Evidence that Ubiquinone Is a Required Intermediate for Rhodoquinone Biosynthesis in *Rhodospirillum rubrum*\(^\dagger\)

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*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 (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 activity of oxygen as a terminal oxidant is limiting (23). Another recent finding is that RQH\(_2\) is capable of inducing Q-cycle activity of oxygen as a terminal oxidant is limiting (23).

Based on the results presented, we have demonstrated that RQ is a required intermediate for the biosynthesis of RQ in *R. rubrum.*

*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 their lifecycle.
a polyisoprenyl diphosphate (compound 5) is assembled from dimethylallyl diphosphate (compound 3) and isopentyl diphosphate (compound 4).

Known Coq (from *S. cerevisiae*) and Ubi (from *E. coli*) gene products required for the biosynthesis of ubiquinone (Q, compound 2) are labeled.

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 polyprenyl diphosphate (compound 5) is assembled from dimethylallyl diphosphate (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 (DDMQₙ, compound 9). RQ (compound 1) has been proposed to arise from compound 9, demethoxyubiquinone (DMQₙ; compound 10), demethylubiquinone (DMeQₙ; 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-¹⁴C]-p-hydroxybenzoate, ¹⁴C was incorporated into both Q₁₀ and RQ₁₀ (50). In their growth experiments, the specific activity of Q₁₀ was measured at its maximal value 15 h after inoculation and then began to decrease. However, the specific activity of RQ₁₀ continued to increase for 40 h before declining. These results suggested that Q₁₀ was a biosynthetic precursor of RQ₁₀, although this was not directly demonstrated using radiolabeled Q₁₀; hence, the possibility remained that the labeled RQ₁₀ was derived from another radiolabeled lipid species. We have done this feeding experiment with a synthetic analog of Q where n = 3 (Q₃) and monitored for the production of RQ₃. 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 RQₙ, i.e., demethyldemethoxyubiquinone-3 (DDMQₙ; compound 9), demethoxyubiquinone-3 (DMQₙ; compound 10), and demethylubiquinone-3 (DMeQₙ; compound 11).

In *S. cerevisiae* and *E. coli*, the last O-methylation step in Q biosynthesis is catalyzed by the 5′-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. 384724 Rru_A0742) that had 41% and 59% sequence identity with Coq3 and UbiG, respectively, was identified in *R. rubrum*. 5′-Adenosyl-L-homocysteine (SAH) is a well-known inhibitor of SAM-dependent methyl-
transferrases (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-homo-cysteine 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). Prior to inoculation, KH2PO4 buffer (pH 7, 0.15 mM) was added. All elements FeSO4·7H2O (0.5 mM), Na2EDTA·5H2O (16 mM), MnCl2 (1.5 mM), and CoCl2·6H2O (0.1 mM) were added as an internal standard, and water (37°C) was added to all assay mixtures to give final concentrations of 5, 1, and 0.2 mM; the same volume of DMSO (0.6 mM) was added to the 0 mM SAH controls. rR-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 rR-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 rR-Homocysteine thiolactone of 5, 1, and 0.2 mM. Following preincubation, using a 125-ml separatory funnel, the tubes were flushed briefly with N2 gas and sealed. The mass of the wet pellets were obtained before they were stored at −85°C. Prior to extraction, the pellets were thawed and Q3 (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) 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 petroleum ether (containing 1 μM BHT). The ether layer was transferred to 5-ml glass centrifuge tubes, and the methanol layer was extracted with another 2 ml of petroleum ether and water solutions sequentially (3730 M BHT). To the methanol solution, sample tubes were stored on ice during the extraction procedure and kept away from direct light. The combined etheral layers were dried under a steady stream of N2 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.

