

Yeast and Rat Coq3 and *Escherichia coli* UbiG Polypeptides Catalyze Both O-Methyltransferase Steps in Coenzyme Q Biosynthesis*

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Ubiquinone (coenzyme Q or Q) is a lipid that functions in the electron transport chain in the inner mitochondrial membrane of eukaryotes and the plasma membrane of prokaryotes. Q-deficient mutants of *Saccharomyces cerevisiae* harbor defects in one of eight *COQ* genes (*coq1–coq8*) and are unable to grow on nonfermentable carbon sources. The biosynthesis of Q involves two separate O-methylation steps. In yeast, the first O-methylation utilizes 3,4-dihydroxy-5-hexaprenylbenzoic acid as a substrate and is thought to be catalyzed by Coq3p, a 32.7-kDa protein that is 40% identical to the *Escherichia coli* O-methyltransferase, UbiG. In this study, farnesylated analogs corresponding to the second O-methylation step, demethyl-Q₃ and Q₃, have been chemically synthesized and used to study Q biosynthesis in yeast mitochondria *in vitro*. Both yeast and rat Coq3p recognize the demethyl-Q₃ precursor as a substrate. In addition, *E. coli* UbiGp was purified and found to catalyze both O-methylation steps. Furthermore, antibodies to yeast Coq3p were used to determine that the Coq3 polypeptide is peripherally associated with the matrix-side of the inner membrane of yeast mitochondria. The results indicate that one O-methyltransferase catalyzes both steps in Q biosynthesis in eukaryotes and prokaryotes and that Q biosynthesis is carried out within the matrix compartment of yeast mitochondria.

the isoprenoid tail functions to anchor Q in the membrane. In eukaryotes, Q functions to shuttle electrons from either Complex I or Complex II to Complex III/bc₁ complex. The transfer of electrons from Q to the bc₁ complex is coupled to proton-translocation via the Q cycle mechanism that was first proposed by Mitchell (2). A number of studies support such a mechanism (for a review, see Ref. 1) including the recently determined complete structure of the bc₁ complex (3).

The redox properties of Q also allow it to function as a lipid soluble antioxidant. Q functions by either directly scavenging lipid peroxyl radicals (4) or indirectly reducing α -tocopherol radicals to regenerate α -tocopherol (5, 6). Additionally, Q protects cells from oxidative damage generated by the autoxidation of polyunsaturated fatty acids (7). Q is found in many eukaryotic intracellular membranes, including the plasma membrane, where, in conjunction with a plasma membrane electron transport system, it functions to scavenge ascorbate free radicals (8, 9). In the plasma membrane of prokaryotes, Q participates in the maintenance of the enzymatic activity of DsbA/DsbB disulfide bond forming proteins (10), and Q-deficient *Escherichia coli* strains are hypersensitive to thiol exposure (11).

In both eukaryotes and prokaryotes, the first committed step in the biosynthesis of Q begins with the precursors *p*-hydroxybenzoic acid (pHB) and isoprenoid diphosphate, in which the isoprenoid is covalently attached to the aromatic ring. The pathway derives from the characterization of accumulating Q biosynthetic intermediates in studies with *Saccharomyces cerevisiae* (12) and *E. coli* (13) Q-deficient mutants. In yeast, Q mutant strains have been classified into eight complementation groups, and five *COQ* genes have been characterized. The *COQ1* and *COQ2* genes encode the polyprenyl diphosphate synthase and the pHB:polyprenyldiphosphate transferase, respectively (14, 15). The *COQ3* gene encodes the O-methyltransferase thought to catalyze the first O-methylation step (16, 17), and the *COQ5* gene encodes the C-methyltransferase in Q biosynthesis (18, 19). Finally, the *COQ7* gene encodes a protein that localizes to yeast mitochondria (20) and is required for the final monooxygenase step in Q biosynthesis (21), but has also been implicated in aging and development in *C. elegans* (22).

The Q biosynthetic pathway in *E. coli* has been carefully worked out by analyzing *ubi* mutant strains (23) for accumulating Q intermediates at the blocked metabolic steps, and many of the bacterial genes have been characterized (24). These include *ubiC*, *ispB*, and *ubiA*, which encode the chorismate pyruvate lyase (25), octaprenyl synthase (26), and the pHB:octaprenyltransferase (27), respectively. Genes encoding the hydroxylase (*ubiH*) (28) and the O-methyltransferase (*ubiG*) (29, 30) have also been reported, and recently, the gene encoding the C-methyltransferase gene in *E. coli* was charac-

Ubiquinone is an essential lipid in the electron transport chain that is found in the inner mitochondrial membranes of eukaryotes and in the plasma membrane of prokaryotes (1). The structure of Q¹ consists of a quinone head group and a hydrophobic isoprenoid tail that can vary in length depending on the species in which it is found. The quinone group undergoes reversible single electron transfers, interchanging between the quinone, semiquinone, and hydroquinone, whereas

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¹ The abbreviations used are: Q, ubiquinone or coenzyme Q; pHB, *p*-hydroxybenzoic acid; AdoMet, *S*-adenosyl-L-methionine; demethyl-Q_n, demethyl-Q or 5-polyprenyl-2-hydroxy-3-methoxy-6-methyl-1,4-benzoquinone, where *n* indicates the number of isoprenoids; COMT, catechol O-methyltransferase.

terized (*ubiE*) (31). Although eukaryotes and prokaryotes share many similar steps in Q biosynthesis, the pathway diverges after the prenylation step (16, 32, 33). In prokaryotes, decarboxylation, hydroxylation, and methylation follow prenylation, whereas in eukaryotes, the sequence is hydroxylation, methylation, and then decarboxylation. Recent evidence suggests that the Q biosynthetic pathway in higher eukaryotes is similar to *S. cerevisiae*. Both rat and human *COQ3* and *COQ7* homologs can complement the corresponding defect in yeast (34–36).²

