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Over-expression of the Coq8 kinase in *Saccharomyces cerevisiae coq* null mutants allows for accumulation of diagnostic intermediates of the Coenzyme Q₆ biosynthetic pathway*

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*Running Title: Novel Q-intermediates in *coq* null yeast over-expressing Coq8

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Keywords: lipid metabolism, mitochondria; ubiquinone; yeast

Background: Several steps of eukaryotic coenzyme Q biosynthesis are still in question.

Results: Yeast *coq* null mutants over-expressing the Coq8 kinase have stable Coq polypeptides and accumulate new Q-intermediates that help diagnose the blocked step.

Conclusion: New functions for Coq polypeptides are proposed.

Significance: Identification of the blocked step allows for the use of alternate ring precursors that rescue Q biosynthesis in some mutants.

SUMMARY

Most of the Coq proteins involved in coenzyme Q (ubiquinone or Q) biosynthesis are interdependent within a multi-protein complex in the yeast *Saccharomyces cerevisiae*. Lack of only one Coq polypeptide, as in Δcoq strains, results in the degradation of several Coq proteins. Consequently, Δcoq strains accumulate the same early intermediate of the Q₆ biosynthetic pathway; this intermediate is therefore not informative about the deficient

biosynthetic step in a particular Δcoq strain. In this work, we report that the over-expression of the protein Coq8 in Δcoq strains restores steady state levels of the unstable Coq proteins. Coq8 has been proposed to be a kinase and we provide evidence that the kinase activity is essential for the stabilizing effect of Coq8 in the Δcoq strains. This stabilisation results in the accumulation of several novel Q₆ biosynthetic intermediates. These Q-intermediates identify chemical steps impaired in cells lacking Coq4 and Coq9 polypeptides, for which no function has been established to date. Several of the new intermediates contain a C4-amine and provide information on the deamination reaction that takes place when para-aminobenzoic acid is used as a ring precursor of Q₆. Finally, we used synthetic analogues of 4-hydroxybenzoic acid to bypass deficient biosynthetic steps and we show here that 2,4-dihydroxybenzoic acid is able to restore Q₆ biosynthesis and respiratory growth in a $\Delta coq7$ strain over-expressing Coq8. The over-expression of Coq8 and the use of 4-

hydroxybenzoic acid analogues represent innovative tools to elucidate the Q biosynthetic pathway.

INTRODUCTION

Coenzyme Q (ubiquinone or Q)⁷ is a redox-active lipid essential for electron and proton transport in the mitochondrial respiratory chain. Q is also important in the mitochondrial inner membrane because it serves as an antioxidant, it modulates the function of the mitochondrial membrane transition pore and is a cofactor of uncoupling proteins (1). Q is composed of a fully-substituted benzoquinone ring which is attached to a polyisoprenyl tail of various length (six isoprenyl units in *Saccharomyces cerevisiae* hence Q₆, ten units in humans, hence Q₁₀). The eukaryotic Q biosynthetic pathway has been studied most thoroughly in *S. cerevisiae* where it implicates at least eleven proteins, Coq1-Coq9, Arh1, and Yah1 (2,3).

Genetic and biochemical studies have shown that most Coq proteins are present in a high molecular mass, multi-subunit Q₆ biosynthetic complex in *S. cerevisiae* (4,5). The absence of a single Coq polypeptide from the complex causes a drastic diminution of the steady state levels of some Coq proteins. For example, the steady state levels of Coq4, Coq6, Coq7 and Coq9 are decreased in each of the $\Delta coq1$ - $\Delta coq9$ null strains (6). As a result, the same early intermediate 3-hexaprenyl-4-hydroxybenzoic acid (HHB; Fig. 1, path 1), accumulates in each of the $\Delta coq3$ - $\Delta coq9$ strains. Certain point mutations resulting in amino acid substitutions seem to have less impact on the integrity of the Q biosynthetic complex than a null mutation; expression of the inactive Coq7-E194K polypeptide in a $\Delta coq7$ strain caused accumulation of the expected intermediate demethoxy-Q₆ (DMQ₆, Fig. 1) (7,8). Apart from this example, the absence of accumulation of biosynthetic intermediates downstream of HHB in Δcoq strains has hindered our understanding of the Q biosynthetic pathway. Therefore the precise order of certain biosynthetic steps is still elusive and the function of Coq4 and Coq9 is not defined.

The yeast *COQ8* gene was formerly called *ABC1* and was thought to be essential for complex III function (9,10). However, it was later shown that *COQ8* was required for Q₆ biosynthesis and,

as such, its deletion only affected complex III activity indirectly (11). Coq8 is a matrix protein peripherally associated with the mitochondrial inner membrane (12) and belongs to the “atypical kinases” subgroup of the protein-kinase-like superfamily (13). Mutations in *ADCK3*, the human ortholog of *COQ8*, were shown to cause Q₁₀ deficiency and cerebellar ataxia (13,14). In yeast, Coq8 is essential for phosphorylation of Coq3 and for its association with the Q biosynthetic complex (12,15). In addition, several phosphorylated forms of Coq5 and Coq7 disappear in a yeast strain expressing the G130D mutant form of Coq8 (12), which mimics the pathogenic G272D mutation found in *ADCK3* (14). Therefore Coq8 appears to be a kinase essential for the phosphorylation of several conserved Coq polypeptides and some of these phosphorylated forms likely play a role in the assembly or maintenance of the Q₆ biosynthetic complex. Recent studies indicate that over-expression of *COQ8* can have profound effects on Q₆ biosynthesis. Indeed, the over-expression of Coq8 (from now on referred to as Coq8 OE) in a $\Delta coq7$ strain promoted the accumulation of DMQ₆ (16), implying that all Coq proteins acting upstream of Coq7 in the biosynthetic pathway were stable and active. The effect of Coq8 OE is likely post-transcriptional since *COQ4* mRNA levels in the $\Delta coq7$ strain were not dependent on the level of Coq8 OE (16). Recently, the low steady-state level of Coq4 encountered in $\Delta coq2$, $\Delta coq3$, $\Delta coq5$, and $\Delta coq7$ strains was shown to be restored to wild-type levels by Coq8 OE (17). In the case of a $\Delta coq6$ strain, Coq8 OE allowed the specific accumulation of 3-hexaprenyl-4-hydroxyphenol (4-HP; Fig. 1) which led us to identify Coq6 as the monooxygenase responsible for the C5-hydroxylation step (18). This example demonstrates that the accumulation of Q₆ biosynthetic intermediates in Δcoq strains and the identification of their chemical structure are important for understanding the function of Coq proteins.

In addition to the classic Q biosynthetic pathway emanating from 4-hydroxybenzoic acid (4-HB), *S. cerevisiae* also makes use of para-aminobenzoic acid (pABA) as a ring precursor for Q₆ biosynthesis (Fig. 1, path 2) (3,19). Coq2 is able to catalyze the prenylation of 4-HB to yield 3-

hexaprenyl-4-hydroxybenzoic acid (HHB), as well as the prenylation of pABA to yield 3-hexaprenyl-4-aminobenzoic acid (HAB). We have hypothesized that Coq3-Coq9 enzymes modify both HAB and HHB, and that the C4-amino group must be removed from the HAB derived intermediates in order to produce Q₆ (3,19). The deamination reaction likely occurs before the C6-hydroxylation step catalyzed by Coq7 because 4-imino-DMQ₆ (IDMQ₆) was proposed to be a precursor of DMQ₆ (19). Other ring precursors than 4-HB and pABA can also be used *in vivo* by *S. cerevisiae* to synthesize Q₆. Indeed, 3,4-dihydroxybenzoic acid and vanillic acid bypass a deficiency in the Coq6-mediated C5-hydroxylation reaction and restore Q₆ biosynthesis in *coq6* or *yah1* mutant strains (18).

