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# Coenzyme Q Biosynthesis: Coq6 Is Required for the C5-Hydroxylation Reaction and Substrate Analogs Rescue Coq6 Deficiency

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## SUMMARY

**Coenzyme Q (Q), an essential component of eukaryotic cells, is synthesized by several enzymes from the precursor 4-hydroxybenzoic acid. Mutations in six of the Q biosynthesis genes cause diseases that can sometimes be ameliorated by oral Q supplementation. We establish here that Coq6, a predicted flavin-dependent monooxygenase, is involved exclusively in the C5-hydroxylation reaction. In an unusual way, the ferredoxin Yah1 and the ferredoxin reductase Arh1 may be the in vivo source of electrons for Coq6. We also show that hydroxylated analogs of 4-hydroxybenzoic acid, such as vanillic acid or 3,4-dihydroxybenzoic acid, restore Q biosynthesis and respiration in a *Saccharomyces cerevisiae coq6* mutant. Our results demonstrate that appropriate analogs of 4-hydroxybenzoic acid can bypass a deficient Q biosynthetic enzyme and might be considered for the treatment of some primary Q deficiencies.**

## INTRODUCTION

Coenzyme Q or ubiquinone (Q) is a redox active lipid present in most organisms and in all tissues of multicellular eukaryotes where it shuttles electrons from complex I and II to complex III of the mitochondrial respiratory chain and acts as an important lipid-soluble antioxidant. Moreover, Q participates in the control of the mitochondrial membrane transition pore and functions with uncoupling proteins in the mitochondrial inner membrane (Bentinger et al., 2010). Q contains a polyprenyl tail with six isopentenyl units in *Saccharomyces cerevisiae* ($Q_6$) and 10 in human ($Q_{10}$) (Kawamukai, 2009) (Figure 1). In *S. cerevisiae*, the biosynthesis of Q is accomplished by multiple conserved mitochondrial matrix enzymes (termed Coq1-Coq9) some of which are assembled in a large Q biosynthetic complex associated with the inner membrane (Tran and Clarke, 2007). Mutations affecting five genes involved in Q biosynthesis have been described and result in primary Q deficiencies that cause clinically heterogeneous diseases (Quinzii and Hirano, 2010). Oral $Q_{10}$ supplementation of patients yields significant improvement in some cases (Quinzii and Hirano, 2010). Very recently, mutations in a sixth gene, *COQ6*, were shown to cause nephrotic syndrome with sensorineural deafness (Heeringa et al., 2011).

The initial stage of Q biosynthesis involves Coq2. In *S. cerevisiae*, Coq2 prenylates 4-hydroxybenzoic acid (4-HB) or the newly identified precursor para-aminobenzoic acid (pABA) to yield 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB), respectively (Figure 1) (Marbois et al., 2010; Pierrel et al., 2010). The C4-amine originating from pABA is subsequently converted into an hydroxyl at an unidentified step of $Q_6$ biosynthesis (Figure 1). *S. cerevisiae* cells depleted for either the mitochondrial ferredoxin Yah1 or the mitochondrial ferredoxin reductase Arh1 synthesize almost no $Q_6$ and accumulate 3-hexaprenyl-4-aminophenol (4-AP) upon culturing in the presence of pABA or 3-hexaprenyl-4-hydroxyphenol (4-HP) in the presence of 4-HB (Figure 1) (Pierrel et al., 2010). This result established that Yah1 and Arh1 are absolutely required for the C5-hydroxylation step. Arh1 and Yah1 form a well established electron transfer complex necessary in *S. cerevisiae* for iron sulfur cluster (ISC) and heme A biosynthesis (Barros et al., 2002; Lange et al., 2000; Li et al., 2001). Yah1 has two human homologs, Fdx1 and Fdx2. Fdx1 functions in steroid biogenesis by transferring electrons to mitochondrial cytochrome P450 enzymes whereas Fdx2 transfers electrons for the biogenesis of heme A and ISC (Sheftel et al., 2010).

Coq6 is required for $Q_6$ biosynthesis in *S. cerevisiae* (Gin et al., 2003) and, based on its amino acid sequence, has been predicted to belong to the class A flavoprotein monooxygenase family suggesting that it contains a FAD cofactor (van Berkel et al., 2006). Coq6 has been postulated to function in the C1- and/or the C5-hydroxylation reactions (Gin et al., 2003) (Figure 1). This ambiguity has not been resolved partly because a *S. cerevisiae* mutant lacking the entire *COQ6* gene (Δ*coq6*) accumulates only the early $Q_6$ biosynthetic intermediate, HHB when grown in the presence of 4-HB (Gin et al., 2003). In fact, the Δ*coq3-9* mutants all accumulate HHB because most Coq polypeptides (excluding Coq1, Coq2, Coq5, and Coq8) are interdependent

 

