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# Coq6 Is Responsible for the C4-deamination Reaction in Coenzyme Q Biosynthesis in *Saccharomyces cerevisiae*\*

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**Background:** A deamination reaction is necessary for yeast to synthesize coenzyme Q from *para*-aminobenzoic acid.

**Results:** The deamination requires dioxygen and an active Coq6 enzyme.

**Conclusion:** The flavin monooxygenase Coq6 is responsible for the substitution of the amino group with a hydroxyl group.

**Significance:** Coq6 hydroxylates two positions of its amino substrate and partly determines whether an organism can synthesize coenzyme Q from *para*-aminobenzoic acid.

The yeast *Saccharomyces cerevisiae* is able to use *para*-aminobenzoic acid (pABA) in addition to 4-hydroxybenzoic acid as a precursor of coenzyme Q, a redox lipid essential to the function of the mitochondrial respiratory chain. The biosynthesis of coenzyme Q from pABA requires a deamination reaction at position C4 of the benzene ring to substitute the amino group with an hydroxyl group. We show here that the FAD-dependent monooxygenase Coq6, which is known to hydroxylate position C5, also deaminates position C4 in a reaction implicating molecular oxygen, as demonstrated with labeling experiments. We identify mutations in Coq6 that abrogate the C4-deamination activity, whereas preserving the C5-hydroxylation activity. Several results support that the deletion of Coq9 impacts Coq6, thus explaining the C4-deamination defect observed in  $\Delta coq9$  cells. The vast majority of flavin monooxygenases catalyze hydroxylation reactions on a single position of their substrate. Coq6 is thus a rare example of a flavin monooxygenase that is able to act on two different carbon atoms of its C4-aminated substrate, allowing its deamination and ultimately its conversion into coenzyme Q by the other proteins constituting the coenzyme Q biosynthetic pathway.

Coenzyme Q (ubiquinone or Q)<sup>5</sup> is a redox-active lipid essential for electron and proton transport in the respiratory

chain of mitochondria and some bacteria (1–3). Q also serves as a membrane antioxidant and a cofactor of uncoupling proteins (1) and has recently been implicated in osmoprotection in *Escherichia coli* (4). Q is composed of a fully substituted benzoquinone ring that is attached to a polyisoprenyl tail of various length (six isoprenyl units in *Saccharomyces cerevisiae* hence Q<sub>6</sub>, 10 units in humans, hence Q<sub>10</sub>). In *S. cerevisiae*, the biosynthesis of Q takes place in the mitochondrial matrix and implicates at least 12 proteins, Coq1–Coq9, Coq11, Arh1, and Yah1 (5, 6), most of them being conserved in humans. Most Coq proteins form a multisubunit biosynthetic complex in *S. cerevisiae* (termed the CoQ-synthome), which is destabilized by the absence of a single Coq polypeptide, causing a drastic diminution of the steady state levels of some Coq proteins in  $\Delta coq$  deletion mutants (7–9). The instability of several Coq proteins in such mutants can be corrected through overexpression of the Coq8 kinase (10) or by supplementing the growth medium with Q<sub>6</sub> (11). Under such stabilizing conditions, the Coq proteins are functional and allow accumulation of Q biosynthetic intermediates in  $\Delta coq$  strains (10), which provide information on the reaction deficient in a given  $\Delta coq$  mutant.

In 2010, Clarke's group and ourselves (5, 12) discovered that *S. cerevisiae* is able to use *para*-aminobenzoic acid (pABA) in addition to 4-hydroxybenzoic acid (4-HB) as a precursor of Q<sub>6</sub> (Fig. 1). Endogenous pABA and 4-HB originate from chorismate and are limiting for Q<sub>6</sub> biosynthesis because their addition to the growth medium increases Q<sub>6</sub> levels in *S. cerevisiae* (5). pABA and 4-HB enter the Q<sub>6</sub> biosynthetic pathway via the prenylation reaction catalyzed by Coq2 and then multiple enzymes modify the aromatic ring to yield Q<sub>6</sub> (Fig. 1). Competition experiments demonstrated that pABA and 4-HB provided

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<sup>5</sup> The abbreviations used are: Q, coenzyme Q; pABA, *para*-aminobenzoic acid; FMO, flavin monooxygenase; 4-HB, 4-hydroxybenzoic acid; 3,4-diHB, 3,4-dihydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; HAB,

3-hexaprenyl-4-aminobenzoic acid; DHHB, 3-hexaprenyl-4,5-dihydroxybenzoic acid; 4-AP<sub>6</sub>, 3-hexaprenyl-4-aminophenol; 4-HP<sub>6</sub>, 3-hexaprenyl-4-hydroxyphenol; HHAB, 3-hexaprenyl-4-amino-5-hydroxybenzoic acid; IDMQ<sub>6</sub>, 4-imino-demethoxy-Q<sub>6</sub>; DDMQ<sub>6</sub>, demethyl-demethoxy-Q<sub>6</sub>; DMQ<sub>6</sub>, demethoxy-Q<sub>6</sub>; pHBH, *para*-hydroxybenzoate hydroxylase; ECD, electrochemical detection; YNB, yeast nitrogen base; 3H4AB, 3-hydroxy-4-aminobenzoic acid; VA, vanillic acid; IDDMQ<sub>6</sub>, 4-imino-demethyl-demethoxy-Q<sub>6</sub>; huCoq6, human Coq6.

## Dioxygen-mediated Deamination in Coenzyme Q Biosynthesis

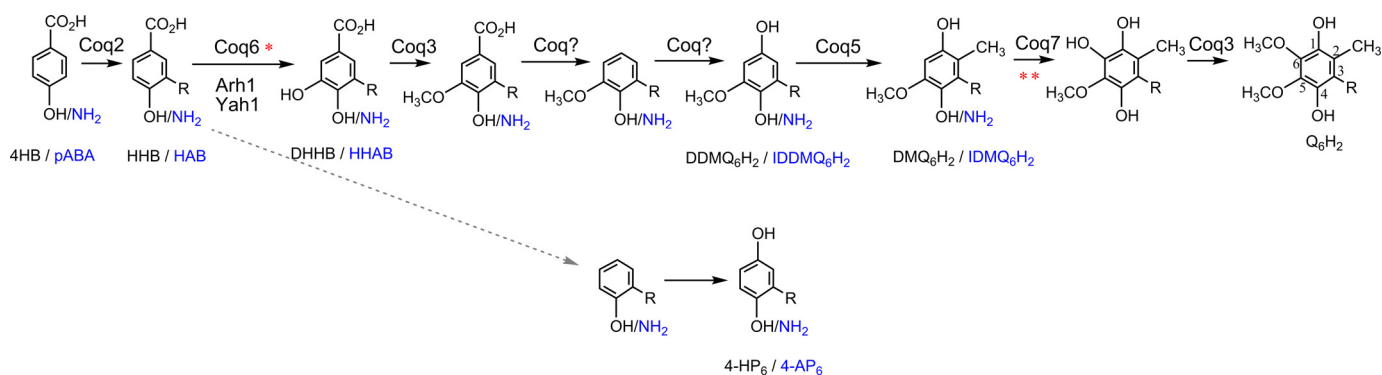


FIGURE 1. *S. cerevisiae*  $Q_6$  biosynthetic pathway. 4-HB and pABA differ by the presence of a hydroxyl (black) or an amino group (blue) at position C4. The numbering of the aromatic carbon atoms used on all  $Q_6$  biosynthetic intermediates mentioned in this study is shown on the reduced form of  $Q_6$ ,  $Q_6H_2$ . 4-HB and pABA serve as precursors for  $Q_6$  biosynthesis and are prenylated by Coq2 to form HHB and HAB, respectively. *R* represents the hexaprenyl tail. The presence of a hydroxyl or an amino group at position C4 of intermediates is represented by OH/ $NH_2$  and the respective names are indicated: DHHB, HHAB,  $DDMQ_6H_2$ , and  $DMQ_6H_2$  are the reduced forms of demethyl-demethoxy- $Q_6$  ( $DDMQ_6$ ) and demethoxy- $Q_6$  ( $DMQ_6$ );  $IDDMQ_6H_2$  and  $IDMQ_6H_2$  are the reduced forms of 4-imino-demethyl-demethoxy- $Q_6$  ( $IDDMQ_6$ ) and 4-imino-demethoxy- $Q_6$  ( $IDMQ_6$ ). The C4-deamination reaction occurs at an undefined step and  $IDMQ_6$  is the most downstream amino-containing intermediate identified to date. Upon inactivation of *coq6*, HHB and HAB are decarboxylated (dashed arrow) and hydroxylated at position C1, yielding 4- $HP_6$  and 4- $AP_6$ . Steps impaired in the  $\Delta coq9$  strain are designated with a red asterisk (\*) for partial inactivation of the reaction, and double asterisk (\*\*\*) for complete inactivation.

exogenously are equally efficient at promoting  $Q_6$  biosynthesis (12). We also showed that several synthetic analogs of 4-HB (2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid (3,4-diHB), and vanillic acid (VA)) can serve as precursors of  $Q_6$  and can bypass deficient biosynthetic steps (10, 13). In particular, addition of VA or 3,4-diHB to the growth medium restores  $Q_6$  biosynthesis in  $\Delta coq6$  cells deficient for the C5-hydroxylation reaction (13). Nevertheless, the effect of VA was annihilated by minute amounts of 4-HB. This result could either reflect an inefficient transport of VA to the mitochondrial matrix and/or a higher affinity of Coq2 for 4-HB than for VA (13).

