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Genetic evidence for a multi-subunit complex in the *O*-methyltransferase steps of coenzyme Q biosynthesis

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Abstract

Coq3 *O*-methyltransferase carries out both *O*-methylation steps in coenzyme Q (ubiquinone) biosynthesis. The degree to which Coq3 *O*-methyltransferase activity and expression are dependent on the other seven *COQ* gene products has been investigated. A panel of yeast mutant strains harboring null mutations in each of the genes required for coenzyme Q biosynthesis (*COQ1–COQ8*) have been prepared. Mitochondria have been isolated from each member of the yeast *coq* mutant collection, from the wild-type parental strains and from respiratory deficient mutants harboring deletions in *ATP2* or *COR1* genes. These latter strains constitute Q-replete, respiratory deficient controls. Each of these mitochondrial preparations has been analyzed for *COQ3*-encoded *O*-methyltransferase activity and steady state levels of Coq3 polypeptide. The findings indicate that the presence of the other *COQ* gene products is required to observe normal levels of *O*-methyltransferase activity and the Coq3 polypeptide. However, *COQ3* steady state RNA levels are not decreased in any of the *coq* mutants, relative to either wild-type or respiratory deficient control strains, suggesting either a decreased rate of translation or a decreased stability of the Coq3 polypeptide. These data are consistent with the involvement of the Coq polypeptides (or the Q-intermediates formed by the Coq polypeptides) in a multi-subunit complex. It is our hypothesis that a deficiency in any one of the *COQ* gene products results in a defective complex in which the Coq3 polypeptide is rendered unstable.

Keywords: Coenzyme Q; *S*-Adenosyl-L-methionine dependent methyltransferase; Ubiquinone; Yeast; *Saccharomyces cerevisiae*

1. Introduction

Coenzyme Q (ubiquinone or Q) is a prenylated benzoquinone lipid that is found in membranes throughout the cell, and functions in a wide variety of enzyme-mediated redox reactions. Q is best appreciated for its role in respiratory metabolism, where it functions in the inner mitochondrial membrane of eukaryotic cells as a transporter of electrons and protons in complexes II and III [1]. Q also functions in a *trans*-plasma membrane electron transport, where it enables cells to reduce extracellular compounds, such as ascorbyl radicals [2,3]. This plasma membrane redox function of Q appears to play an important role in cells with defects in mitochondrial respiration, where it participates in pathways that oxidize NADH, in order to regenerate NAD^+ for glycolytic metabolism [4]. In the inner membrane of *Escherichia coli*, Q functions in the *trans*-plasma

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membrane electron transport system that plays a dual role in both respiration and in the introduction of disulfide bonds in periplasmic proteins mediated by the *dsb* system [5,6]. The redox chemistry of Q also enables it to function as a potent antioxidant, where it has the capacity to act directly as a chain terminating antioxidant or can act in concert with α-tocopherol by reducing α-tocopheroxyl radicals [7].

In general, cells rely on de novo biosynthesis for their supply of Q [8]. The yeast *Saccharomyces cerevisiae* provides a valuable model for characterizing the eukaryotic Q biosynthetic pathway. The availability of eight complementation groups of Q deficient mutants has greatly facilitated the recovery and characterization of the genes required for Q biosynthesis [9,10]. As shown in Fig. 1, Q is synthesized from the precursors 4-hydroxybenzoic acid and polyisoprene diphosphate [8]. The 4-HB:polyprenyldiphosphate transferase, encoded by the *COQ2* gene [11], generates compound **5** in both the yeast and rat Q biosynthetic pathway. Further modifications of the aromatic ring involve a decarboxylation step, three hydroxylation steps and three methylation steps to produce the fully substituted benzoquinol ring, QH_2. Both the *C*- and *O*-methyltransferases involved in Q biosynthesis have been identified [12–14]. The Coq3 polypeptide carries out both *O*-methylation steps in Q biosynthesis [15].

The Coq3 polypeptide is located within the matrix of the mitochondria, where it is peripherally associated with the inner membrane [15]. The other Coq polypeptides are also localized to mitochondria [10]. Attempts to solubilize Coq3p in an active form from yeast mitochondria have not yet been successful [15]. In addition, expression of yeast Coq3p in *E. coli ubiG* mutants (deficient in both *O*-methyltransferase steps of Q biosynthesis; [16]) does not restore Q biosynthesis, nor does it generate detectable *O*-methyltransferase activity with either the farnesylated analog of **6** (Fig. 1) or with the prokaryotic farnesylated catechol substrate (A.Y. Hsu, unpublished data). Together, these data indicate that Coq3p may require other yeast polypeptide components for its activity or stability.