We have been examining the enzymes that catalyze the O-methylations in prokaryotic and eukaryotic Q biosynthesis. *E. coli* strains harboring null mutations in the *ubiG* gene are defective in the first O-methylation step (conversion of compound **1** to **2**, Fig. 1) (30). Surprisingly, strains harboring leaky mutant alleles of *ubiG* accumulate demethyl-Q₈, the last intermediate in Q biosynthesis (Fig. 1, compound **5**), and are unable to carry out the last O-methylation step (37, 38). The analysis of both null and leaky mutant alleles of *ubiG* suggested that the *ubiG* gene product was required for both of the O-methylations in Q biosynthesis (30). Unlike the *E. coli ubi* mutants, analysis of accumulating Q intermediates in yeast *coq* mutants has been less informative. Yeast strains harboring *coq3*, *coq4*, *coq5*, *coq6*, *coq7*, or *coq8* mutant alleles all accumulate the same single predominant intermediate, 3-hexaprenyl-4-hydroxybenzoic acid (39, 40). For this reason, it has often been instructive to compare the yeast *COQ* genes with the *E. coli ubi* gene counterparts. The encoded amino acid sequence of yeast *COQ3* is 40% identical with the *E. coli UbiG* protein and both sequences contain the four motifs identified in a large family of S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases (41). In this study, *in vitro* assays have been developed that facilitate the study the catalytic role of both the UbiG and Coq3 proteins in O-methylation reactions. These assays demonstrate that each enzyme is active at all three O-methylation steps shown in Fig. 1. Mitochondria subfractionation studies indicate that the Coq3 polypeptide is a peripherally associated inner membrane protein, located on the matrix side. The results presented suggest that both the first and last O-methylation steps in the yeast Q biosynthetic pathway occur within the mitochondria matrix compartment.

EXPERIMENTAL PROCEDURES

General Synthetic Procedures—All reagents were used as received from Aldrich Chemical Co. unless otherwise noted. Unless specified as dry, the solvents were of unpurified reagent grade. Diethyl ether was distilled from sodium using benzophenone as an indicator. All air- or water-sensitive reactions were carried out under positive pressure of argon. Reactions were followed by TLC using Whatman precoated plates of silica gel 60 with fluorescent indicator. Reactions forming quinones were followed by leucomethylene blue stain. Normal phase flash chromatography was performed on Davisil Grade 643 silica gel (230–400 mesh). NMR spectra were measured on a Bruker ARX400 or ARX500 MHz spectrometer. Low and high resolution mass spectra were determined on a VG Autospec. Synthetic procedures used to generate farnesylated analogs of compounds **1**, **2**, **3**, and **4** (Fig. 1) were described previously (30, 42, 43).

3-Hydroxy-4,5-dimethoxy-2-acetyltoylene (8)—In a glove bag under N₂, AlCl₃ (3.29 g, 24.7 mmol) was placed into a 100-ml round-bottomed flask. The flask was sealed and transferred to an argon atmosphere before anhydrous ether (15 ml) was slowly added, followed by 3,4,5-trimethoxytoluene (**7**) (2.8 ml, 16.6 mmol) and acetyl chloride (1.5 ml, 17.3 mmol). The reaction mixture turned dark and murky and was stirred for 20 h at room temperature. Following the addition of water (10 ml) and concentrated HCl (1 ml), the mixture was extracted with ether (three times, 15 ml). The combined ether layers were extracted with 1 M NaOH (three times, 20 ml), and the resulting aqueous layers were acidified by dropwise addition of concentrated HCl and then cooled in an ice-bath for 1 h. The product crystallized and was filtered using a

Buchner funnel with Whatman No. 50 paper to give 1.56 g (44.6% yield) of pale yellow solid **8**. ¹H NMR (CDCl₃, 400 MHz) δ 2.54 (s, ³H), 2.62 (s, ³H), 3.86 (s, ³H), 3.91 (s, ³H), 6.31 (s, ³H), 11.97 (s, ³H); ¹³C NMR (CDCl₃, 100 MHz) δ 204.33, 156.62, 155.87, 135.91, 134.46, 117.15, 106.90, 60.63, 55.84, 33.00, 24.37; LRMS *m/z* (relative intensity) EI 210.1 (72), 195.1 (100), 180.0 (17); HRMS *m/z* calculated for C₁₁H₁₄O₄ (M⁺), 210.089067; found, 210.089209.

2,3-Dihydroxy-4,5-dimethoxytoluene (9)—Compound **8** (180 mg, 0.86 mmol) was dissolved in a solution of sodium hydroxide (68 mg, 1.7 mmol) and water (4 ml). Hydrogen peroxide (0.12 ml, 30% in H₂O) was added dropwise to the reaction mixture via an addition funnel over 10 min. The mixture was then heated at 45 °C for 2 h. Five minutes after the heating was initiated, the solution darkened from a pale yellow to a deep violet. The reaction was quenched by the addition of 1 M HCl (15 ml) and then extracted with dichloromethane (3 × 20 ml). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated, by rotary evaporation. Flash chromatography using hexane:ethyl acetate (9:1) gave yellow solid **9** (80 mg, 51% yield). ¹H NMR (CDCl₃, 400 MHz) δ 3.22 (s, ³H), 3.23 (s, ³H), 3.89 (s, ³H), 4.95 (s, 1H), 5.61 (s, 1H), 6.26 (s, 1H); ¹³C NMR (CDCl₃, 100 MHz) 145.36, 136.39, 136.31, 134.06, 118.80, 105.41, 61.13, 56.25, 15.46; LRMS *m/z* (relative intensity) EI 184.1 (100), 169.0 (96), 154.1 (74), 139.0 (20), 126.0 (25), 111.0 (83); HRMS *m/z* calculated for C₉H₁₂O₄ (M⁺), 184.073485; found, 184.073559.

2-Hydroxy-3-methoxy-6-methyl-1,4-benzoquinone or Fumigatin (10)—Compound **9** (73.4 mg, 0.40 mmol) was dissolved in a 1:1 mixture of dichloromethane:acetonitrile (4 ml). A solution of ammonium cerium (IV) nitrate (655.4 mg, 1.20 mmol) in dichloromethane:acetonitrile (1:1, 2 ml) was then added dropwise to the reaction mixture over 5 min. The solution color changed from yellow to a turbid maroon. Stirring was continued for 5 min before the reaction was quenched by the addition of 10 ml of water. The reaction mixture was extracted with dichloromethane (three times, 15 ml) and the combined organic layers were concentrated by rotary evaporation. The crude residue was redissolved in ether (20 ml) and then treated with 1 M NaHCO₃ (20 ml). The aqueous layer became a bright violet color. Following two washes with ether, the aqueous layer was slowly acidified using concentrated HCl until the solution color changed from deep violet to orange. The aqueous layer was then extracted three times with ether. The combined ether extracts were dried over MgSO₄, filtered, and concentrated by rotary evaporation to give fumigatin (56.5 mg, 84.4% yield), a red crystalline solid. ¹H NMR (CDCl₃, 400 MHz) δ 2.06 (d, J = 1.7 Hz, ³H), 4.09 (s, ³H), 6.39 (q, J = 1.7 Hz, 1H), 6.44 (br. s, 1H); ¹³C NMR (CDCl₃, 100 MHz) δ 184.73, 183.45, 141.56, 140.02, 137.51, 132.44, 60.35, 14.93; LRMS *m/z* (relative intensity) EI 168.0 (100), 127.0 (48), 97.0 (33); HRMS *m/z* calculated for C₈H₈O₄ (M⁺), 168.042536; found, 168.042259.