Preparation of standards. LC-MS standards were prepared containing Q2 (0.05, 0.25, 1, 2.5, 5, or 10 pmol/10 μl injection), Q3 (0.0015, 0.075, 0.15, 0.75, 1.5, or 3 pmol/10 μl injection), and Q4 (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 a ternary solvent containing ammonium carbonate-deoxycortisol-acetonitrile. The column was maintained at 60°C. Chromatography was performed using a pentadecylsilane propyl column [Luna PFP(2), 5.0 by 2.0 mm, 3 μm, 100 Å; Phenomenex, Torrance, CA]. Farnesylated and geranylated quinones were eluted between 0.02 M and 0.06 M of each was added to assay mixtures to give final concentra-

Bacterial growth. Wild-type R. rubrum (ATCC 11170) was cultivated under lighted anaerobic conditions at 32°C in an Innova 4403 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; Verilux, Inc., Waitsfield, VT). Cultures were grown in screw-capped medium bottles (600 ml) filled to capacity for 4 to 6 days. The medium recipe was adapted from reference (37) and contained NH4Cl (37 mM), tri-malic acid (30 mM), yeast extract (2 g/liter; total nitrogen, 9.8%; amino nitrogen, 5.1%, amino N/total N ratio, 0.52; Sigma-Aldrich, St. Louis, MO); Design and building of the medium to ensure anaerobic growth. Controls were also prepared without DMo3Q and Q3 at final SAH concentrations of 5 and 0 μM. 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 centri-

Materials and methods

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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 was used 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 Student’s t-test.

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

The DMeQ3 feeding assays revealed that _Rhodospirillum rubrum_ is capable of converting DMeQ3 to both Q3 and RQ3 in vivo (Fig. 3A), with the amount of Q3 produced being between 100 and 400 times greater than that of RQ3 from 24 to 4 h, respectively. A small amount of Q3 (0.07 ± 0.02 pmol/mg wet pellet weight) was detected in the control samples at 24 h. The amounts of Q3 and RQ3 (pmol/mg wet pellet weight) produced from DMeQ3 in 24 h. The white bars indicate quantities of Q3, and the striped bars represent quantities of RQ3. Panel B shows a logarithmic plot of Q3 and RQ3 production as a function of incubation time. The following global conditions were used for MS/MS analysis of each quinone precursor ion ([M]+): declustering potential (V) 27.00 23.00 25.00 31.00; collision cell exit potential (V) 71.00 71.00 76.00 71.00; collision energy (V) 16.00 16.00 16.00 14.00; entrance potential (V) 10.00 10.00 10.00 10.00; curtain gas pressure, 20 lb/in2; nebulizer gas pressure, 50 lb/in2; turbo gas pressure, 60 lb/in2; 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.

<table>
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<tr>
<th>MS parameter</th>
<th>Q3</th>
<th>DMeQ3</th>
<th>Q1</th>
<th>RQ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP (V)</td>
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<td>71.00</td>
<td>76.00</td>
<td>71.00</td>
</tr>
<tr>
<td>CE (V)</td>
<td>27.00</td>
<td>23.00</td>
<td>25.00</td>
<td>31.00</td>
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<tr>
<td>CXP (V)</td>
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<td>16.00</td>
<td>16.00</td>
<td>14.00</td>
</tr>
<tr>
<td>Precursor mass [M+H]+ (m/z)</td>
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<td>387.19</td>
<td>372.21</td>
</tr>
<tr>
<td>Ion product mass [M]+ (m/z)</td>
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<td>183.20</td>
<td>197.10</td>
<td>182.00</td>
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<tr>
<td>LC retention time (min)</td>
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<td>1.64</td>
<td>2.72</td>
<td>1.96</td>
</tr>
</tbody>
</table>

* DP, declustering potential.
* CE, collision energy.
* CXP, collision cell exit potential.

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 [RQ3 + H]+ precursor ion (C23H34NO3); exact mass, 343.2) and the DDMQ tropylium ion (C8H9O3; exact mass, 387.2) and the O tropylium ion (C9H11O3; exact mass, 372.2) and the RQ tropylium ion (C9H12NO3; exact mass, 357.2) and the DMQ tropylium ion (C10H13O4; exact mass, 373.2) and the DMeQ tropylium ion (C9H11O4; exact mass, 357.2) and the base peak for this compound is highly collision energy dependent.