Several lines of evidence argue for an interdependence of the *COQ* gene products: (1) a single early

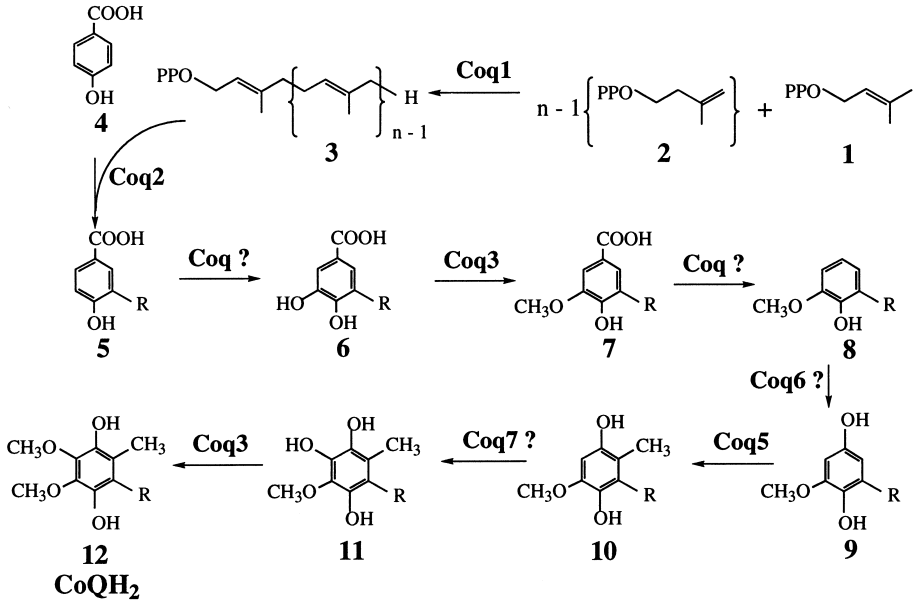

Fig. 1. The eukaryotic Q biosynthetic pathway. Dimethylallyl diphosphate (**1**) and isopentenyl diphosphate (**2**) provide the precursors for the assembly of all-*trans* polyprenyl diphosphate (**3**), as catalyzed by Coq1. The prenylation of 4-hydroxybenzoic acid (**4**) is catalyzed by Coq2 to form 3-polyprenyl-4-hydroxybenzoic acid (**5**). The other intermediates in the pathway are 3,4-dihydroxy-5-polyprenylbenzoic acid (**6**), 3-methoxy-4-hydroxy-5-polyprenylbenzoic acid (**7**), 2-polyprenyl-6-methoxy-phenol (**8**), 2-polyprenyl-6-methoxy-1,4-benzoquinol (**9**), 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol or 5-demethoxyubiquinol (**10**), 2-polyprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol or demethyl-QH_2 (**11**), coenzyme Q_nH_2 (**12**). In *S. cerevisiae*, $n=6$ and compound **5** is referred to as 3-hexaprenyl-4-hydroxybenzoate (HHB). *S. cerevisiae* gene products are identified as Coq.

intermediate in the Q biosynthetic pathway (**5**) accumulates in strains harboring mutations in *COQ3*, *COQ4*, *COQ5*, *COQ6*, *COQ7* or *COQ8*, suggesting that the absence of one *COQ* gene product prevents the function of the others [17]; (2) a yeast strain harboring a point mutation in the *COQ7* gene (*coq7-1*; $G_{104}D$) accumulates intermediate **10**, while yeast strains harboring null alleles of *coq7* produce only **5**, the earlier intermediate [18]; and (3) yeast strains harboring certain point mutations in the *COQ5* gene are rescued by expression of the *E. coli* Coq5 homologue, UbiE, however, the presence of *E. coli* UbiE does not rescue mutant strains harboring null alleles of *coq5* [19]. These findings are compatible with the coordinate regulation of the *COQ* gene products, in a manner that is similar to the synthesis of flagella proteins [20]. Alternatively, the distinct phenotypes manifested by yeast strains harboring either null or point mutations are compatible with a multi-subunit complex of the *COQ*-encoded enzymes. Precedent for this second scenario is provided by the multi-subunit complexes of the respiratory electron transport chain. For example, yeast strains harboring mutations in one of the structural genes of complex IV, cytochrome oxidase, contain markedly decreased steady state levels of several of the other components of complex IV, due to their rapid proteolytic degradation [21]. Such rapid proteolytic degradation of the unassembled components is also observed in strains containing mutations in genes encoding subunits of complex III (QH_2-cytochrome *c* reductase) [22], complex II (succinate dehydrogenase) [23] and in complex V (F_1/F_0 ATPase) [9].

The degree to which *O*-methyltransferase activity and expression are dependent on the other *COQ* gene products is the subject of this present study. A complete panel of yeast *coq* null mutants have been prepared, and the effect of the null mutations on the *COQ3*-encoded *O*-methyltransferase activity, polypeptide and RNA levels has been evaluated. The data presented here indicate that Coq3 *O*-methyltransferase activity and polypeptide levels depend on the presence of each of the other *COQ* gene products. At present, we favor the model that the low steady state level of the Coq3 polypeptide results from its enhanced degradation, resulting from either a defective or unassembled complex of Q biosynthetic enzymes.