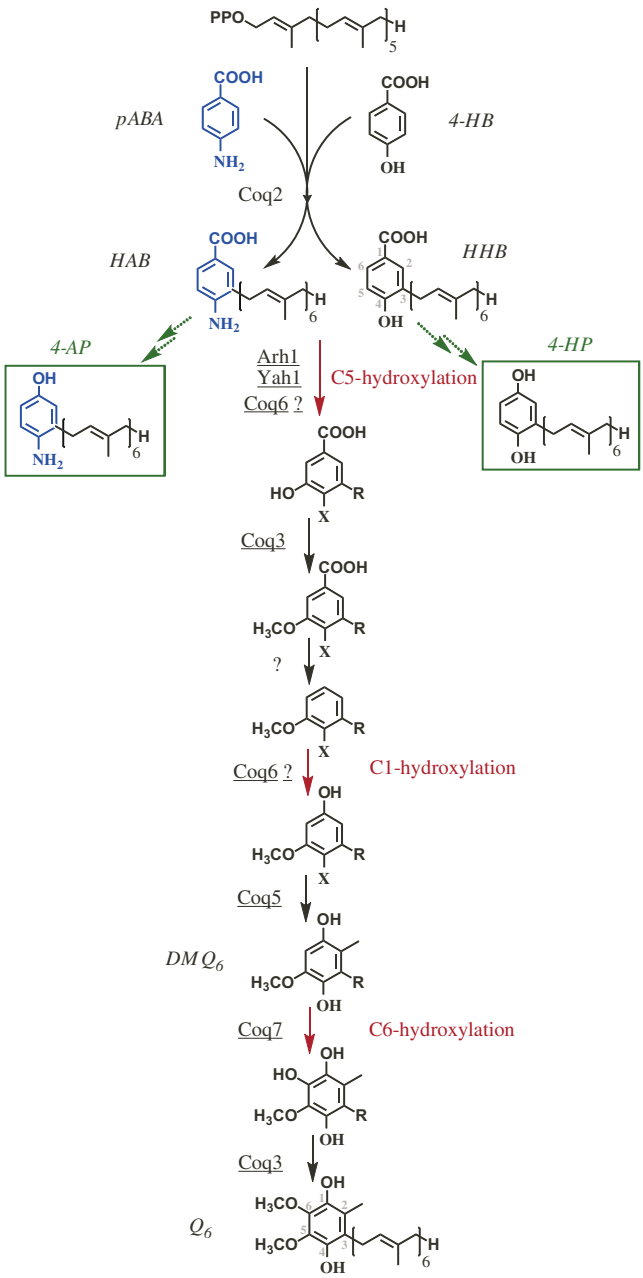


**Figure 1. Current Model of the *S. cerevisiae* Coenzyme Q Biosynthetic Pathway**

The names of proteins (underlined) and intermediates (italicized) relevant to this study are for *S. cerevisiae*. The numbering of the aromatic carbon atoms used throughout this study is shown on HHB and $Q_6$. Prenylation of 4-hydroxybenzoic acid (4-HB) or para-aminobenzoic acid (pABA) by Coq2 yields 3-hexaprenyl-4-hydroxybenzoic acid (HHB) or 3-hexaprenyl-4-aminobenzoic acid (HAB). In subsequent reactions, R stands for the hexaprenyl tail and X designates $NH_2$ or OH. $NH_2$ is converted into OH prior to demethoxyubiquinone ($DMQ_6$) formation (Marbois et al., 2010; Pierrel et al., 2010). The three hydroxylation reactions necessary to yield $Q_6$ are shown in red and the potential implication of Coq6 in the C5- and/or the C1-hydroxylation reactions is indicated (Coq6?). In cells depleted for Yah1 or Arh1, the C5-hydroxylation is deficient and results in synthesis of 3-hexaprenyl-4-aminophenol (4-AP) from HAB and of 3-hexaprenyl-4-hydroxyphenol (4-HP) from HHB (green dashed arrows and green boxes) (Pierrel et al., 2010).

for their stability within the Q biosynthetic complex (Hsieh et al., 2007). Indeed, the absence of a single Coq polypeptide causes the degradation of other Coq proteins (Tran and Clarke, 2007), thus preventing the biosynthesis of $Q_6$ intermediates diagnostic of the altered step in Δ*coq* mutants. Consequently, it has been difficult to elucidate the precise function of Coq4, Coq6, and Coq9. Coq8, a predicted protein kinase, is essential for $Q_6$ biosynthesis and participates in the phosphorylation of Coq3, Coq5, and Coq7 (Tauche et al., 2008; Xie et al., 2011). Overexpression of Coq8 has been shown to restore the levels of Coq3 and Coq4 in most Δ*coq* mutants (Zampol et al., 2010). In addition, overexpression of Coq8 in a Δ*coq7* strain causes the accumulation of demethoxyquinone ($DMQ_6$) (Padilla et al., 2009), the substrate of the C6-hydroxylase, Coq7 (Stenmark et al., 2001) (Figure 1). This result implies that overexpression of Coq8 in the Δ*coq7* strain prevents the degradation of the Coq polypeptides involved in $Q_6$ biosynthetic steps upstream of Coq7. $DMQ_6$ also forms in a Δ*coq7* strain expressing the inactive allele *COQ7-E233K* even in the absence of Coq8 overexpression (Padilla et al., 2004). It therefore seems that single mutations in a given Coq protein have less impact on the integrity of the $Q_6$ biosynthetic complex than null mutations.

In the current study, we sought to define which hydroxylation step of $Q_6$ biosynthesis is dependent on Coq6. We show that a Δ*coq6* strain expressing inactive *COQ6* alleles or overexpressing Coq8 accumulates products of the $Q_6$ biosynthetic pathway that establish that the monooxygenase Coq6 is specifically required for the C5-hydroxylation reaction. The functional combination of Yah1, Arh1, and Coq6 in the C5-hydroxylation reaction is discussed. In addition, we demonstrate that hydroxylated forms of 4-HB like vanillic acid or 3,4-dihydroxybenzoic acid are able to restore $Q_6$ biosynthesis and respiration in a *S. cerevisiae* strain deficient for Coq6. This represents the first indication that the use of 4-HB analogs might be considered as a strategy to bypass defective steps in the Q biosynthetic pathway.

## RESULTS

### Coq6 Is Required for the C5- But Not the C1-Hydroxylation Reaction of Q Biosynthesis

Regular synthetic yeast growth medium contains pABA that is a precursor of $Q_6$ (Marbois et al., 2010; Pierrel et al., 2010). In order to control the nature of the precursors employed for $Q_6$ biosynthesis, we used a synthetic medium without pABA for all our experiments. Electrochemical detection (ECD) of cell lipid extracts separated by HPLC revealed that addition of pABA or 4-HB to the growth medium increased the $Q_6$ and $DMQ_6$ content of *S. cerevisiae* (see Figure S1A available online) consistent with our previous conclusion that endogenous 4-HB is limiting for $Q_6$ biosynthesis (Pierrel et al., 2010). Overexpression of Coq8 in the Δ*coq6* mutant strain led to an accumulation of 4-AP or 4-HP in cells cultured in the presence of pABA or 4-HB, respectively, whereas no products were formed in the absence of Coq8 overexpression (Figure 2A). Identities of 4-AP and 4-HP were established previously (Pierrel et al., 2010) and here confirmed by their retention time in HPLC chromatograms, their ultraviolet-visible spectra (Figure S1B) and the expected ion transitions (m/z: 518.3/122 for 4-AP and 519.3/123 for 4-HP) in mass spectrometry coupled to HPLC (data not shown). Without pABA or

