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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 Q_6 biosynthetic pathway*

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

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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 Δ*coq* strains, results in the degradation of several Coq proteins. Consequently, Δ*coq* strains accumulate the same early intermediate of the Q_6 biosynthetic pathway; this intermediate is therefore not informative about the deficient biosynthetic step in a particular Δ*coq* strain. In this work, we report that the over-expression of the protein Coq8 in Δ*coq* 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 Δ*coq* strains. This stabilisation results in the accumulation of several novel Q_6 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 Q_6. 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 Q_6 biosynthesis and respiratory growth in a Δ*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 Q_6, ten units in humans, hence Q_{10}). 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 Q_6 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 Δ*coq1*- Δ*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 Δ*coq3*-Δ*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 Δ*coq7* strain caused accumulation of the expected intermediate demethoxy-Q_6 (DMQ_6, Fig. 1) (7,8). Apart from this example, the absence of accumulation of biosynthetic intermediates downstream of HHB in Δ*coq* 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 Q_6 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 Q_{10} 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 Q_6 biosynthetic complex. Recent studies indicate that over-expression of *COQ8* can have profound effects on Q_6 biosynthesis. Indeed, the over-expression of Coq8 (from now on referred to as Coq8 OE) in a Δ*coq7* strain promoted the accumulation of DMQ_6 (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 *Δcoq7* strain were not dependent on the level of Coq8 OE (16). Recently, the low steady-state level of Coq4 encountered in Δ*coq2*, Δ*coq3*, Δ*coq5*, and Δ*coq7* strains was shown to be restored to wild-type levels by Coq8 OE (17). In the case of a Δ*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 Q_6 biosynthetic intermediates in Δ*coq* 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 Q_6 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 Q_6 (3,19). The deamination reaction likely occurs before the C6-hydroxylation step catalyzed by Coq7 because 4-imino-DMQ_6 ($IDMQ_6$) was proposed to be a precursor of DMQ_6 (19). Other ring precursors than 4-HB and pABA can also be used *in vivo* by *S. cerevisiae* to synthesize Q_6. Indeed, 3,4-dihydroxybenzoic acid and vanillic acid bypass a deficiency in the Coq6-mediated C5-hydroxylation reaction and restore Q_6 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 Δ*coq* strains. The stabilization of the Coq polypeptides leads to the accumulation of Q_6 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 Δ*coq4* and Δ*coq9* strains and to gain insights into the deamination reaction. Finally, the use of alternate ring precursors promoted the restoration of Q_6 biosynthesis and respiratory growth for a Δ*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 OD_{600} 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 ^{o}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 difuloride (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 Q_4 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 Q_4 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 µl/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 Q_6-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 Q_6, 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 (lcCoq8) 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 Q_6 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 DMQ_6 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 Q_6 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 Q_6 intermediates in conjunction with the corresponding product ion base peak. A Δ*coq7* strain with Coq8 OE revealed that DMQ_6 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 DMQ_6 from pABA is consistent with our previous observations that DMQ_6, but not its imino-counterpart $IDMQ_6$, 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 DMQ_6 was accumulated in a Δ*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 ($DDMQ_6$) ($[M+H]^+$ = $C_{37}H_{55}O_3^+$: 547.4151; –0.2 ppm), the expected substrate of Coq5. The identification of $DDMQ_6$ is further supported by the trap scan spectrum which showed a tropylium-like ion at m/z 153 characteristic of Q_6-related compounds (Fig. S1A). $DDMQ_6$ was synthesized by the Δ*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 $^{13}C_6$-$DDMQ_6$ in Δ*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 Q_6 biosynthetic intermediates besides HHB or HAB were detected in either Δ*coq5* or Δ*coq7* strains with Coq8-G130D OE (data not shown). Our results show that Coq8 OE in Δ*coq5* and Δ*coq7* strains leads to the biosynthesis of the expected Q_6 intermediates, $DDMQ_6$ and DMQ_6 respectively.

Bypass of the Q biosynthetic deficiency in Δ*coq7 cells by 4-HB analogue*—Based on our recent demonstration of the bypass of the deficient C5-hydroxylation reaction in a Δ*coq6* strain by 3,4-dihydroxybenzoic acid (3,4-diHB) and vanillic acid (VA) (Fig. 1) (18), we grew Δ*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 Q_6 whereas growth in the presence of 4-HB only produced DMQ_6 (Fig. 6A). The product eluting at 780 sec in 2,4-diHB treated cells is not DMQ_6 since its UV-vis spectrum has a maximum at 265 nm different from that of DMQ_6 at 271nm (data not shown). Q_6-deficient strains have a growth defect on respiratory carbon sources because the mitochondrial electron transport chain is interrupted. The quantity of Q_6 synthesized in the presence of 2,4-diHB was sufficient to allow respiratory growth on lactate-glycerol (Fig. 6B). DMQ_6 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 Δ*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 Δ*coq7* strain with 2,4-diHB provided that Coq polypeptides are stabilized by Coq8 OE.