2. Materials and methods

2.1. Yeast strains and growth media

The genotypes and sources of the mutant and wild-type yeast strains are shown in Table 1. Media for growth of yeast were prepared as described [12]. Yeast null mutant strains were generated by the one-step gene replacement procedure [28]. Segments of DNA containing either disruption or deletion mutations of the targeted genes were either generated from plasmids containing the null mutation or were PCR-amplified from the genomic DNA of designated null mutants. The deletion mutation in the *COR1* gene was constructed by replacing a 0.25 kb *Kpn*I/*Xba*I fragment internal to the *COR1* open reading frame (ORF) (1.37 kb) with a 1.7 kb *HIS3* cassette as described [25]. A deletion in the *ATP2* gene was prepared by removal of the 0.84 kb *Bam*HI/*Kpn*I fragment internal to the *ATP2* ORF and insertion of a 2.9 kb *LEU2* cassette [26]. Disruption mutations were introduced in the *COQ1* and *COQ2* genes by the insertion of a 1.15 kb *HIS3* into *Cvn*I and *Pst*I sites of the respective ORFs. Deletion of either the *COQ3*, *COQ5* or *COQ7* gene was performed as described [14,12,18,27]. Disruption of the *COQ4* gene was generated by the insertion of a 0.85 kb *TRP1* cassette into the *Sph*I site of *COQ4* ORF (accession number AF005742, GenBank) (A.Y. Hsu, unpublished data). A 0.414 kb *Bgl*II fragment containing part of the *COQ6* ORF (−146 to +268, accession number AF003698, GenBank) was replaced with a 1.7 kb *HIS3* cassette to construct both W303ΔG63 and CENΔCOQ6 (A. Tzagoloff, unpublished data). W303ΔCOQ8 and CENΔCOQ8 were generated by replacement of a 1.1 kb *Afl*II/*Sun*I fragment internal to the *COQ8* ORF with a 1.15 kb *HIS3* cassette as described (T.Q. Do and A.Y. Hsu, unpublished data). The presence of the gene disruption allele in each strain was indicated by the lack of growth on non-fermentable carbon sources, rho^+ status and verification of the presence of the disrupted allele by either Southern blotting or by PCR analysis of genomic DNA.

2.2. Preparation of mitochondria

Yeast strains were grown at 30°C in YPGal (1%

yeast extract, 2% peptone and 2% galactose) to an OD_{600} of 4.0, and mitochondria were isolated according to Yaffe [29]. The final mitochondrial pellets were resuspended in mitochondria isolation buffer (0.6 M mannitol, 20 mM HEPES–KOH, pH 7.4) with 0.5 mM PMSF and 10% glycerol (v/v) and stored in aliquots at −80°C. In selected preparations, protease inhibitors were included during cell lysis, mitochondrial isolation and storage at the following final concentrations: benzamidine, 1.0 mM; pepstatin, 2 μg/ml; and leupeptin, chymostatin, aprotinin and antipain each at 1 μg/ml. The protein concentrations of the mitochondria samples were determined by the BCA method (Pierce), and the same concentration of glycerol was used in preparing a standard curve to correct for the glycerol interference with the BCA assay.

2.3. In vitro O-methyltransferase assay

Each reaction mixture (250 μl) contained 50 mM sodium phosphate, pH 7.0, 1.0 mM $ZnSO_4$, 0.2 mM 3,4-dihydroxy-5-farnesylbenzoic acid (in 5 μl methanol), 100 μl of the mitochondrial suspension (containing from 0.70 to 1.00 mg protein) and 60 μM of radiolabeled S-adenosyl-L-[*methyl*-^{3}H]methionine (NEN-DuPont, specific activity was adjusted to 560 mCi/mmol with unlabeled *S*-adenosyl-L-methionine, Sigma). The reactions were carried out at 30°C for 60 min with mitochondria isolated from *coq* null mutant strains, and 30 min for reactions containing mitochondria isolated from wild-type, *atp2* or *cor1* mutants. These times were found to be within the linear range of the *O*-methyltransferase assay. To terminate the reaction, 2 μl of glacial acetic acid was added, and lipids were extracted twice with 0.5 ml of chloroform. The extract was then dried under a stream of N_2 gas, resuspended in methanol and analyzed by an isocratic reverse phase high performance liquid chromatography system with C18 column (Alltech Lichrosorb, 5 μM, 4.6×250 mm) and 9:1 methanol/water as mobile phase at a flow rate of 1 ml/min as described by Poon et al. [15,30]. One ml fractions were collected, mixed with 10 ml of fluor (Safety