In this study, we show that Coq8 OE restores the steady state levels of the Coq proteins in most Δcoq strains. The stabilization of the Coq polypeptides leads to the accumulation of Q₆ biosynthetic intermediates which allow the diagnosis of the impaired step. We have used this property to demonstrate that several biosynthetic steps are impaired in the $\Delta coq4$ and $\Delta coq9$ strains and to gain insights into the deamination reaction. Finally, the use of alternate ring precursors promoted the restoration of Q₆ biosynthesis and respiratory growth for a $\Delta coq7$ strain.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions – *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* strains were transformed with lithium acetate as described (20,21). YNB without pABA and folate (–pABA –folate) was purchased from MP Biomedicals. Rich YP medium was prepared as described (22). Dextrose or lactate-glycerol was used at 2%. In preparation for analyses by HPLC-ECD, yeast cells were cultured as described (3) and grown for 18 h at 30°C. Stock solutions of 4-HB analogues at 100 mM were prepared by slowly titrating NaOH (care was taken not to exceed pH 9) until complete dissolution. The solutions were then filter-sterilized and aliquots were kept at –20°C for several months. The 4-HB analogues were added to –pABA –folate growth medium at the indicated concentrations. Alternatively, in preparation for analyses by HPLC-MS/MS, yeast cells were cultured in Drop Out Galactose medium

(DOGAL) (19) and labelled as described. Briefly, 100 OD₆₀₀ cells were collected from overnight culture and transferred to fresh medium in the presence of various aromatic ring precursors for 2-4 hours at 30 °C. Cells were then collected by centrifugation and subject to LC/MS/MS analysis.

Plasmids – Plasmids used in this study are listed in Table 2. *COQ7* ORF with its own promoter and terminator was cloned into pRS425 using HindIII and XhoI. Sequencing was used to confirm cloning products.

SDS-PAGE and Immunoblot analysis –

Whole cell lysate and isolation of mitochondria were performed as described (12). Proteins were transferred from SDS-polyacrylamide gels to polyvinylidene difluoride (PVDF) membrane and then incubated with primary antibodies at the following dilutions: Coq1, 1:10,000; Coq3, 1:1000; Coq4, 1:1000; Coq5, 1:10,000; Coq6, 1:200; Coq7, 1:1000; Coq8, 1:100; and Coq9, 1:1000 and Pda1 1:1000 ; oat anti-rabbit IgG secondary antibodies (Calbiochem) were used at 1:10,000.

Lipid Extraction and Detection of Electroactive Compounds by HPLC-ECD –

Cellular lipid extraction after addition of the Q₄ standard and detection of electroactive compounds by HPLC-ECD with a 5011A analytical cell (E1, –550 mV; E2, +550 mV) were conducted as described (3). Hydroquinones present in samples were oxidized with a precolumn 5020 guard cell set in oxidizing mode (E, +650 mV).

Lipid Extraction and RP-HPLC-MS/MS –

Lipid extractions of cells made use of Q₄ as internal standard and all LC-MS/MS analysis were performed as previously described (19). Briefly, a 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used. Applied Biosystem software, Analyst version 1.4.2, was used for data acquisition and processing. A binary HPLC solvent delivery system was used with a phenyl-hexyl column (Luna 5u, 100 x 4.60 mm, 5 micron, Phenomenex) for yeast extracts. The mobile phase consisted of Solvent A (methanol:isopropanol, 95:5, with 2.5 mM ammonium formate) and Solvent B (isopropanol, 2.5 mM ammonium formate). The percentage of Solvent B was increased linearly from 0% to 5% over 6 min, and the flow rate was increased from 600 µl/min to 800 µl/min. The flow rate and mobile phase were changed back to

initial condition linearly by 7 min. All samples were analyzed in multiple reaction monitoring mode (MRM).

Purification of Q biosynthetic intermediates and high resolution mass spectrometry measurements – The compounds were purified from yeast cells as previously described (3). Samples in methanol were diluted with 90% acetonitrile and 0.2% formic acid and were infused into the nanospray source of a discovery ORBITRAP instrument (Thermo Fischer Scientific) at a flow rate of 0.5 μ l/min for high-resolution mass spectrometry analyses (3).

RESULTS

Over-expression of Coq8 restores steady state levels of Coq proteins in mitochondria of Δ coq strains— Immunodetection on whole cell lysates revealed that Coq4, Coq7, and Coq9 steady state levels were increased by Coq8 OE in Δ coq3 and Δ coq5 strains (Fig. 2A-B). Similarly, Coq8 OE increased steady state levels of Coq9 and Coq7 in the Δ coq4 strain, and Coq9 and Coq4 in the Δ coq7 strain (Fig. 2A). Even though Coq8 OE resulted in comparable levels of Coq8 in all Δ coq strains tested, it failed to increase the steady-state levels of Coq7 and Coq9 in Δ coq1 and Δ coq2 (Fig. 2B). The inefficiency of Coq8 OE in these two strains likely results from the absence of synthesis of prenylated Q₆-intermediates which have been hypothesized to be important for the stability of Coq4 and Coq6 (23). In the Δ coq9 strain, Coq8 OE did not restore the steady-state levels of either Coq4 or Coq7 (Fig. 2A).

Since the yeast Coq polypeptides localize to mitochondria (2), we verified that the proteins stabilized by Coq8 OE were present in this organelle. The Coq4, Coq7 and Coq9 proteins were readily detected in mitochondria prepared from Δ coq5 and Δ coq6 cells with Coq8 OE (Fig. 3A). Although previous studies indicated that the Coq3 polypeptide was labile in Δ coq strains (6), subsequent experiments performed in the presence of both protease and phosphatase inhibitors preserved steady-state levels of Coq3 (12,15). In agreement, Coq3 levels were unaffected in Δ coq5 or Δ coq6 cells (Fig. 3A). The Coq6 antibody reacts with many nonspecific polypeptides in yeast cell extracts and therefore immunoblotting for

Coq6 is best carried out on isolated mitochondria. The steady state level of Coq6 was also increased by Coq8 OE in Δ coq3, Δ coq4, and Δ coq7 although it did not reach wild-type level (Fig. 3B). Steady state levels of the Coq1 polypeptide provide a loading control, since Coq1 is unaffected by null mutations in Δ coq2- Δ coq9 (6).

Moderate over-expression of kinase-active Coq8 is sufficient for the stabilization of Coq polypeptides— The yeast W222 mutant (24) has a mutated *COQ8* gene which encodes a Coq8-G130D polypeptide that mimics the human G272D pathogenic mutation found in ADCK3 (14). W222 does not synthesize Q₆, has low levels of Coq4, Coq7 and Coq9 and has no detectable phosphorylated form of Coq3, suggesting that the G130D mutation completely abolishes the kinase activity of Coq8 (12). We tested the importance of the kinase activity of Coq8 for the stabilisation of Coq proteins in Δ coq strains by over-expressing Coq8-G130D. The levels of Coq7 and Coq9 were restored in Δ coq6 cells with Coq8 OE but remained undetectable with Coq8-G130D OE (Fig. 4). Similarly, Coq9 steady state levels were restored in Δ coq7 cells with Coq8 OE but not with Coq8-G130D OE. Coq8-G130D steady-state level was lower than that of wild-type Coq8 over-expressed from the same high copy vector (Fig. 4). Transformation of Δ coq6 and Δ coq7 cells with a centromeric plasmid containing the *COQ8* gene (1cCoq8) resulted in moderate expression of Coq8 which was sufficient to restore steady-state levels of the Coq7 and Coq9 polypeptides (Fig. 4). Since the Coq8-G130D polypeptide also accumulates to a moderate level upon over-expression, we conclude that the kinase activity of Coq8 is necessary to cause the stabilization of the Coq polypeptides.

Over-expression of Coq8 promotes the accumulation of diagnostic intermediates in Δ coq5 and Δ coq7 strains—Based on our observation that Coq8 OE stabilizes most Coq proteins in the collection of the Δ coq strains, we reasoned that these mutants may now accumulate Q₆ biosynthetic intermediates downstream of HHB. In fact, it has been shown that a Δ coq7 strain known to accumulate HHB, can be induced to proceed further and to accumulate DMQ₆ upon Coq8 OE (16). In the present study, we have used HPLC separation coupled to either electrochemical

detection (ECD) or mass spectrometry (MS) to detect Q₆ intermediates in lipid extracts of the different strains tested. In our HPLC-ECD system, a precolumn electrode oxidizes the reduced hydroquinone intermediates present in the lipid extracts into their oxidized quinone form, accounting for the presence of only oxidized products in the HPLC-column eluate. In the HPLC-MS/MS detection, the redox state of the intermediates is detected and affects the elution position. Multiple reaction monitoring (MRM) allows the detection of the molecular ion of Q₆ intermediates in conjunction with the corresponding product ion base peak. A $\Delta coq7$ strain with *Coq8* OE revealed that DMQ₆ was produced in a synthetic medium supplemented with either pABA or 4-HB whereas no electroactive compound was detected in the absence of *Coq8* OE (Fig. 5A and data not shown). The synthesis of DMQ₆ from pABA is consistent with our previous observations that DMQ₆, but not its imino-counterpart IDMQ₆, was the main intermediate synthesized when strains expressing either a partially inactive form of *Coq7* (3) or a *coq7* point mutant (19) were grown in pABA containing medium.