Yeast Δ*coq3 and* Δ*coq4 cells over-expressing Coq8 accumulate early Q biosynthetic intermediates*—We detected only HAB and HHB in a Δ*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 Δ*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 DMQ_6 in the Δ*coq3* strain with Coq8 OE (Fig. 7A). This result demonstrates that the C1-decarboxylase, the C1-hydroxylase and Coq5 are active in the Δ*coq3* strain.

In a Δ*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 $^{13}C_6$ 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 $^{13}C_6$-4HB, the corresponding anticipated intermediate, $^{13}C_6$-DHHB was not detected in Coq4-deficient strains, only $^{13}C_6$-HHB was detected (data not shown). We also failed to detect DHHB in the Δ*coq4* strain with Coq8 OE even when the medium was supplemented with 3,4-diHB (100 ug/ml; data not shown). The accumulation of HHAB suggested a deficient O5-methyltransferase activity, even though the Coq3 polypeptide is stable in the Δ*coq4* strain. Addition of VA to Δ*coq4* strain with Coq8 OE failed to generate either DMQ_6 (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 Q 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 Δ*coq3* (Fig. 7A) or Δ*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 Q_6 pathway is impaired. This situation is consistent with a more general functional/structural role for Coq4 in the Q_6 biosynthetic complex (5).

Yeast Δcoq9 cells over-expressing Coq8 accumulate intermediates diagnostic of a deficiency in C5 and C6-hydroxylation reactions— Coq9 is essential for Q_6 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 Δ*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 DMQ_6 and of the oxidized form of 3-hexaprenyl-4-hydroxyphenol (4-HP). The identity of these intermediates was further confirmed by labelling with $^{13}C_6$-4-HB and by comparing the lipid extracts of Δ*coq9* cells to those of Δ*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 Δ*coq7* cells that contain DMQ_6 (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 ($IDMQ_6$) based on its observed mass ($M+H^+$) m/z 560.44458 ($IDMQ_6$; $[M+H]^+ = C_{38}H_{58}NO_2^+$: 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 M+6 (m/z) upon labelling with $^{13}C_6$-pABA (Fig. S4B). The $^{13}C_6$-labeled form of 4-AP (524.4 to 128 transition at 2.86 min, Fig. 8D; trap scan spectra Fig. S5) was detected in Δ*coq6* and Δ*coq9* cells and $^{13}C_6$-$IDMQ_6$ (566.6 to 172 transition at 4.76 min, Fig. 8E) was present in Δ*coq7* and Δ*coq9* cells. The ion with a 525.4 to 129 transition at 2.86 min in Δ*coq6* cells with Coq8 OE (Fig. 8B) is attributed to the +1 isotope of $^{13}C_6$-4-AP (compare Figs. 8B and 8D). Likewise, the signal at 4.76 min (Fig. 8C) actually corresponds to the +1 isotope of $^{13}C_6$-$IDMQ_6$ (566.6 to 172 transition; compare Figs. 8C and 8E). Our experiments show that Δ*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 DMQ_6 / $IDMQ_6$ 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 Q_6 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 Δ*coq9* cells. Supplementation of the growth medium with VA caused the accumulation of DMQ_6 (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 Q_6 could be detected but instead a new electroactive compound at 730 sec appeared (Fig. S6). The absence of Q_6 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 Δ*coq9* strain is lower than that obtained in Δ*coq6* and Δ*coq7* strains (Fig. 8B-D) which have higher levels of Coq polypeptides compared to the Δ*coq9* strain (Fig. 2A). Finally, 2,3,4-trihydroxybenzoic acid which could in theory bypass both C5 and C6-hydroxylations was found to inhibit Q_6 biosynthesis in WT cells and was therefore not tested further (data not shown).

DISCUSSION

Most of the Coq polypeptides involved in Q_6 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 Q_6 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 (Δ*coq1*- Δ*coq9* strains), assembly of the Q_6 biosynthetic complex is impaired which results in the degradation of Coq4, Coq6, Coq7 and Coq9. This situation is not unique for multi-protein 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 Δ*coq3*-Δ*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 Q_6 biosynthetic intermediates in several Δ*coq* strains, suggesting that the Q_6 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 Q_6 biosynthetic complex. An obvious candidate is Coq3 whose association with the Q_6 biosynthetic complex was shown to be dependent on Coq8 (15). However, stable Coq proteins are detected in a Δ*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 Q_6 biosynthesis since it seems to favour assembly of the Q_6 biosynthetic complex which should lead to increased Q_6 biosynthesis. On the contrary, a regulatory phosphorylation with a negative impact on Q_6 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 Q_6 and an increased level of DMQ_6, 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 Q_6

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 Δ*coq9* and Δ*coq4* cells points to a role of these proteins in the general function or organization of the Q_6 biosynthetic complex rather than to a role in the catalysis of a particular biosynthetic step. In Δ*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 $IDMQ_6$ / DMQ_6. 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 DMQ_6 without any production of detectable Q_6 or demethyl-Q_6 (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 Δ*coq9* cells with Coq8 OE. Nevertheless, Coq9 is not absolutely required for the stability of the Q_6 complex to which it has been shown to belong (6), because the accumulation of DMQ_6 implies that the Coq enzymes implicated in the Q_6 pathway upstream of Coq7 are stable and active, at least partially.

