addition/elimination reaction could also occur *in vivo*. However, a methoxyquinone is not a typical substrate for transamination by an amido- or aminotransferase; instead, an alcohol or ketone functional group is usually required, respectively (51, 56). It is possible that there is another unstable intermediate formed during the interconversion of Q to RQ. Using the more sensitive technique of LC-MS with MRM analysis, we have observed several unknown farnesylated products that were generated from the Q_3 feeding assays that have the same MRM transition as $DMeQ_3$ (373.2 > 183.2 *m/z*) but with longer retention times than the standard (1.8 to 2.0 min versus 1.64 min). We have yet to fully characterize these compounds, as they degrade rapidly and the sample sizes are too small for other methods of structural elucidation, such as nuclear magnetic resonance analysis. In order for these unknown compounds to possess the same mass transition as $DMeQ_3$, they can differ only in the regiochemistry of substituents or in oxidation state. Photodegradation of Q_{10} to $DMeQ_{10}$ (and iso-$DMeQ_{10}$) has been previously reported (27, 43), and this analogous degradation was observed in our no-cell time course assays with Q_3. In the Q_3 no-cell assays, $DMeQ_3$ was detected by MS (presumably both the 2- and 3-hydroxyquinone regioisomers) with the same retention time as the standard. A small amount of Q_3 was also detected from RQ_3 and $DMeQ_3$ in the no-cell assays. It is unlikely that this photodegradation occurs *in vivo*, as no degradation was observed in the dead-cell assays. In the presence of cells, most light is absorbed by pigments from the light-harvesting antennae of phototrophic bacteria (15, 70).

A possible structure of one of the unknown compounds formed in the Q_3 assay is the unstable orthoquinone of $DMeQ_3$ (*o*-$DMeQ_3$, compound 14), which could participate in a transamination with a PLP-dependent aminotransferase to form RQ_3 (Fig. 8). Interestingly, these unknown compounds are not detected in the $DMeQ_3$ assays, where, for example, a direct tautomerization to the orthoquinone seems more feasible. We are currently investigating the synthesis of the proposed tautomer *o*-$DMeQ_3$ for use as a standard to further characterize these unknown products. A possible method for generation of the orthoquinone *in vivo* is through an O-dem-

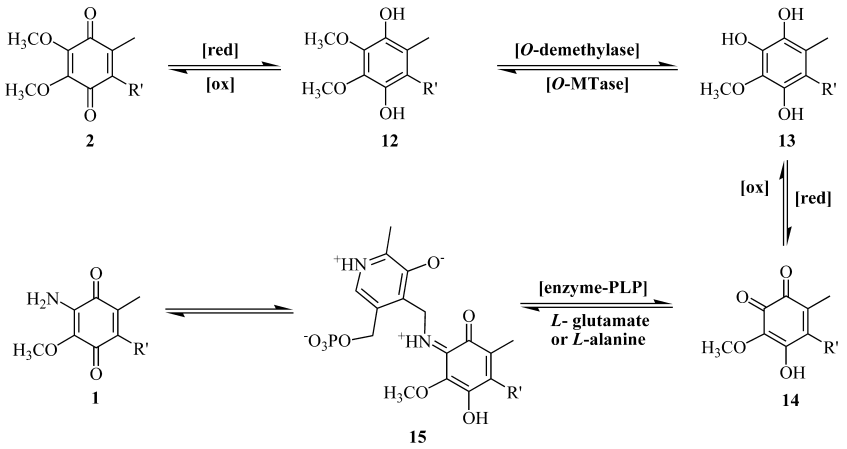

FIG. 8. Possible route for reversible conversion of Q to RQ via a PLP-dependent aminotransferase. Reduction of Q to QH_2 (compound 12) provides an aromatic substrate for an *O*-demethylase. Demethylation of compound 12 to give the catechol intermediate compound 13, followed by oxidation, gives *o*-DMeQ (compound 14). A ketimine intermediate (compound 15) is proposed to form with an amino donor such as glutamate or alanine and compound 14, assisted by a PLP-dependent aminotransferase. Subsequent cleavage of the PLP group would provide RQ (compound 1). All of the steps in this mechanism are proposed to be reversible. R′ denotes the polyprenyl tail group.