An electro-active compound that eluted earlier than DMQ₆ was accumulated in a $\Delta coq5$ strain only upon *Coq8* OE (Fig. 5B). The observed mass of the purified compound ($M+H^+$) m/z 547.4150 corresponds to that of demethyl-demethoxyubiquinone (DDMQ₆) ($[M+H]^+ = C_{37}H_{55}O_3^+$: 547.4151; -0.2 ppm), the expected substrate of *Coq5*. The identification of DDMQ₆ is further supported by the trap scan spectrum which showed a tropylium-like ion at m/z 153 characteristic of Q₆-related compounds (Fig. S1A). DDMQ₆ was synthesized by the $\Delta coq5$ strain with *Coq8* OE when either 4-HB or pABA were used as ring-precursors (Fig. 5B). This result was further confirmed by detecting $^{13}C_6$ -DDMQ₆ in $\Delta coq5$ with *Coq8* OE grown either in the presence of $^{13}C_6$ -pABA or $^{13}C_6$ -4-HB (Fig. 5C, Fig. S1B). As expected, no Q₆ biosynthetic intermediates besides HHB or HAB were detected in either $\Delta coq5$ or $\Delta coq7$ strains with *Coq8*-G130D OE (data not shown). Our results show that *Coq8* OE in $\Delta coq5$ and $\Delta coq7$ strains leads to the biosynthesis of the expected Q₆ intermediates, DDMQ₆ and DMQ₆ respectively.

Bypass of the Q biosynthetic deficiency in $\Delta coq7$ cells by 4-HB analogue—Based on our recent demonstration of the bypass of the deficient C5-hydroxylation reaction in a $\Delta coq6$ strain by 3,4-dihydroxybenzoic acid (3,4-diHB) and vanillic acid (VA) (Fig. 1) (18), we grew $\Delta coq7$ cells with *Coq8* OE in the presence of 2,4-dihydroxybenzoic acid (2,4-diHB). In the presence of 2,4-diHB, cells contained a significant amount of Q₆ whereas growth in the presence of 4-HB only produced DMQ₆ (Fig. 6A). The product eluting at 780 sec in 2,4-diHB treated cells is not DMQ₆ since its UV-vis spectrum has a maximum at 265 nm different from that of DMQ₆ at 271nm (data not shown). Q₆-deficient strains have a growth defect on respiratory carbon sources because the mitochondrial electron transport chain is interrupted. The quantity of Q₆ synthesized in the presence of 2,4-diHB was sufficient to allow respiratory growth on lactate-glycerol (Fig. 6B). DMQ₆ was incompetent at re-establishing the flow of electrons in the respiratory chain as shown by the absence of growth on respiratory medium containing 4-HB (Fig. 6B). Likewise, *Coq8*-G130D OE failed to rescue the respiratory growth defect of the $\Delta coq7$ strain in the presence of 2,4-diHB. These results show that it is possible to bypass the deficient C6-hydroxylation reaction in a $\Delta coq7$ strain with 2,4-diHB provided that *Coq* polypeptides are stabilized by *Coq8* OE.

*Yeast $\Delta coq3$ and $\Delta coq4$ cells over-expressing *Coq8* accumulate early Q biosynthetic intermediates*—We detected only HAB and HHB in a $\Delta coq3$ strain with *Coq8* OE (data not shown). This was surprising to us since *Coq4*, *Coq7*, *Coq9* (Fig. 2A) and *Coq6* (Fig. 3B) are stable in a $\Delta coq3$ strain with *Coq8* OE and we therefore expected the accumulation of 3-hexaprenyl-4,5-dihydroxybenzoic acid (DHHB), the product of *Coq6*. VA bypassed the requirement for the C5-hydroxylase (*Coq6*) and the O5-methyltransferase (*Coq3*) and led to the accumulation of DMQ₆ in the $\Delta coq3$ strain with *Coq8* OE (Fig. 7A). This result demonstrates that the C1-decarboxylase, the C1-hydroxylase and *Coq5* are active in the $\Delta coq3$ strain.

In a $\Delta coq4$ strain grown in the presence of $^{13}C_6$ -pABA, *Coq8* OE caused the accumulation of a product whose mass is consistent with the $^{13}C_6$ labelled form of 3-hexaprenyl-4-amino-5-

hydroxybenzoic acid (HHAB; Fig. 7B). HHAB was also detected in the *coq4-1* point mutant (Fig. 7B) and trap scan spectra confirmed both the unlabeled and $^{13}\text{C}_6$ labelled forms of HHAB (Fig. S2A-B). The HHAB intermediate is not observed in pABA-labelled wild-type yeast (data not shown). Upon culture with $^{13}\text{C}_6$ -4HB, the corresponding anticipated intermediate, $^{13}\text{C}_6$ -DHHB was not detected in *Coq4*-deficient strains, only $^{13}\text{C}_6$ -HHB was detected (data not shown). We also failed to detect DHHB in the $\Delta coq4$ strain with *Coq8* OE even when the medium was supplemented with 3,4-diHB (100 $\mu\text{g/ml}$; data not shown). The accumulation of HHAB suggested a deficient O5-methyltransferase activity, even though the *Coq3* polypeptide is stable in the $\Delta coq4$ strain. Addition of VA to $\Delta coq4$ strain with *Coq8* OE failed to generate either DMQ₆ (Fig. 7A) or the predicted prenylation product, 3-hexaprenyl-4-hydroxy-5-methoxybenzoic acid (data not shown). Addition of 3-methoxy-4-aminobenzoic acid (AMB) to the growth medium of the *coq4-1* point mutant, led to the accumulation of 3-hexaprenyl-4-amino-5-methoxybenzoic acid (HMAB; Fig. 7C and Fig. S3). HMAB is expected to accumulate in cells deficient for the C1-decarboxylation reaction. However, it is important to note that HMAB is also observed in wild-type yeast incubated in the presence of AMB (Fig. 7C). Therefore, a possible defect in the C1-decarboxylation reaction in the *coq4-1* point mutant can not be probed by using AMB. Collectively, our data show that only early amino-intermediates of the Q₆ pathway accumulate in *coq4* yeast mutants and that the use of analogues of 4-HB or pABA does not cause the accumulation of downstream intermediates contrary to what we obtained in $\Delta coq3$ (Fig. 7A) or $\Delta coq7$ (Fig. 6A) mutants. Therefore, in addition to the O5-methylation which we proved to be deficient in a *coq4* mutant, at least one other downstream biosynthetic step of the Q₆ pathway is impaired. This situation is consistent with a more general functional/structural role for *Coq4* in the Q₆ biosynthetic complex (5).

*Yeast $\Delta coq9$ cells over-expressing *Coq8* accumulate intermediates diagnostic of a deficiency in C5 and C6-hydroxylation reactions*—*Coq9* is essential for Q₆ biosynthesis but its molecular function is unknown (25). Upon *Coq8* OE, we observed the accumulation of distinct