5-Farnesyl-2-hydroxy-3-methoxy-6-methyl-1,4-benzoquinone or Demethyl-Q₃ (5)—The Freidel-Crafts allylation of **10** was performed as described (44) with the following modifications. Fumigatin (30.1 mg, 0.177 mmol) was dissolved in 1:1 ether:ethanol (6 ml), and then Na₂S₂O₄ (10% in H₂O) was added dropwise to the stirred solution until decolorization of the mixture was achieved. Ether (5 ml) was added to the decolorized mixture and the organic layer was washed three times with brine, dried over MgSO₄, and concentrated *in vacuo*. The resulting hydroquinone of fumigatin was dissolved in freshly distilled 1,4-dioxane (6 ml) under an argon atmosphere. *Trans-trans*-farnesol (118 mg, 0.53 mmol) was added to the solution, followed by BF₃·OEt₂ (79.6 ml, 0.63 mmol), and the reaction was allowed to proceed for 18 h at room temperature. The reaction mixture was washed with brine and extracted three times with ether. The combined organic layers were dried over MgSO₄, filtered, and concentrated *in vacuo*. The crude product was dissolved in ether (10 ml) and treated with excess FeCl₃ in a 1:1 mixture of water:methanol for 30 min. The resulting mixture was extracted three times with ether, dried over MgSO₄, filtered, and concentrated by rotary evaporation. The crude product was purified on a Florisil column with the following gradient system: 4:1 hexane/ethyl acetate, 1:1 hexane/ethyl acetate, 100% ethyl acetate, 4:1 hexane/ethyl acetate, 4:1 hexane/ethyl acetate with 1% glacial acetic acid. As described by Moore and Folkers (44), the desired product (demethyl-Q₃) was retained as a bright purple compound at the top of the column until the final wash containing 1% glacial acetic acid was performed. Upon treatment with acetic acid, the color of the desired compound changed from purple to red-orange, and it was then eluted from the column. A yellow-orange oil (**5**) (18.5 mg, 28% yield) was obtained. ¹H NMR (CDCl₃, 500 MHz) δ 1.57 (s, ³H), 1.59 (s, ³H), 1.67 (s, ³H), 1.74 (s, ³H), 1.98 (m, 8H), 2.04 (s, ³H), 3.20 (d, J = 7 Hz, 2H), 4.06 (s, ³H), 4.92 (t, J = 1 Hz, 1H), 5.06 (m, 2H), 6.48 (br. s, 1H); ¹³C NMR (CDCl₃, 125 MHz) δ 185.14, 183.16, 143.36, 139.17, 137.82, 137.04, 136.16, 135.22, 131.30, 124.29, 123.81, 118.64, 60.26, 39.68, 26.74, 26.42, 35.68, 25.42, 17.65, 16.33, 16.01, 11.59;

² T. Jonassen and C. F. Clarke, unpublished data.

LRMS m/z (relative intensity) EI 372.2 (40), 267.0 (11), 236.1 (58), 221.1 (100), 183.1 (48), 162.0 (20), 121.1 (30); HRMS m/z calculated for $C_{25}H_{32}O_4$ (M^+), 372.230403; found, 372.230060.

Strains and Growth Media—The strains of *S. cerevisiae* used in the *in vitro* studies were JM45 (MATa, *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*, *his4-580*) (45), a parent strain possessing Q synthesis, and JM45 Δ coq3 (MATa, *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*, *his4-580*, *coq3::LEU2*) (17). The yeast strains used in the localization studies were wild-type, W3031A (MATa, *leu2-3*, *leu2-112*, *ura3-1*, *trp1-1*, *his3-11*, *ade2-1*) (46), and the Δ coq3 strain, CC3031B (MATa, *leu2-3*, *leu2-112*, *ura3-1*, *trp1-1*, *his3-11*, *ade2-1*, *coq3::LEU2*) (7). The *E. coli* strain used was DH5 α , which was obtained from Life Technologies, Inc. Growth media for yeast were prepared as described (47) and included YPD (1% yeast extract, 2% peptone, 2% dextrose), YPG (1% yeast extract, 2% peptone, 3% glycerol), YPGal (1% yeast extract, 2% peptone, 2% galactose) and SD-Ura (0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4 , 0.5% $(NH_4)_2SO_4$), with complete supplement minus uracil. The complete supplement was modified so that the final concentration of each component was as follows: 80 mg/liter, adenine sulfate, uracil, tryptophan, histidine, methionine, and cysteine; 40 mg/liter, arginine and tyrosine; 120 mg/liter, leucine; 60 mg/liter, isoleucine, lysine, and phenylalanine; 100 mg/liter, glutamic acid and aspartic acid; 150 mg/liter, valine; 200 mg/liter, threonine; and 400 mg/liter, serine). *S. cerevisiae* and *E. coli* were grown at 30 and 37 °C, respectively.

Plasmid Construction—DNA constructions were performed as described (48). pTHG was constructed to express UbiG as a fusion protein with a 33-amino acid N-terminal extension, containing 6 His residues (His_6 -UbiG) to provide for metal affinity column purification. A DNA segment containing the complete *ubiG* ORF (851–1572) was generated by a polymerase chain reaction with Vent DNA Polymerase (New England Biolabs) using pRPB (29) as the DNA template with the primers, pGB, (5'-GCGGATCCGATGAATGCCGAAAAATCGCCGTA-3') and pCC4K (30). The resulting 723-base pair product was inserted after digestion with *Bam*HI and *Kpn*I into the similarly digested vector pTrcHisB (Invitrogen) to generate pTHG. The plasmids, pQM and pCHQ3, were described previously (30).

Purification of His_6 -UbiG Fusion Protein—Purification of His_6 UbiG was done with the TALON metal affinity resin (CLONTECH) as described by the manufacturer. The *E. coli* strain, DH5 α :pTHG, containing the His_6 -UbiG was grown in LB+Amp (50 μ g/ml) and induced with isopropyl-1-thio- β -D-galactopyranoside (final concentration, 0.4 mM), and cells were disrupted by the French press method. His_6 -UbiG was purified on a TALON column under native conditions. The resin was washed with 15 mM imidazole to remove nonspecifically bound proteins, His_6 -UbiG was eluted from the resin with 250 mM imidazole, and the imidazole was removed by dialysis against 0.05 M sodium phosphate, pH 7.0.