Table 1
Genotypes and sources of *S. cerevisiae* strains

Strains	Relevant genotype	Source or reference
D273-10B	MATα, *met6*	[24]
W303.1A	MATα, *ade2-1, his3-11, leu2-3,112, trp1-1, ura3-1*	R. Rothstein[a]
W303.1B	MATα, *ade2-1, his3-11, leu2-3,112, trp1-1, ura3-1*	R. Rothstein[a]
αW303ΔCOR1	W303-1B, *cor1::HIS3*	[25]
CC304.1	W303-1B, *atp2::LEU2*	[26]
W303ΔCOQ2	W303-1A, *coq2::HIS3*	[11]
CC303	W303-1B, *coq3::LEU2*	[26]
W303ΔCOQ4	W303-1A, *coq4::TRP1*	This work
W303ΔCOQ5	W303-1B, *coq5::HIS3*	[12]
W303ΔG63	W303-1B, *coq6::HIS3*	A. Tzagoloff[b]
W303ΔCOQ7	W303-1B, *coq7::LEU2*	[18]
W303ΔCOQ8	W303-1A, *coq8::HIS3*	This work
CEN.PK2-1C	MATα, *ura3, his3, leu2, trp1*	[27]
CENΔCOR1	CEN.PK2-1C, *cor1::HIS3*	This work
CENΔATP2	CEN.PK2-1C, *atp2::LEU2*	This work
CENΔCOQ1	CEN.PK2-1C, *coq1::HIS3*	This work
CENΔCOQ2	CEN.PK2-1C, *coq2::HIS3*	This work
CENΔCOQ3	CEN.PK2-1C, *coq3::LEU2*	This work
CENΔCOQ4	CEN.PK2-1C, *coq4::TRP1*	This work
CENΔCOQ5	CEN.PK2-1C, *coq5::HIS3*	This work
CENΔCOQ6	CEN.PK2-1C, *coq6::HIS3*	This work
CEN.MP3-1A	CEN.PK2-1C, *coq7/cat5::HIS3*	[27]
CENΔCOQ8	CEN.PK2-1C, *coq8::HIS3*	This work

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[b]Department of Biological Sciences, Columbia University, New York, USA.

Solve, RPI, Mount Prospect, IL, USA) and subjected to scintillation counting. The counting efficiency of ^3H was 40.5%. Radioactivity present in fractions 7–9 (or in some analyses 8–10) represented material that co-eluted with the methylated product standard, 3-methoxy-4-hydroxy-5-farnesylbenzoic acid. The sum of the radioactivity in these fractions, minus the background radioactivity (defined as fractions 6, 11 and 12), was used to calculate the Coq3 *O*-methyltransferase activity (expressed as cpm/mg protein/h).

2.4. Western analysis of Coq3 polypeptide

Equal amounts of protein from isolated mitochondria were separated by electrophoresis on 12% polyacrylamide sodium dodecyl sulfate (SDS)–Tris–glycine gels 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 washing buffer. Affinity-purified antibodies to a GST-Coq3 fusion protein [15] were used at a final concentration of 1.0 μg/ml, and alkaline phosphate-conjugated goat anti-rabbit secondary antibodies (Amersham Pharmacia Biotech) were used at a 1:2000 dilution.

2.5. RNA isolation and Northern analysis

Yeast total RNA was isolated from the yeast strains prepared from the CEN.PK2-1C background as listed in Table 1. Yeast was grown in YPGal media, incubated at 30°C with shaking, harvested when cell density reached an OD_{600} of 1.0 and RNA was isolated by the hot acidic phenol method as described [31]. RNA was analyzed by electrophoresis on formaldehyde gels [32], transferred to Biodyn (ICN) nylon membranes and baked for 2 h at 80°C. A DNA segment containing the *COQ3* gene was amplified by PCR with primers pBC3-1 (5′-TAAATTTCTGAGCTCGCCCCCGGGTATTTCATTTG-3′) and pBC3-2 (5′-CGCGGGATCCATTCAGTCTCTGAATAGCCA-3′) and genomic DNA from the wild-type yeast strain D273-10B. The resulting PCR product, containing the entire *COQ3* ORF and 650 bp 5′ non-coding sequence, was labeled by random priming with [α-^{32}P]dCTP (3000 Ci/mmol, ICN Biochemicals) and the Oligolabeling kit (Amersham Pharmacia Biotech). Unincorporated nucleotides were eliminated with NucTrap push column (Stratagene). An actin specific probe was prepared similarly by PCR with forward primer pACT1 (5′-ATGTGTAAAGCCGGTTTTGC-3′) and reverse primer pACT2 (5′-TTAGAAACACTTGTGGTGAA-3′) to generate 1.083 kb yeast actin DNA product containing the entire ORF for labeling. Hybridization was performed at 57°C for 18 h as described [33]. Blots were washed twice with 2×SSC, 0.1% SDS at room temperature, and twice for 15 min each (55°C) with 0.2×SSC, 0.1% SDS, and analyzed with a phosphorimager (Molecular Dynamics, version 4.0).