electroactive compounds depending on whether pABA or 4-HB was added to the growth medium of a $\Delta coq9$ strain (Fig. 8A). In the presence of 4-HB, two compounds were detected with UV-visible spectra (not shown) and retention times characteristic of DMQ₆ and of the oxidized form of 3-hexaprenyl-4-hydroxyphenol (4-HP). The identity of these intermediates was further confirmed by labelling with $^{13}\text{C}_6$ -4-HB and by comparing the lipid extracts of $\Delta coq9$ cells to those of $\Delta coq6$ cells which are known to contain 4-HP (18) (525.4 to 129 transition at 2.66 min, Fig. 8B) and to those of $\Delta coq7$ cells that contain DMQ₆ (567.6 to 173 transition at 4.86 min, Fig. 8C). In the presence of pABA (Fig. 8A), the compound eluting at 600 sec corresponds to the oxidized form of 3-hexaprenyl-4-aminophenol (4-AP). The compound eluting at 860 sec (Fig. 8A) was identified as 4-imino-demethoxyquinone (IDMQ₆) based on its observed mass ($\text{M}+\text{H}^+$) m/z 560.44458 (IDMQ₆; $[\text{M}+\text{H}]^+ = \text{C}_{38}\text{H}_{58}\text{NO}_2^+$: 560.44675; -3.90 ppm) and its fragmentation spectrum with tropylium-like and chromenylium-like ions at m/z 166 and 206 (Fig. S4A) which are shifted with $\text{M}+6$ (m/z) upon labelling with $^{13}\text{C}_6$ -pABA (Fig. S4B). The $^{13}\text{C}_6$ -labeled form of 4-AP (524.4 to 128 transition at 2.86 min, Fig. 8D; trap scan spectra Fig. S5) was detected in $\Delta coq6$ and $\Delta coq9$ cells and $^{13}\text{C}_6$ -IDMQ₆ (566.6 to 172 transition at 4.76 min, Fig. 8E) was present in $\Delta coq7$ and $\Delta coq9$ cells. The ion with a 525.4 to 129 transition at 2.86 min in $\Delta coq6$ cells with *Coq8* OE (Fig. 8B) is attributed to the +1 isotope of $^{13}\text{C}_6$ -4-AP (compare Figs. 8B and 8D). Likewise, the signal at 4.76 min (Fig. 8C) actually corresponds to the +1 isotope of $^{13}\text{C}_6$ -IDMQ₆ (566.6 to 172 transition; compare Figs. 8C and 8E). Our experiments show that $\Delta coq9$ cells with *Coq8* OE accumulate 4-AP / 4-HP which are diagnostic of a deficiency in the C5-hydroxylation catalyzed by *Coq6* (18) and DMQ₆ / IDMQ₆ which are formed consequently to a defect in the C6-hydroxylation reaction catalyzed *Coq7*. Moreover, the C4-deamination (black dotted arrows in Fig. 1) of Q₆ biosynthetic intermediates originating from pABA is not efficiently catalyzed in the absence of *Coq9*. In conclusion, deletion of *coq9* impacts on the activity of multiple *Coq* proteins: *Coq6*, *Coq7* and the putative deaminase.

We next attempted to bypass the biosynthetic defects of $\Delta coq9$ cells. Supplementation of the growth medium with VA caused the accumulation of DMQ₆ (Fig. S6), showing that even though the Coq6 deficiency could be bypassed, a strong block subsists at the level of Coq7. With 2,4-diOH, no Q₆ could be detected but instead a new electroactive compound at 730 sec appeared (Fig. S6). The absence of Q₆ biosynthesis may be caused by the poor stability of several Coq polypeptides even with Coq8 OE (Fig. 2A). Indeed, the quantity of intermediates accumulated in the $\Delta coq9$ strain is lower than that obtained in $\Delta coq6$ and $\Delta coq7$ strains (Fig. 8B-D) which have higher levels of Coq polypeptides compared to the $\Delta coq9$ strain (Fig. 2A). Finally, 2,3,4-trihydroxybenzoic acid which could in theory bypass both C5 and C6-hydroxylations was found to inhibit Q₆ biosynthesis in WT cells and was therefore not tested further (data not shown).

DISCUSSION

Most of the Coq polypeptides involved in Q₆ biosynthesis in *S. cerevisiae* are part of a multiprotein complex. Among Coq proteins, only Coq2 is predicted to possess transmembrane domains. Organization of the Coq polypeptides in a Q₆ biosynthetic complex associated with the mitochondrial inner membrane helps rationalize how these proteins can gain access to their substrates which are hexaprenylated lipophilic compounds likely imbedded in the membrane (26). In the absence of any one of the Coq polypeptides ($\Delta coq1$ - $\Delta coq9$ strains), assembly of the Q₆ biosynthetic complex is impaired which results in the degradation of Coq4, Coq6, Coq7 and Coq9. This situation is not unique for multiprotein complexes in *S. cerevisiae* and is also encountered for example with cytochrome *c* oxidase. Indeed most Cox proteins are degraded in the absence of any of the three mitochondrially-encoded core subunits Cox1-Cox3 (27). Our present study establishes that Coq8 OE stabilizes the levels of Coq4, Coq6, Coq7 and Coq9 in $\Delta coq3$ - $\Delta coq7$ strains and therefore generalizes to most Coq polypeptides this stabilizing effect which had previously been described for Coq4 (17). The stabilized proteins are active since they allowed accumulation of novel Q₆ biosynthetic intermediates in several Δcoq strains, suggesting

that the Q₆ biosynthetic complex is at least partially assembled.

Coq8 has been proposed to possess a kinase activity (11) and has been shown to be necessary for the existence of phosphorylated forms of Coq3, Coq5 and Coq7 *in vivo* (12,15). A G130D mutation in Coq8 abolishes the phosphorylated form of Coq3 suggesting that this mutation impairs the kinase activity (12). Our data with the G130D mutant indicate that the kinase activity of Coq8 is also important for the stabilization of Coq6 and Coq7 (Fig. 4) and probably also of Coq4 and Coq9. How does Coq8 OE stabilize Coq polypeptides which are otherwise degraded? A direct phosphorylation by Coq8 of the polypeptides could occur, especially in the case of Coq7 for which Coq8-dependent phosphorylated forms have been detected (12). However, a direct phosphorylation seems unlikely in the case of Coq4 since no phosphorylated forms of this protein could be detected (12). In addition, we do not currently know whether Coq6 and Coq9 are phosphorylated *in vivo* (12). So to explain the effect of Coq8, we favor a hypothesis in which Coq8 OE increases the phosphorylation state of a particular Coq protein which modulates the assembly and/or stability of the Q₆ biosynthetic complex. An obvious candidate is Coq3 whose association with the Q₆ biosynthetic complex was shown to be dependent on Coq8 (15). However, stable Coq proteins are detected in a $\Delta coq3$ strain with Coq8 OE (Fig. 2A), establishing that phosphorylated Coq3 is not the only factor promoting the stabilization of the Coq polypeptides. Coq8-dependent phosphorylation(s) may be considered a positive regulator of Q₆ biosynthesis since it seems to favour assembly of the Q₆ biosynthetic complex which should lead to increased Q₆ biosynthesis. On the contrary, a regulatory phosphorylation with a negative impact on Q₆ biosynthesis has recently been described for Coq7 (28). In that study, an increase in the phosphorylated forms of Coq7 correlated with a decreased level of Q₆ and an increased level of DMQ₆, the substrate of Coq7. It was therefore concluded that phosphorylated Coq7 has diminished activity (28). It remains to be established which kinase is responsible for the phosphorylation of the regulatory sites of Coq7.

We exploited the stabilizing effect of Coq8 OE to understand more precisely the Q₆

biosynthetic pathway, especially the role played by Coq4 and Coq9. We observed the accumulation of several intermediates in strains lacking Coq9 or Coq4 contrary to strains lacking Coq5 or Coq7 which accumulate a single late-stage intermediate, the substrate of the missing enzyme. In consequence, the impairment of several biosynthetic steps in $\Delta coq9$ and $\Delta coq4$ cells points to a role of these proteins in the general function or organization of the Q₆ biosynthetic complex rather than to a role in the catalysis of a particular biosynthetic step. In $\Delta coq9$ cells with Coq8 OE, the accumulation of 4-AP / 4-HP establishes that the C5-hydroxylation step catalyzed by Coq6 is limiting but this C5-hydroxylation occurs to some extent as demonstrated by the accumulation of IDMQ₆ / DMQ₆. These later intermediates reveal that the C6-hydroxylation catalyzed by Coq7 is also deficient. In fact, the C6-hydroxylation is completely impaired because addition of VA which bypasses the C5-hydroxylation resulted in increased accumulation of DMQ₆ without any production of detectable Q₆ or demethyl-Q₆ (Fig. S6). The role of Coq9 in the C5- and C6-hydroxylation reactions is not clear but Coq9 appears to be important for the stability of Coq7 because steady-state levels of Coq7 are not restored in $\Delta coq9$ cells with Coq8 OE. Nevertheless, Coq9 is not absolutely required for the stability of the Q₆ complex to which it has been shown to belong (6), because the accumulation of DMQ₆ implies that the Coq enzymes implicated in the Q₆ pathway upstream of Coq7 are stable and active, at least partially.