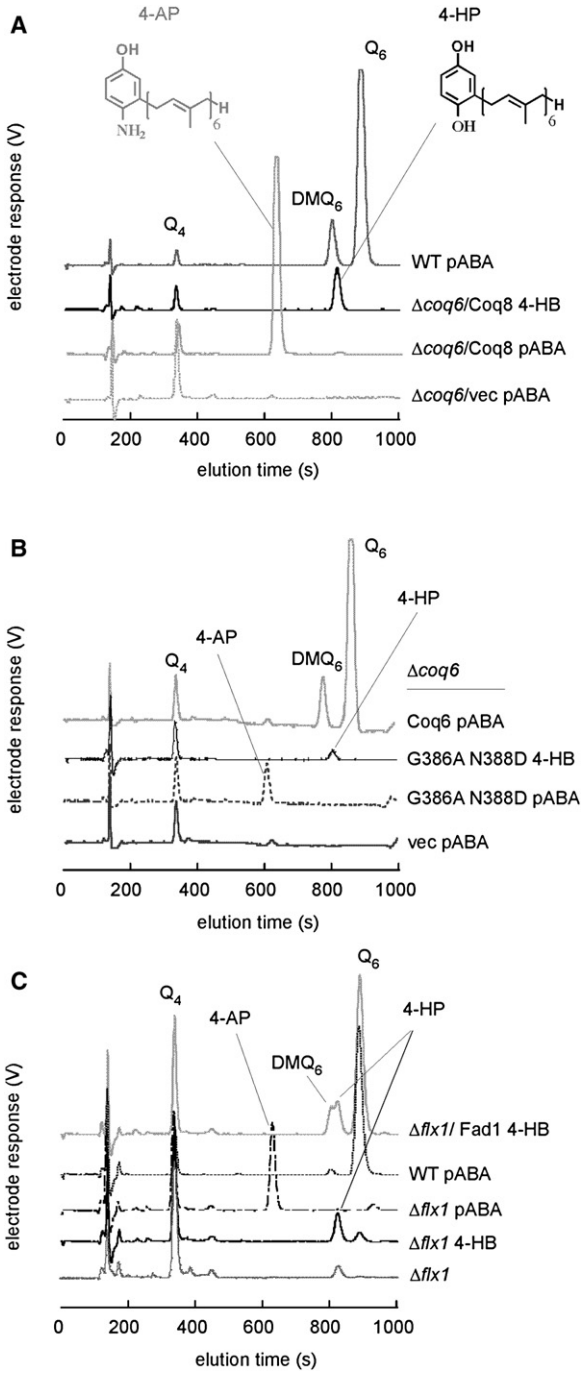


**Figure 2. Coq6 Is Required for the C5 but Not for the C1-Hydroxylation of $Q_6$ Biosynthesis**

(A) WT cells or Δ*coq6* cells transformed with an empty vector (vec) or with an episomal vector coding for Coq8 were grown in 2% glucose synthetic medium without pABA containing or not 100 μM 4-hydroxybenzoic acid (4-HB) or 100 μM pABA. Lipid extracts of 1 mg of cells (WT), 2 mg of cells (/Coq8), or 8 mg of cells (/vec) were analyzed by HPLC-ECD. Chemical structures of 3-hexaprenyl-4-aminophenol (4-AP, eluting at 610 s) and of 3-hexaprenyl-4-hydroxyphenol (4-HP, eluting at 810 s) are displayed. The peaks corresponding to coenzyme $Q_6$ ($Q_6$), demethoxyquinone ($DMQ_6$), and to the $Q_4$ standard are indicated.

(B) Δ*coq6* cells transformed with an empty vector (vec) or centromeric vectors coding either for Coq6 or Coq6-G386A-N388D were grown in glucose synthetic medium without pABA containing 100 μM pABA or 100 μM 4-HB. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

(C) WT and Δ*flx1* cells transformed or not with an episomal vector coding for Fad1 were grown in glucose synthetic medium without pABA supplemented or not with pABA or 4-HB at 100 μM. Lipid extracts of 4 mg of cells (Δ*flx1*) and 1 mg of WT were analyzed by HPLC-ECD.

See also Figures S1 and S2.

4-HB supplementation of the growth medium, the Δ*coq6* strain overexpressing Coq8 accumulated 4-HP suggesting that endogenous 4-HB enters the Q biosynthetic pathway preferentially over endogenous pABA (Figure S1C). We also mutated residues in Coq6 (G202, G386, N388) selected on the basis of their conservation in other flavoprotein monooxygenases (Figure S2) in which they are implicated in the binding of NADPH and FAD, as shown in the case of the parahydroxybenzoate hydroxylase PobA (Eppink et al., 1997; Palfey et al., 1994). As expected, a vector coding for Coq6 restored $Q_6$ biosynthesis in the Δ*coq6* strain whereas G202A and G386A-N388D mutant alleles of *COQ6* were inactive as proven by the absence of $Q_6$ and $DMQ_6$ (Figure 2B; Figure S1C). However, these mutants caused an accumulation of 4-AP in Δ*coq6* cells cultured in the presence of pABA, and of 4-HP upon growth in the presence of 4-HB (Figure 2B). Together, these results show that a Δ*coq6* strain either expressing a Coq6 protein inactivated by point mutations or overexpressing Coq8 accumulates 4-AP and 4-HP. This finding demonstrates that the C1- but not the C5-hydroxylation reaction is still catalyzed in the absence of Coq6 activity (Figure 1). Because Coq6 is a predicted FAD-dependent monooxygenase (Gin et al., 2003; van Berkel et al., 2006), we checked whether the C5-hydroxylation is dependent on the availability of FAD in mitochondria. Deletion of the gene *FLX1* that codes for a mitochondrial FAD transporter results in decreased activity of FAD-dependent mitochondrial enzymes (Bafunno et al., 2004; Tzagoloff et al., 1996). The Δ*flx1* strain showed markedly diminished levels of $Q_6$ and accumulated 4-AP and 4-HP when cultured in the presence of pABA and 4-HB respectively (Figure 2C). Furthermore, we observed that overexpression of Fad1, the flavin adenine dinucleotide synthetase, restored $Q_6$ biosynthesis in the Δ*flx1* strain, however without completely abolishing the formation of 4-HP (Figure 2C). This result is in agreement with the overexpression of Fad1 being able to partially complement the mitochondrial FAD deficiency of a *flx1* mutant strain (Wu et al., 1995). Collectively, our results show that the C5-hydroxylation depends on mitochondrial FAD and that Coq6, a predicted FAD-dependent monooxygenase, is essential for the C5- but not for the C1-hydroxylation reaction of $Q_6$ biosynthesis.