3. Results and discussion

3.1. Coq3 O-methyltransferase activity is decreased in the coq1, coq2, coq4, coq5, coq6, coq7 and coq8 null mutants

Yeast strains were constructed that harbored null mutations in one of the *COQ* genes, or in either the *ATP2* or *COR1* gene (Table 1). The latter two genes encode subunits of either complex III (the Cor1 subunit of the bc_1 complex) or complex V (the β-subunit of the F_1 ATPase), and provide respiratory deficient control strains [25,34]. Two different laboratory 'wild-type' yeast backgrounds were employed, in order to assess whether the genetic background might introduce variability in the assays of *O*-methyltransferase activity.

Mitochondria were prepared and assayed for the amount of *O*-methyltransferase activity with a farnesylated analog of compound **6** (Fig. 1) as substrate, as described in Section 2. As shown in Fig. 2A, **6**:*O*-methyltransferase activity was greatly decreased in each of the *coq* null mutants. The **6**:*O*-methyltransferase activity was also decreased in the *atp2* and *cor1* null mutant strains relative to wild-type, however, this decrease was not nearly as dramatic as that observed for the *coq* mutants. The second *O*-methylation step of Q biosynthesis was also assayed in the *coq4* null mutant strain. As observed in assays of the first *O*-methyltransferase step, the *coq4* null mutant strains were found to have only 4% of the wild-type level of **11**:*O*-methyltransferase activity (data not

shown). A similar degree of impairment in these two steps is not surprising, since the Coq3 *O*-methyltransferase is responsible for both *O*-methylation steps of Q biosynthesis [15].

Assays of **6**:*O*-methyltransferase activity were also performed on mitochondria isolated from mutants constructed in a different background (CEN.PK2-1C). As shown in Fig. 2B, there was a consistent trend among the mutant strains; *atp2* and *cor1* had the highest levels of *O*-methyltransferase activity, followed by *coq6* and *coq4*. *O*-Methyltransferase activity levels in the *coq1*, *coq2*, *coq7* and *coq8* mutant strains were significantly decreased relative to the *cor1* null mutant. However, considerable variation in the level of *O*-methyltransferase activity was observed in mitochondria isolated from both yeast strains, resulting in the large S.D.s shown. We then investigated the source of this variability. As shown in the inset of Fig. 2B, the variability cannot be attributed to the *O*-methyltransferase assay itself, since measurements of activity within a given preparation of mitochondria are reproducible. Purification of mitochondria from the *coq6* mutant strain in the presence of various protease inhibitor cocktails was carried out to address the possibility that mitochondria isolated from the *coq* mutants exhibit an enhanced susceptibility to proteases. However, the inhibitors tested (see Section 2) had no effect on reducing the large variation observed in measurements of **6**:*O*-methyltransferase activity. The *coq* mutants are known to be sensitive to certain types of oxidative stress [26,17] and it seemed possible that the *O*-methyltransferase activity might be prone to oxidative damage. To test this idea, either 0.5 mM trolox (a water soluble analog of α-tocopherol) or 15 μM Q_6 (a concentration which restores growth of each of the *coq* mutant strains on non-fermentable carbon sources) was added to the growth media. In addition, the effect of freezing and thawing on the samples was studied. However, none of these adjustments solved the problem of the inherent high variability experienced in assaying the amount of *O*-