FIG. 3. _Rhodospirillum rubrum_ time course assays with DMeQ3. DMeQ3 was added at a 1 μM final concentration to a 650-ml _R. rubrum_ culture (with a starting O660 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 a logarithmic plot of Q3 and RQ3 production as a function of incubation time.
was already formed from DMeQ3 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 RQ3 was detected. RQ3 was first detected after 4 h, when the amount of Q3 present had increased by about 36 times (2.5 ± 0.8 pmol/mg wet pellet weight). The amount of Q3 formed from DMeQ3 reached a maximal level after 12 h (8.3 ± 1.8 pmol/mg wet pellet weight), while the amount of RQ3 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 Q3 versus RQ3 with live cultures. No farnesylated products were observed in the dead-cell assays (again, due to the absence of a pellet, data not shown). Other, unexplained small amounts of RQ3 detected from Q3 at the 0-h time point; however, the amount decreased over time. No Q3 was detected from either assay after 4 days of growth (data not shown). However, a small amount of Q3 was detected in both assays (~0.02 pmol/mg wet pellet weight), and DMeQ3 was also formed from DDMQ3 (0.2 pmol/mg wet pellet weight). Since the starting concentrations of DDMQ3 and DMeQ3 in these experiments were five times greater than in the reported DMeQ3 and Q3 assays, the data are not directly comparable.

SAH inhibition assays. The results presented here suggest that R. rubrum can synthesize Q3 when provided either Q3 or DMeQ3. To investigate whether RQ3 could be produced directly from DMeQ3 in the absence of Q3, 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 DMeQ3 to Q3 (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 DMeQ3 to form Q3 (0.2 to 5 mM SAH). The average yield of Q3 from DMeQ3 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 RQ3 formed from DMeQ3; the average yield of RQ3 was reduced by 89% with 0.2 mM SAH, and no RQ3 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 RQ3 biosynthesis, another control experiment was performed. Q3 was used as the substrate, and the production of RQ3 was monitored at the same three concentrations of SAH. Under 0.2

FIG. 4. Comparison of RQ3 produced from DMeQ3 and that produced from Q3. DMeQ3 or Q3 was added at a 1 μM final concentration to R. rubrum cultures (with a starting OD660 of 1.7) as described in the legend to Fig. 3. The dotted bars correspond to RQ3 from Q3, and the striped bars indicate amounts of RQ3 produced from DMeQ3 (in pmol/mg pellet wet weight).
mM SAH inhibition conditions, the amount of RQ₃ produced from Q₃ was reduced by 31% compared to that produced by the 0 mM SAH control. The average yield of RQ₃ was reduced by 81% at 1 mM SAH and by 91% at 5 mM SAH. A comparison of the levels of RQ₃ produced from DMeQ₃ versus Q₃ at various concentrations of SAH is shown in Fig. 7. Even though the yields were reduced, RQ₃ was produced under all of the assay conditions with Q₃ as the substrate.