Generation of Yeast Coq3p Antibodies—A plasmid encoding a glutathione S-transferase-Coq3p fusion protein was constructed by subcloning the 1.7-kilobase *Eco*RI fragment of pRS12A (17) into the *Eco*RI site of pGEX-2T (Amersham Pharmacia Biotech). The fusion protein contained amino acids 64–316 of yeast Coq3p as a C-terminal fusion to glutathione S-transferase and was produced in *E. coli* and the insoluble fraction was separated by preparative SDS-polyacrylamide gel electrophoresis. The 50-kDa fusion protein was visualized by copper staining (49) and eluted from the gel by diffusion (50). The protein was injected into rabbits, and antibodies were affinity purified according to standard techniques (51).

In Vitro Assays—Assays for O-methyltransferase activities were determined with the three synthetic methyl-acceptors, compounds **1**, **3**, and **5**. Stocks of **1**, **3**, and **5** were stored undiluted at –20 °C under argon. In assays with either **1** or **3**, the substrates were redissolved into methanol, and each reaction mixture (250 μ l) contained 0.05 M sodium phosphate, pH 7.0, 1.0 mM $ZnSO_4$, and 50 μ l of crude yeast mitochondria (0.25–0.50 mg of protein) (52) or purified *E. coli* protein, His_6 -UbiG (1.2 ng). The final concentration of compound **1** or **3** in each assay was 50 μ M unless otherwise stated. Reactions were started with the addition of S-adenosyl-L-[methyl- 3H]methionine to a final concentration of 60 μ M (NEN Life Science Products, 84.1 Ci/mmol; specific activity was adjusted to 560 mCi/mmol with unlabeled S-adenosyl-L-methionine). The concentration of S-adenosyl-L-methionine was determined by its absorbance at 256 nm (ϵ 15, 200 $M^{-1} cm^{-1}$) (53). After incubation, the reaction was stopped by addition of glacial acetic acid (5 μ l), and the lipids were extracted with chloroform, concentrated and analyzed by high performance liquid chromatography as described (30). *In vitro* assays with compound **5** were the same as described above except that **5** was redissolved into hexane and NADH (3 mM) was included in the

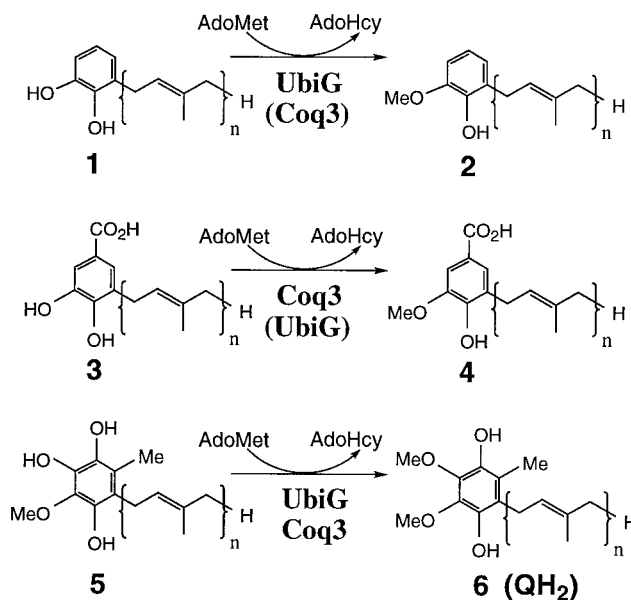


FIG. 1. O-Methyltransferase steps in ubiquinone biosynthesis. The proposed biosynthetic pathway for Q in eukaryotes and prokaryotes is thought to diverge following prenylation of *p*-hydroxybenzoic acid and in both cases involves two O-methylation steps. In *E. coli*, the first O-methylation step requires UbiG, and involves the O-methylation of 2-polyprenyl-6-hydroxyphenol (compound **1**), to form 2-polyprenyl-6-methoxyphenol (compound **2**). In eukaryotes, the first O-methylation step requires the Coq3 polypeptide for the conversion of 3,4-dihydroxy-5-polyprenylbenzoic acid (compound **3**) to 3-methoxy-4-hydroxy-5-polyprenylbenzoic acid (compound **4**). In both eukaryotes and prokaryotes, the second O-methylation of 2-polyprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone (compound **5**) forms ubiquinol-*n* (compound **6**). *E. coli*, $n = 8$; *S. cerevisiae*, $n = 6$.

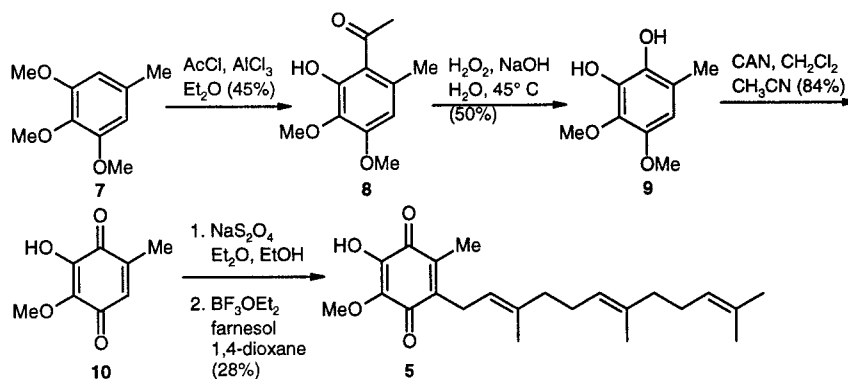
assays with yeast mitochondria in order to form the hydroquinone. In assays with purified His_6 -UbiG, **5** was reduced with 10% sodium dithionite, and prior to addition, the sodium dithionite was removed by centrifugation. Following incubation, reactions were terminated by the addition of excess ammonium cerium (IV) nitrate to oxidize the methylated product, and lipids were extracted with hexane (two times, 0.5 ml), concentrated, and analyzed by high performance liquid chromatography as described above.