Coq6 catalyzes the C5-hydroxylation reaction (13) and according to its primary sequence, it belongs to the family of class A flavoprotein monooxygenases (FMOs) (14). Class A FMOs contain a flavin adenine dinucleotide (FAD) and we recently demonstrated the presence of FAD in purified Coq6.<sup>6</sup> The prototype enzyme of class A FMOs is *para*-hydroxybenzoate hydroxylase (16), which catalyzes the *ortho*-hydroxylation of 4-HB in some bacteria (17). In *para*-hydroxybenzoate hydroxylase the FAD cofactor is reduced by NADPH and then reacts with dioxygen to form a FAD-hydroperoxide intermediate, which transfers a hydroxyl group onto the substrate (17, 18). The resulting FAD-hydroxide then eliminates water, which results in oxidized FAD, the starting point of the next catalytic cycle. All class A FMOs proceed similarly and thus use one oxygen atom of the dioxygen molecule for hydroxylation of the substrate, whereas the other oxygen atom is reduced into water (16). UbiI, UbiH, and UbiF are class A FMOs used by *E. coli* to catalyze the three hydroxylation reactions of  $Q_8$  biosynthesis (19) and early *in vivo* labeling experiments indeed demonstrated the incorporation of molecular oxygen into three hydroxyl groups of  $Q_8$  (20). In *S. cerevisiae*, only two hydroxylases are presently known (Fig. 1). The FMO Coq6 catalyzes the C5-hydroxylation (13), whereas Coq7 catalyzes the C6-hydrox-

ylation (21, 22) and uses a dinuclear iron center to activate dioxygen (23).

To convert pABA into  $Q_6$ , *S. cerevisiae* must replace the C4-amino group with a C4-hydroxyl group in a reaction termed C4-deamination (5, 10). Coq6 and Coq9 are thought to be important for the C4-deamination reaction because cells lacking either protein accumulate C4-amino containing intermediates when grown with exogenous pABA (10, 13, 24).  $\Delta coq6$  cells overexpressing Coq8 (+ *COQ8*) form 3-hexaprenyl-4-amino-phenol (4- $AP_6$ ) in the presence of pABA and 3-hexaprenyl-4-hydroxyphenol (4- $HP_6$ ) when 4-HB is used as a precursor (13) (Fig. 1). The accumulation of 4- $HP_6$  and 4- $AP_6$  shows that the C1-decarboxylation and C1-hydroxylation reactions can take place in the absence of a C5-methoxy group.  $\Delta coq9$  + *COQ8* cells produce 4- $AP_6$  and 4-imino-demethoxy- $Q_6$  ( $IDMQ_6$ ) with pABA, and 4- $HP_6$  and demethoxy- $Q_6$  ( $DMQ_6$ ) with 4-HB (10) (Fig. 1). Thus deletion of Coq9 causes a partial inactivation of the C5-hydroxylation reaction catalyzed by Coq6 and a complete impairment of the C6-hydroxylation reaction catalyzed by Coq7 (10). Accordingly, human Coq9 was demonstrated to bind lipids and to associate with Coq7, leading to the suggestion that Coq9 may present  $DMQ_{10}$  to Coq7 (25). The role played by Coq6 and Coq9 in the yeast C4-deamination reaction is unclear and the step at which this reaction takes place is not defined (Fig. 1). The formation of  $IDMQ_6$  from pABA in  $\Delta coq9$  + *COQ8* cells shows that all Coq biosynthetic enzymes up to Coq7 can accommodate substrates with a C4-amino group. However, the predominant accumulation of demethyl-demethoxy- $Q_6$  ( $DDMQ_6$ ) and  $DMQ_6$  in  $\Delta coq5$  + *COQ8* and  $\Delta coq7$  + *COQ8* cells grown in the presence of pABA suggests that the C4-deamination reaction may take place prior to the C2-methyltransferase reaction catalyzed by Coq5 (10).

In this study, we address the question of the C4-deamination step allowing pABA conversion into  $Q_6$  in *S. cerevisiae*. We unambiguously show that this reaction is achieved by Coq6. We provide evidence supporting that the C4-deamination occurs on an early intermediate of  $Q_6$  biosynthetic pathway, namely 3-hexaprenyl-4-amino-5-hydroxybenzoic acid (HHAB) and

<sup>6</sup> A. Ismail, V. Leroux, M. Smaja, L. Gonzalez, M. Lombard, F. Pierrel, C. Mellot-Draznieks, and M. Fontecave, *Coenzyme Q biosynthesis: evidence for a substrate access channel in the FAD-dependent monooxygenase Coq6*. submitted for publication.

# Dioxygen-mediated Deamination in Coenzyme Q Biosynthesis

**TABLE 1**  
Plasmids used in this study

Name	Vector base	Plasmid relevant genes	Copy number	Source
pCOQ8	pRS423	<i>S. cerevisiae</i> COQ8 under its own promoter	High copy	This work
pCOQ6 G386A-N388D	pRS416	<i>S. cerevisiae</i> COQ6 G386A-N388D under <i>MET25</i> promoter	Low copy	13
pCOQ6	pCM189	<i>S. cerevisiae</i> COQ6 under its own promoter	Low copy	36
pCOQ6 M469X	pCM189	<i>S. cerevisiae</i> COQ6 M469X under its own promoter	Low copy	36
pCOQ6 F420C	pCM189	<i>S. cerevisiae</i> COQ6 F420C under its own promoter	Low copy	36
pCOQ6 A361D	pCM189	<i>S. cerevisiae</i> COQ6 A361D under its own promoter	Low copy	36
pCOQ6 G248R	pCM189	<i>S. cerevisiae</i> COQ6 G248R under its own promoter	Low copy	36
pCOQ6 L382E	pCM189	<i>S. cerevisiae</i> COQ6 L382E under its own promoter	Low copy	15
phuCOQ6	pCM189	Human COQ6 under <i>CYC1</i> promoter	Low copy	36

**TABLE 2**  
*S. cerevisiae* strains used in this study

Strain	Genotype	Source
W303	<i>MAT</i> $\alpha$ , <i>leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>	R. Rothstein
$\Delta$ <i>coq6</i> ( $\alpha$ W303 COQ6-2)	<i>MAT</i> $\alpha$ , <i>coq6::LEU2 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>	29
W303 COQ6-2	<i>MAT</i> $\alpha$ , <i>coq6::LEU2 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>	29
$\Delta$ <i>coq6</i> $\Delta$ <i>coq9</i>	<i>MAT</i> $\alpha$ , <i>coq6::LEU2 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 coq9::kanMX</i>	This work
$\Delta$ <i>coq9</i>	<i>MAT</i> $\alpha$ , <i>ylr201c::kanMX4 his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	Euroscarf

that the reaction proceeds via hydroxylation of the C4-amino carbon atom of HHAB and subsequent loss of the amino group. We further show that Coq9 plays only an indirect role in the C4-deamination reaction, likely by affecting the C-terminal region of Coq6. Collectively, our results define Coq6 as an FMO that catalyzes two sequential hydroxylation reactions on two adjacent aromatic carbon atoms when pABA is used as a precursor of Q<sub>6</sub> by *S. cerevisiae*.

## Experimental Procedures

**Plasmids and Chemicals**—Plasmids used in this study are listed in Table 1. pCOQ8 was constructed by amplifying the *S. cerevisiae* COQ8 gene from genomic DNA using the primers COQ8\_3Xho (5'-GCTATTGGCAGAAAGctcgagCGTTGCTAAG) and COQ8\_5Eco (5'-GGTCTgaattcGATCCGGGTGTTCCGG) for the PCR. The PCR product and the pRS423 plasmid were digested with EcoRI and XhoI, purified from agarose gel, and ligated. One plasmid containing the insert was checked by DNA sequencing. All chemicals were from Sigma. The synthesis of <sup>13</sup>C<sub>7</sub>-pABA has been described (5).

**Yeast Strains and Culture Conditions**—*S. cerevisiae* strains used in this study are listed in Table 2. DNA transformations were performed with the PEG-lithium acetate method as previously reported (26).

The cells were grown in yeast nitrogen base (YNB) without pABA and folate (YNB-p) from MP Biomedicals. YNB-p was supplemented with 10  $\mu$ M FeCl<sub>3</sub>, with carbon sources (glucose, galactose, or lactate/glycerol at 2% (w/v)) and with amino acids and nucleobases to cover the yeast auxotrophies except for the selectin of plasmids. For solid media, Bacto-agar (Euromedex) was added at 1.6% (w/v). Typically, strains were incubated overnight at 30 °C, 180 rpm in 2 ml of medium containing 2% (w/v) galactose and 0.2% (w/v) glucose. This preculture was diluted 100-fold in 5 ml of medium containing 2% (w/v) galactose and the cells were cultured at 30 °C at 180 rpm until A<sub>600</sub> reached ~2. The cells were either used for serial dilution growth assay or for quinone content analysis. For mitochondrial preparations, yeast cells were grown at 30 °C in rich YPGal medium containing 2% (w/v) bacto-peptone (Difco), 1% (w/v) yeast extract (Difco), and 2% (w/v) galactose.

The  $\Delta$ *coq6* $\Delta$ *coq9* strain was constructed by transforming the W303 COQ6-2 strain with a PCR product corresponding to the kanMX4 cassette flanked by the COQ9 promoter and terminator regions. This PCR product was obtained with primers COQ9\_5 (5'-GGCGGAAGAAAAATAGC) and COQ9\_3 (5'-CCACTGGCCCAGGAAGG) used on the genomic DNA from the  $\Delta$ *coq9* strain. Transformants were selected on YPD-agar plates containing G418 and clones were checked by *coq9* locus PCR and phenotypic characterization.