The $\Delta coq4$ strain was diagnosed to be impaired in multiple biosynthetic steps: Coq3, which catalyses both the O5- and O6-methylation steps, does not function in the $\Delta coq4$ strain as established by the accumulation of HHAB. Furthermore, addition of VA did not generate DMQ₆ in the $\Delta coq4$ strain, contrary to what we observed in the $\Delta coq3$ strain (Fig. 7A), revealing that at least another biosynthetic step downstream of the O5-methylation is impaired. In agreement with these results, Coq4 was hypothesized to serve as an anchor for the Q₆ biosynthetic complex (5) and was recently proposed to bind the polyisoprenyl tail of Q₆-intermediates, therefore allowing sequential modification of the aromatic head group (29). It is of interest to note that a

diploid yeast carrying a deletion of one allele of *COQ4* showed a diminished Q₆ content demonstrating that wild-type level of Coq4 is crucial for the function of the Q₆ biosynthetic complex (30). Human Coq4 may play an analogous role in organizing Q₁₀ biosynthesis since *COQ4* haploinsufficiency was recently shown to cause Q₁₀ deficiency in a patient (30).

It is interesting that Q₆ intermediates containing a catechol moiety (DHHB and demethyl-Q₆) could not be detected in this study despite the fact that we expected their synthesis in some of the strains tested. Indeed, we anticipated the formation of DHHB in a $\Delta coq4$ strain grown in the presence of 4-HB because this strain produced HHAB from pABA. Also, the $\Delta coq3$ strain synthesized DMQ₆ but not demethyl-Q₆ from VA. In consequence, the catechol-containing Q₆ intermediates may be unstable and hence can not accumulate in detectable amount in *S. cerevisiae*.

pABA is a precursor of Q₆ in *S. cerevisiae* and the C4-amine must be replaced by a C4-hydroxyl along the Q₆ pathway (3,19). Our data reveal several new elements regarding this C4-deamination (or deimination) reaction. First, $\Delta coq6$ and $\Delta coq9$ cells with Coq8 OE synthesized C4-aminated intermediates (4-AP and IDMQ₆) in the presence of pABA; but synthesized the C4-hydroxylated intermediates (4-HP and DMQ₆) in the presence of 4-HB. This result supports the notion that HHB and HAB follow the same biosynthetic steps up to DMQ₆ / IDMQ₆ and are modified by the same Coq enzymes which thus accommodate both C4-hydroxylated and C4-aminated Q₆ biosynthetic intermediates (path 1 and path 2, Fig. 1). Second, the deamination reaction does not proceed via non-enzymatic hydrolysis because in this case, IDMQ₆, which was previously established to be a precursor of DMQ₆, should be converted into DMQ₆. Third, the C4-deamination/deimination reaction occurs efficiently in some mutants ($\Delta coq5$ and $\Delta coq7$), but not in others ($\Delta coq6$, $\Delta coq9$ and possibly $\Delta coq4$). The deamination reaction may implicate Coq6 or Coq9 or an uncharacterized protein which may be inactive or unstable in $\Delta coq6$ and $\Delta coq9$ cells. In this latter case, a differential proteomic analysis of $\Delta coq5$ and $\Delta coq7$ cells on the one hand and $\Delta coq6$ and $\Delta coq9$ cells on the other hand, may reveal the identity of the C4-deaminase. Fourth,

the deamination reaction can occur prior to the C2-methylation catalyzed by Coq5 as supported by the accumulation of DDMQ₆ and not 2-demethyl-4-amino-demethoxy-Q₆ (IDDMQ₆) by the $\Delta coq5$ strain cultured in the presence of pABA. The step at which the deamination reaction occurs in a WT strain is still in question. Indeed, it could take place prior to the C2-methylation or prior to the C6-hydroxylation catalyzed by Coq7 as suggested by the results establishing that IDMQ₆ is a precursor of DMQ₆ (19). In that study, the *coq7-1* point mutant and also wild-type yeast grown in the presence of pABA, accumulated lower quantities of IDMQ₆ compared to DMQ₆ (19), in agreement with results presented in this present study. The real question now concerns the reasons of the synthesis of the minute amounts of IDMQ₆ detected in WT cells: Is it a “normal” intermediate which only accumulates in small quantities because it is efficiently converted into DMQ₆ or does IDMQ₆ represent a byproduct formed when

the deamination reaction that may occur prior to Coq5 is rate-limiting? Further experiments will address this question.

The accumulation of Q₆ intermediates in $\Delta coq4$ and $\Delta coq9$ cells with Coq8 OE shed some light on the biosynthetic defects in these strains and is a significant advance in our understanding of Q₆ biosynthesis. Furthermore, the use of the 2,4-diHB aromatic ring precursor together with Coq8 OE was shown to restore Q₆ biosynthesis in yeast $\Delta coq7$ cells. Finally, the accumulation of C4-aminated intermediates in some Δcoq strains provided new information regarding the deamination reaction. Overall, our study describes that Coq8 OE and the use of 4-HB analogues represent valuable tools to advance our comprehension of the Q₆ biosynthetic pathway by allowing for the unprecedented molecular dissection of the defect of particular Δcoq strains.

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FOOTNOTES

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⁷The abbreviations used are: 4-AP, 3-hexaprenyl-4-aminophenol; DDMQ₆, the oxidized form of demethyl-demethoxy-Q₆H₂; DHHB, 3-hexaprenyl-4,5-dihydroxybenzoic acid; DIDMQ₆H₂, 2-demethyl-4-amino-demethoxy-Q₆H₂; 3,4-diHB, 3,4-dihydroxybenzoic acid; 2,4-diHB, 2,4-dihydroxybenzoic acid; DMQ₆, demethoxy-Q₆; DMQ₆H₂, demethoxy-Q₆H₂; ECD, electrochemical detection; 4-HB, 4-hydroxybenzoic acid; HAB, 3-hexaprenyl-4-aminobenzoic acid; HHAB, 3-hexaprenyl-4-amino-5-hydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; HMAB, 3-hexaprenyl-4-amino-5-methoxybenzoic acid; 4-HP, 3-hexaprenyl-4-hydroxyphenol; IDDMQ₆H₂, 2-demethyl-4-amino-demethoxy-Q₆H₂; IDMQ₆, 4-imino-demethoxy-Q₆; MRM, multiple reaction monitoring; OE, over-expression; pABA, *para*-aminobenzoic acid; Q, ubiquinone or coenzyme Q; Q₆H₂, ubiquinol or coenzyme Q₆H₂; RP-HPLC-MS/MS; Reverse phase-high performance liquid chromatography-tandem mass spectrometry; VA, vanillic acid (4-hydroxy-3-methoxybenzoic acid).

FIGURE LEGENDS

Figure 1. *S. cerevisiae* Q₆ biosynthetic pathway: accumulation of Q₆ biosynthetic intermediates caused by the over-expression of *Coq8* in Δcoq strains. The classic Q biosynthetic pathway is shown in path 1 emanating from 4-hydroxybenzoic acid (4-HB). *Coq1* (not shown) synthesizes the hexaprenyl-diphosphate tail which is transferred by *Coq2* to 4-HB to form 3-hexaprenyl-4-hydroxybenzoic acid (HHB). R represents the hexaprenyl tail present in all intermediates from HHB to Q₆. Alternatively, in path 2, *para*-aminobenzoic acid (pABA) is prenylated by *Coq2* to form 3-hexaprenyl-4-aminobenzoic acid (HAB). Both HHB and HAB are early Q₆-intermediates, readily detected in each of the *coq* null strains ($\Delta coq3$ - $\Delta coq9$). The numbering of the aromatic carbon atoms used throughout this study is shown on the reduced form of Q₆, Q₆H₂. *Coq8* OE in certain Δcoq strains leads to the accumulation of the following compounds: 4-AP, 3-hexaprenyl-4-aminophenol; 4-HP, 3-hexaprenyl-4-hydroxyphenol; HHAB, 3-hexaprenyl-4-amino-5-hydroxybenzoic acid; HMAB, 3-hexaprenyl-4-amino-5-methoxybenzoic acid; DDMQ₆H₂, the reduced form of demethyl-demethoxy-Q₆; IDMQ₆, 4-imino-demethoxy-Q₆; IDMQ₆H₂, 4-amino-demethoxy-Q₆H₂; DMQ₆, demethoxy-Q₆; DMQ₆H₂, demethoxy-Q₆H₂. IDDMQ₆H₂, 2-demethyl-4-amino-demethoxy-Q₆H₂ and DHHB, 3-hexaprenyl-4,5-dihydroxybenzoic acid are shown in parentheses to indicate that they have not been detected in this study. Black dotted arrows (from path 2 to

path 1) designate the replacement of the C4-amine with a C4-hydroxyl and correspond to the C4-deamination/deimination reaction. A putative mechanism to replace the C4-imino group with the C4-hydroxy group is shown in blue brackets for IDMQ₆ but could also occur on IDDMQ₆ (not shown). 4-AP and 4-HP which are formed upon inhibition of the C5-hydroxylation catalyzed by Coq6 are shown in red. Analogues of 4-HB and pABA allowing the bypass of certain steps in Q biosynthesis are indicated in green. Steps defective in the $\Delta coq9$ strain are designated with a *red asterisk*.