The Δ*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 Δ*coq4* strain as established by the accumulation of HHAB. Furthermore, addition of VA did not generate DMQ_6 in the Δ*coq4* strain, contrary to what we observed in the Δ*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 Q_6 biosynthetic complex (5) and was recently proposed to bind the polyisoprenyl tail of Q_6-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 Q_6 content demonstrating that wild-type level of Coq4 is crucial for the function of the Q_6 biosynthetic complex (30). Human Coq4 may play an analogous role in organizing Q_{10} biosynthesis since *COQ4* haploinsufficiency was recently shown to cause Q_{10} deficiency in a patient (30).

It is interesting that Q_6 intermediates containing a catechol moiety (DHHB and demethyl-Q_6) 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 Δ*coq4* strain grown in the presence of 4-HB because this strain produced HHAB from pABA. Also, the Δ*coq3* strain synthesized DMQ_6 but not demethyl-Q_6 from VA. In consequence, the catechol-containing Q_6 intermediates may be unstable and hence can not accumulate in detectable amount in *S. cerevisiae*.

pABA is a precursor of Q_6 in *S. cerevisiae* and the C4-amine must be replaced by a C4-hydroxyl along the Q_6 pathway (3,19). Our data reveal several new elements regarding this C4-deamination (or deimination) reaction. First, Δ*coq6* and Δ*coq9* cells with Coq8 OE synthesized C4-aminated intermediates (4-AP and $IDMQ_6$) in the presence of pABA; but synthesized the C4-hydroxylated intermediates (4-HP and DMQ_6) in the presence of 4-HB. This result supports the notion that HHB and HAB follow the same biosynthetic steps up to DMQ_6 / $IDMQ_6$ and are modified by the same Coq enzymes which thus accommodate both C4-hydroxylated and C4-aminated Q_6 biosynthetic intermediates (path 1 and path 2, Fig. 1). Second, the deamination reaction does not proceed via non-enzymatic hydrolysis because in this case, $IDMQ_6$, which was previously established to be a precursor of DMQ_6, should be converted into DMQ_6. Third, the C4-deamination/deimination reaction occurs efficiently in some mutants (Δ*coq5* and Δ*coq7*), but not in others (Δ*coq6*, Δ*coq9* and possibly Δ*coq4*). The deamination reaction may implicate Coq6 or Coq9 or an uncharacterized protein which may be inactive or unstable in Δ*coq6* and Δ*coq9* cells. In this latter case, a differential proteomic analysis of Δ*coq5* and Δ*coq7* cells on the one hand and Δ*coq6* and Δ*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 $DDMQ_6$ and not 2-demethyl-4-amino-demethoxy-Q_6 ($IDDMQ_6$) by the Δ*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 $IDMQ_6$ is a precursor of DMQ_6 (19). In that study, the *coq7-1* point mutant and also wild-type yeast grown in the presence of pABA, accumulated lower quantities of $IDMQ_6$ compared to DMQ_6 (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 $IDMQ_6$ detected in WT cells: Is it a "normal" intermediate which only accumulates in small quantities because it is efficiently converted into DMQ_6 or does $IDMQ_6$ 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 Q_6 intermediates in Δ*coq4* and Δ*coq9* cells with Coq8 OE shed some light on the biosynthetic defects in these strains and is a significant advance in our understanding of Q_6 biosynthesis. Furthermore, the use of the 2,4-diHB aromatic ring precursor together with Coq8 OE was shown to restore Q_6 biosynthesis in yeast Δ*coq7* cells. Finally, the accumulation of C4-aminated intermediates in some Δ*coq* 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 Q_6 biosynthetic pathway by allowing for the unprecedented molecular dissection of the defect of particular Δ*coq* strains.