Localization of Yeast Coq3p—Yeast (W3031A or CC3031B) was grown in YPGal media to saturation density ($A_{600} \sim 10.0$), and a crude mitochondria fraction was isolated and further purified over a linear Nycodenz gradient as described (52). Subfractionation of purified mitochondria was carried out by generating mitoplasts as described (20), and fractionation of mitoplasts was accomplished by either sonication (four 10-s pulses, 20% duty cycle, 2.5 output setting Sonifier W350, Branson Sonic Power Co.) or alkaline carbonate extraction (54, 55). Protease protection experiments were carried out as described (56). Equal amounts of protein as determined by the BCA method (Pierce) were separated by electrophoresis on 12% polyacrylamide gels (57) and subsequently transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech). Western analysis with the ECL system was carried out as described by Amersham Pharmacia Biotech except that 10 mM Tris, pH 8.0, 154 mM NaCl, 0.1% Triton X-100 was used as the Western washing buffer. The primary antibodies were used at the following dilutions: α -Coq3, 1:1,000; Hsp 60, 1:10,000; $F_1\beta$ ATPase, 1:10,000; cytochrome c_1 , 1:400; cytochrome b_2 , 1:5000. Horseradish peroxidase-linked secondary antibodies to rabbit IgG (Amersham Pharmacia Biotech) were used in a 1:1000 dilution.

RESULTS

Chemical Synthesis of Ubiquinone Biosynthetic Analogs as Methyl-acceptor Substrates for *in Vitro* Assays—We have chemically synthesized farnesylated analogs of Q-intermediates **1–4** in Fig. 1 ($n = 3$) (30, 43). Fig. 2 shows the synthetic scheme for the last O-methylation substrate, demethyl-Q₃ (compound **5**). Fumigatin (compound **10**) was generated by a modification of the synthesis described by Baker and Raistrick (58). Friedel-Crafts allylation (59) of the hydroquinone form of **10** with a

FIG. 2. Chemical synthesis of demethyl- Q_3 . Details of the synthesis are described under "Experimental Procedures."



prenyl tail forms demethyl- Q_3 (Fig. 2, compound 5). A tin-assisted allylation of Q_0 was carried out as described by Naruta (60) to form the farnesylated product standard for the final O-methylation step, Q_3 (Fig. 1, compound 6).

Coq3p Is Required for Both O-Methylation Steps in Ubiquinone Biosynthesis—Our previous O-methyltransferase *in vitro* assays indicated that multiple steps may be catalyzed by the same enzyme (30). Specifically, *in vitro* assays with cell free extracts of *E. coli* showed that the *ubiG* gene was required for the methylation of both compounds 1 and 3. These results indicated that UbiG was involved in both O-methylation steps of Q biosynthesis, because Leppik *et al.* (38) showed that UbiG was required for the methylation of 5 to 6. By analogy, it seemed likely that the *COQ3* gene product may also be required for both O-methylation steps in eukaryotic Q biosynthesis. To test this idea, *in vitro* O-methylation assays were performed with the synthetic Q-intermediate analog 5 ($n = 3$) as substrate. The methyl donor was [methyl- 3H]AdoMet, and mitochondria were isolated from three yeast strains: 1) a wild-type respiratory competent strain (JM45), 2) the *coq3* deletion mutant harboring the plasmid vector as a control (JM45 Δ coq3:pQM), and 3) a rescued mutant with a multicopy plasmid encoding yeast *COQ3* expressed from the *CYC1* promoter (JM45 Δ coq3:pCHQ3) (Fig. 3A). Mitochondria from respiratory competent yeast produced a radioactive product that co-migrated with the Q_3 standard (compound 6) on reverse-phase high performance liquid chromatography (Fig. 3A) (fraction 17). This activity (40.2 pmol/mg of protein/h) required the reducing agent, NADH, because omitting NADH resulted in no O-methyltransferase activity. No activity was detected in mitochondria isolated from a *coq3* null mutant (JM45 Δ coq3:pQM). However, transformation of this strain with the *COQ3* gene (JM45 Δ coq3:pCHQ3) restored activity (161 pmol/mg of protein/h). Thus, a functional Coq3 polypeptide is required for both the first (43) and second O-methylation steps in yeast Q biosynthesis.

Similar *in vitro* assays were carried out to determine whether Coq3p was required for the methylation of the farnesylated analog of the *E. coli* substrate (compound 1). As shown in Fig. 3B, mitochondria from wild-type yeast contained high activity (22.8 pmol/mg of protein/h) and produced a radiolabeled product that co-migrated with the farnesylated analog of 2. This activity was not detected in the *coq3* null mutant (JM45 Δ coq3:pQM), but the activity (16.3 pmol/mg of protein/h) was again restored when mitochondria from the rescued strain were examined (JM45 Δ coq3:pCHQ3). These results suggest that the Coq3p O-methyltransferase is capable of methylating multiple Q precursor analogs.

Conservation of Function between Yeast and Rat O-Methyltransferase Activity—To examine whether the *in vitro* assays described above could be used to study Q biosynthetic steps in higher eukaryotes, the plasmid pAB2 (34), which contains the

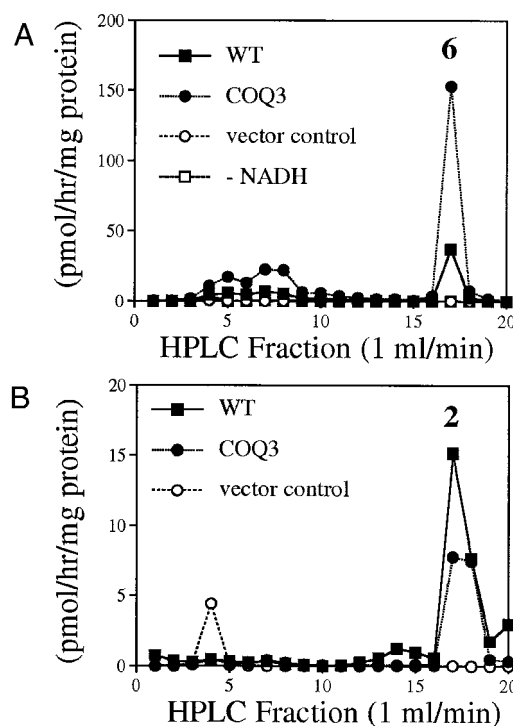


FIG. 3. Coq3p is required for the O-methylation of demethyl- Q_3 (compound 5) and 2-farnesyl-6-hydroxyphenol (compound 3). Crude mitochondrial extracts were prepared from JM45 (wild-type (WT), ■ and □), JM45 Δ coq3:pQM (vector control, ○), and JM45 Δ coq3:pCHQ3 (*COQ3* gene on a multiple copy plasmid, ●) as described, and incubated with demethyl- Q_3 (compound 5) (A) or 2-farnesyl-6-hydroxyphenol (1 mM) (compound 3) (B), and S-adenosyl-L-[methyl- 3H]methionine. In A, 3 mM NADH was included in all incubations except for that indicated (□). Following incubation for 1 h, lipids were extracted and analyzed by reverse-phase high performance liquid chromatography (Alltech Lichrosorb C-18, 5 mm, 4.6 \times 250 mm) with 9:1 methanol/water as the mobile phase and a flow rate of 1 ml/min.

rat *COQ3* cDNA, was transformed into JM45 Δ coq3. Mitochondria were isolated from this strain and assayed for O-methylation activity with farnesylated analogs of 1 (Fig. 4A), 5 (Fig. 4B) or 3 (Fig. 4C). In each case, the radioactive methylated products were detected that eluted with chemically synthesized methylated products (2, 6, and 4, respectively). The activities were 174.2, 42.5, and 54.1 pmol/mg of protein/h, respectively. These assays demonstrate that farnesylated analogs of Q biosynthetic intermediates can be used to study Q biosynthesis in higher eukaryotes. Additionally, these results indicate that both O-methylation steps in rats also require Coq3p and that this O-methyltransferase has a wide substrate specificity.