**DISCUSSION**

The time course studies presented here show that DMeQ₃ is a precursor of both Q₃ and RQ₃ in *Rhodospirillum rubrum*. However, formation of RQ₃ 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 DMeQ₃ as the immediate precursor of RQ₃. These assays indicated that no RQ₃ is produced from DMeQ₃ in the absence of Q₃. This observation further supports the results from the time course assay where Q₃ from DMeQ₃ was detected prior to that from RQ₃. 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 Q₁₀ to RQ₁₀ (and iso-RQ₁₀) has been observed by treatment of Q₁₀ with NH₄OH 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 Q₃ feeding assays that have the same MRM transition as DMeQ₃ (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 DMeQ₃, they can differ only in the regiochemistry of substituents or in oxidation state. Photodegradation of Q₁₀ to DMeQ₁₀ (and iso-DMeQ₁₀) has been previously reported (27, 43), and this analogous degradation was observed in our no-cell time course assays with Q₃. In the Q₃ no-cell assays, DMeQ₃ was detected by MS (presumably both the 2- and 3-hydroxyquinone regioisomers) with the same retention time as the standard. A small amount of Q₃ was also detected from RQ₃ and DMeQ₃ 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 Q₃ assay is the unstable orthoquinone of DMeQ₃ (o-DMeQ₃, compound 14), which could participate in a transamination with a PLP-dependent aminotransferase to form RQ₃ (Fig. 8). Interestingly, these unknown compounds are not detected in the DMeQ₃ assays, where, for example, a direct tautomerization to the orthoquinone seems more feasible. We are currently investigating the synthesis of the proposed tautomer o-DMeQ₃ 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-
eral species, such as *Pseudomonas maltophilia*, recently reported to consist of a three-component enzyme complex involving a reductase, a ferredoxin, and an oxygenase (8, 20). The vanillate *O*-demethylase of *S. paucimobilis* (1) 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 monoxygenase (69) or peroxidase (39). A blastp screening of the *R. rubrum* genome identified candidates with sequence similarities to the *O*-demethylase oxidoreductase (YP_425621.1, expect value of 7 e-10) and the vanillate *O*-demethylase oxygenase subunit (YP_426436.1, expect value of 7 e-10). 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 RQ10 are substantially lower than the levels of Q10; however, when *R. rubrum* is grown anaerobically, the amount of RQ10 surpasses that of Q10 (54, 55). To investigate the possible reversibility of the interconversion between Q and RQ, RQ3 was fed to *R. rubrum* in a control experiment and monitored for the production of Q3. This experiment did, in fact, show the production of Q3, as well as a new compound with a slightly shorter retention time but with the same mass transition as Q3. The level of Q3 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 Q3 was converted back to RQ3 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 Q10 and RQ10 de novo (66, 68). These conclusions are based on the different number of isoprenoid units in the tail between the host’s quinone (Q10) and that of the parasite (Q10 and RQ10). If Q is a precursor of RQ, the authors expected that RQ would be detected from catabolism of the host’s Q10; however, this was not observed. It was also demonstrated that *F. hepatica* could incorporate [2,14C]mevalonic acid into Q10 and RQ10, 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 Q10. 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 Q9 but did not detect any RQ9; however, experimental conditions were not provided. It is possible that small quantities of RQ9 were not detected due to degradation or lack of sensitivity of the UV analysis method. We observed that low concentrations of RQ3 (1 to 10 nM) rapidly decomposed in the lipid matrix, and our analysis

![FIG. 8. Possible route for reversible conversion of Q to RQ via a PLP-dependent aminotransferase. Reduction of Q to QH2 (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.](http://jb.asm.org/)
proved initially difficult. We were able to stabilize RQ3 in the lipid extracts using BHT during extraction (~400 μM final concentration in the LC-MS sample). Furthermore, the quantities of RQ3 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 RQ3 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 Q8 biosynthesis and require dietary Q from E. coli; in fact, the only detectable Q in the clk-1 mutants is Q6. The form produced by E. coli (32, 33, 40). E. coli does not make or require RQ, and no RQ6 is detected in the clk-1 mutant from the proposed catabolism of Q6. However, an interesting finding is that the clk-1 mutants are still capable of producing RQ6. In addition, these mutants produce increased levels of RQ6 compared to those produced by the wild-type species (32, 33). The clk-1 mutant has been found to accumulate the Q precursor DMO6 (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 DMQ6 to DMeQ6 (or o-DMeQ6) under anaerobic conditions to allow the synthesis of RO6. 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 RQ6 directly from Q8, since no Q6 is detected. In contrast, evidence suggests that RQ6 biosynthetic pathways have evolved similarly in prokaryotes and eukaryotes has been reported by Pows and Hemming (53). It was shown with the single-celled eukaryote E. gracilis that the kinetics of labeling with [U-14C]Cl-hydroxybenzoic acid is consistent with a precursor-product relationship between Q6 and RO6 (53). These results agree with our R. rubrum feeding experiments with farnesylated substrates, as well as the radiolabeling experiments performed by Parsons and Rudney, where RO10 appeared to be catabolized from Q10 (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 (DDMO6 [compound 9], DMQ6 [compound 10], and DMeQ6 [compound 11]). Even though RQ was detected in assays from DMeQ6, it was not observed in the absence of Q6, 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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REFERENCES


of rhodiquinone and ubiquinone in the activity preparations of chromato-