**Analysis of the Quinone Content**—The cultures were placed on ice for 30 min, then the cells were collected by centrifugation, washed once with ice-cold water, and their wet weight was determined in pre-weighted Eppendorf tubes before freezing at -20 °C. For lipid extraction, glass beads (100  $\mu$ l), 50  $\mu$ l of KCl (0.15 M), a Q<sub>4</sub> solution (4  $\mu$ M in methanol, 2  $\mu$ l/mg of wet weight), and 0.6 ml of methanol were added to cell pellets (10–30 mg wet weight) and the tubes were vortexed for 10 min. Neutral lipids were extracted by adding 0.4 ml of petroleum ether (40–60 °C boiling range) and by vortexing for 3 min. The phases were separated by centrifugation (3 min, 1000  $\times$  g at room temperature). The upper petroleum ether layer was transferred to a fresh tube. Petroleum ether (0.4 ml) was added to the glass beads and methanol-containing tube, and the extraction was repeated once more. The petroleum ether layers were combined and dried under argon. The lipids were resuspended in 100  $\mu$ l of methanol, and aliquots were analyzed by reversed-phase high-pressure liquid chromatography (HPLC) on a Dionex U3000 system equipped with a C18 column (Betabasic-18, 5  $\mu$ m, 4.6  $\times$  150 mm, Thermo Scientific) at a flow rate of 1 ml/min with a mobile phase composed of 75% (98% (v/v) methanol, 2% (v/v) 1 M ammonium acetate), 5% isopropyl alcohol, and 20% acetonitrile. Hydroquinones present in injected samples were oxidized with a precolumn 5020 guard cell set in oxidizing mode (*E*, +600 mV). Electrochemical detection (ECD) was performed with a Coulochem III (ESA) equipped with a 5011A analytical cell (*E*<sub>1</sub>, -550 mV; *E*<sub>2</sub>, 550 mV). The standard Q<sub>4</sub> solution was injected in the same conditions to generate a standard curve that was used to correct for sample loss during the organic extraction (on the basis of the

recovery of the Q<sub>4</sub> internal standard) and to quantify Q<sub>6</sub>. When mass spectrometry (MS) detection was needed, the flow was split after the diode array detector with an adjustable split valve (Analytical Scientific Instruments) to allow simultaneous EC (60% of the flow) and MS (40% of the flow) detections. MS detection was achieved with an MSQ Plus spectrometer (Thermo) used in positive mode with electrospray ionization. The probe temperature was 450 °C and the cone voltage was 80 V. Because of the precolumn guard cell, the following compounds were detected by single ion monitoring in their oxidized state: 4-AP<sub>6</sub> (M + H<sup>+</sup>), *m/z* 515.9–516.9, 7–9 min, scan time 0.2 s; <sup>13</sup>C<sub>6</sub>-4-AP<sub>6</sub> (M + H<sup>+</sup>), *m/z* 521.9–522.9, 7–9 min, scan time 0.2 s; IDMQ<sub>6</sub> (M + H<sup>+</sup>), *m/z* 559.9–560.9, 9.5–14 min, scan time 0.2 s; <sup>13</sup>C<sub>6</sub>-IDMQ<sub>6</sub> (M + H<sup>+</sup>), *m/z* 565.9–566.9, 9.5–14 min, scan time 0.2 s; DMQ<sub>6</sub> (M + NH<sub>4</sub><sup>+</sup>), *m/z* 577.9–578.9, 7–12 min, scan time 0.6 s; <sup>13</sup>C<sub>6</sub>-DMQ<sub>6</sub> (M + NH<sub>4</sub><sup>+</sup>), *m/z* 583.9–584.9, 7–12 min, scan time 0.6 s; Q<sub>6</sub> (M + NH<sub>4</sub><sup>+</sup>), *m/z* 607.9–608.9, 9–11.5 min, scan time 0.2 s. MS spectra were also recorded between *m/z* 500 and 700 or 450 and 650 with a scan time of 0.4 s.

**Mitochondrial Preparation and Western Blotting**—Yeast cells grown on YPGal were harvested in the late log phase (A<sub>600</sub> near 5). Mitochondria were prepared as described by Daum *et al.* (27). Briefly, spheroplasts obtained after enzymatic digestion of the cell wall by Zymolyase 20T (Seikagaku) were disrupted by Dounce homogenization in 0.6 M mannitol, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% (w/v) BSA, and 1 mM PMSF. Mitochondria were isolated by differential centrifugation, washed in the same buffer devoid of BSA and PMSF, and stored in liquid nitrogen. The protein concentrations were determined using a BCA protein assay kit (Sigma) and bovine serum albumin as a standard. 20 μg of mitochondrial proteins were heated at 95 °C for 5 min in Laemmli buffer and separated on a 12.5% acrylamide SDS-PAGE gel. Antibodies were used at the following dilutions: anti-C-terminal peptide of Anc2p (28), 1/3000; anti-SDS-treated Por1 (28), 1/2000; and anti-Coq6 (29), 1/500. Immunodetection was performed using horseradish peroxidase-coupled protein A and the ECL-enhanced chemiluminescence system (Amersham Biosciences).

**<sup>18</sup>O<sub>2</sub> Labeling Experiment**—In a 10-ml culture flask, 1 ml of lactate/glycerol YNB-p medium supplemented with 20 μM 4-HB or 20 μM pABA was inoculated with 10 μl of an overnight preculture of W303 in the same medium without precursors. The culture flask was extensively degassed with N<sub>2</sub> and sealed with a septum prior to the introduction of 2.5 ml of <sup>18</sup>O<sub>2</sub> (97% enrichment, eurisotop) with a syringe. The culture was incubated at 30 °C, 200 rpm for 24 h until the cells reached saturation. The cells were then placed on ice for 30 min before opening the flask to harvest the cells.

**Model of Coq6**—The homology model of wild-type Coq6 was taken from our recent experimental and computational study of the Coq6-FAD complex.<sup>6</sup> This model was constructed using MODELLER (30) on the basis of three functionally related flavoprotein monooxygenase PDB structures: 2X3N (pqsL, an alkylquinolone hydroxylase from *Pseudomonas aeruginosa*), 4N9X (a ubiquinone biosynthesis hydroxylase from *Erwinia carotovora*), and 4K22 (ubiL, a ubiquinone biosynthesis hydroxylase from *E. coli*). Structural models of mutated Coq6 enzyme

were generated on the basis of this wild-type model. All models were subjected to molecular dynamics using the AMBER99SB-ILDN force field (31) as implemented in GROMACS 4.6.5 (32). 20-ns production trajectories were analyzed with CAVER (33) and VMD (34) to detect channels leading from the protein surface to the active site.

## Results

**The Hydroxyl Group on C4 of Q<sub>6</sub> Originates from Dioxygen in pABA-grown Cells**—It was previously hypothesized that the C4-deamination reaction may occur via Schiff base chemistry with a water molecule attacking the C4-imino group of an oxidized Q<sub>6</sub> biosynthetic intermediate (12). To test this hypothesis, we cultivated wild-type *S. cerevisiae* W303 cells in medium containing 75% H<sub>2</sub><sup>18</sup>O (v/v) and supplemented with either 50 μM 4-HB or pABA. In both cases, no labeling was detected in Q<sub>6</sub> (C<sub>39</sub>H<sub>58</sub>O<sub>4</sub>) because the main ions of the mass spectrum were at *m/z* 608 (M + NH<sub>4</sub><sup>+</sup>) and *m/z* 591 (M + H<sup>+</sup>) (Fig. 2A and data not shown). This result establishes that water is not the source of the C4-hydroxyl group of Q<sub>6</sub> in pABA-grown cells. We then cultured the W303 strain in medium supplemented with 50 μM 4-HB or pABA in an atmosphere containing ~80% N<sub>2</sub> (v/v) and 20% <sup>18</sup>O<sub>2</sub> (v/v). From the 4-HB culture, the mass spectrum of Q<sub>6</sub> showed two main ions at *m/z* 614.3 (M + NH<sub>4</sub><sup>+</sup>) and 597.3 (M + H<sup>+</sup>) (Fig. 2B), which correspond to an increase of 6 units of the mass of Q<sub>6</sub> (C<sub>39</sub>H<sub>58</sub><sup>16</sup>O<sup>18</sup>O<sub>3</sub>) compared with unlabeled Q<sub>6</sub> (C<sub>39</sub>H<sub>58</sub><sup>16</sup>O<sub>4</sub>) (Fig. 2A). This result demonstrates for the first time that the three hydroxylation reactions of the *S. cerevisiae* Q<sub>6</sub> biosynthetic pathway use dioxygen as a substrate in agreement with results previously obtained in *E. coli* (20). In the case of the pABA culture, the mass spectrum of Q<sub>6</sub> showed prominent ions at *m/z* 616.3 (M + NH<sub>4</sub><sup>+</sup>) and 599.3 (M + NH<sub>4</sub><sup>+</sup>) corresponding to a Q<sub>6</sub> molecule in which all four oxygen atoms are labeled (C<sub>39</sub>H<sub>58</sub><sup>18</sup>O<sub>4</sub>) (Fig. 2C). These results unambiguously show that an additional <sup>18</sup>O atom is incorporated into Q<sub>6</sub> when pABA is used as a precursor as compared with 4-HB and establish that dioxygen is the source of the OH group on C4, which substitutes the amino group originally present on pABA. This reaction thus relies on a protein that has the capacity to activate dioxygen.