Figure 2. Over-expression of Coq8 in several Δcoq strains restores steady-state levels of Coq4, Coq7, and Coq9. Whole cell lysates were prepared from W303-1B wild-type yeast (WT) or from the indicated Δcoq strains (all except $\Delta coq9$ in W303 genetic background) with (+) or without (–) p4HN4, a multi-copy plasmid with yeast *COQ8* (Coq8). Yeast cells cultured in YPGal medium to mid-log (2–3 OD_{600nm}) were harvested and aliquots of 20 OD_{600nm} were lysed and analyzed by SDS-PAGE (10% acrylamide) followed by transfer to PVDF membrane and immunoblotting with antibodies to the designated Coq polypeptides or to Pda1, the alpha subunit of pyruvate dehydrogenase. (A) Pda1 serves as a loading control; (B) Coq1 and/or Coq5 serve as loading controls.

Figure 3. Over-expression of Coq8 restores steady state levels of Coq polypeptides in isolated mitochondria. Isolated mitochondria were purified with Opti-Prep gradients from W303-1B wild-type yeast (WT) or from the designated Δcoq strains (W303 genetic background) with (+) or without (–) p4HN4 (Coq8). Aliquots of mitochondrial protein (20 µg) were analyzed by SDS-PAGE followed by immunoblot analyses with antisera to the designated Coq polypeptides (A) or with antisera to Coq6 (B). Coq1 serves as a loading control.

Figure 4. Over-expression of Coq8-G130D does not restore steady-state levels of Coq polypeptides. Whole cell lysates were prepared as described in Figure 2 from W303-1B wild-type yeast (WT) or from $\Delta coq6$ or $\Delta coq7$ strains (W303 genetic background) harboring either no plasmid; pFL44, a multi-copy plasmid expressing Coq8 (hcCoq8); G130D, a multi-copy plasmid with Coq8 containing the G130D-mutation (G130D); or p3HN4, a low copy plasmid expressing Coq8 (lcCoq8). Yeast cells were cultured in SD–Ura medium to mid-log (2–3 OD_{600nm}), harvested and aliquots of 20 OD_{600nm} were lysed and subjected to immunoblotting analyses with antisera to the designated Coq polypeptides. Pda1 serves as a loading control.

Figure 5. $\Delta coq7$ and $\Delta coq5$ strains over-expressing Coq8 accumulate the respective Q-intermediates, DMQ₆ and DDMQ₆. (A) $\Delta coq7$ cells (W303 genetic background) transformed with pFL44 an episomal vector encoding Coq8 (Coq8) were grown overnight in YNB–pABA –folate 2% dextrose with no additions or with 5 µM of 4-HB or pABA. Lipid extracts of 4 mg of cells (no addition) or of 1.5 mg of cells (4-HB, pABA) were analyzed by HPLC-ECD. The peaks corresponding to DMQ₆ and to the Q₄ standard are marked. (B) $\Delta coq5$ cells (W303 genetic background) transformed either with pFL44 (Coq8) or an empty vector (vec) were grown overnight in YNB–pABA –folate 2% dextrose containing either no addition or 5 µM of 4-HB or pABA. Lipid extracts of 10 mg of cells were analyzed by HPLC-ECD. (C) $\Delta coq5$ cells (W303 genetic background) containing either no plasmid or p4HN4, an episomal vector encoding Coq8 (Coq8) were first grown in YPGal + 0.1% dextrose and then cultured in DOGAL+0.1% dextrose –pABA –folate –tyrosine overnight. Finally cells were collected and transferred to fresh DOGAL+0.1% dextrose –pABA –folate in the presence of either ¹³C₆-4HB or ¹³C₆-pABA (10 µg/ml; 3 ml) and incubated for 2 h. Cells were collected (150 OD_{600nm}) and lipid extracts were subjected to RP-HPLC-ESI/MS-MS as described in Experimental Procedures and detection of the precursor-to-product ion transition (553.4/159.0) was performed with MRM. The traces indicate arbitrary units and the scale is the same for all traces within a panel.

Figure 6. Bypass of the respiratory growth defect of the $\Delta coq7$ strain with alternate ring precursors. (A) $\Delta coq7$ cells (W303 genetic background) transformed with an episomal vector coding for Coq8 (pFL44) were grown in YNB–pABA –folate 2% dextrose containing 1 mM 2,4-dihydroxybenzoic acid (2,4-diHB) or not (–). Lipid extracts of 2 mg of cells were analyzed by HPLC-ECD. (B) WT W303 cells or $\Delta coq7$ cells transformed either with an empty vector (vec), an episomal vector (pFL44) encoding Coq8 or Coq8-G130D, or with an episomal vector encoding Coq7 were grown in YNB–pABA –folate 2% dextrose for 24 h and serial dilutions were spotted onto agar plates. The plates contained either YP 2% dextrose (Glu) or synthetic medium –pABA –folate supplemented with 2% lactate-2% glycerol (LG) containing either 4-HB or 2,4-dihydroxybenzoic acid (2,4-diHB) at 1 mM. The plates were incubated for 2 days (Glu) or 4 days (LG) at 30 °C.

Figure 7. $\Delta coq3$ and $\Delta coq4$ strains over-expressing Coq8 or a *coq4-1* strain accumulate early Q₆ biosynthetic intermediates. (A) Yeast $\Delta coq3$ (BY4741 genetic background) and $\Delta coq4$ cells (BY4742 genetic background) transformed with pFL44, an episomal vector encoding Coq8 (Coq8) were grown in YNB–pABA –folate 2% dextrose containing 100µM vanillic acid (VA) or 100µM 4-HB. Lipid extracts of 4 mg of cells were analyzed by HPLC-ECD. (B) Yeast $\Delta coq4$ cells (W303 genetic background) transformed with p4HN4, an episomal vector encoding Coq8, and *coq4-1* cells were grown in YPGal+0.1% dextrose overnight and labelled in DOGAL+0.1% dextrose –folate –pABA in the presence of either ¹³C₆-4HB or ¹³C₆-pABA (20 µg/ml; 3 ml) for 2 hours. Lipid extracts of 100 OD_{600nm} of cells were analyzed by RP-HPLC-MS/MS as described in Experimental Procedures and detection of the precursor-to-product ion transition (568.0/172.0) was performed with MRM. (C) Yeast *coq4-1* mutant cells or wild-type yeast cells were grown in YPGal+0.1% dextrose overnight and transferred into 3 ml fresh DOGAL+0.1% dextrose –folate –pABA in the presence of 100 µg/ml 4-amino-3-methoxybenzoic acid (AMB). Lipid extracts of 100 OD_{600nm} of cells were analyzed by RP-HPLC-ESI/MS-MS as described in Experimental Procedures and detection of the precursor-to-product ion transition (576.0/180.0) was performed with MRM.

Figure 8. Coq9 is important for Coq6 and Coq7 hydroxylation steps and for removal of the amino substituent. (A) $\Delta coq9$ cells (BY4742 genetic background) transformed either with an episomal vector coding for Coq8 (pFL44), or an empty vector (vec), were grown in YNB–pABA –folate 2% dextrose containing or not 5 µM 4-hydroxybenzoic acid (4-HB) or pABA. Lipid extracts of 10 mg of cells were analyzed by HPLC-ECD. The peaks corresponding to demethoxyubiquinone (DMQ₆), to imino-demethoxyubiquinone (IDMQ₆), or to the oxidized forms of 3-hexaprenyl-4-aminophenol (4-AP) and of 3-hexaprenyl-4-hydroxyphenol (4-HP) are marked. (B) – (E) Yeast $\Delta coq6$, $\Delta coq7$, $\Delta coq9$, in the absence or presence of p4HN4 (an episomal plasmid encoding Coq8) were cultured and prepared as described in Figure 5. Yeast cell pellets (150 OD_{600nm} of cells) were subjected to RP-HPLC-MS/MS as described in Experimental Procedures and detection of the designated precursor-to-product ion transitions (525.4/129.0, 524.4/128.0, 567.6/173.0, 566.6/172.0) were performed with MRM.