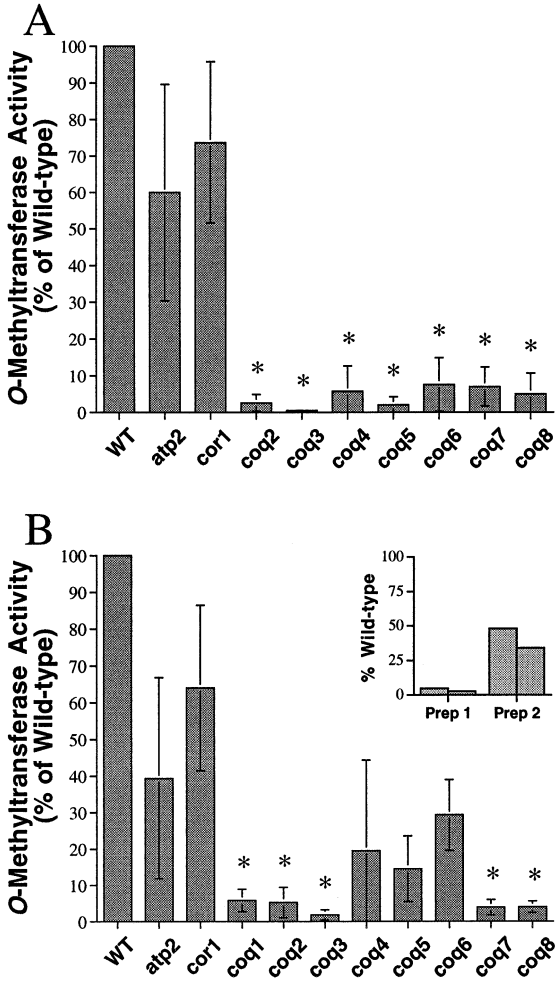

Fig. 2. Yeast mutants harboring deletions in any *COQ* gene have decreased levels of Coq3 *O*-methyltransferase activity. Yeast *coq*, *atp2* and *cor1* null mutants were generated in two yeast genetic backgrounds, W303 (A) and CEN.PK2-1C (B). The activity of the Coq3-mediated **6**:*O*-methyltransferase in mitochondrial preparations from these yeast strains was measured. The in vitro assays were performed with 3,4-dihydroxy-5-farnesylbenzoic acid (**6**) as the methyl acceptor substrate and *S*-adenosyl-L-[*methyl*-^{3}H]methionine as described in Section 2. Following incubation, lipids were extracted and analyzed by reverse phase high performance liquid chromatography and the radioactivity was determined in each 1 ml fraction. The radioactivity co-eluting with the 3-methoxy-4-hydroxy-5-farnesylbenzoic acid standard, compound **7**, provided a measure of *O*-methyltransferase activity (expressed as pmol methyl groups/h/mg protein). The *O*-methyltransferase activity from each strain is expressed as percentage of the wild-type activity. A and B represent the average values (±S.D.) from three sets of mitochondrial preparations. The average wild-type activity (100%) in A was 24.48±4.53 pmol/h/mg protein and in B was 7.20±2.37 pmol/h/mg protein. An asterisk (*) designates that activity is significantly different from the *cor1*Δ control strain; $P=0.05$. The inset in B shows duplicate determinations of *O*-methyltransferase activity for each of two separate preparations of mitochondria from the *coq4* null mutant strain CENΔCOQ4.

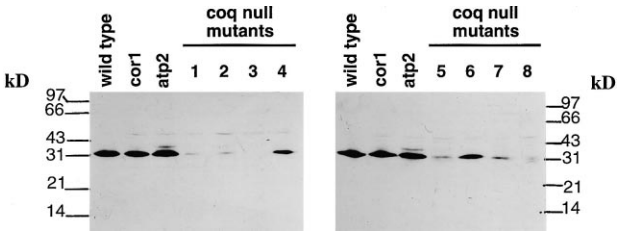

Fig. 3. Analysis of the levels of steady state Coq3 polypeptide in yeast strains harboring deletions in *COR1*, *ATP2* or one of the *COQ* genes. Mitochondria were prepared from wild-type and mutant yeast strains of the CEN.PK2-1C background as described in Fig. 2. Samples of mitochondria (100 μg of protein) were separated by SDS–polyacrylamide gel electrophoresis and then analyzed by Western blotting with antibodies to Coq3p as described in Section 2. The following values designate the respective levels of *O*-methyltransferase activity and Coq3 polypeptide expressed as percentage of wild-type (100%): *cor1*Δ, (65/95); *atp2*Δ, (39/108); *coq1*Δ, (5.0/7.3); *coq2*Δ, (3.0/9.9); *coq3*Δ, (1.0/0); *coq4*Δ, (7.8/47); *coq5*Δ, (9.1/20); *coq6*Δ, 27.1/61); *coq7*Δ, (2.8/22) and *coq8*Δ, (5.0/9.2).

methyltransferase activity in the respiration defective yeast strains. Similar problems with variability in assays of NADH oxidase and NADH-cytochrome *c* reductase have been reported by others when studying respiration defective yeast mutants [35].

3.2. The steady state level of Coq3 polypeptide is decreased in coq mutant strains

The above results indicate that the presence of the other *COQ* genes is required to observe normal levels of *O*-methyltransferase activity. Such a requirement could reflect the involvement of the other *COQ* gene products in the expression or stability of the Coq3 polypeptide. In order to investigate this possibility, levels of the Coq3 polypeptide were evaluated by Western analysis with an antibody specific for yeast Coq3p [15]. Fig. 3 shows that decreased levels of the Coq3 polypeptide are present in mitochondria prepared from the *coq* null mutants. Within the *coq* mutant panel, Coq3p levels are present at highest levels in the *coq4* and *coq6* mutants. When this analysis was performed with two other sets of independently isolated mitochondria from the CEN.PK2-1C background, the same trend was observed. Coq3p levels were dramatically decreased in the *coq* mutant panel relative to wild-type, *cor1* or *atp2* mutants, but were highest in the *coq4* and *coq6* null mutants (Fig. 4). However, there does not appear to be a strict correlation between the amount of Coq3 polypeptide and the level of *O*-methyltransferase activity. This is apparent from the values presented in Fig. 3, legend; when the *O*-methyltransferase activity was normalized to the amount of Coq3p, the *O*-methyltransferase activity is still greatly reduced. Figs. 2–4 show that the *atp2*Δ and *cor1*Δ respiratory deficient control mutant strains appear to have decreased *O*-methyltransferase activity but not a decreased level of Coq3p. Perhaps in the absence of respiration there may be a decreased need for coenzyme Q, and the activity of enzymes in the Q biosynthetic pathway is subject to repression. In summary, two classes of *coq* null mutants can be identified: (1) those with very low or absent Coq3p and *O*-methyltransferase activity (e.g. *coq1*, *coq2*, *coq5*, *coq7* and *coq8*); and (2) those with relatively high Coq3p and detectable *O*-methyltransferase activity (*coq4* and *coq6*).