**Metabolism of 3-Hydroxy-4-aminobenzoic Acid into Q<sub>6</sub> Requires an Active Coq6**—To verify whether the C4-deamination defect is a consequence or not of the C5-hydroxylation defect in *coq6*-deficient cells, 3-hydroxy-4-aminobenzoic acid (3H4AB) was used as substrate. Indeed, if prenylated by Coq2, 3H4AB will yield HHAB with a C5-hydroxyl group without assistance by Coq6. First, we established that exogenous 3H4AB is indeed prenylated by Coq2 because its addition at 1 mM in the growth medium was able to increase the cellular Q<sub>6</sub> content of W303 cells grown in pABA-free medium to levels similar to those obtained with 10 μM 4-HB (Fig. 3, A and B). Therefore, 3H4AB is a precursor of Q<sub>6</sub> in WT cells. Besides Q<sub>6</sub>, only a minute amount of DMQ<sub>6</sub> was detected by HPLC-ECD in extracts from 3H4AB-treated cells (Fig. 3A), showing that the C4-amino group does not perturb a particular biosynthetic step. In fact, C4-deamination of compounds derived from 3H4AB also implicated O<sub>2</sub> because cells grown with 3H4AB in 80% N<sub>2</sub> and 20% <sup>18</sup>O<sub>2</sub> synthesized mostly <sup>18</sup>O<sub>3</sub>-Q<sub>6</sub> (Fig. 3C),

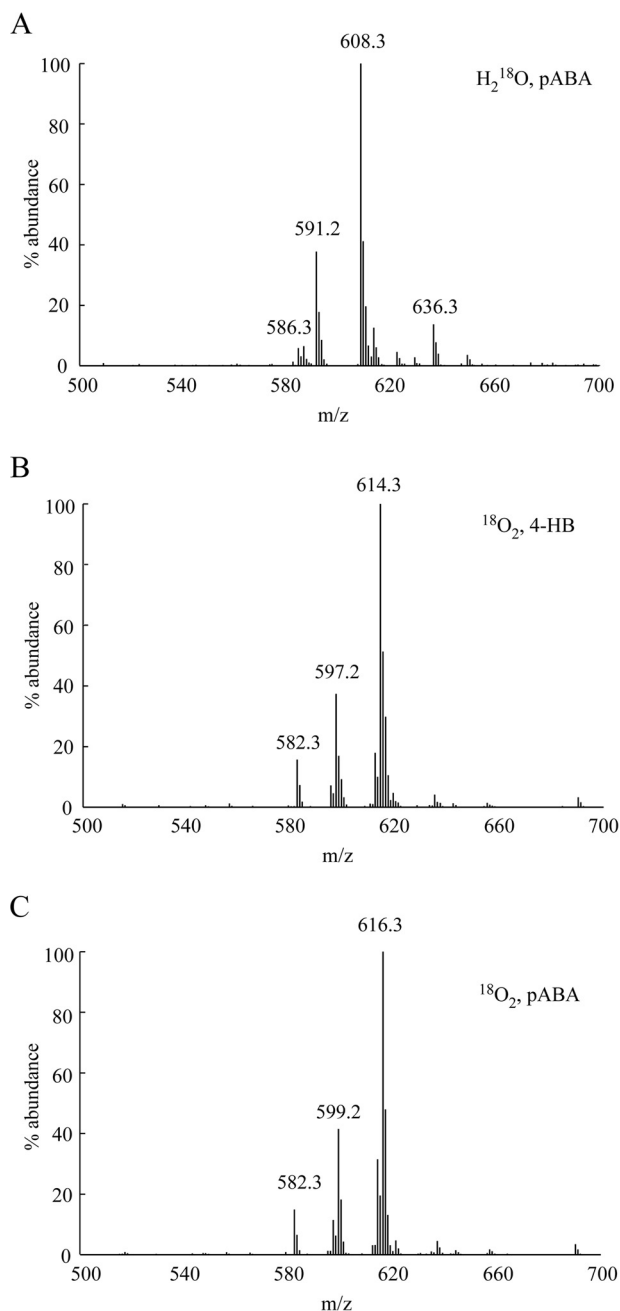
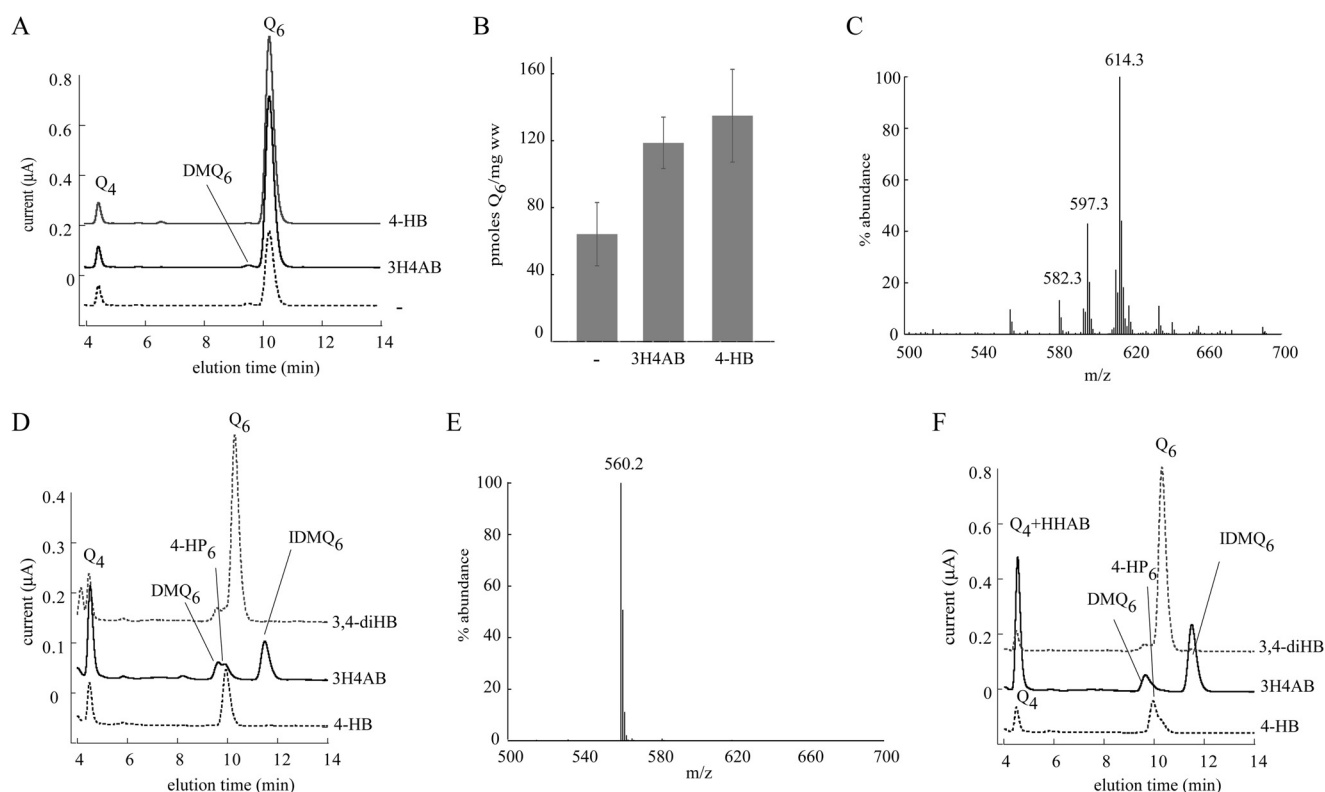


FIGURE 2. **Isotopic labeling of Q<sub>6</sub> in W303 cells.** A, MS spectrum of Q<sub>6</sub> eluting at 10.4 min in the HPLC analysis of lipid extracts from W303 cells grown in YNB-p, 2% lactate (w/v), 2% glycerol (w/v) medium containing 50  $\mu$ M pABA and 75% H<sub>2</sub><sup>18</sup>O (v/v). B and C, MS spectra of Q<sub>6</sub> from cells grown under <sup>18</sup>O<sub>2</sub> atmosphere in YNB-p, 2% lactate, 2% glycerol medium containing 50  $\mu$ M 4-HB (B) or 50  $\mu$ M pABA (C).

consistent with <sup>18</sup>O labeling at positions C1, C4, and C6 and with the absence of labeling on the C5-hydroxyl derived from 3H4AB. In  $\Delta$ coq6 + pCOQ8 cells, 4-HB caused the accumulation of 4-HP<sub>6</sub>, whereas 3,4-diHB restored Q<sub>6</sub> biosynthesis, as already shown (Fig. 3D) (13). In contrast, 3H4AB was unable to yield any Q<sub>6</sub> but several compounds were detected instead: 4-HP<sub>6</sub>, DMQ<sub>6</sub>, and IDMQ<sub>6</sub> (Fig. 3D). 4-HP<sub>6</sub> likely originates from endogenous 4-HB, showing that exogenous 3H4AB cannot completely outcompete endogenous 4-HB, as already established for vanillic acid (13). The most prominent com-

pound that eluted at 11.6 min (Fig. 3D) was identified as IDMQ<sub>6</sub> based on its mass spectrum (M + H<sup>+</sup>) m/z 560.2 (Fig. 3E) and on its co-elution with the previously characterized IDMQ<sub>6</sub> molecule from  $\Delta$ coq9 + COQ8 cells grown in the presence of pABA (10). The accumulation of IDMQ<sub>6</sub> demonstrates that the C4-deamination is impaired in  $\Delta$ coq6 + pCOQ8 cells but not completely because DMQ<sub>6</sub> was also detected (Fig. 3D). DMQ<sub>6</sub> and IDMQ<sub>6</sub> are the most downstream product of the Q<sub>6</sub> pathway detected in  $\Delta$ coq6 + pCOQ8 cells grown in the presence of 3H4AB, showing that the C6-hydroxylation by Coq7 is inhibited in these conditions. IDMQ<sub>6</sub> may be responsible for this inhibition in agreement with the proposition that an intermediate derived from prenyl-pABA has an inhibitory effect on the C6-hydroxylation of DMQ<sub>6</sub> (12). To verify that the results obtained in  $\Delta$ coq6 cells were independent from the overexpression of Coq8, we next tested the effect of 3H4AB in cells expressing Coq6 G386A-N388D. These point mutations inactivate Coq6 but do not destabilize the protein that allows for assembly of the CoQ-synthome without Coq8 overexpression (13). Like in  $\Delta$ coq6 + pCOQ8 cells, 3,4-diHB resulted in Q<sub>6</sub> biosynthesis in  $\Delta$ coq6 + pCOQ6 G386A/N388D but 3H4AB failed and caused mostly the accumulation of IDMQ<sub>6</sub> (Fig. 3F). The electroactive compound co-eluting with Q<sub>4</sub> at 4.3 min in 3H4AB-treated cells is likely to be oxidized prenylated 3H4AB based on its mass spectrum (M + H<sup>+</sup>) m/z 560.1 (data not shown) and on its short retention time on the reverse phase column caused by the polarity of the carboxyl group. Overall, the accumulation of IDMQ<sub>6</sub> in coq6-deficient cells grown in the presence of 3H4AB shows that (i) the conversion of this substrate analog into Q<sub>6</sub> is dependent of an active Coq6 enzyme, (ii) the presence of a methoxy group on C5 is not sufficient to promote efficient C4-deamination in the absence of an active Coq6 enzyme. These results establish that the lack of C4-deamination in coq6-deficient cells grown in pABA (13) is not a mere consequence of the absence of the methoxy group on C5 but rather support that Coq6 itself is required for efficient C4-deamination.