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FOOTNOTES

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[7]The abbreviations used are: 4-AP, 3-hexaprenyl-4-aminophenol; $DDMQ_6$, the oxidized form of demethyl-demethoxy-Q_6H_2; DHHB, 3-hexaprenyl-4,5-dihydroxybenzoic acid; $DIDMQ_6H_2$, 2-demethyl-4-amino-demethoxy-Q_6H_2; 3,4-diHB, 3,4-dihydroxybenzoic acid; 2,4-diHB, 2,4-dihydroxybenzoic acid; DMQ_6, demethoxy-Q_6; DMQ_6H_2, demethoxy-Q_6H_2; 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; $IDDMQ_6H_2$, 2-demethyl-4-amino-demethoxy-Q_6H_2; $IDMQ_6$, 4-imino-demethoxy-Q_6; MRM, multiple reaction monitoring; OE, over-expression; pABA, *para*-aminobenzoic acid; Q, ubiquinone or coenzyme Q; Q_6H_2, ubiquinol or coenzyme Q_6H_2; 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* Q_6 biosynthetic pathway: accumulation of Q_6 biosynthetic intermediates caused by the over-expression of Coq8 in Δ*coq* 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 Q_6. 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 Q_6-intermediates, readily detected in each of the *coq* null strains (Δ*coq3*-Δ*coq9*). The numbering of the aromatic carbon atoms used throughout this study is shown on the reduced form of Q_6, Q_6H_2. Coq8 OE in certain Δ*coq* 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; $DDMQ_6H_2$, the reduced form of demethyl-demethoxy-Q_6; $IDMQ_6$, 4-imino-demethoxy-Q_6; $IDMQ_6H_2$, 4-amino-demethoxy-Q_6H_2; DMQ_6, demethoxy-Q_6; DMQ_6H_2, demethoxy-Q_6H_2. $IDDMQ_6H_2$, 2-demethyl-4-amino-demethoxy-Q_6H_2 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 $IDMQ_6$ but could also occur on $IDDMQ_6$ (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 Δ*coq9* strain are designated with a *red asterisk*.

Figure 2. Over-expression of Coq8 in several Δ*coq* 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 Δ*coq* strains (all except Δ*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 Δ*coq* 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 Δ*coq6* or Δ*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. Δ*coq7* and Δ*coq5* strains over-expressing Coq8 accumulate the respective Q-intermediates, DMQ_6 and $DDMQ_6$. (*A*) Δ*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 DMQ_6 and to the Q_4 standard are marked. (*B*) Δ*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*) Δ*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 $^{13}C_6$-4HB or $^{13}C_6$-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 Δ*coq7* strain with alternate ring precursors. (*A*) Δ*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 Δ*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. Δ*coq3* and Δ*coq4* strains over-expressing Coq8 or a *coq4-1* strain accumulate early Q_6 biosynthetic intermediates. (*A*) Yeast Δ*coq3* (BY4741 genetic background) and Δ*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 Δ*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 $^{13}C_6$-4HB or $^{13}C_6$-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*) Δ*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 (DMQ_6), to imino-demethoxyubiquinone ($IDMQ_6$), 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 Δ*coq6*, Δ*coq7*, Δ*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

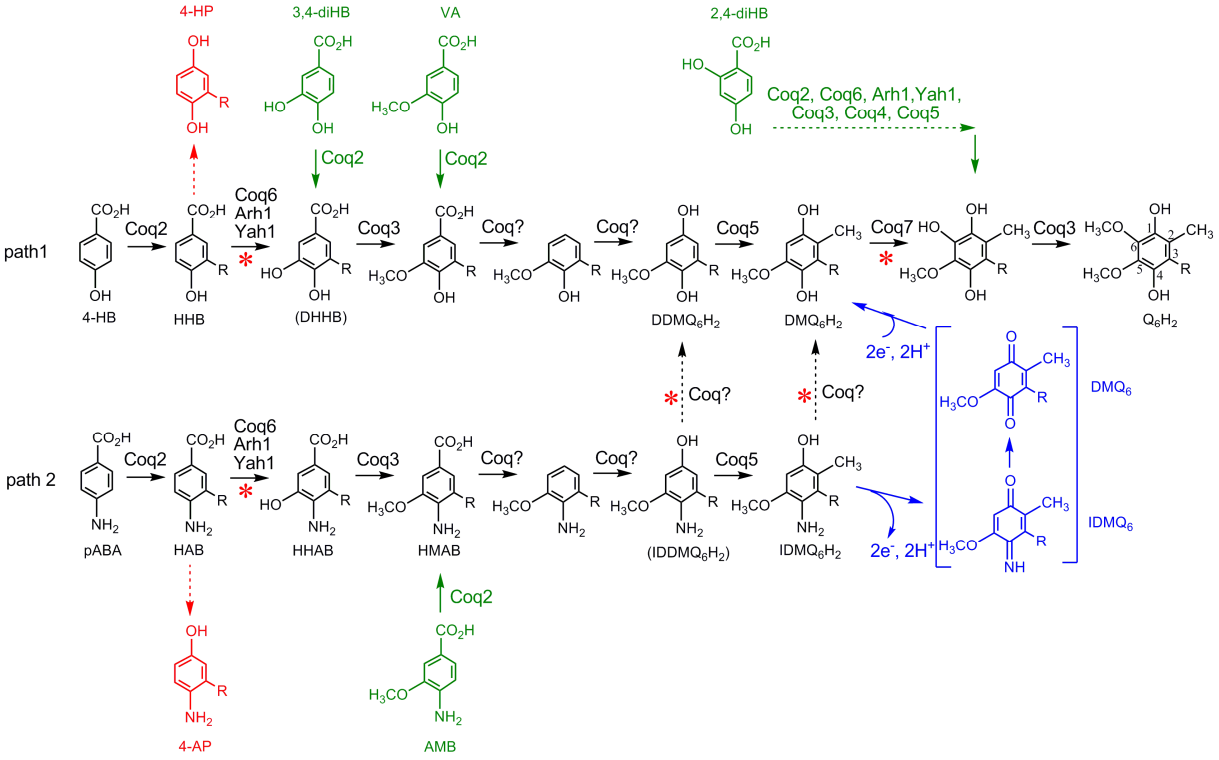
Strain	Genotype	Source
W303-1A	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303Δ*coq1*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq1::LEU2*	(23)
W303Δ*coq2*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq2::HIS3*	(31)
CC303	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1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