3.3. COQ3 RNA levels are not decreased in the panel of coq mutants

To examine the possibility that a lack of one of the *COQ* gene products results in a lower level of *COQ3*

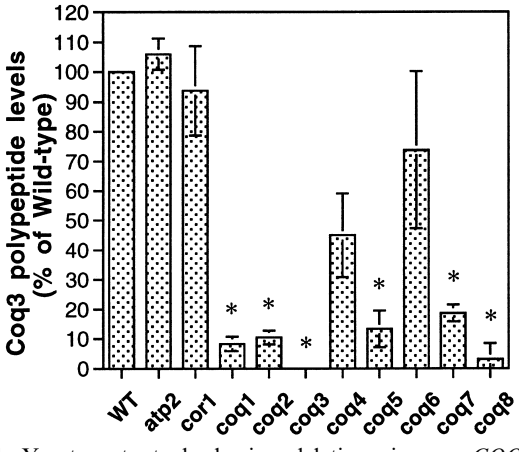

Fig. 4. Yeast mutants harboring deletions in any *COQ* gene have decreased levels of steady state Coq3 polypeptide. Three independent preparations of mitochondria isolated from wild-type and mutant yeast strains of the CEN.PK2-1C background were subjected to Western blotting as described in Fig. 3. The relative amounts of Coq3 polypeptide were quantified by densitometric scanning with an Alpha Imager 2000 system. The average ± S.D. (in arbitrary units) is plotted as percent of the signal present in wild-type yeast. An asterisk (*) designates that activity is significantly different from the *cor1*Δ control strain; $P = 0.05$.

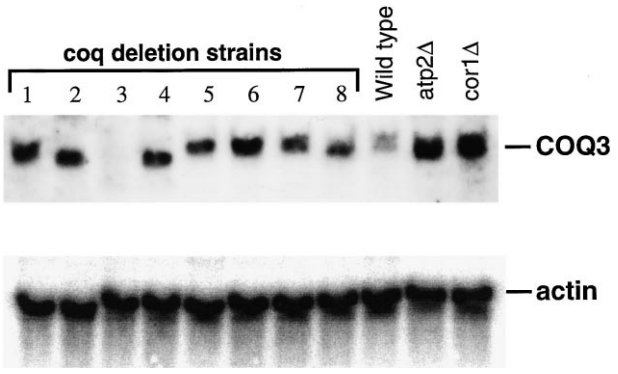

Fig. 5. *COQ3* RNA levels are not decreased in the other yeast *coq* null mutants. Yeast total RNA was prepared as described in Section 2 and 20 μg was analyzed on each lane of a denaturing agarose gel and blotted to a nylon membrane. Hybridizations were performed as described in Section 2 with the ^{32}P-labeled probes corresponding to *COQ3* (upper panel) or to yeast actin (lower panel).

RNA, Northern blot analysis was performed. As shown in Fig. 5, *COQ3* steady state RNA levels are not decreased in any of the *coq* mutants, relative to either wild-type or respiratory deficient control strains. In fact, the level of the *COQ3* mRNA is significantly increased in the *coq* deletion mutant panel (*coq1*, *2*, *4–8*) and even more so in the *atp2* and *cor1* deletion mutant strains. These results are quite striking and are in the opposite direction as might be predicted from the activity and protein levels of Coq3p. The finding of elevated levels of *COQ3* RNA suggests that either the translation of the Coq3 polypeptide is decreased or that Coq3 polypeptide is produced at normal levels but is less stable.

3.4. Is a multi-subunit complex involved in Q biosynthesis?

The data presented here show that Coq3p *O*-methyltransferase activity is greatly decreased in *coq1*, *coq2*, *coq4*, *coq5*, *coq6*, *coq7* or *coq8* null mutant strains. The *coq* null strains also have greatly decreased levels of the Coq3 polypeptide, yet levels of *COQ3* RNA are not decreased. The data are consistent with two models: (1) an involvement of the *COQ* gene products in regulating the Coq3 polypeptide at the level of translation; and (2) an involvement of the Coq polypeptides (or their lipid products) in a multi-subunit complex. In the latter case, a deficiency in any one of the *COQ* gene products would result in a defective complex in which the Coq3 polypeptide is rendered unstable. Analysis of the rates of Coq3 polypeptide synthesis and degradation should distinguish between these two models. Our attempts to solubilize Coq3 activity and determine the native molecular weight by sonication or detergent treatment have not yet met with success [15].