**L382E and M469X Mutations in Coq6 Affect the C4-deamination While Preserving Partially the C5-hydroxylation**—We next tested the efficiency of five Coq6 point mutants to synthesize Q<sub>6</sub> from 4-HB or pABA. In this last case, pABA added in excess in the medium competes efficiently with endogenous 4-HB to enter the Q biosynthetic pathway (5). The mutations G248R, A361D, F420C, and M469X (truncation of the last 11 residues) in *S. cerevisiae* Coq6 correspond to pathological mutations found in the human Coq6 protein (huCoq6) (35, 36) and decrease the Q<sub>6</sub> content to different extents in cells grown in rich medium (36). We recently constructed a L382E mutant that retains significant C5-hydroxylase activity with 4-HB added to the growth medium.<sup>6</sup> In our molecular model of Coq6,<sup>6</sup> the Ala-361 and Phe-420 residues are far from the FAD molecule that is buried inside the protein and from the predicted active site that faces the isoalloxazine ring of FAD (Fig. 4A). Conversely, the Leu-382 and Gly-248 residues face each other across the substrate access channel (Fig. 4B), which leads to the active site and likely accommodates the hexaprenyl tail of Coq6 substrates.<sup>6</sup> The last 11 residues of Coq6, which are truncated in the M469X mutant, are localized on the protein surface



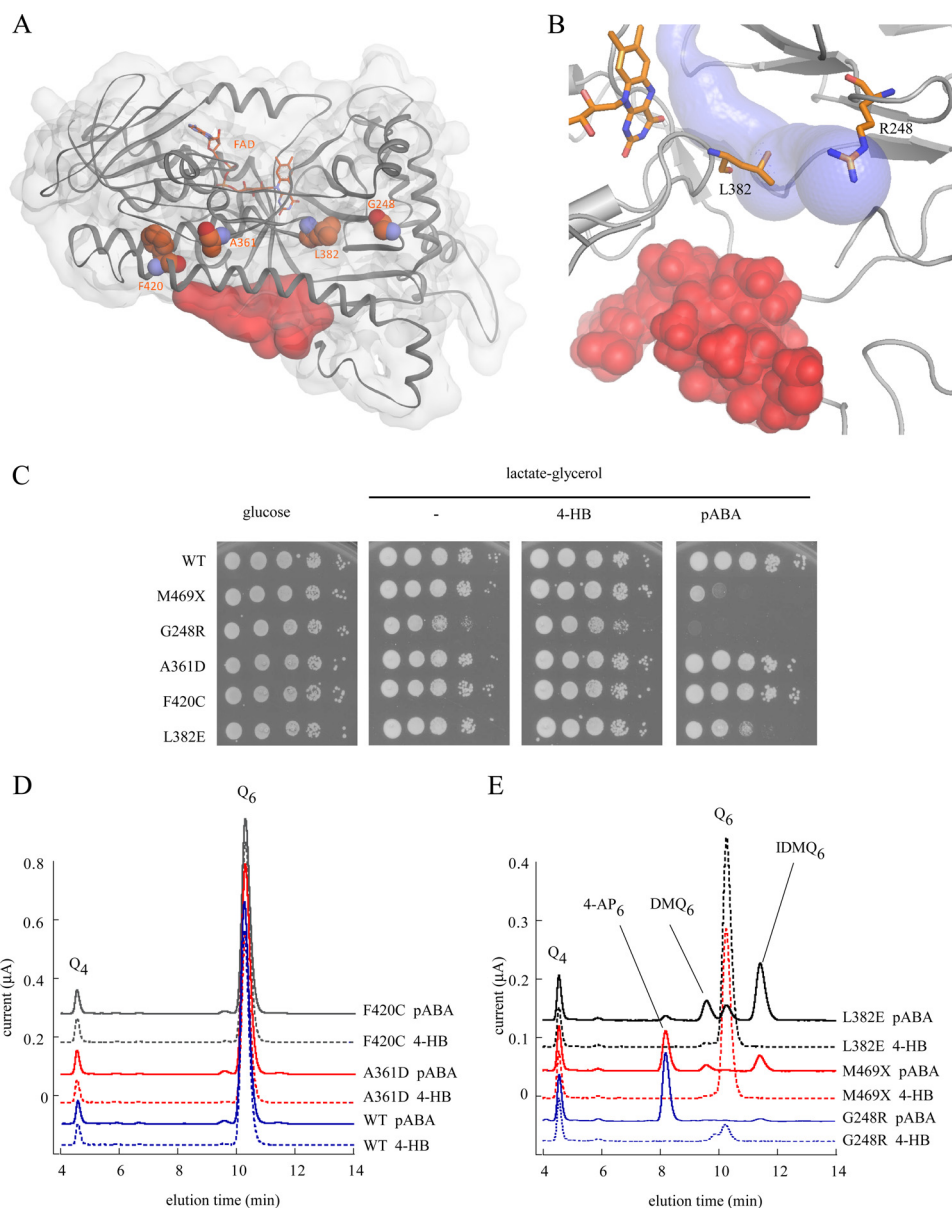
**FIGURE 3. Conversion of 3H4AB into  $Q_6$  requires an active Coq6.** *A*, HPLC-ECD analysis of lipid extracts from 2 mg of WT (W303) cells grown in YNB-p, 2% galactose containing or not 10  $\mu$ M 4-HB or 1 mM 3H4AB. The peaks corresponding to  $Q_6$ , DMQ<sub>6</sub>, and to the internal standard  $Q_4$  are marked. The chromatograms are shifted for better visualization but respect the scale of the *y* axis with the baseline corresponding to 0  $\mu$ A. *B*, quantification of cellular  $Q_6$  content ( $n = 5$ ) of the same cells as in *A* in picomoles per mg of wet weight, *error bars* represent standard deviation. *C*, HPLC-MS analysis of  $Q_6$  from WT cells grown under  $^{18}O_2$  atmosphere in YNB-p, 2% lactate, 2% glycerol medium containing 1 mM 3H4AB. *D*, HPLC-ECD analysis of lipid extracts from 10 mg of  $\Delta coq6$  cells overexpressing COQ8 and grown in YNB-p, 2% galactose containing 10  $\mu$ M 4-HB or 1 mM 3H4AB or 3,4-diHB. The peaks corresponding to DMQ<sub>6</sub>, IDMQ<sub>6</sub>, and 4-HP<sub>6</sub> are marked. *E*, HPLC-MS analysis of IDMQ<sub>6</sub> eluting at 11.6 min in the analysis of lipid extracts from  $\Delta coq6 + pCOQ8$  cells grown with 1 mM 3H4AB. *F*, HPLC-ECD analysis of lipid extracts from 10 mg of  $\Delta coq6$  cells expressing Coq6 G386A-N388D and grown in the same media as in *D*. The electrochromatograms are representative of 3 (*D*), 4 (*F*), or 5 (*A*) independent experiments.

(volume highlighted in red, Fig. 4A) and are also in close proximity to the substrate access channel (Fig. 4B). The growth of *S. cerevisiae* on a respiratory medium is dependent on the function of the mitochondrial respiratory chain and thus on the biosynthesis of  $Q_6$ .  $\Delta coq6$  strains containing pCOQ6, pCOQ6 A361D, or pCOQ6 F420C grew equally well on the respiratory medium lactate/glycerol containing either 4-HB or pABA (Fig. 4C). In contrast, the growth of strains with the plasmids pCOQ6 M469X, pCOQ6 G248R, or pCOQ6 L382E was superior when the medium was supplemented with 4-HB as compared with pABA (Fig. 4C). The growth pattern of all strains was similar whether media were supplemented or not with 4-HB (Fig. 4C), showing that  $Q_6$  synthesized from endogenous substrates is not limiting for respiratory growth and supporting that the endogenous substrate predominantly used for  $Q_6$  biosynthesis is 4-HB. This last point is in line with the major accumulation of 4-HP<sub>6</sub> over 4-AP<sub>6</sub> in  $\Delta coq6 + pCOQ8$  cells grown in pABA-free medium (13). HPLC-ECD analysis showed that pABA or 4-HB yielded a comparable  $Q_6$  content in  $\Delta coq6$  cells expressing Coq6, Coq6 A361D, or Coq6 F420C (Fig. 4D). In contrast, Coq6 M469X led to the synthesis of an appreciable amount of  $Q_6$  when 4-HB was added to the growth medium but  $Q_6$  was almost undetectable with pABA as a precursor (Fig. 4E). Instead, 4-AP<sub>6</sub>, IDMQ<sub>6</sub>, and a small quantity of DMQ<sub>6</sub> were detected (Fig. 4E). The accumulation of 4-AP<sub>6</sub> shows that the

C5-hydroxylation of HAB is perturbed and the predominant synthesis of C4-amino intermediates over C4-hydroxy intermediates demonstrates that the C4-deamination is almost completely abrogated by the M469X truncation. Similarly, the L382E mutant was efficient at synthesizing  $Q_6$  from 4-HB but the main compound accumulated from pABA was IDMQ<sub>6</sub> (Fig. 4E), demonstrating that the C5-hydroxylation was functional and C4-deamination was limiting in this strain. The G248R mutation had more drastic effects because the amount of  $Q_6$  synthesized from 4-HB was minimal, whereas from pABA, 4-AP<sub>6</sub> and a minute amount of IDMQ<sub>6</sub> were detected (Fig. 4E). Based on the synthesis of  $Q_6$  and IDMQ<sub>6</sub> in the presence of 4-HB and pABA, respectively, we conclude that Coq6 M469X and Coq6 L382E are competent for C5-hydroxylation of HHB and HAB. In contrast, the C4-deamination is limiting because IDMQ<sub>6</sub> is accumulated predominantly over DMQ<sub>6</sub> and  $Q_6$  in cells grown in the presence of pABA (Fig. 4E). Altogether, these results show that the M469X truncation and the L382E mutation impact slightly the C5-hydroxylation but strongly the C4-deamination.