[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 *ABC1/COQ8*	Low copy	(11)
p4HN4	Yeast *ABC1/COQ8*	Multi copy	(42)
pFL44 Coq8	Yeast *ABC1/COQ8*	Multi copy	(9)
pFL44 Coq8 G130D	Yeast *ABC1/COQ8 G130D*	Multi copy	(14)
pRS425 Coq7	Yeast *COQ7*	Multi copy	This work

Figure 1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3,4-diHB
VA
2,4-diHB
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Coq3, Coq4, Coq5
Coq2
Coq2
path1
Coq2
Coq6
Arh1
Yah1
Coq3
Coq?
Coq?
Coq5
Coq7
Coq3
4-HB
HHB
(DHHB)
DDMQ6H2
DMQ6H2
Q6H2
2e-, 2H+
Coq?
Coq?
DMQ6
path 2
Coq2
Coq6
Arh1
Yah1
Coq3
Coq?
Coq?
Coq5
pABA
HAB
HHAB
HMAB
(IDDMQ6H2)
IDMQ6H2
2e-, 2H+
IDMQ6
Coq2
4-AP
AMB

Figure 2

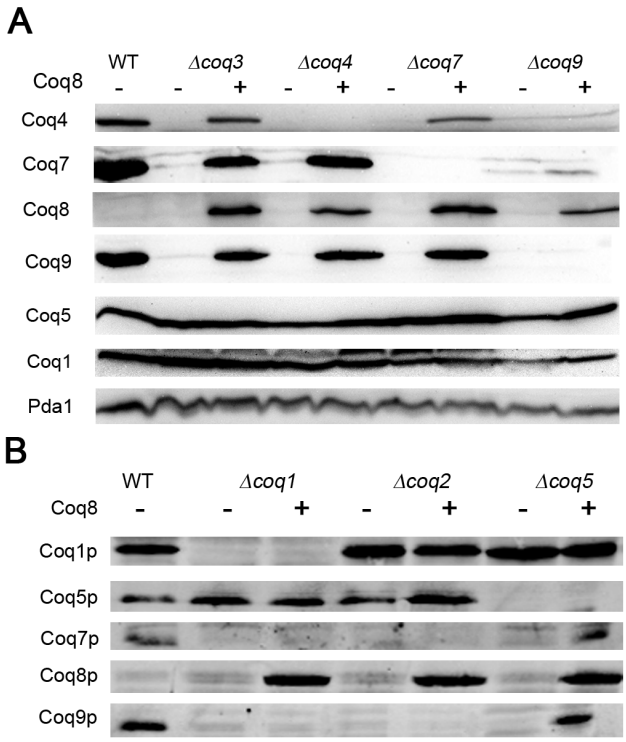


Figure 3

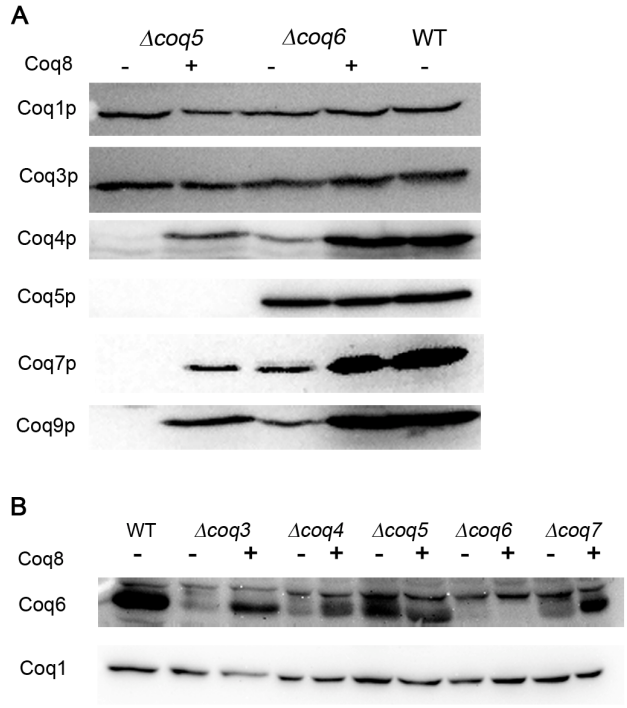


Figure 4

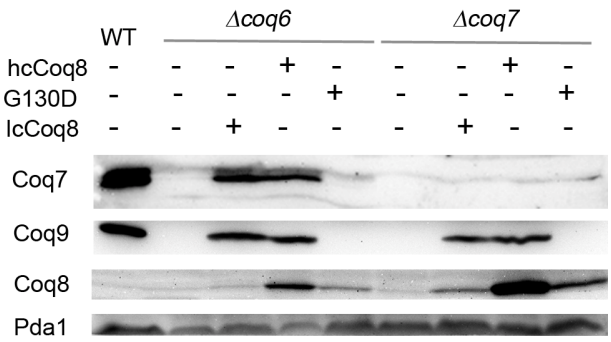


Figure 5

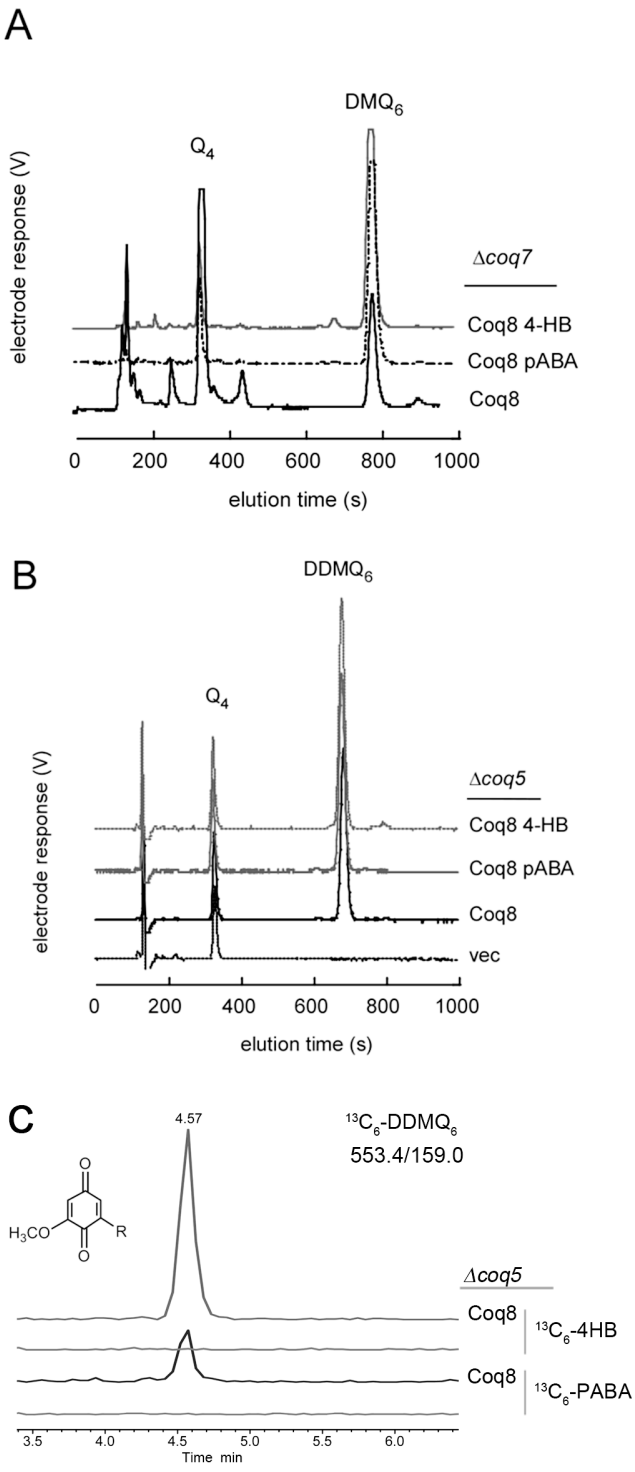

A
DMQ6
Q4
electrode response (V)
Δcoq7
Coq8 4-HB
Coq8 pABA
Coq8
0
200
400
600
800
1000
elution time (s)
B
DDMQ6
Q4
electrode response (V)
Δcoq5
Coq8 4-HB
Coq8 pABA
Coq8
vec
0
200
400
600
800
1000
elution time (s)
C
4.57
13C6-DDMQ6
553.4/159.0
H3CO
R
Δcoq5
Coq8
13C6-4HB
Coq8
13C6-PABA
3.5
4.0
4.5
5.0
5.5
6.0
Time min