The UbiG Polypeptide Catalyzes Both O-Methylation Steps in E. coli Q Biosynthesis—A direct test of the hypothesis that Coq3p and UbiGp catalyze both O-methyltransferase steps re-

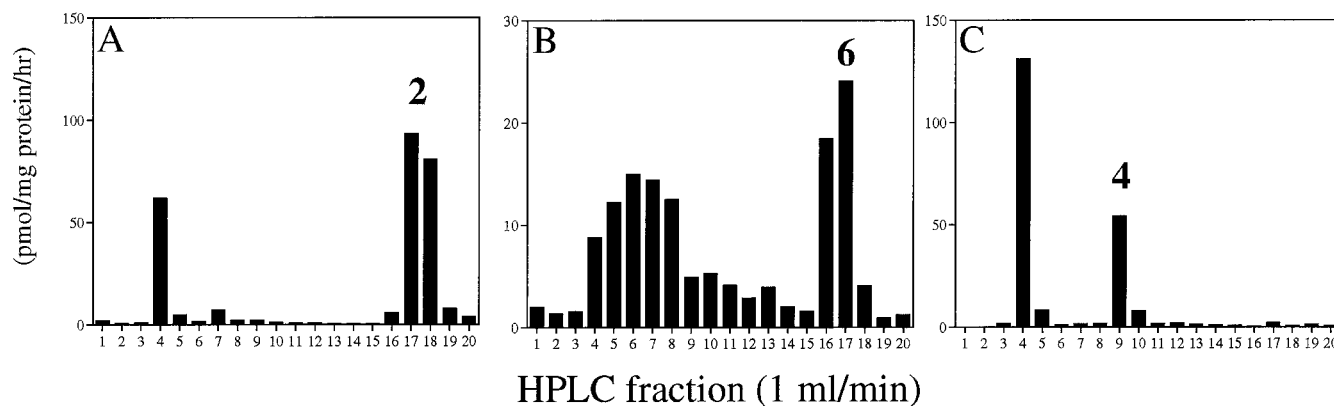


FIG. 4. The rat *COQ3* gene restores *O*-methyltransferase activity in *coq3* null mutant yeast. Yeast crude mitochondrial extracts were prepared from JM45 Δ *coq3*:pAB2 (rat *COQ3* gene) and *in vitro* *O*-methylation assays were carried out as described in Fig. 5. Three different analogs of Q-intermediates were used as substrates: A, 2-farnesyl-6-hydroxyphenol (compound 1); B, demethyl-Q₃ (compound 5); C, 3,4-dihydroxy-5-farnesylbenzoic acid (compound 3). In each assay, *O*-methyltransferase activity required the rat *COQ3* gene because no activity was detected in its absence (see Fig. 3, A and B). The elution positions of methylated farnesylated standards (2, 6, and 4) are indicated.

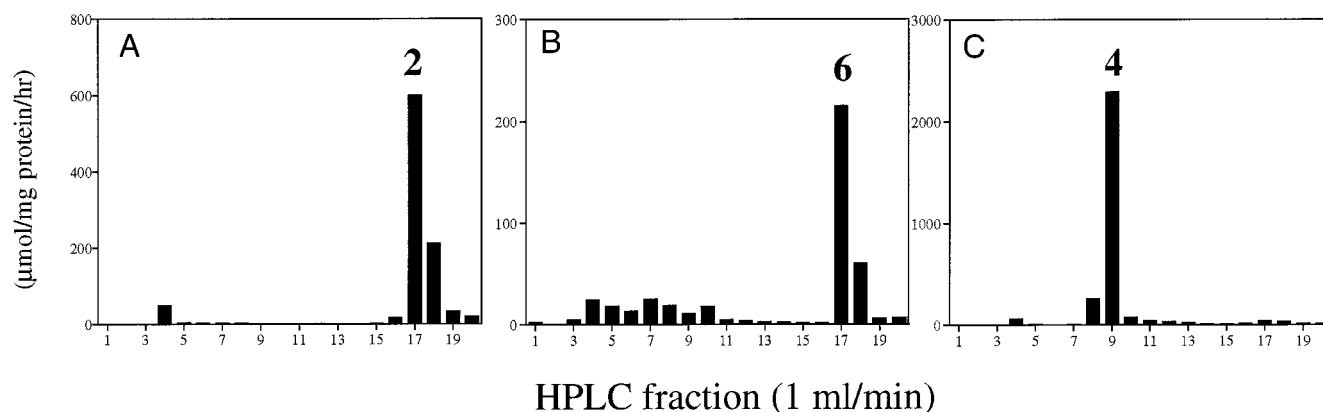


FIG. 5. Purified His₆-UbiG catalyzes all *O*-methylation steps in Q biosynthesis. *In vitro* *O*-methyltransferase assays were carried out with the purified His₆-UbiG enzyme using the three synthetic analogs previously described. A, 2-farnesyl-6-hydroxyphenol (compound 1); B, the hydroquinone form of demethyl-Q₃ (compound 5); C, 3,4-dihydroxy-5-farnesylbenzoic acid (compound 3). The elution position of the methylated products are indicated (2, 6, and 4).

quired preparations of the pure polypeptides. To facilitate purification, the UbiG polypeptide was expressed as a fusion protein containing an N-terminal His₆ sequence. The N-terminal extension does not interfere with activity, as the expression of this fusion protein in the *E. coli ubiG* disruption mutant GD1 (30) restores growth on succinate and results in a 50-fold increase in 1:*O*-methyltransferase activity in *E. coli* whole cell extracts. The His₆-UbiG fusion protein was purified as described under "Experimental Procedures" and used in *in vitro* *O*-methylation assays. Farnesylated analogs of 1 (Fig. 5A), 5 (Fig. 5B) or 3 (Fig. 5C) were tested as methyl-acceptor substrates. In each case, radioactive methylated products were detected that co-eluted with chemically synthesized methylated products 2 (813 μ mol/mg of protein/h), 6 (275 μ mol/mg of protein/h), and 4 (2, 290 μ mol/mg of protein/h), respectively. Methylation of 5 required that it be reduced prior to addition (data not shown). Thus, the purified UbiG polypeptide is sufficient for the catalysis of both *O*-methylation steps in the biosynthesis of Q in *E. coli*, and is capable of methylating the eukaryotic substrate.