## Vanillic Acid and 3,4-Dihydroxybenzoic Acid Restore $Q_6$ Biosynthesis in Strains Lacking Coq6 Activity

We tested whether hydroxylated forms of 4-HB like 3,4-dihydroxybenzoic acid (3,4-diHB) or vanillic acid (VA) (Figure S3A) may enter the $Q_6$ biosynthetic pathway and therefore bypass the C5-hydroxylation step that is deficient in *coq6* mutants. Addition of 3,4-diHB or VA to the growth medium restored $Q_6$ biosynthesis in a dose-dependent manner in the Δ*coq6* strain overexpressing Coq8 (Figure 3A; Figure S3B) or expressing the inactive Coq6 mutant G386A-N388D (Figure S3C). The Δ*coq6*

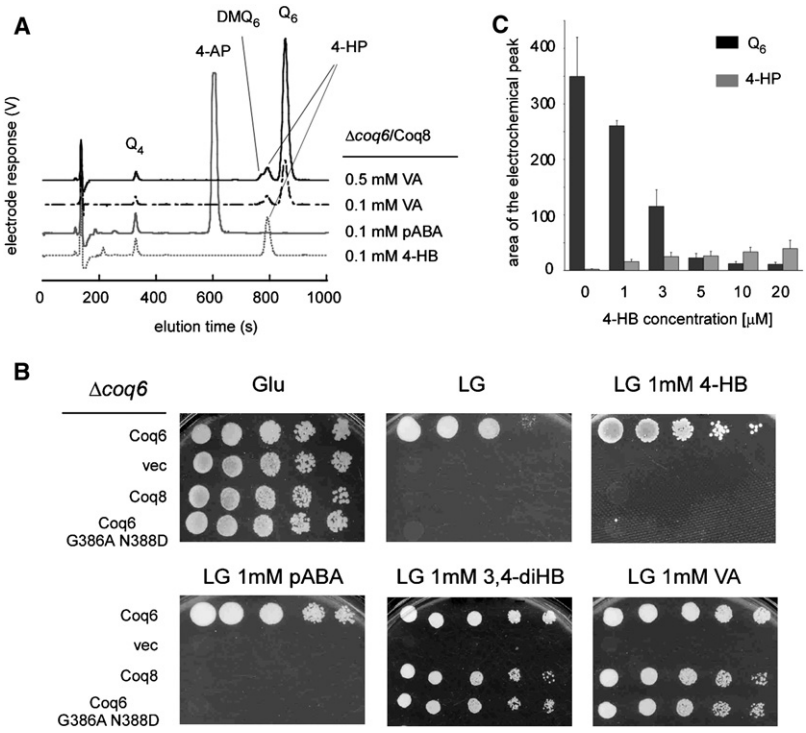


**Figure 3. Vanillic Acid and 3,4-Dihydroxybenzoic Acid Restore $Q_6$ Biosynthesis and Respiration in *coq6* Mutant Cells**

(A) Δ*coq6* cells transformed with an episomal vector coding for Coq8 were grown in 2% glucose synthetic medium without pABA containing the indicated concentrations of 4-HB, pABA, or vanillic acid (VA). Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

(B) Δ*coq6* cells transformed either with an episomal vector coding for Coq8, an empty vector (vec) or with centromeric vectors coding for WT Coq6 or Coq6-G386A-N388D were grown in glucose synthetic medium for 24 hr and serial dilutions were spotted onto agar plates. The plates contained synthetic medium without pABA supplemented with 2% glucose (Glu) or 2% lactate-2% glycerol (LG) and either vanillic acid (VA), pABA, or 3,4-dihydroxybenzoic acid (3,4-diHB). The plates were incubated for 2 days (Glu) or 4 days (LG) at 30°C.

(C) Quantification of $Q_6$ and 4-HP formed in Δ*coq6* cells overexpressing Coq8 grown in glucose synthetic medium without pABA with 1mM VA and the indicated concentration of 4-HB. Error bars are standard deviation (n = 2). See also Figure S3.

strain containing only an empty vector synthesized no $Q_6$ from VA showing that the integrity of the Q biosynthetic complex is required to convert VA in $Q_6$ (Figure S3B). The Δ*coq6* strain overexpressing Coq8 or expressing Coq6-G386A-N388D was able to grow on respiratory carbon sources supplemented with 3,4-diHB or VA, showing that $Q_6$ biosynthesized from these substrate analogs is physiologically functional (Figure 3B). When pABA or 4-HB were added in place of VA or 3,4-diHB, the cells were not able to grow on respiratory substrates (Figure 3B), thus showing that 4-AP or 4-HP are not competent for electron transfer in the respiratory chain. VA also restored $Q_6$ biosynthesis in the Δ*flx1* mutant to 70% of wild-type (WT) (Figure S3D).