**Coq9 Is Indispensable to the Activity of Coq6 M469X**—We reported a defect in C4-deamination not only in cells lacking Coq6, but also in cells lacking Coq9 (10). However, the absence of Coq9 affects Coq6 and Coq7 activities leading to accumulation of 4-HP<sub>6</sub> and DMQ<sub>6</sub> in the presence of 4-HB and to 4-AP<sub>6</sub>

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**FIGURE 4. Point mutations in Coq6 affect the C4-deamination reaction.** *A*, structural model of yeast Coq6<sup>6</sup> as prepared with Discovery Studio Visualizer (Accelrys Software Inc.). Mutation sites are shown as *spheres* and the FAD is shown as *sticks*. The *red* corresponds to the 11 C-terminal residues, which are truncated in the M469X mutant. *B*, closer view of the active site (in a slightly different orientation compared with *A*) and of the residues important for deamination as prepared with PyMol. The substrate access tunnel is *blue* and C-terminal 11 residues are *red*. FAD, Leu-382, and G248R are shown as *sticks* and Arg-248 is superposed from the model of the G248R mutant.<sup>6</sup> *C*, serial dilutions of  $\Delta coq6$  cells expressing WT Coq6 or the indicated mutants. The plates contained YNB-p, 2% glucose or 2% lactate, 2% glycerol and pABA or 4-HB at 20  $\mu$ M as indicated. The plates were imaged after 3 (glucose) or 4 days (lactate/glycerol) at 30 °C. Results are representative of 3 independent experiments. *D* and *E*, HPLC-ECD analysis of lipid extracts from 1 mg of  $\Delta coq6$  cells expressing WT Coq6 or the designated Coq6 mutants and grown in YNB-p, 2% galactose containing 20  $\mu$ M 4-HB or 20  $\mu$ M pABA. The electrochromatograms are representative of 2 (*D*) and 3 (*E*) independent experiments.

and IDMQ<sub>6</sub> in the presence of pABA (10). Therefore, the defect in C4-deamination in  $\Delta coq9$  cells may indirectly result from the perturbation of Coq6. As anticipated, expression of Coq6 in  $\Delta coq6\Delta coq9$  cells grown in the presence of 4-HB caused the biosynthesis of 4-HP<sub>6</sub> and DMQ<sub>6</sub> (Fig. 5A), showing that the C5-hydroxylation catalyzed by Coq6 was partly impaired. Unexpectedly, DMQ<sub>6</sub> was undetectable in  $\Delta coq6\Delta coq9$  cells expressing Coq6 M469X (Fig. 5A), demonstrating that the C5-hydroxylation was completely abrogated. As judged from immunodetection on purified mitochondria, Coq9 is not required for the stability of Coq6 M469X because the steady state levels of Coq6 and Coq6 M469X were comparable whether

Coq9 was present or not (Fig. 5B). Because Coq6 M469X is functional in cells containing Coq9 grown with 4-HB (Fig. 4E), we conclude that the absence of Coq9 and the truncation of Coq6 have an additive negative impact on the C5-hydroxylation. Likewise, in the presence of pABA, IDMQ<sub>6</sub> was synthesized by  $\Delta coq6\Delta coq9$  cells expressing Coq6 but not Coq6 M469X (Fig. 5A). Together, our results show that the presence of Coq9 is indispensable to the C5-hydroxylation activity of Coq6 lacking its C-terminal part, which we also showed to be important for the C4-deamination reaction (Fig. 4E). Thus, the C4-deamination defect observed in  $\Delta coq9$  cells (10) likely results from the perturbation of Coq6.



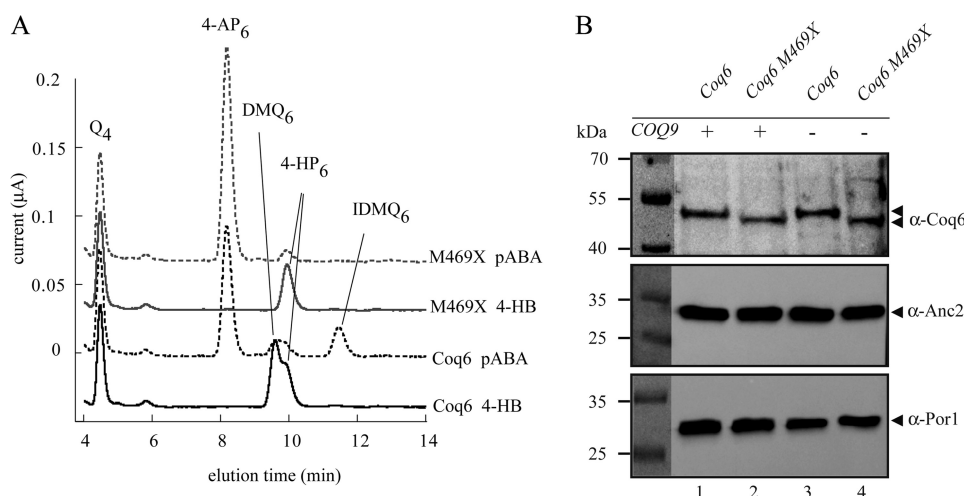


FIGURE 5. **Genetic interaction between Coq6 and Coq9.** *A*, HPLC-ECD analysis of lipid extracts from 4 mg of  $\Delta coq6\Delta coq9$  + pCOQ8 cells expressing WT Coq6 or Coq6-M469X grown in YNB-p, 2% galactose containing 20  $\mu$ M 4-HB or 20  $\mu$ M pABA. The electrochromatograms are representative of 4 independent experiments. *B*, immunodetection of Coq6 in mitochondria prepared from  $\Delta coq6$  (lanes 1 and 2) and  $\Delta coq6\Delta coq9$  + pCOQ8 cells (lanes 3 and 4) expressing WT Coq6 or Coq6-M469X. The mitochondrial proteins Anc2 and Por1 are used as loading control.

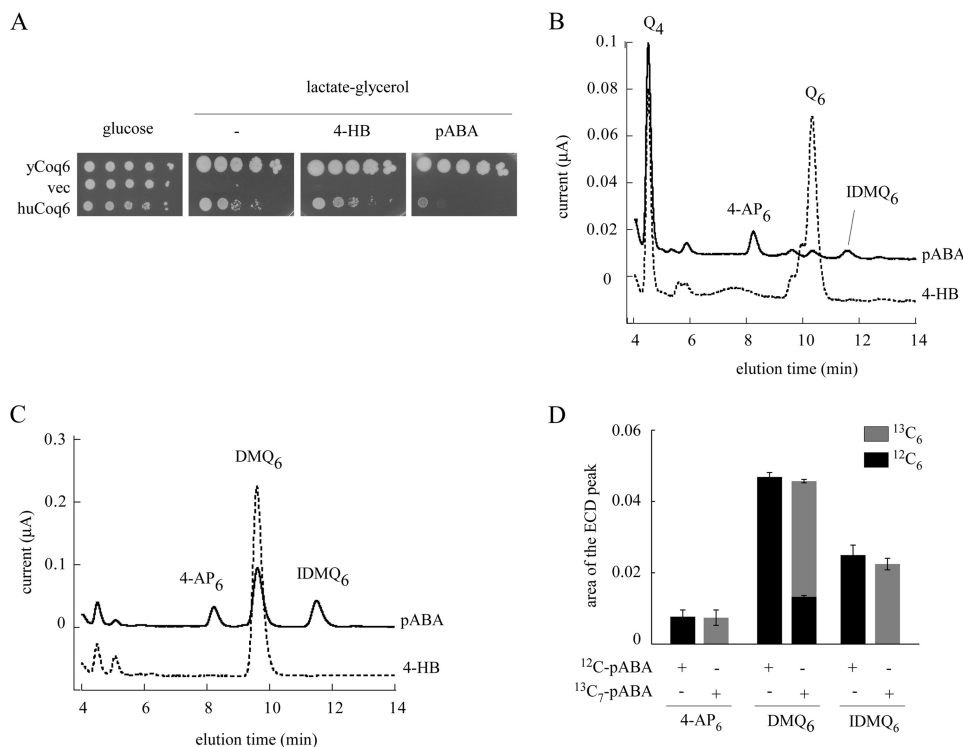
*Human Coq6 Supports C4-deamination in Yeast Cells Lacking Coq9*—Several reports document that pABA is not a precursor of Q<sub>10</sub> in different human cell lines (37–39). Because huCoq6 expressed in *S. cerevisiae*  $\Delta coq6$  cells partially complements the C5-hydroxylation defect (36), we tested the effect of the addition of pABA to the growth medium. The complementation of the respiratory growth defect of  $\Delta coq6$  cells by huCoq6 was efficient when the medium contained 4-HB but not pABA (Fig. 6A). HPLC-ECD analysis revealed that the Q<sub>6</sub> content was indeed very low in  $\Delta coq6$  + phucoq6 cells grown in the presence of pABA compared with 4-HB (Fig. 6B). 4-AP<sub>6</sub> accumulated predominantly but IDMQ<sub>6</sub> was also detected (Fig. 6B), indicating that the C5-hydroxylation took place without C4-deamination. Although huCoq6 is much less efficient than yeast Coq6 at restoring Q<sub>6</sub> biosynthesis in  $\Delta coq6$  cells (Ref. 36 and compare Figs. 4D and 6B), phucoq6 yielded a higher DMQ<sub>6</sub> content than pCoq6 in  $\Delta coq6\Delta coq9$  cells grown in the presence of 4-HB (compare Figs. 5A and 6C). In the presence of pABA, IDMQ<sub>6</sub> was increased in  $\Delta coq6\Delta coq9$  + phucoq6 cells compared with  $\Delta coq6$  + phucoq6 cells (compare Fig. 6, B and C), establishing that the presence of Coq9 is in fact detrimental to the C5-hydroxylase activity of huCoq6. In addition to IDMQ<sub>6</sub> and besides 4-AP<sub>6</sub>, a large peak of DMQ<sub>6</sub> was apparent in pABA-treated  $\Delta coq6\Delta coq9$  + phucoq6 cells (Fig. 6C). To verify the origin of DMQ<sub>6</sub>, we grew these cells in the presence of pABA or <sup>13</sup>C<sub>7</sub>-pABA and analyzed the quinone content and the labeling in each compound by HPLC-ECD-MS. The quantities of 4-AP<sub>6</sub>, DMQ<sub>6</sub>, and IDMQ<sub>6</sub> formed in pABA- or <sup>13</sup>C<sub>7</sub>-pABA-treated cells were comparable and, as expected, 4-AP<sub>6</sub> and IDMQ<sub>6</sub> were almost entirely labeled (99%) in the <sup>13</sup>C<sub>7</sub>-pABA culture (Fig. 6D). In these conditions, <sup>13</sup>C<sub>6</sub>-DMQ<sub>6</sub> represented 71% of the total DMQ<sub>6</sub> (Fig. 6D), proving that the majority of DMQ<sub>6</sub> originated from the exogenously added <sup>13</sup>C<sub>7</sub>-pABA. Altogether, these results establish that Coq9 prevents huCoq6 from functioning efficiently in *S. cerevisiae* and show that, in the absence of Coq9, huCoq6 is able to support both the C5-hydroxylation and the C4-deamination.