Subcellular Localization of the Coq3 Polypeptide—Previous work showed that the yeast Coq3p precursor was imported into mitochondria *in vitro*, and a mitochondrial membrane potential was required for processing to the mature (protease resistant) form (30). To determine the location of Coq3p in yeast, affinity purified polyclonal antibodies were prepared against Coq3p. Fractions of cytosol, crude mitochondria, and Nycodenz-puri-

fied mitochondria were prepared from both the CC3031B null mutant strain and the wild-type parental strain, W3031A. Immunoblot analysis (Fig. 6A) of each fraction indicated that the 33-kDa polypeptide (corresponding to the mature Coq3p) was detected only in the mitochondrial fractions of wild-type yeast but not in the *coq3* null mutant.

Submitochondrial Localization of the Coq3 Polypeptide—To determine the submitochondrial localization of Coq3p, yeast mitochondria were further fractionated (54). Purified mitochondria from W3031A were subjected to treatment with hypotonic buffer, which disrupts the outer membrane and releases soluble proteins of the intermembrane space while keeping the inner membrane intact. Western analysis of the soluble fraction indicated that Coq3p remained associated with the pellet (mitoplast fraction) and did not co-purify with the intermembrane space marker, cytochrome *b*₂ (data not shown). Mitoplasts were further fractionated either by sonication, which releases soluble matrix proteins into the supernatant following centrifugation, or by extraction with alkaline carbonate, which releases both soluble and peripherally bound membrane proteins into the supernatant (61). As shown in Fig. 6B, Coq3p was released by alkaline carbonate extraction, which was similar to the matrix marker, Hsp60 (62), and the peripheral inner membrane protein F₁F₀ATPase (63). In contrast, these conditions did not release the integral membrane marker, cytochrome *c*₁ (64). However, sonication conditions that release Hsp60 into the supernatant fraction, did not re-

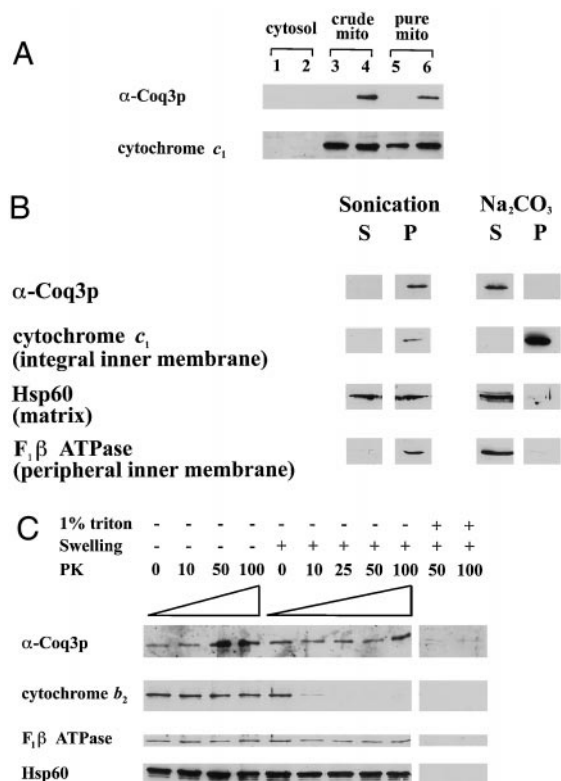


FIG. 6. Coq3p is a peripheral mitochondrial inner membrane protein. *A*, yeast cells from a wild-type strain, W3031A (lanes 2, 4, and 6), or a *coq3* null mutant, CC3031B (lanes 1, 3, and 5), were grown to saturation, and the cells were collected, lysed, and fractionated by standard methods. Crude mitochondria were then purified over Nyco-denz gradients (see under "Experimental Procedures"). Samples (5 μ g of protein) from the cytoplasmic fractions (lanes 1 and 2), the crude mitochondrial fractions (crude mito) (lanes 3 and 4), and the Nyco-denz purified mitochondrial fractions (pure mito) (lanes 5 and 6) were separated by SDS-polyacrylamide gel electrophoresis. The gel was transferred to nitrocellulose for Western analysis by chemiluminescence detection using antibodies to Coq3p (α Coq3p) and the mitochondrial marker protein, cytochrome *c*₁ (64). *B*, mitoplasts from yeast strain W3031A were generated and either sonicated or treated with 0.1 M Na₂CO₃, pH 11.5, incubated on ice, and centrifuged as described (see under "Experimental Procedures"). 10 μ g of protein from each resultant supernatant (S) and pellet (P) fraction were analyzed by SDS-polyacrylamide gel electrophoresis, transferred for Western analysis, and probed via chemiluminescence detection using antibodies to Coq3p, cytochrome *c*₁, Hsp 60, or F₁βATPase. *C*, intact mitochondria (left four lanes), mitoplasts, or mitoplasts containing 1% Triton X-100 were treated with increasing concentrations of proteinase K (0, 10, 25, 50, and 100 μ g/ml). Samples of each (1 μ g) were analyzed by SDS-polyacrylamide gel electrophoresis, transferred for Western analysis, and probed via chemiluminescence detection using antibodies to Coq3p, cytochrome *b*₂, F₁βATPase, or Hsp 60.

lease Coq3p or the peripheral membrane marker, F₁βATPase. These results indicate that Coq3p is a peripheral membrane protein similar to the F₁βATPase.

To determine whether Coq3p is associated with the matrix-side or the outside of the inner membrane of yeast mitochondria, purified mitochondria or mitoplasts were subjected to increasing concentrations of proteinase K and then subjected to Western analysis (Fig. 6C). The results indicate that Coq3p was protected from protease treatment in both intact mitochondria and mitoplasts. This degree of protease protection is also a property of the inner membrane marker, F₁βATPase, and Hsp60, a matrix marker. However, cytochrome *b*₂, an inter-membrane space protein, was fully digested in mitoplasts as expected. Additionally, treatment of mitoplasts with 1% Triton X-100 detergent rendered all proteins protease-sensitive. These data indicate that the Coq3 polypeptide is peripherally

associated with the matrix side of the inner membrane of mitochondria.

