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, UbiGp. 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.

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. 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 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 prokaryocytes, 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-polyprenylphosphate 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 monoxygenase 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-
O-Methyltransferase Steps in Coenzyme Q Biosynthesis

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, 6H), 2.62 (s, 3H), 3.86 (s, H), 3.91 (s, H), 3.61 (s, H), 11.79 (s, 1H); 13C NMR (CDCl₃, 100 MHz) δ 204.33, 156.62, 155.87, 135.91, 134.66, 115.17, 106.90, 73.15, 54.58, 54.30, 24.37, 21.74 (m/z (relative intensity) EI 210.1 (72), 195.1 (100), 180.0 (17); HRMS m/z calculated for C₁₃H₁₈O₂ (M+) 210.089067, found 210.089209.

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 were 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. cerevisiae. Both rat and human COQ3 and COQ7 homologs can complement the corresponding deficient in yeast (34–36).²

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 benzenophene as an indicator. All air- or water-sensitive reactions were carried out under positive pressure of nitrogen. Reactions were followed by TLC using Whatman precoated silica gel 60 F254 (10:1) plates with the following Gradient system: 4:1 hexane:ethyl acetate, 1:1 hexane:ethyl acetate, 4:1 hexane:ethyl acetate, 100% ethyl acetate. Flash chromatography using hexane:ethyl acetate (3:2) or benzene:ethyl acetate (10:1) gave yellow solid (985.4 mg, 28% yield) 21666.

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O-Methyltransferase Steps in Coenzyme Q Biosynthesis

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 μl) contained 0.05 m sodium phosphate, pH 7.0, and 0.4 mg/ml of crude yeast mitochondria. The reaction mixture was incubated at 37 °C for 20 min. The reaction was stopped by addition of chloroform, and concentrations were measured 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 assay with yeast mitochondria in order to form the hydroquinone. In assays with purified His6-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.

Assays with yeast mitochondria were carried out by generating mitoplasts as described (29), 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).

Generation of Yeast Coq3p Antibodies—A plasmid encoding a glutathione S-transferase-Coq3p fusion protein was constructed by subcloning the 1.7-kilobase EcoRI fragment of pRS12A (17) into the EcoRI 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).

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prenyl tail forms demethyl-Q₃ (Fig. 2, compound 5). A tin-assisted allylation of Q₃ was carried out as described by Naruta (60) to form the farnesylated product standard for the final O-methylation step, Q₄ (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 (JM45Δcoq3:pQM), and 3) a rescued mutant with a multicopy plasmid encoding yeast COQ3 expressed from the CYC1 promoter (JM45Δcoq3:pCHQ3) (Fig. 3A). Mitochondria from respiratory competent yeast produced a radioactive product that co-migrated with the Q₄ 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 (JM45Δcoq3:pQM). However, transformation of this strain with the COQ3 gene (JM45Δcoq3:pCHQ3) restored activity (161 pmol/mg of protein/h). Thus, a functional Coq3p 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 (JM45Δcoq3:pQM). However, this activity (16.3 pmol/mg of protein/h) was again restored when mitochondria from the rescued strain were examined (JM45Δcoq3: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 rat COQ3 cDNA, was transformed into JM45Δcoq3. 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. 2. Chemical synthesis of demethyl-Q₃. Details of the synthesis are described under “Experimental Procedures.”](Image 234x590 to 553x729)

![Fig. 3. Coq3p is required for the O-methylation of demethyl-Q₃ (compound 5) and 2-farnesyl-6-hydroxyphenol (compound 3). Crude mitochondrial extracts were prepared from JM45 (wild-type (WT)), JM45Δcoq3:pQM (vector control, ●), and JM45Δcoq3:pCHQ3 (COQ3 gene on a multiple copy plasmid, ●) as described, and incubated with demethyl-Q₃ (compound 5) 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 × 250 mm) with 9:1 methanol/water as the mobile phase and a flow rate of 1 ml/min.](Image 335x302 to 527x572)
required preparations of the pure polypeptides. To facilitate purification, the UbiG polypeptide was expressed as a fusion protein containing an N-terminal His$_6$ 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 $O$-methyltransferase activity in E. coli whole cell extracts. The His$_6$-UbiG fusion protein was purified as described under “Experimental Procedures” and used in in vitro $O$-methylation assays 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-Q$_3$ (compound 5); C, 3,4-dihydroxy-5-farnesylbenzoic acid (compound 3). 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. 4. The rat COQ3 gene restores $O$-methyltransferase activity in coq3 null mutant yeast.** Yeast crude mitochondrial extracts were prepared from JM48Δcoq3: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-Q$_3$ (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 standards (2, 6, and 4) are indicated.

**Fig. 5. Purified His$_6$-UbiG catalyzes all $O$-methylation steps in Q biosynthesis.** In vitro $O$-methyltransferase assays were carried out with the purified His$_6$-UbiG enzyme using the three synthetic analogs previously described. A, 2-farnesyl-6-hydroxyphenol (compound 1); B, the hydroquinone form of demethyl-Q$_3$ (compound 5); C, 3,4-dihydroxy-5-farnesylbenzoic acid (compound 3). The elution position of the methylated products are indicated (2, 6, and 4).
lease Coq3p or the peripheral membrane marker, FβATPase. These results indicate that Coq3p is a peripheral membrane protein similar to the FβATPase.

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 protease 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, FβATPase, and Hsp60, a matrix marker. However, cytochrome b₆, an intermembrane 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. 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 submitchondrial 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 hexaprenyl-diphosphate synthase and the pHB:polyprenyldiphosphate...
transference 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/Ckcl-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 (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 mutants that may lack the required “additional” proteins. In Nocardia lactamurans, the biosynthesis of cehamycin C involves the interaction of two proteins, a hydroxylase and a methyltransferase, encoded by the genes cmcl and cmcJ, respectively, that are required for function (81). The sequence of hydroxylation and methylation in cehamycin 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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O-Methyltransferase Steps in Coenzyme Q Biosynthesis

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