We have previously postulated the involvement of a multi-subunit complex in converting **5** to Q [18]. This model is consistent with the accumulation of **5** as the sole predominant intermediate detected in each of the *coq* null mutants (*coq3–coq8*) [17]. This model is also consistent with the observation that the activity of the *COQ1* and *COQ2* encoded enzymes is independent of the other *COQ* gene products [36]. However, it is notable that both the *coq1* and *coq2* null mutants each have profoundly decreased levels of *O*-methyltransferase activity and Coq3 polypeptide (Fig. 2–4). Are these polypeptides also part of the postulated multi-subunit complex? While possible, this scenario does not seem likely, because *COQ1* homologues from a diverse array of species have been shown to restore Q synthesis in yeast *coq1* null mutants [37,38]. For example, the *Haemophilus influenzae ispB* gene (GenBank accession no. U32770; 28% amino acid identity with *S. cerevisiae* Coq1) encodes a heptaprenyl diphosphate synthase in demethyl-menaquinone biosynthesis but does not participate in Q biosynthesis because *H. influenzae* does not produce Q [39]. Similarly, the *ddsA* gene from *Gluconobacter suboxydans* encodes a decaprenyl diphosphate synthase with relatively low sequence identity to yeast Coq1 (DDBJ accession no. AB006850; 26% amino acid identity with *S. cerevisiae* Coq1) [40]. Expression of either the *H. influenzae ispB* gene or the *G. suboxydans ddsA* gene in a *coq1* null mutant strain of *S. cerevisiae* restores respiration and the synthesis of either Q_7 or Q_{10}, respectively [38]. These and other experiments by Okada et al. [37,38] show that the Q isoform produced (Q_5 to Q_{10}) depends solely on the type of *COQ1* homologue being expressed. It seems unlikely that each of these distinct Coq1 homologues would function to reconstitute a multi-subunit complex. An alternate, more probable model is that the lipid product formed by Coq1 and Coq2 (e.g. compound **5**) may provide the component required for the assembly or stability of

the complex. In this model, the low level of Coq3p and *O*-methyltransferase activity detected in the *coq1* and *coq2* yeast mutant strains may result from the absence of **5**, as opposed to the absence of the Coq1 or Coq2 polypeptides.

Compound **5** is readily detected in wild-type yeast [30]. In fact, when cells are cultured in glucose containing media in the presence of [U-^{14}C]-4-hydroxybenzoic acid and harvested at log phase, there is 4-fold more radioactivity recovered in **5** than in Q [30]. Compound **5** is also the predominant Q-intermediate in rat heart mitochondria [41]. While the accumulation of this intermediate may serve as a stockpile of a biosynthetic intermediate committed to Q, it may also act as an important component of the Q biosynthetic complex and aid in either the assembly or stability of such a complex. Precedent for this idea derives from the work by Knoell [42,43] in the study of Q biosynthesis in *E. coli*. Although the *ubi* mutants of *E. coli* each accumulate distinct intermediates that are diagnostic of the blocked step [44], there is evidence that the Ubi polypeptides form a complex together with a pool of the prokaryotic Q biosynthetic precursor, 2-octaprenyl phenol [42,43].

The genetic analysis of the different types of *coq* yeast mutants indicates that some components may play relatively peripheral roles in complex assembly or stability. For example, the yeast *COQ3* gene can be functionally replaced by human, rat, *Arabidopsis* or *E. coli COQ3* homologues [45–47,16]. In each case, the rescue is less optimal than with the wild-type yeast gene; for example, rescue with the human homologue requires its presence on a multi-copy plasmid, yet growth is much slower and levels of Q are decreased about 70% [45]. Similarly, the *COQ7* gene can be functionally replaced by human, rat and *Caenorhabditis elegans* homologues, when present on multi-copy plasmids [48–50]. These studies suggest that certain polypeptide components are readily replaced by homologues from different species, and that their function in the complex assembly or stability may not be critical, or may be indirect (as postulated for Coq1p above). While the rescue of each of the other *coq* mutants is still under investigation, it is clear that the yeast *COQ5* gene cannot be replaced by its *E. coli* homologue, *ubiE*. In fact, from the analysis of specific point mutant alleles, it is clear that the yeast *COQ5* gene product plays a second function in stabilizing yeast Coq3p [19]. Thus, some of the components may provide crucial roles in the assembly or stability of the putative complex, while others may require the complex for their activity but are themselves only peripheral components.

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