DISCUSSION

This study demonstrates that both *O*-methylation steps in Q biosynthesis are catalyzed by the same enzyme. The *in vitro* *O*-methylation assays employ farnesylated analogs of compounds 1, 3, and 5 as substrates, [*methyl*-³H]AdoMet, and the detection of radiolabeled methylated products corresponding to compounds 2, 4, and 6. Such assays have been performed with isolated yeast mitochondria containing yeast Coq3p (Fig. 3) (43), yeast mitochondria containing rat Coq3p (Fig. 4), cell free extracts of *E. coli* (30), and with purified UbiG polypeptide (Fig. 5). In each case, the presence of either Coq3 or UbiG is required to observe *in vitro* *O*-methylation, and both Coq3p and UbiG methylate all three substrates.

These assays showed that methylation of 5 by yeast mitochondria required NADH. A similar requirement was observed for the *O*-methylation of 5 by *E. coli* extracts (38) and rat liver mitochondria (65). It is likely that NADH provides the reducing equivalents for the generation of the hydroquinone. Accordingly, the purified UbiG *O*-methyltransferase also requires 5 to be present in the reduced form (Fig. 5). All three compounds thus contain a similar catechol functional group.

The *O*-methylation of the farnesylated analogs of Q-intermediates by yeast and rat Coq3 and *E. coli* UbiG is interesting because the naturally occurring quinone species in each of these organisms is different. In yeast, the prenyl tail length (*n*) is 6; in *E. coli*, *n* = 8; and in rats, *n* = 9 or 10. Additionally, Q biosynthesis can be restored in *coq3* null mutants by the human *COQ3* homolog.² Therefore, it is likely that the human Coq3p recognizes the farnesylated species as well. Such promiscuity is not uncommon in Q biosynthesis because the pHB: polyprenyldiphosphate transferase from rats can recognize other aromatic precursors (66, 67), and in yeast, it can utilize polyprenyl groups ranging from *n* = 5 to *n* = 10 (68). Also, the *C*-methyltransferase enzyme in *E. coli* carries out steps in both Q and menaquinone biosynthesis (31).

A low degree of substrate specificity is also seen for the enzyme, catechol-*O*-methyltransferase (COMT). COMT is known to methylate numerous neurotransmitters (dopamine, norepinephrine, and epinephrine), their hydroxylated derivatives, and other analogs (69). Both COMT and Coq3/UbiG enzymes require a divalent cation, but comparison of their primary amino acid sequences fails to reveal any homology aside from the AdoMet-dependent methyltransferase motifs. The recent structure of COMT from rat liver (70) provides insight into the mechanism for the *O*-methylation reaction. The *O*-methyltransferase in Q biosynthesis may rely on a similar mechanism as the one reported for COMT.

Subcellular fractionation localizes Coq3p to the mitochondria. These data confirm and extend previous results that demonstrated import of the yeast Coq3p precursor into the mitochondria *in vitro*, and showed that such import required a membrane potential (30). The N terminus of the precursor Coq3p contains a putative mitochondrial leader sequence (71, 72), which is proteolytically cleaved upon import to produce the mature form (30). The submitochondrial localization of Coq3p was also determined (Fig. 6). Mitochondrial fractionation and protease protection experiments coupled with Western analysis demonstrated that Coq3p was a peripherally associated protein of the inner mitochondrial membrane. This evidence localizes Coq3p and therefore the site for both *O*-methylation steps of Q biosynthesis within the mitochondrial matrix.

The intracellular site(s) for Q biosynthesis in eukaryotes is still not elucidated. Studies in yeast show that the hexaprenyldiphosphate synthase and the pHB:polyprenyldiphosphate

transferase activities reside in mitochondria (73), and both proteins contain typical mitochondrial leader sequences (13, 14). Recently, the yeast *COQ5* gene encoding the C-methyltransferase was localized to mitochondria (18, 19). The Coq7 (Cat5/Clk-1) polypeptide, which is required in one or more hydroxylase steps in Q biosynthesis (21), was also found in the mitochondria (20). The *COQ3* gene product from *Arabidopsis* was recently localized to the membrane fraction of mitochondria (74). Also, it was previously shown that the O-methyltransferase responsible for converting 5 to Q in rat liver was localized to the inner membrane of the mitochondria (65). However, studies with rat liver show Q biosynthesis occurring in the endoplasmic reticulum-Golgi system (75–77). These results conflict with earlier studies that indicate that Q is synthesized solely in the mitochondria (65, 78, 79). The ability of the rat Coq3p to rescue a yeast *coq3* mutant (34) suggests that it must be present in the mitochondria of yeast and of rats as well. This conclusion is further supported by the rescue of a *coq3Δ* mutant with the *E. coli* homolog, *ubiG*, on a single copy plasmid that required that UbiG contain a mitochondrial targeting sequence at the N terminus (30). Although redistribution of the mitochondrial targeted protein fumarase has been reported (80), this requires a cotranslational insertion mechanism that is not required for Coq3p.

UbiG can function as a soluble enzyme. Earlier studies showed that UbiG activity was associated with the *E. coli* plasma membrane, but it could be solubilized (30, 38). This differs from yeast and higher eukaryotes, in which the corresponding homolog, Coq3, appears tightly associated with the inner mitochondrial membrane. Our attempts to solubilize Coq3p activity by sonication or detergent treatments have been unsuccessful. However, activity for the second O-methyltransferase in rat liver mitochondria was solubilized by treatment with Triton X-100 (65). The native molecular weight for the enzyme in those studies was not determined.

Unlike UbiG, which is readily purified as an active soluble enzyme, overexpression of Coq3p in *E. coli* produced no active enzyme and failed to rescue the *ubiG* growth defect in *E. coli*. These observations suggest that Coq3p may require additional polypeptides that 1) may function to keep it peripherally associated with the membrane, or 2) may function in a possible uncharacterized regulatory manner not present in prokaryotes. In either case, these additional polypeptides evidently are required for activity. The evidence for a possible complex in Q biosynthesis in eukaryotes is further supported by the lack of O-methyltransferase activity in other *coq* null mutants³ that may lack the required “additional” proteins. In *Nocardia lactamdurans*, the biosynthesis of cephamycin C involves the interaction of two proteins, a hydroxylase and a methyltransferase, encoded by the genes *cmcI* and *cmcJ*, respectively, that are required for function (81). The sequence of hydroxylation and methylation in cephamycin C biosynthesis is similar to Q biosynthesis. The possibility of a protein complex involved in Q biosynthesis will require further study.

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