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para-Aminobenzoic Acid Is a Precursor in Coenzyme Q_6 Biosynthesis in *Saccharomyces cerevisiae*[*]

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Beth Marbois[‡§], Letian X. Xie[‡], Samuel Choi[‡], Kathleen Hirano[‡], Kyle Hyman[‡], and Catherine F. Clarke[‡§1]

From the [‡]Department of Chemistry and Biochemistry and the [§]Molecular Biology Institute, University of California, Los Angeles, California 90095-1569

Coenzyme Q (ubiquinone or Q) is a crucial mitochondrial lipid required for respiratory electron transport in eukaryotes. 4-Hydroxybenzoate (4HB) is an aromatic ring precursor that forms the benzoquinone ring of Q and is used extensively to examine Q biosynthesis. However, the direct precursor compounds and enzymatic steps for synthesis of 4HB in yeast are unknown. Here we show that *para*-aminobenzoic acid (*p*ABA), a well known precursor of folate, also functions as a precursor for Q biosynthesis. A hexaprenylated form of *p*ABA (prenyl-*p*ABA) is normally present in wild-type yeast crude lipid extracts but is absent in yeast *abz1* mutants starved for *p*ABA. A stable $^{13}C_6$-isotope of *p*ABA (*p*-amino[*aromatic*-$^{13}C_6$]benzoic acid ([$^{13}C_6$]*p*ABA)), is prenylated in either wild-type or *abz1* mutant yeast to form prenyl-[$^{13}C_6$]*p*ABA. We demonstrate by HPLC and mass spectrometry that yeast incubated with either [$^{13}C_6$]*p*ABA or [$^{13}C_6$]4HB generate both $^{13}C_6$-demethoxy-Q (DMQ), a late stage Q biosynthetic intermediate, as well as the final product $^{13}C_6$-coenzyme Q. Pulse-labeling analyses show that formation of prenyl-*p*ABA occurs within minutes and precedes the synthesis of Q. Yeast utilizing *p*ABA as a ring precursor produce another nitrogen containing intermediate, 4-imino-DMQ_6. This intermediate is produced in small quantities in wild-type yeast cultured in standard media and in *abz1* mutants supplemented with *p*ABA. We suggest a mechanism where Schiff base-mediated deimination forms DMQ_6 quinone, thereby eliminating the nitrogen contributed by *p*ABA. This scheme results in the convergence of the 4HB and *p*ABA pathways in eukaryotic Q biosynthesis and has implications regarding the action of *p*ABA-based antifolates.

Coenzyme Q (Q)[2] is an essential polyprenylated benzoquinone lipid in cellular energy metabolism. The prenyl tail anchors Q in cellular membranes, whereas the redox chemistry of the benzoquinone ring plays a crucial role in respiratory electron transport, in catabolic and biosynthetic metabolism (1), as a co-antioxidant able to recycle vitamin E, and as a chain-terminating antioxidant (2). In these reactions the quinone ring of Q thus cycles between oxidized and reduced (QH_2, or hydroquinone) states.

Cells rely on *de novo* synthesis for an adequate supply of Q. Studies in *Escherichia coli*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* have made use of Q-deficient mutants to elucidate the biosynthetic pathway (3–5). In *S. cerevisiae*, nine *COQ* genes are required, and each of the yeast *coq* mutants (*coq1* through *coq9*) lack Q_6 and are unable to grow on media containing non-fermentable carbon sources such as glycerol or ethanol. The dedicated precursors in the biosynthesis of Q are polyisoprene diphosphate, which provides the tail (*S. cerevisiae* synthesizes Q_6, with a tail containing six isoprene units), and 4-hydroxybenzoic acid (4HB) (6, 7). Studies in animal cells and in *E. coli* indicate that different metabolic pathways are used to produce 4HB. Animals (*e.g.* rats and humans) generate 4HB from the essential dietary amino acid tyrosine (6–8). Phenylalanine also acts as a precursor for 4HB, however, the incorporation is thought to proceed primarily following its conversion to tyrosine via phenylalanine hydroxylase (8). The biosynthetic steps leading from 4-hydroxyphenylpyruvate to 4HB in animal cells are not yet characterized (see Fig. 1). *E. coli* relies on shikimate biosynthesis, the formation of chorismate, and chorismate pyruvate lyase (encoded by the *ubiC* gene) to synthesize 4HB (9, 10). *E. coli ubiC* mutants lack Q unless 4HB is provided in the growth media (9). *E. coli* mutants lacking shikimate or chorismate also require exogenous 4HB to synthesize Q (11). Thus, *E. coli* cells are unable to convert tyrosine or phenylalanine to Q and rely exclusively on the *de novo* synthesis of 4HB from chorismate.