## Discussion

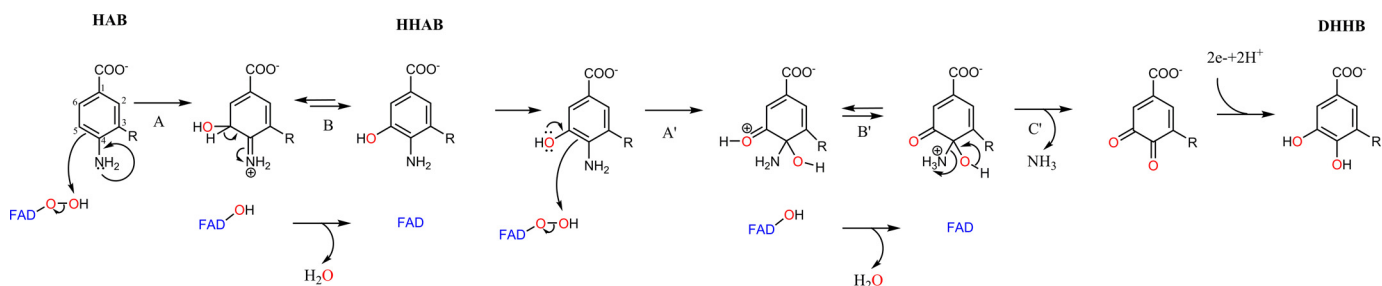
*Coq6 Is Responsible for the C4-deamination*—In 2010, it was discovered that *S. cerevisiae* is able to use pABA in addition to 4-HB as a precursor of Q<sub>6</sub> (5, 12). The conversion of pABA into Q<sub>6</sub> requires a C4-deamination reaction in which the C4-amino group is replaced by a C4-hydroxyl group. The results presented in this study strongly support that Coq6, the monooxygenase responsible for the C5-hydroxylation, fulfills also the C4-deamination. This conclusion relies on the following observations: (i) coq6 mutant cells are defective not only in C5-hydroxylation but also in C4-deamination whether pABA or its C5-hydroxylated analog, 3H4AB, are provided as a substrate in the growth medium (Fig. 3). (ii) Mutation of Leu-382, a residue buried inside Coq6, severely affects the C4-deamination reaction, whereas the C5-hydroxylation is mostly maintained (Fig. 4). (iii) huCoq6 is able to complement the C5-hydroxylation and the C4-deamination defects of *S. cerevisiae*  $\Delta coq6$  cells, albeit only in the absence of the Coq9 protein (Fig. 6). The function of Coq9 with respect to Coq6 activity is discussed below. (iv) The oxygen atom introduced at C4 exclusively derives from molecular oxygen (Fig. 2), in agreement with the involvement of an O<sub>2</sub> activating enzyme in the deamination reaction.

*Proposed Mechanism of the Dioxygen-mediated Deamination*—Our <sup>18</sup>O<sub>2</sub> *in vivo* labeling experiments confirm that dioxygen is the substrate of the three hydroxylation reactions (on C1, C5, and C6) necessary to synthesize Q<sub>6</sub> from 4-HB, which implies that the unknown C1-hydroxylase is likely also a dioxygen-activating enzyme. The <sup>18</sup>O<sub>2</sub> labeling experiment with pABA as a substrate unambiguously shows that Q<sub>6</sub> incorporates four <sup>18</sup>O atoms, demonstrating that dioxygen is also the source of the oxygen atom at C4, which is introduced during the C4-deamination reaction. We thus propose a unifying mechanism for the C5-hydroxylation and the C4-deamination by Coq6 in agreement with the classical mechanism of class A FMOs to which Coq6 belongs (18). FAD is first reduced by NAD(P)H and reacts with dioxygen to form a flavin C4a-hy-

## Dioxygen-mediated Deamination in Coenzyme Q Biosynthesis



**FIGURE 6. Human Coq6 supports C4-deamination in yeast cells lacking Coq9.** *A*, serial dilutions of  $\Delta coq6$  cells containing an empty vector (*vec*) or a centromeric vector expressing yeast Coq6 (*yCoq6*) or human Coq6 isoform 1 (*huCoq6*). The plates contained YNB-p glucose or lactate glycerol and pABA or 4-HB at  $20 \mu\text{M}$  as indicated. The plates were imaged after 3 (glucose) or 10 days (lactate/glycerol) at  $30^\circ\text{C}$ . Results are representative of 5 independent experiments. *B*, HPLC-ECD analysis of lipid extracts from 9 mg of  $\Delta coq6$  cells expressing human Coq6 grown in YNB-p, 2% galactose containing  $20 \mu\text{M}$  4-HB or  $20 \mu\text{M}$  pABA. The electrochromatograms are representative of 4 independent experiments. *C*, HPLC-ECD analysis of lipid extracts from 5 mg of  $\Delta coq6\Delta coq9 + pCOQ8$  cells expressing human Coq6 grown in YNB-p, 2% galactose containing  $20 \mu\text{M}$  4-HB or  $20 \mu\text{M}$  pABA. The electrochromatograms are representative of 6 independent experiments. *D*, area of the electrochemical peaks (arbitrary units) corresponding to 4-AP<sub>6</sub>, DMQ<sub>6</sub>, and IDMQ<sub>6</sub> in lipid extracts from 7 mg of  $\Delta coq6\Delta coq9 + pCOQ8 + \text{phuCoq6}$  cells grown in YNB-p 2% galactose containing  $20 \mu\text{M}$  pABA or  $20 \mu\text{M}$  <sup>13</sup>C<sub>7</sub>-pABA as indicated. The proportion of each compound, labeled (gray) or unlabeled (black), as determined by MS analysis is shown ( $n = 3$ ), error bars represent S.D.



**SCHEME 1. Proposed mechanism for the C5-hydroxylation and C4-deamination reaction catalyzed by Coq6.** Nucleophilic attack of HAB onto the flavin C4a-hydroperoxide (*FAD-O-OH*) results in the formation of the nonaromatic C5-hydroxylated product (step *A*). Rearomatization (step *B*) leads to HHAB. A second round of hydroxylation proceeds on carbon C4 according to a similar mechanism. Nucleophilic attack of HHAB onto *FAD-O-OH* results in the formation of the nonaromatic C4-hydroxylated product (step *A'*). Elimination of ammonia (step *C'*) facilitated by protonation of the amino group (step *B'*) leads to the formation of an intermediate *o*-quinone, which is then reduced into DHHB by 2 electrons and 2 protons. In Coq6 L382E or Coq6 M469X, hydroxylation at C4 is not efficient (see text for details) and the amino group on C4 is therefore not eliminated. *R* represents the hexaprenyl tail and the numbering of the carbon atoms is shown on HAB.

droperoxide adduct. The hydroxylation reaction proceeds via a nucleophilic attack of the C5 carbon atom of HAB onto the distal oxygen of the peroxide (Scheme 1, step *A*). Then, aromatization of the product occurs by deprotonation leading to the final phenol product (HHAB), whereas the oxidized flavin is regenerated by elimination of a water molecule from the flavin C4a-hydroxide (Scheme 1, step *B*). This reaction mechanism shown for the C4-amino substrate (HAB) (Scheme 1) also applies to the C4-hydroxy substrate (HHB) produced when 4-HB and not pABA is prenylated by Coq2 (Fig. 1). Next, the C4-deamination proceeds also via a nucleophilic attack of the

C4 carbon atom of the HHAB intermediate onto the distal oxygen of the *FAD*-hydroperoxide (Scheme 1, step *A'*). The geminal amino and hydroxy groups at C4 easily convert to a keto function by elimination of  $\text{NH}_3$  facilitated by protonation of the amino group, thus generating an ortho-quinone intermediate (Scheme 1, steps *B'* and *C'*). The latter is easily reducible into DHHB. Although the reducing system for this reaction is unknown, we raise the possibility that the reduced *FAD* cofactor of Coq6 is involved via hydride transfer to the quinone. Reduced flavins have indeed been extensively studied as quinone reducing agents (40–42). Alternatively, the ferredoxin/

ferredoxin reductase system (Yah1/Arh1) (5) may be implicated in the reduction of DHHB. Overall, our results seem to indicate that Coq6 is a unique multifunctional redox enzyme responsible for O<sub>2</sub>-dependent oxygen atom insertion at two different positions of an aromatic ring as well as possibly for reduction of a quinone. The proposed mechanism also accounts for the labeling obtained when 3H4AB was provided as substrate (Fig. 3C) because prenylation of this compound yields HHAB in which the C5-hydroxyl will not be labeled *in vivo* when derived from 3H4AB. Our proposed mechanism ought to be confirmed by *in vitro* experiments. Despite extensive efforts, we have not so far obtained *in vitro* activity for purified Coq6, possibly because interacting partners of the *in vivo* CoQ-synthome (6, 7) may be required for Coq6 to adopt its functional tridimensional structure, contrary the other class A FMOs studied to date.