Figure 6

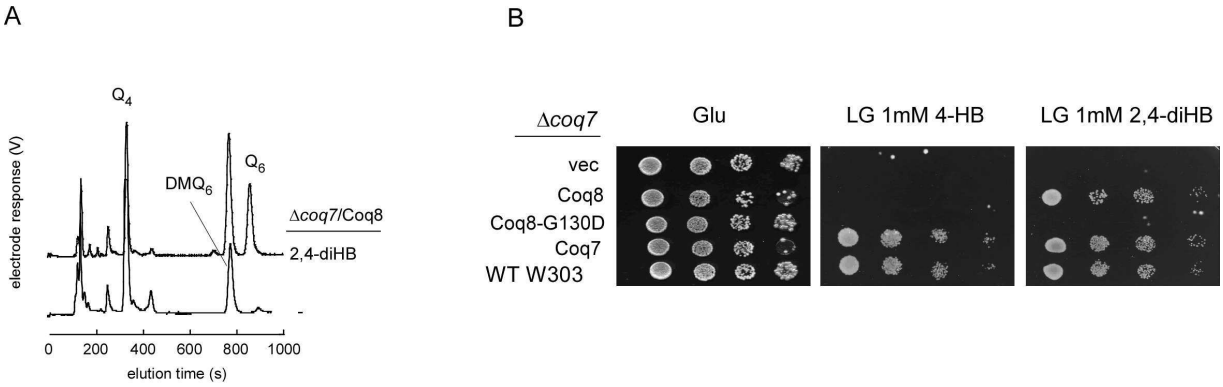

A
B
Q4
DMQ6
Q6
Δcoq7/Coq8
2,4-diHB
electrode response (V)
0
200
400
600
800
1000
elution time (s)
Δcoq7