Addition of increasing concentrations of 4-HB to the growth medium containing 1mM VA gradually decreased $Q_6$ levels and promoted the accumulation of 4-HP in the Δ*coq6* strain overexpressing Coq8 (Figure 3C). pABA had a similar impact on $Q_6$ levels at concentrations comparable to those of 4-HB (Figure S3E). Altogether our data show that exogenous pABA and 4-HB compete with VA to enter the $Q_6$ biosynthetic pathway at the prenylation step catalyzed by Coq2 and that prenylated VA is converted into $Q_6$, therefore bypassing the deficient C5-hydroxylation reaction in *coq6* mutant cells.

## Coq6 and Yah1 Are Functionally Coupled in the C5-Hydroxylation Reaction

Our results show that the phenotypes of cells depleted for Yah1 (Pierrel et al., 2010) or deficient for Coq6 are strikingly similar with regard to $Q_6$ biosynthesis suggesting a mechanism in which Coq6 and Yah1 work together in the C5-hydroxylation step. The following experiments establish indeed that depletion of Yah1 directly impacts Coq6 activity. First, we demonstrate that Coq6 is stable when Yah1 is depleted. We constructed a Gal-*YAH1 COQ6-3HA* strain that contains a chromosomal insertion of a sequence coding for a triple hemagglutinin (3HA) epitope tag on the 3′ end of *COQ6* and allows for regulated expression of Yah1. Indeed, the native *YAH1* promoter has been replaced by the *GAL1-10* promoter allowing for expression of Yah1 in a culture medium containing galactose and for depletion of Yah1 in a medium containing glucose (Lange et al., 2000). The Coq6-3HA protein was detected by immunoblotting at the expected size of 57 kDa (Figure 4A). Depletion of Yah1 by culturing the Gal-*YAH1 COQ6-3HA* strain in the presence of glucose had no effect on the steady-state level of Coq6 (Figure 4A). The presence of Coq6 in Yah1-depleted cells is also confirmed by the observation that VA or 3,4-diHB restores $Q_6$ biosynthesis in the Yah1-depleted cells regardless of Coq8 overexpression (Figure S4A), thus showing that the Q biosynthetic complex is intact and therefore that Coq6 is present. Second, overexpression of Fad1 did not complement the $Q_6$ biosynthetic defect in Yah1-depleted cells (Figure S4B), suggesting that this defect does not result from a depletion of mitochondrial FAD. These data together show that Yah1 depletion, although having a negative effect on $Q_6$ biosynthesis, has no effect on Coq6 stability or mitochondrial FAD content.

Human Fdx2 was recently shown to complement the ISC biosynthetic defect of Yah1-depleted cells (Sheftel et al., 2010). Surprisingly, none of the two human homologs of Yah1, Fdx1, or Fdx2 complemented the $Q_6$ defect of Yah1-depleted cells as shown from the observation that the cells contained almost no $Q_6$ and furthermore accumulated 4-AP upon culture in the presence of pABA (Figure 4B). Growth of the Gal-*YAH1* strain on synthetic medium containing the respiratory carbon source lactate was limited unless it contained a plasmid carrying the *YAH1* gene (Figure 4C). With a plasmid expressing Fdx2, growth was restored upon addition of VA or 3,4-diHB to the lactate medium whereas addition of 4-HB was without any effect

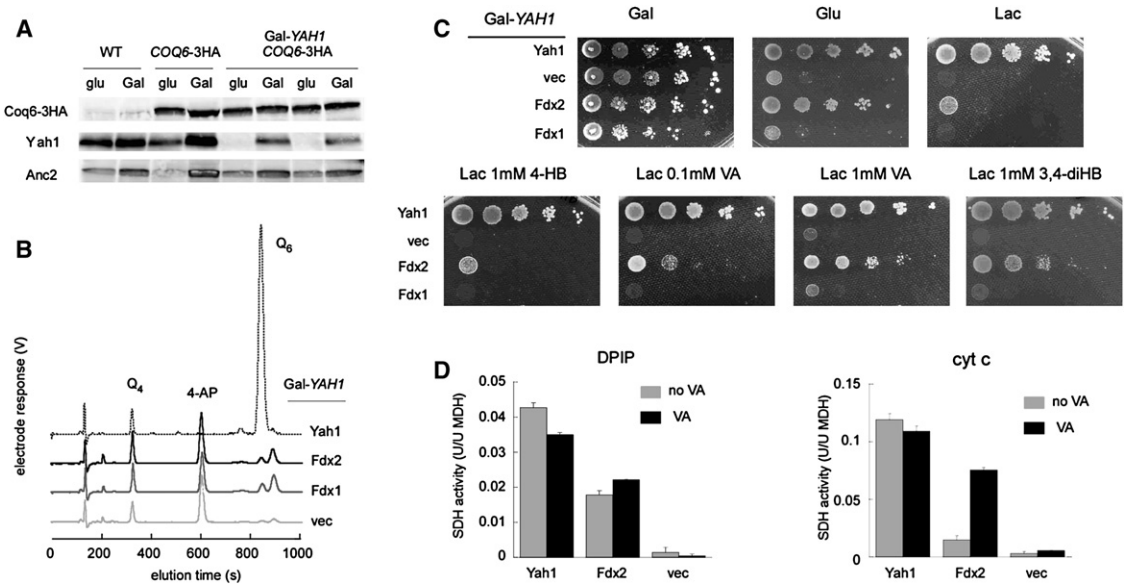


**Figure 4. $Q_6$ Is Limiting for Respiratory Growth of Yah1-Depleted Cells Expressing Fdx2**

(A) Immunoblot of whole-cell lysates from WT, *COQ6-3HA*, and Gal-*YAH1 COQ6-3HA* (two clones) cells grown in YP rich medium containing either 2% glucose (Glu) or 2% galactose (Gal). Coq6-3HA was detected at 57 kDa with an anti-HA antibody and as expected was absent from extracts of the WT strain. Yah1 was detected around 16 kDa and Anc2, the major ADP/ATP carrier of the mitochondrial inner membrane, at 35 kDa.