ethylation reaction of the Q hydroquinone (QH_2, compound 12), followed by oxidation of the catechol product (compound 13). This is perhaps more plausible than a nonenzymatic degradation process. It has been shown that degradation of Q often involves destruction of the polyprenyl tail (6, 10). In our experiments, we have found that the farnesyl tail remains intact and only selective modifications of the head group are observed. Furthermore, as demonstrated in our feeding control experiments, *R. rubrum* cannot produce farnesylated quinones de novo.

Aromatic *O*-demethylases have been characterized in several species, such as *Pseudomonas maltophilia* (8, 20), *Moorella thermoacetica* (45), and *Sphingomonas paucimobilis* (1). The O-demethylation reactions have been proposed to occur through several different pathways. The dicamba *O*-demethylase from *P. maltophilia* was recently reported to consist of a three-component enzyme complex involving a reductase, a ferredoxin, and an oxygenase (8, 20). The vanillate *O*-demethylase from the anaerobic bacterium *M. thermoacetica* has also been reported to contain three components and is tetrahydrofolate dependent (45), as is the *O*-demethylase of *S. paucimobilis* (1). Other O-demethylation reactions have been reported that involve a cytochrome P450-dependent monooxygenase (69) or peroxidase (39). A blastp screening of the *R. rubrum* genome identified candidates with sequence similarities to the vanillate *O*-demethylase oxidoreductase (YP_425621.1, expect value of 9×10^{-5}) and the vanillate *O*-demethylase oxygenase subunit (YP_426436.1, expect value of 7×10^{-2}). It is possible that an *O*-demethylase required for demethylation of Q is part of an enzyme complex that acts in concert with an aminotransferase, therefore not requiring DMeQ as a direct substrate.

As proposed in Fig. 8, it is conceivable that reversible interconversion between intermediates is possible and could be regulated by environmental conditions (e.g., oxygen availability). It has been shown in the helminth parasites that the amount of RQ present is dependent on whether or not the parasite is living inside the host organism (35, 67). A similar phenomenon is observed with *R. rubrum*. When the bacteria are grown aerobically, the levels of RQ_{10} are substantially lower than the levels of Q_{10}; however, when *R. rubrum* is grown anaerobically, the amount of RQ_{10} surpasses that of Q_{10} (54, 55). To investigate the possible reversibility of the interconversion between Q and RQ, RQ_3 was fed to *R. rubrum* in a control experiment and monitored for the production of Q_3. This experiment did, in fact, show the production of Q_3, as well as a new compound with a slightly shorter retention time but with the same mass transition as Q_3. The level of Q_3 decreased over time, possibly due to the decrease in available oxygen. Assays were prepared under aerobic conditions and required about 1.5 h to become fully anaerobic. It is possible that Q_3 was converted back to RQ_3 due to the reduction in oxygen. Further experiments with controlled oxygen levels must be performed to investigate the dependence of RQ biosynthesis on oxygen availability.

It has been reported by Van Hellemond et al. that *F. hepatica* and *Schistosoma mansoni* synthesize both Q_{10} and RQ_{10} de novo (66, 68). These conclusions are based on the different number of isoprenoid units in the tail between the host's quinone (Q_9) and that of the parasite (Q_{10} and RQ_{10}). If Q is a precursor of RQ, the authors expected that RQ_9 would be detected from catabolism of the host's Q_9, and this was not observed. It was also demonstrated that *F. hepatica* could incorporate [2-^{14}C]mevalonic acid into Q_{10} and RQ_{10}, suggesting that each is synthesized de novo. The results reported by Van Hellemond et al. suggest that Q from the host is not catabolized by the parasites to form RQ; however, these experiments only address the host's Q supply. These results do not eliminate the possibility that the parasites are catabolizing their own supply of Q_{10}. Because the tail lengths are the same, it is not possible to differentiate the two in this experiment. The authors also performed *in vitro* assays with Q_9 but did not detect any RQ_9; however, experimental conditions were not provided. It is possible that small quantities of RQ_9 were not detected due to degradation or lack of sensitivity of the UV analysis method. We observed that low concentrations of RQ_3 (1 to 10 nM) rapidly decomposed in the lipid matrix, and our analysis