Glu
LG 1mM 4-HB
LG 1mM 2,4-diHB
vec
Coq8
Coq8-G130D
Coq7
WT W303

Figure 7

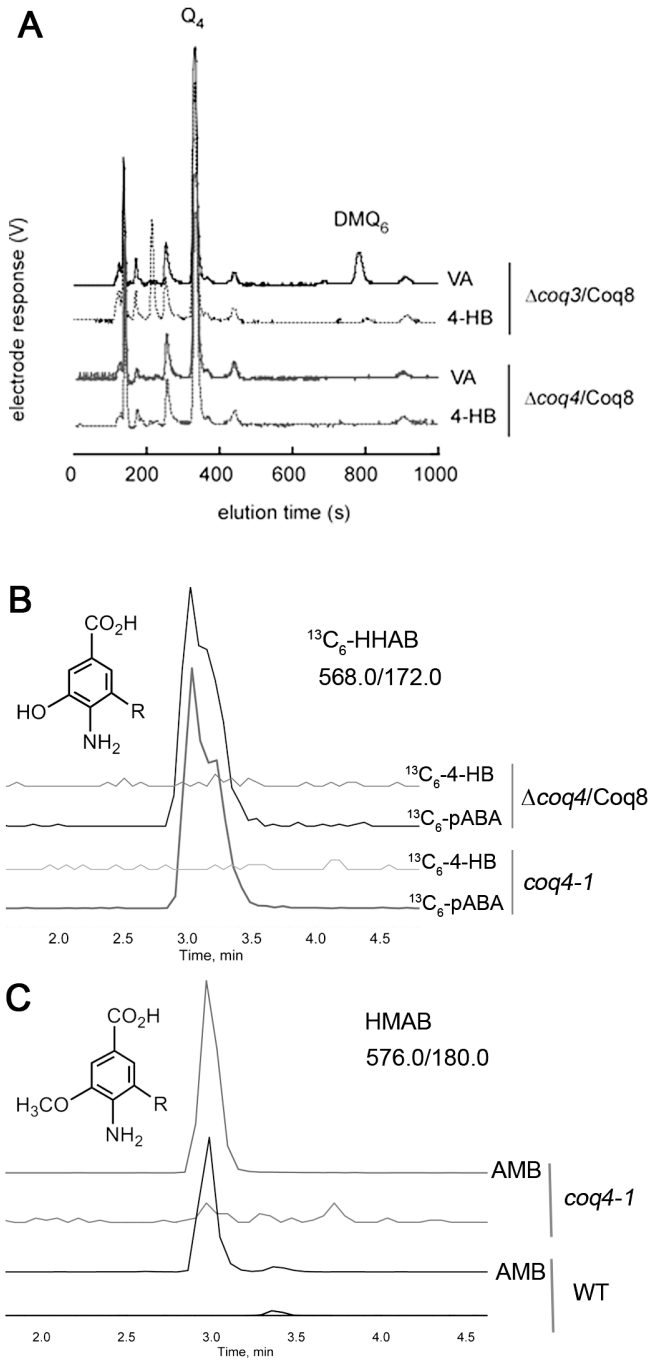
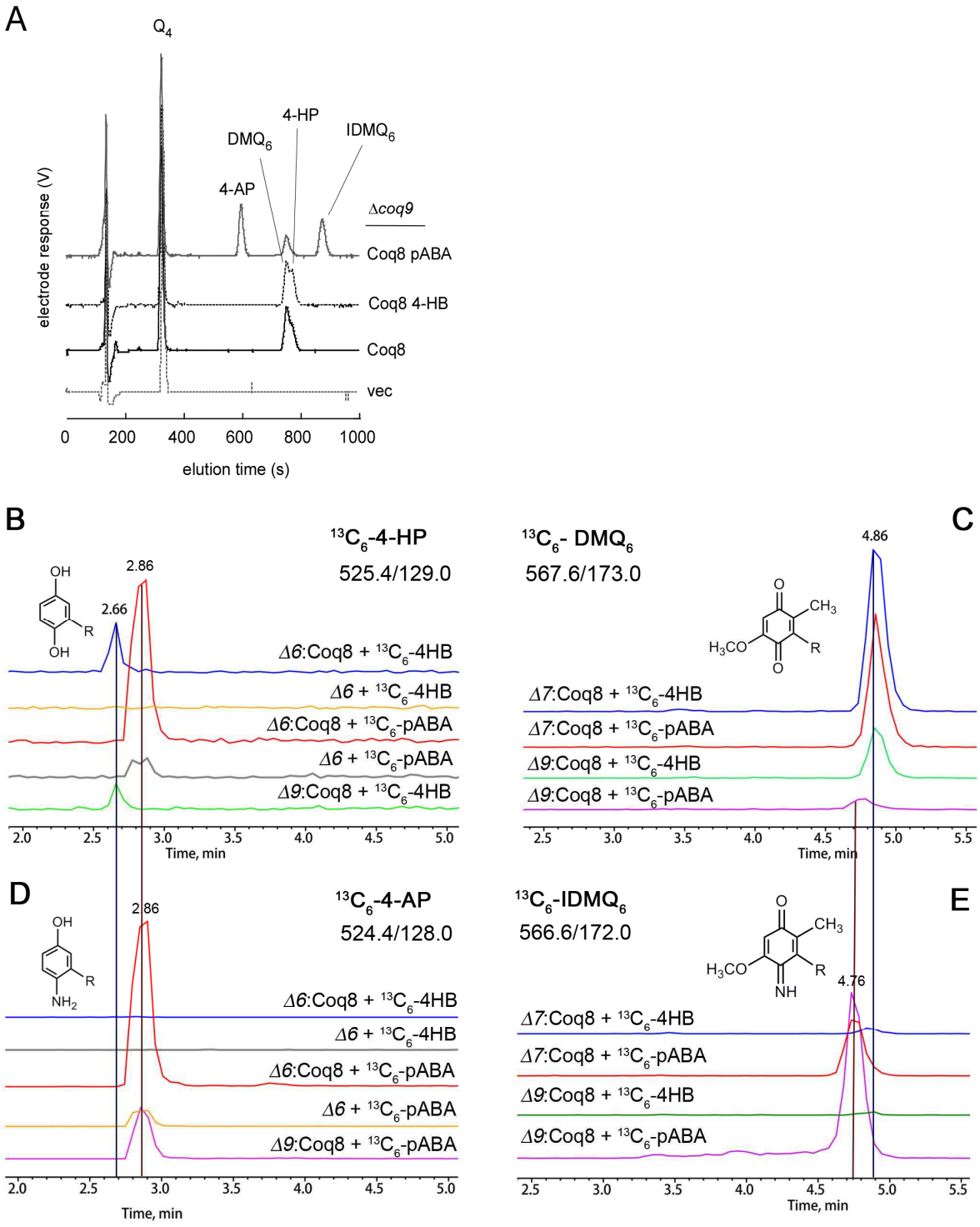