(B) Gal-*YAH1* cells transformed with an empty vector (vec), a centromeric vector coding for Yah1 or episomal vectors coding for mitochondrially targeted human ferredoxin 1 or 2 (Fdx1 or Fdx2) were grown for 24 hr in synthetic medium supplemented with glucose and 1mM pABA. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD.

(C) Same cells as in (B) were grown in glucose synthetic medium without pABA for 24 hr and serial dilutions were spotted onto agar plates. The plates contained synthetic medium without pABA supplemented at 2% with either glucose (Glu), galactose (Gal), or lactate (Lac) and either vanillic acid (VA), 3,4-dihydroxybenzoic acid (3,4-diHB), or 4-HB. The plates were incubated for 4 days at 30°C.

(D) Gal-*YAH1* cells containing an empty vector (vec), a centromeric vector coding for Yah1 (Yah1) or an episomal vector coding for mitochondrially-targeted human ferredoxin 2 (Fdx2) were cultivated in glucose synthetic medium for 26 hr and finally in glucose synthetic medium without pABA in the presence or absence of 1 mM vanillic acid (VA) for 14 hr. Mitochondria were isolated and the enzyme activities of SDH (succinate to dichlorophenol-indophenol [DPIP]) and SDH in combination with complex III (succinate to cytochrome *c* [cyt c]) were determined and normalized to malate dehydrogenase activity (MDH). Error bars are standard deviation (n = 3).

See also Figure S4.

(Figure 4C). Our results thus suggest that, in the absence of VA or 3,4-diHB, $Q_6$ is limiting for respiratory growth of the Gal-*YAH1* strain expressing Fdx2. This was confirmed by measuring the in vitro activity of succinate dehydrogenase (SDH), a multiprotein complex that contains three ISC and one FAD, all required for activity. As expected from the complementation of the ISC biosynthetic defect of Yah1-depleted cells by Fdx2 (Sheftel et al., 2010), Fdx2 significantly restored reduction of the chemical dichlorophenol-indophenol (DPIP) by SDH in Yah1-depleted cells, although not to WT levels (Figure 4D). $Q_6$ is required to transfer electrons from SDH to the cytochrome $bc_1$ complex that reduces cytochrome c (cyt c). Fdx2 restored SDH-cyt c reductase activity in Yah1-depleted cells only when VA was added to the culture medium (Figure 4D). This result shows that the limiting factor in transferring electrons from succinate to cyt c in Yah1-depleted cells expressing human Fdx2 is $Q_6$, the level of which is restored by addition of VA to the growth medium (Figure S4A).

## DISCUSSION

Our current work addresses the precise functional role of the Coq6 monooxygenase in the hydroxylation reactions of the Q biosynthetic pathway. As shown previously in the case of the Δ*coq7* strain (Padilla et al., 2004, 2009), overexpression of Coq8 or expression of inactive alleles of *COQ6* allow for preservation of the integrity of the $Q_6$ biosynthetic complex in a Δ*coq6* strain. We show here for the first time that under these conditions, the Δ*coq6* strain grown in the presence of pABA or 4-HB accumulates significant amounts of 4-AP and 4-HP, respectively. The accumulation of 4-AP and 4-HP is diagnostic of an impaired C5-hydroxylation but of a functional C1-hydroxylation. As a consequence, the predicted FAD-dependent monooxygenase Coq6 is required for the C5-hydroxylation but not for the C1-hydroxylation, definitively resolving the uncertainty regarding which hydroxylation reaction is catalyzed by Coq6. As a result, the C1-hydroxylase is still unknown (Figure 5) and is unlikely to be any of the Coq proteins identified to date because among these, only Coq6 and Coq7 display sequence homologies with monooxygenases and Coq7 participates exclusively in the C6-hydroxylation (Behan and Lippard, 2010; Padilla et al., 2004). Some experiments reported here suggest that the C1-hydroxylase is unlikely to be a FAD-dependent monooxygenase. A Δ*flx1* strain, characterized by impaired FAD-dependent mitochondrial activities (Bafunno et al., 2004; Tzagoloff et al., 1996), accumulates 4-AP and 4-HP indicating

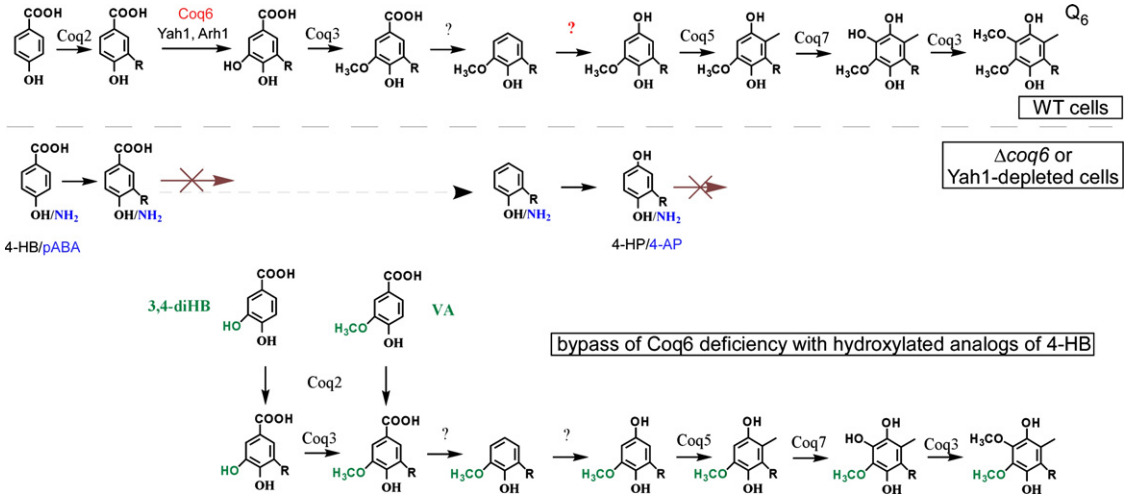


**Figure 5. Coq6 Is Required for the C5-Hydroxylation of $Q_6$ Biosynthesis But Coq6 Deficiency Can Be Bypassed by Using Analogs of 4-HB**