In contrast, *S. cerevisiae* may utilize either shikimate or tyrosine to synthesize the aromatic ring precursor of Q (6, 12). Yeast preferentially utilize shikimate to produce Q, and tyrosine is utilized only when the synthesis of shikimate is blocked (12). Thus, yeast *aro1C* mutants (unable to synthesize shikimate), and yeast *aro2* mutants (unable to synthesize chorismate) still synthesize Q *de novo*, because they are able to utilize exogenously added tyrosine (Fig. 1). The steps producing 4HB from tyrosine have not been identified, although the pathway may be similar to that described for the catabolism of *p*-coumarate to 4HB in *Acinetobacter bayli* (13). Although it has been assumed that yeast may generate 4HB via chorismate pyruvate lyase activity, *S. cerevisiae* lack a homolog of UbiC. This raises the

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[1] To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of California, Los Angeles, 607 Charles E. Young Drive East, Los Angeles, CA 90095-1569. Tel.: 310-825-0771; Fax: 310-206-5213; E-mail: cathy@chem.ucla.edu.

[2] The abbreviations used are: Q, coenzyme Q; [$^{13}C_6$]4HB, *p*-hydroxy[*aromatic*-$^{13}C_6$]benzoic acid; [$^{13}C_6$]*p*ABA, *p*-amino[*aromatic*-$^{13}C_6$]benzoic acid; DMQ, demethoxy-Q; ESI-MS/MS, electrospray ionization mass spectrometry; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; 4HB, 4-hydroxybenzoic acid; 4-imino-DMQ_6, 3-hexaprenyl-4-imino-5-methoxy-2-methylcyclohexa-2,5-dienone; *p*ABA, 4-aminobenzoic acid; prenyl-*p*ABA, 3-hexaprenyl-4-aminobenzoic acid; psig, pound-force per square inch gauge; Q_6, coenzyme Q_6 with a tail of 6 isoprene units; Q_6H_2, reduced or hydroquinone form of Q_6; RP, reverse phase.

question: how do yeast utilize chorismate to produce a ring precursor of Q?

Here we describe our surprising findings that *para*-aminobenzoic acid (*p*ABA), a known precursor of folates, is also an aromatic precursor for Q biosynthesis, via the synthesis of 3-hexaprenyl-4-aminobenzoic acid (prenyl-*p*ABA). These pathways are described in Fig. 1. The biosynthetic steps in yeast necessary for the production of *p*ABA are catalyzed by the *ABZ1* and *ABZ2* gene products. Abz1 amidates chorismate to make the 4-aminodeoxychorismate intermediate (14, 15), and the Abz2 lyase forms free *p*ABA (16). Import of *p*ABA into the mitochondria is necessary for further folate synthesis; the *FOL1* gene product is required for this import and also performs multiple enzymatic functions in pteroglutamoyl synthesis (17). Immunogold particle labeling and a Fol1-GFP fusion localized the tri-functional polypeptide Fol1p in yeast to mitochondrial membranes (17).