Table 1
Genotype and Source of Yeast Strains

Strain	Genotype	Source
W303-1A	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i>	R. Rothstein ^a
W303Δ <i>coq1</i>	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (23) <i>coq1::LEU2</i>	
W303Δ <i>coq2</i>	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (31) <i>coq2::HIS3</i>	
CC303	MAT α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (32) <i>coq3::LEU2</i>	
W303Δ <i>coq4</i>	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (33) <i>coq4::TRP1</i>	
W303Δ <i>coq5</i>	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (34) <i>coq5::HIS3</i>	
W303Δ <i>coq6</i>	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (35) <i>coq6::LEU2</i>	
W303Δ <i>coq7</i>	MAT α <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (36) <i>coq7::LEU2</i>	
W303Δ <i>coq8</i>	MAT a <i>ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</i> (33) <i>abc1/coq8::HIS3</i>	
BY4741	MAT a <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(37)
BY4741Δ <i>coq9</i>	MAT a <i>coq9Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0</i> (38)b <i>ura3Δ0</i>	
C9-E1	MAT a <i>ade2-1, coq4-1, trp1-1, ura3-1</i>	(39)
BY4741Δ <i>coq3</i>	MAT a <i>coq3Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0</i> (38)b <i>ura3Δ0</i>	
BY4742Δ <i>coq4</i>	MAT alpha <i>coq4Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0</i> (38)b <i>ura3Δ0</i>	
BY4742Δ <i>coq9</i>	MAT alpha <i>coq9Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0</i> (38)b <i>ura3Δ0</i>	

^a Dr. Rodney Rothstein, Department of Human Genetics, Columbia University.

^b European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF), available on-line.

Table 2

Plasmid constructs used in this study

Plasmid	Relevant Genes	Copy number	Source
pRS316	Yeast shuttle vector	Low copy	(40)
pRS426	Yeast shuttle vector	Multi copy	(41)
p3HN4	Yeast <i>ABC1/COQ8</i>	Low copy	(11)
p4HN4	Yeast <i>ABC1/COQ8</i>	Multi copy	(42)
pFL44 Coq8	Yeast <i>ABC1/COQ8</i>	Multi copy	(9)
pFL44 Coq8 G130D	Yeast <i>ABC1/COQ8 G130D</i>	Multi copy	(14)
pRS425 Coq7	Yeast <i>COQ7</i>	Multi copy	This work