The pathway leading to $Q_6$ biosynthesis in WT *S. cerevisiae* cells is shown (above dashed line) with Coq6 implicated together with Yah1 and Arh1 in the C5-hydroxylation, whereas the C1-hydroxylation is catalyzed by an unidentified protein (?). In Yah1-depleted cells or in Δ*coq6* cells overexpressing either *COQ8* or an inactive *COQ6* allele (below dashed line), the C5-hydroxylation does not take place (crossed arrow) but the C1-decarboxylation (dashed arrow) and the C1-hydroxylation proceed efficiently, leading to the accumulation of 4-HP when 4-HB is prenylated, or the accumulation of 4-AP when pABA is prenylated (blue). 3,4-diHB and VA contain an additional C5-hydroxyl (green) or C5-methoxyl (green) compared to 4-HB. 3,4-diHB and VA that correspond to the unprenylated products of the reactions catalyzed by Coq6 and Coq3 are prenylated in vivo by Coq2 and restore $Q_6$ biosynthesis in cells deficient for Coq6.

that the C1-hydroxylase is functional in this mutant (Figure 2C). Furthermore, restoration of $Q_6$ biosynthesis in Δ*flx1* strain by VA (Figure S3D) is consistent with the C1-hydroxylation proceeding normally in the Δ*flx1* strain.

Our results show that VA and 3,4-diHB can be used as $Q_6$ precursors and thus restore $Q_6$ biosynthesis and respiration in Δ*coq6* cells overexpressing Coq8 or expressing the Coq6 inactive alleles (Figure 5). This proves that analogs of 4-HB added to the growth medium of *S. cerevisiae* can reach the mitochondrial matrix, where $Q_6$ biosynthesis takes place, and then enter the $Q_6$ biosynthetic pathway via prenylation by Coq2 (Figure 5). The strong impact of minor quantities of pABA or 4-HB observed on the levels of $Q_6$ synthesized from VA (Figure 3C and Figure S3E) may be explained by two nonexclusive hypotheses. VA may not be as efficiently transported to the mitochondrial matrix as pABA and 4-HB or Coq2 may have a higher affinity for pABA and 4-HB compared to VA. In any case, Coq2 has a broad substrate specificity in agreement with early in vitro studies that showed that mitochondrial preparations from rat heart and liver were able to prenylate VA and 3,4-diHB (Nambudiri et al., 1976). In fact, these analogs of 4-HB meet the structural requirements for prenylation by the polyprenyl transferase Coq2, i.e., an electron-donating substituent at position 4 of the aromatic ring combined with a carboxylic acid, a strong electron-attracting group, at position 1 (Alam et al., 1975). On the contrary, 4-nitrobenzoic acid that harbors the strong electron-attracting nitro group at position 4 inhibits Coq2 and as a consequence acts as an inhibitor of Q biosynthesis in mammalian cells cultures (Forsman et al., 2010). We would like to suggest that 4-HB analogs compatible with prenylation by Coq2 may also bypass deficient Q biosynthetic steps downstream of the C5-hydroxylation reaction. It is interesting to note that most mutations identified so far in humans to cause primary Q deficiency are not found in genes encoding enzymes that catalytically modify the prenylated aromatic ring except for *COQ6* (Heeringa et al., 2011). In fact they are found in (1) *PDSS1* and *PDSS2* (Lopez et al., 2006; Mollet et al., 2007), which are the relatives of *COQ1* catalyzing the synthesis of the polyprenyl tail; (2) the polyprenyl transferase *COQ2* (Mollet et al., 2007); (3) *ADCK3/CABC1* (Lagier-Tourenne et al., 2008; Mollet et al., 2008), the *COQ8* homolog; and (4) *COQ9,* a gene with no specific function assigned (Duncan et al., 2009). Our results suggest that VA may be efficient at promoting $Q_{10}$ biosynthesis in patients with *COQ6* mutations. Practically, as vanillin (3-methoxy-4-hydroxybenzaldehyde), a common nontoxic food additive, is converted in the liver to VA (Muskiet and Groen, 1979; Sayavongsaa et al., 2007), vanillin may represent an interesting therapeutic molecule to try in patients with *COQ6* mutations.

Finally, we previously demonstrated that Yah1 and Arh1 are essential for the C5-hydroxylation reaction (Pierrel et al., 2010). The similar impact on $Q_6$ biosynthesis of Yah1 or Arh1 depletion or Coq6 inactivation raises the question of how these three enzymes are functionally coupled. Here we provide preliminary data aiming at understanding this link. Yah1/Arh1 form a ferredoxin/ferredoxin reductase system that transfers electrons for different mitochondrial processes, in particular ISC assembly (Lill, 2009). The fact that Fdx2, a human homolog of Yah1, complements the ISC biosynthetic defect but not the $Q_6$ biosynthetic defect of Yah1-depleted cells excludes that Coq6 and thus $Q_6$ metabolism may indirectly be affected by an impairment of ISC biosynthesis. Coq6 is classified among Class A flavoprotein monooxygenases that have been described to contain a tightly bound FAD cofactor and to depend on NAD(P)H as a coenzyme for reduction of FAD (van Berkel et al., 2006). We checked whether inactivation of Yah1 could indirectly cause a depletion of mitochondrial NAD(P)H or FAD that would result in decreased