A
Q_4
DMQ_6
electrode response (V)
VA
4-HB
Δcoq3/Coq8
VA
4-HB
Δcoq4/Coq8
0
200
400
600
800
1000
elution time (s)
B
CO_2H
HO
R
NH_2
$^{13}C_6$-HHAB
568.0/172.0
$^{13}C_6$-4-HB
$^{13}C_6$-pABA
Δcoq4/Coq8
$^{13}C_6$-4-HB
$^{13}C_6$-pABA
coq4-1
2.0
2.5
3.0
3.5
4.0
4.5
Time, min
C
CO_2H
H_3CO
R
NH_2
HMAB
576.0/180.0
AMB
coq4-1
AMB
WT
2.0
2.5
3.0
3.5
4.0
4.5
Time, min

Figure 8


A
Q4
4-HP
DMQ6
IDMQ6
4-AP
Δcoq9
Coq8 pABA
Coq8 4-HB
Coq8
vec
electrode response (V)
0
200
400
600
800
1000
elution time (s)
B
13C6-4-HP
525.4/129.0
2.86
2.66
Δ6:Coq8 + 13C6-4HB
Δ6 + 13C6-4HB
Δ6:Coq8 + 13C6-pABA
Δ6 + 13C6-pABA
Δ9:Coq8 + 13C6-4HB
Time, min
C
13C6- DMQ6
567.6/173.0
4.86
Δ7:Coq8 + 13C6-4HB
Δ7:Coq8 + 13C6-pABA
Δ9:Coq8 + 13C6-4HB
Δ9:Coq8 + 13C6-pABA
Time, min
D
13C6-4-AP
524.4/128.0
2.86
Δ6:Coq8 + 13C6-4HB
Δ6 + 13C6-4HB
Δ6:Coq8 + 13C6-pABA
Δ6 + 13C6-pABA
Δ9:Coq8 + 13C6-pABA
Time, min
E
13C6-IDMQ6
566.6/172.0
4.76
Δ7:Coq8 + 13C6-4HB
Δ7:Coq8 + 13C6-pABA
Δ9:Coq8 + 13C6-4HB
Δ9:Coq8 + 13C6-pABA
Time, min