proved initially difficult. We were able to stabilize RQ_3 in the lipid extracts using BHT during extraction (~400 μM final concentration in the LC-MS sample). Furthermore, the quantities of RQ_3 generated from our *in vivo* feeding assays were in the low fmol/μl range and also required the sensitivity of a triple-quadrupole mass spectrometer with MRM for detection. We were unable to detect RQ_3 from our *in vivo* assays using UV analysis.

Pathways for RQ biosynthesis may have evolved separately in eukaryotic and prokaryotic species. Evidence to support this hypothesis has come from experiments performed with the *C. elegans clk-1* mutant, which exhibits slow developmental growth and behavior and has an increased life span compared to that of wild-type *C. elegans* (14). The *clk-1* mutants are deficient in Q_9 biosynthesis and require dietary Q from *E. coli*; in fact, the only detectable Q in the *clk-1* mutants is Q_8, the form produced by *E. coli* (32, 33, 40). *E. coli* does not make or require RQ, and no RQ_8 is detected in the *clk-1* mutant from the proposed catabolism of Q_8. However, an interesting finding is that the *clk-1* mutants are still capable of producing RQ_9. In addition, these mutants produce increased levels of RQ_9 compared to those produced by the wild-type species (32, 33). The *clk-1* mutant has been found to accumulate the Q precursor DMQ_9 (compound 10, Fig. 2) in its mitochondria (32, 33). It is possible that an alternative hydroxylase is present in the worm that catalyzes the conversion of DMQ_9 to $DMeQ_9$ (or *o*-$DMeQ_9$) under anaerobic conditions to allow the synthesis of RQ_9. A precedent for bypass hydroxylase mechanisms has been shown in *E. coli* for the biosynthesis of Q under anaerobic conditions using mutants (*ubiH*, *ubiF*, and *ubiB*) blocked in hydroxylation reactions of the aerobic pathway (3). However, it is unlikely that the mutant worms are synthesizing RQ_9 directly from Q_9, since no Q_9 is detected. In contrast, evidence suggesting that RQ biosynthetic pathways have evolved similarly in prokaryotes and eukaryotes has been reported by Powls and Hemming (53). It was shown with the single-celled eukaryote *E. gracilis* that the kinetics of labeling with [U-^{14}C]*p*-hydroxybenzoic acid is consistent with a precursor-product relationship between Q_9 and RQ_9 (53). These results agree with our *R. rubrum* feeding experiments with farnesylated substrates, as well as the radiolabeling experiments performed by Parson and Rudney, where RQ_{10} appeared to be catabolized from Q_{10} (50).

In summary, we have clearly demonstrated that Q is a biosynthetic intermediate of RQ in *R. rubrum*. Results from our *in vivo* feeding experiments render it unlikely that RQ is derived directly from the products ($DDMQ_3$ [compound 9], DMQ_3 [compound 10], and $DMeQ_3$ [compound 11]). Even though RQ was detected in assays from $DMeQ_3$, it was not observed in the absence of Q_3, as demonstrated in the SAH *O*-methyltransferase inhibition assays. Further characterization of pathway D (Fig. 1) is currently under way in our laboratories. Identification of new intermediates, the metabolic source of the amino group in RQ, and the enzyme which catalyzes the amino transfer, is the focus of our research. Complete characterization of the amination step may permit regulation of RQ and would provide a parasite-specific enzyme target for drug development.

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