Coq6 activity. Our data summarized below indicate that, in the absence of Yah1, the Coq6 polypeptide is not degraded, mitochondrial FAD and NAD(P)H are available, and yet Coq6 is unable to perform the C5-hydroxylation, implying that Yah1 itself plays a role in this reaction. First, the Coq6-3HA polypeptide is detected in Yah1-depleted cells. Second, a shortage of mitochondrial FAD, the predicted cofactor of Coq6, is unlikely. Indeed, Yah1-depleted cells expressing Fdx2 are impaired for the C5-hydroxylation reaction but display significant SDH activity, known to depend on FAD. In addition, overexpression of *FAD1* restored $Q_6$ biosynthesis in the Δ*flx1* strain whereas it failed to do so in Yah1-depleted cells. Third, mitochondrial NAD(P)H levels may not be dramatically affected in Yah1-depleted cells expressing Fdx2 because the latter is able to assemble ISC, a process supported by mitochondrial NADPH (Pain et al., 2010). Also in support of this, Yah1-depleted cells are prototroph for arginine (data not shown), the biosynthesis of which requires mitochondrial NADPH produced by Pos5 via phosphorylation of mitochondrial NADH (Outten and Culotta, 2003). As a conclusion, if Coq6 was a classical class A flavoprotein monooxygenase catalyzing reduction of FAD by NAD(P)H, it should be active in Yah1-depleted cells because their levels of mitochondrial FAD and NAD(P)H are not compromised. Consequently, we end up with the hypothesis of an unusual mechanism in which the reducing power of NAD(P)H may transit via the Yah1/Arh1 system before reaching Coq6. The β-cyclohexenyl carotenoid epoxidase, another class A flavoprotein monooxygenase, has been shown to require a ferredoxin/ferredoxin reductase system for in vitro activity (Bouvier et al., 1996) and thus represents a precedent for such an electron transfer pathway from a ferredoxin to a flavin-monoxygenase. Unequivocal demonstration that Coq6 may also use an unconventional reducing system like Arh1/Yah1 will necessitate the development of an in vitro assay with purified proteins; a challenging task given that Coq6 likely interacts with several Coq polypeptides in the $Q_6$ biosynthetic complex (Marbois et al., 2005). Nonetheless, our study has unambiguously established that Coq6 is required exclusively for the C5-hydroxylation of $Q_6$ biosynthesis and that hydroxylated analogs of 4-HB can be used as precursors of $Q_6$, two results that significantly improve our understanding of the biosynthesis of this crucial coenzyme.

## SIGNIFICANCE

**Coenzyme Q (Q) or ubiquinone, an important cellular antioxidant, is essential to electron transport chains and is required for several other cellular processes. Q biosynthesis requires at least 11 proteins in *S. cerevisiae* but the precise function of several of them is not known. Our work establishes that the predicted monoxygenase Coq6 is involved in the C5-hydroxylation reaction and that an unidentified monooxygenase catalyzes the C1-hydroxylation reaction. We further demonstrate the possibility to bypass a deficient Q biosynthetic step in *S. cerevisiae* by providing the defective chemical group within a synthetic 4-hydroxybenzoic acid analog. Indeed, a *coq6* mutant impaired in the C5-hydroxylation reaction recovers $Q_6$ biosynthesis and respiration on addition of two such analogs, 3,4-dihydroxybenzoic acid and vanillic acid. Primary $Q_{10}$ deficiencies have been linked to mutations in six genes of the Q biosynthetic pathway and result in clinically heterogeneous diseases that, if diagnosed early, are improved by $Q_{10}$ supplementation. However, the lipophilicity of $Q_{10}$ may restrain its efficient transport to the mitochondrial inner membrane where $Q_{10}$ functions in the respiratory chain (Quinzii and Hirano, 2010). Our work suggests that hydrophilic analogs of 4-hydroxybenzoic acid may restore $Q_{10}$ biosynthesis in patients with some primary $Q_{10}$ deficiencies by bypassing the altered biosynthetic step. The use of various 4-hydroxybenzoic acid analogs will also contribute to characterize the biosynthetic step(s) blocked in some *S. cerevisiae coq* mutants and will thus help to identify the molecular function of Coq proteins with unknown function. Our work illustrates the importance of a molecular understanding of the Q biosynthetic pathway and warrants the identification of yet unidentified proteins that participates in Q biosynthesis, in particular the monooxygenase responsible for the C1-hydroxylation reaction.**

## EXPERIMENTAL PROCEDURES

### Yeast Strains and Culture Conditions

*S. cerevisiae* strains used in this study are listed in Table S1. *S. cerevisiae* strains were transformed using lithium acetate. A 3HA epitope tag was inserted on the 3′ end of *COQ6* ORF by PCR as described previously (Longtine et al., 1998) to create the *COQ6-3HA* strain. This strain was crossed with the Gal-*YAH1* strain to isolate the Gal-*YAH1 COQ6-3HA* strain by selecting the corresponding markers after tetrad dissection. YNB without pABA and folate was from MP Biomedicals. Rich YP medium was prepared as described (Sherman, 2002). Glucose, galactose, or lactate-glycerol was used at 2%. The Gal-*YAH1* strain was maintained and precultured on galactose medium. Depletion of Yah1 was accomplished by diluting 200-fold the preculture into glucose containing medium and growing the cells for 18 hr at 30°C.

### Plasmids

*COQ6* ORF was cloned into pRS416 under the control of the *MET25* promoter and the *CYC1* terminator using XhoI and XbaI (Mumberg et al., 1994). This vector served as a template to generate the G202V and G386A-N388D Coq6 mutants by site directed mutagenesis. *FAD1* was cloned with its own promoter (370 bp) and terminator (210 bp) into pRS423 using EcoRI and XhoI. Fdx1 and Fdx2 expressing plasmids have been described (Sheftel et al., 2010) and *COQ8* cloned in pFL44 was a kind gift from Dr. Geneviève Dujardin. Sequencing was used to confirm cloning products in all created vectors.

### Miscellaneous Biochemical Analysis

Isolation of mitochondria and immunostaining were performed as described (Diekert et al., 2001; Harlow and Lane, 1988). Cellular lipid extraction after addition of the $Q_4$ standard and quantification of electroactive compounds by HPLC-ECD with a 5011A analytical cell (E1, –420 mV; E2, +380 mV) were as described (Pierrel et al., 2010). Hydroquinones present in samples were oxidized with a precolumn 5020 guard cell set in oxidizing mode (E, +650 mV). Mitochondrial enzymatic activities were measured as previously described (Pierik et al., 2009).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.chembiol.2011.07.008.

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