Figure 1

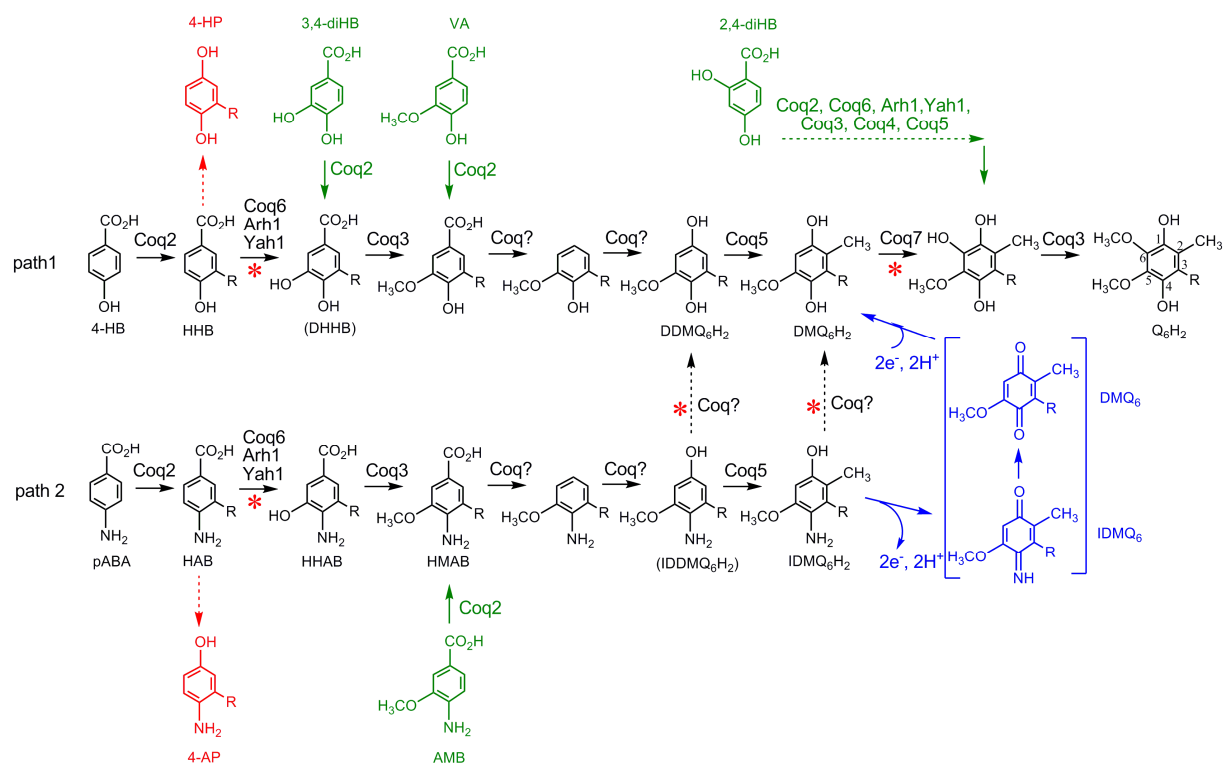


Figure 2

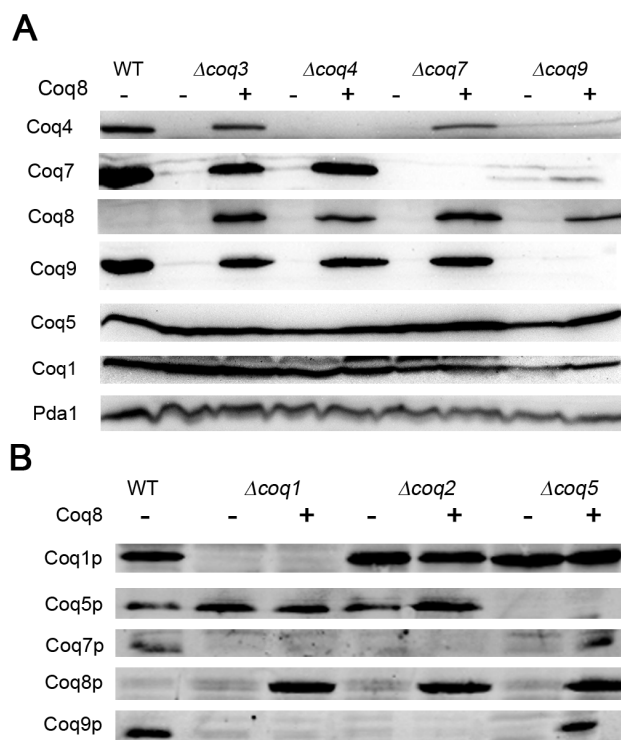


Figure 3

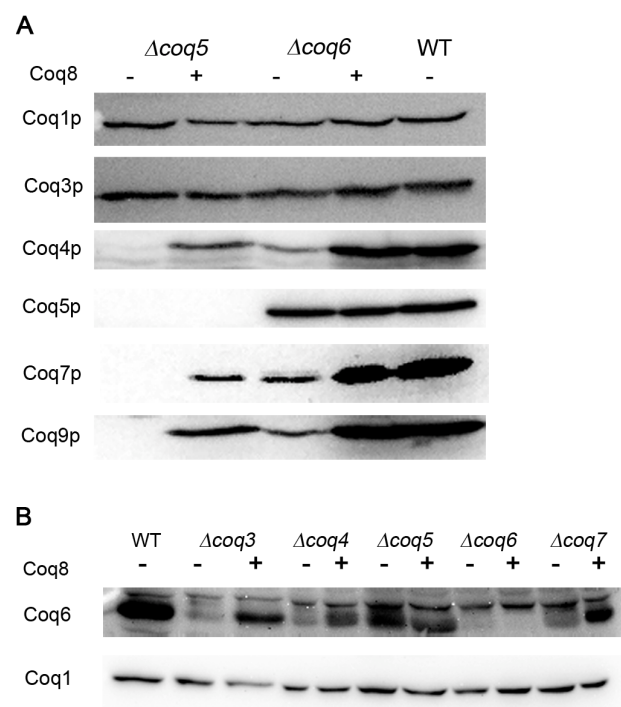


Figure 4

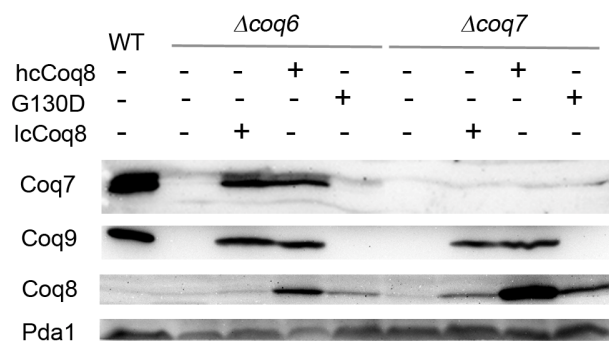


Figure 5

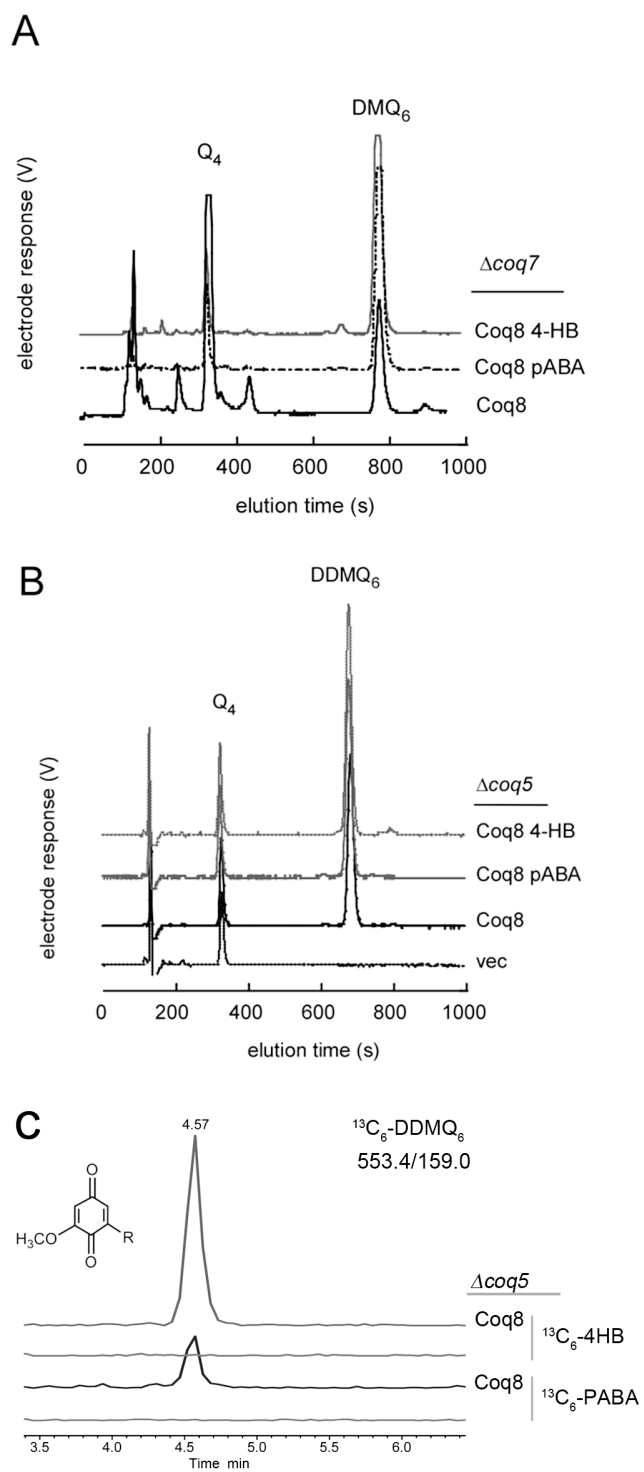


Figure 6

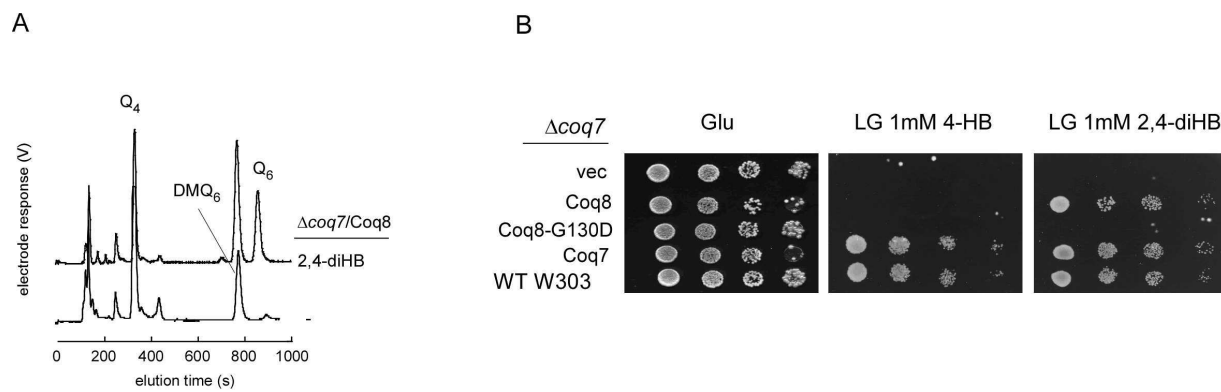


Figure 7

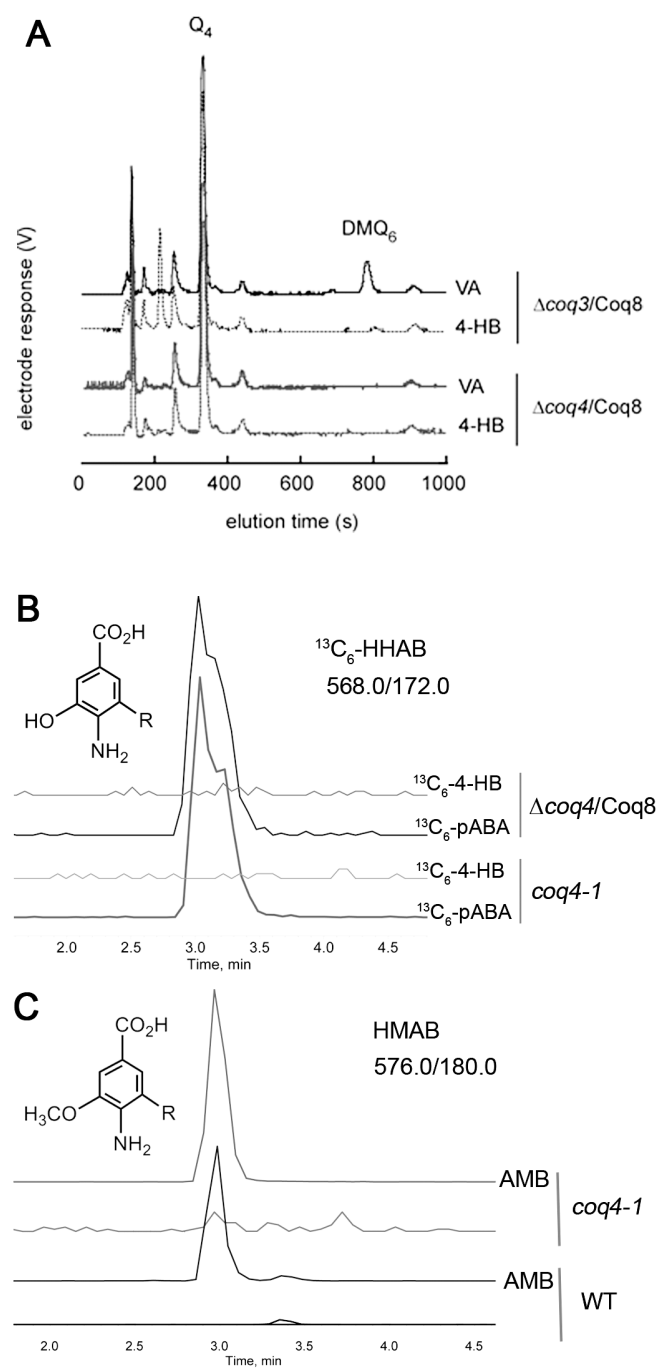


Figure 8