**Different Fates for the Amino Group on Aromatic Substrates of FMOs**—Anthranilate hydroxylase is an FMO that hydroxylates anthranilate at position C3 and at the same time deaminates position C2 in a single reaction, resulting in the introduction of two oxygen atoms and thus formation of 2,3-dihydroxybenzoic acid (43). Labeling experiments established that the C3 hydroxyl derives from dioxygen, whereas the C2 hydroxyl derives from solvent water (43). The deamination reaction catalyzed by anthranilate hydroxylase was therefore proposed to occur via nucleophilic substitution of the amino group by a water molecule during the course of the oxidation (43). Such a mechanism is clearly not possible in the case of Coq6 considering the results of our experiments with <sup>18</sup>O<sub>2</sub> and H<sub>2</sub><sup>18</sup>O. Another FMO, kynurenine 3-monooxygenase, catalyzes an aromatic hydroxylation ortho to an amino group without hydrolyzing it (44). Thus, the amino group ortho to the position hydroxylated by different FMOs encounters different fates: it can either remain unaffected, be hydrolyzed by a water molecule, or be replaced by a hydroxyl group derived from dioxygen as in the case of Coq6. Because all these FMOs utilize the same chemistry for hydroxylation, *i.e.* the nucleophilic attack of the substrate onto the electrophilic FAD-hydroperoxo intermediate, the different outcomes regarding the amino group likely results from subtle differences between the proteins, like solvent accessibility to the active site and positioning of the amino group with respect to the FAD-hydroperoxo.

**Regioselectivity of FMOs**—Most monooxygenases are highly regioselective, however, a few enzymes are known to catalyze sequential hydroxylations at separated sites (45). For example, the FMO PgaE from *Streptomyces* is responsible for two consecutive hydroxylation reactions in the formation of gaudamycin C (46). The large active site cavity revealed by the crystal structure of PgaE is compatible with the two alternative substrates binding in different orientations (46, 47). We have previously demonstrated that in the absence of the C5-hydroxylase UbiI in *E. coli*, the FMO UbiF responsible for the C6-hydroxylation is able to support C5-hydroxylation, albeit not efficiently (19). This result implies that the active site of UbiF accommodates two closely related substrates in different orientations compatible with hydroxylation at C5 and C6. Similarly, our results with Coq6 support that HHB and HAB, the substrates of the first hydroxylation at C5 must adopt a different conforma-

tion than HHAB, the substrate of the second hydroxylation at C4, to place the respective carbon atoms and the FAD-hydroperoxo in positions compatible with catalysis. In fact, Coq6 L382E and Coq6 M469X retained the capacity to hydroxylate at C5 but mostly lost the ability to hydroxylate C4 because deamination did not occur efficiently in these mutants (Fig. 4). Leu-382 lies within the substrate access channel, which may accommodate the hexaprenyl tail of the substrate as supported by our combined *in silico* and *in vivo* mutagenesis studies.<sup>6</sup> The C-terminal part of Coq6, truncated in the M469X mutant, is in close proximity to this channel (Fig. 4B). Thus, we believe that the L382E and M469X mutations may affect differently the positioning of the diverse substrates. The position of HAB and HHB in the active site of these Coq6 mutants would still be compatible with C5-hydroxylation, whereas the position of HHAB would be perturbed and inadequate for hydroxylation at C4. Alternatively, the mutations may accelerate the release of HHAB from the active site and therefore preclude the second hydroxylation at C4. In either case, enzymes downstream of Coq6 convert HHAB into IDMQ<sub>6</sub>H<sub>2</sub> (Fig. 1), which accumulates in the aforementioned Coq6 mutants (Fig. 4E). Actually, sensitive MS-MS detection revealed that IDMQ<sub>6</sub> is also formed in minute amounts by WT *S. cerevisiae* cells cultivated in the presence of pABA (12), showing that the C4-deamination reaction is not always completed even by a WT Coq6 enzyme. Similarly, IDDMQ<sub>6</sub> was recently detected in the yeast *coq5-5* mutant grown in the presence of pABA (24), although DDMQ<sub>6</sub> is clearly the most abundant product in these cells (10, 24). There is no evidence that Coq6 may convert IDDMQ<sub>6</sub> into DDMQ<sub>6</sub> or IDMQ<sub>6</sub> into DMQ<sub>6</sub> and thus these late-stage C4-aminated compounds may actually represent dead end products of the Q biosynthetic pathway.

**Impact of *coq9* Deletion on the C4-deamination Reaction**—In a recent study, Coq9 was proposed to control the deamination reaction (24). The results obtained in this study are compatible with our results that point to an indirect role of Coq9 in the C4-deamination reaction mediated by Coq6. Cells lacking Coq9 cultivated in the presence of pABA form 4-AP<sub>6</sub> and IDMQ<sub>6</sub> (10, 24). Accumulation of the former results from a C5-hydroxylation defect, whereas the latter demonstrates that Coq6 still catalyzes the C5-hydroxylation to some extent, whereas the C4-deamination is impaired (10, 24). We observed that Coq9 is indispensable to the C5-hydroxylase activity of Coq6 M469X (Fig. 5), which reveals that the C-terminal region of Coq6 is more specifically impacted by the absence of Coq9. Because the C-terminal region of Coq6 is important for the C4-deamination but quite dispensable for the C5-hydroxylation (Fig. 4E), we propose that the partial C5-hydroxylation defect and the profound C4-deamination deficit observed in Coq9-deficient cells (10, 24) result from a perturbation of the C-terminal part of Coq6. This hypothesis also accounts for the increased accumulation of IDDMQ<sub>6</sub> in *coq5-5 Δcoq9* cells (24). Our model of Coq6 shows that its C-terminal region is exposed at the protein surface (Fig. 4A), raising the possibility of an interaction with Coq9 via this domain. A direct interaction between Coq6 and Coq9 has yet to be proven, however, both proteins belong to the CoQ-synthome because Coq9 is co-immunoprecipitated and co-purified with Coq4, Coq5, Coq6, and

Coq7 (6, 7). Recently, a direct interaction between human Coq9 and Coq7 proteins was demonstrated (25). A deficiency of Coq9 in mammals seems to impact only Coq7 but not Coq6 unlike in *S. cerevisiae* (10). Indeed, the only biosynthetic intermediate detected in tissues of the Coq9 R239X knock-in mouse was DMQ<sub>9</sub>, supporting a defect in Coq7 activity (48) but a normal activity of Coq6. The likely independence of human Coq6 with regard to Coq9 is in line with our results showing that the C5-hydroxylase activity of huCoq6 expressed in *S. cerevisiae* is actually hampered by the presence of Coq9 (Fig. 6). Notably, the absence of Coq9 also allowed huCoq6 to promote the C4-deamination reaction when expressed in *S. cerevisiae* (Fig. 6). This result definitely proves that Coq9 is not directly implicated in the C4-deamination and collectively, our results support that the C4-deamination defect observed in  $\Delta$ coq9 cells is actually caused by perturbations of Coq6 structure.

**Conservation of the Capacity to Use pABA as a Substrate for Q Biosynthesis**—The respective contribution of endogenous 4-HB and pABA to Q biosynthesis is still unclear, although the predominant accumulation of 4-HP<sub>6</sub> over 4-AP<sub>6</sub> in  $\Delta$ coq6 + pCOQ8 cells suggests that endogenous 4-HB is preferably used over pABA in these growth conditions (13). pABA, is also a precursor in folate metabolism and is produced from chorismate by the action of Abz1, Abz2 (49), although 4-HB is proposed to originate from chorismate as well, but in an unidentified chain of reactions (50). The cellular abundance of 4-HB and pABA, and therefore their contribution to Q biosynthesis, is likely to vary in *S. cerevisiae* depending on growth conditions (12). Conceivably, other organisms than *S. cerevisiae* may use pABA as a precursor of Q if their C5-hydroxylase is able to function like Coq6. Recently, it was shown that *Arabidopsis* does not incorporate pABA into Q, however, it was not established whether pABA is prenylated or not in this organism (15). In *E. coli*, pABA yields several octaprenyl C4-aminated compounds but no Q<sub>8</sub> (39), implying that the C5-hydroxylase Ubil is not capable of catalyzing the C4-deamination. The potential C4-deamination capacity of a given Coq6 homolog is likely linked to the positioning of carbon C4 of HHAB with regard to the FAD as discussed in the case of L382E and M469X mutants. Such fine structural details will only be revealed by a crystal structure of the Coq6 protein, a task that is yet to be achieved.

**Author Contributions**—F. P. conceived and coordinated the study and wrote the manuscript. F. P., M. O., L. P., A. I., and C. M. D performed and analyzed the experiments. M. F. analyzed data and edited the paper. All authors reviewed the manuscript and approved the final version.

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**Coq6 Is Responsible for the C4-deamination Reaction in Coenzyme Q Biosynthesis  
in *Saccharomyces cerevisiae***

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