We recently became aware of similar work identifying *p*ABA and prenyl-*p*ABA as Q biosynthetic precursors (18). These authors identified *p*ABA as a Q precursor in their search for iron-mediated effects on the function of the Coq7 monooxygenase in Q biosynthesis. Our studies independently determined that *p*ABA is a novel coenzyme Q precursor, and we show prenyl-*p*ABA is an endogenously synthesized intermediate in the Q biosynthetic pathway. We further demonstrate the relative contributions of the $^{13}C_6$-isotope of 4HB and *p*ABA under competition conditions with the alternative unlabeled ring precursor. In addition we identify 4-imino-DMQ_6 in wild-type yeast and in *p*ABA-supplemented *abz1* null mutants. Based on our identification of this intermediate, we suggest a possible mechanism for the removal of the nitrogen donated by *p*ABA, and its replacement with an oxygen atom to form the 1,4-quinone moiety in DMQ via Schiff base chemistry.

EXPERIMENTAL PROCEDURES

Yeast Growth Analysis—Yeast strains used in this work are described in Table 1. The *abz1* null mutant (W303Δ*abz1*) was generated as described (19). Yeast colonies from YPD (2% glucose, 1% yeast extract, 2% peptone, 2% agar) plates were inoculated into 18- × 100-mm culture tubes containing 5 ml of *D*rop *O*ut *Gal*actose (Dogal media): 2% galactose, 0.1% dextrose, and 6.8 g/liter Bio101 yeast nitrogen base minus *p*ABA minus folate with ammonium sulfate (MP Biomedicals) and 5.83 mM sodium monophosphate (pH adjusted to 6.0 with NaOH). Amino acids and nucleotides were included at the following final concentrations (milligrams/liter): adenine hemisulfate, 80; arginine hydrochloride, 40; aspartic acid, 100; cysteine hydrochloride, 80; glutamic acid, 100; histidine hydrochloride, 80; isoleucine, 60; leucine, 120; lysine hydrochloride, 60; methionine, 80; phenylalanine, 80; serine, 60; threonine, 400; tryptophan, 200; tyrosine, 40; uracil, 80; and valine, 150. Following overnight incubation, yeast cultures were diluted 1:100 into fresh Dogal minimal media to deplete intrinsic stores of *p*ABA and folate. Alternatively, cultures were diluted into *D*rop *O*ut *G*lycerol *E*thanol media (Doge; made as above, except galactose was

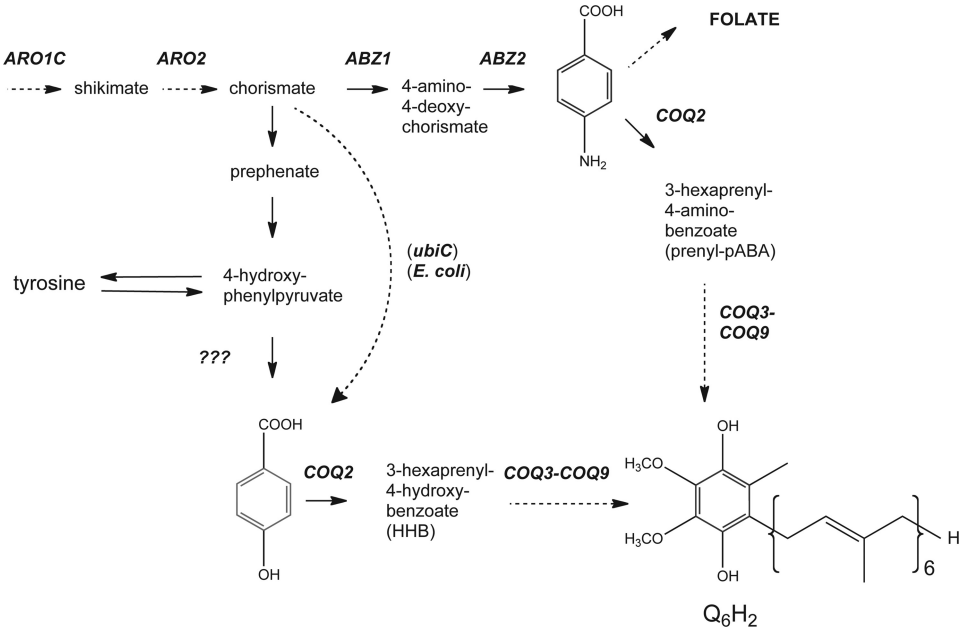

FIGURE 1. **Yeast aromatic ring precursors involved in Q biosynthesis.** We propose that yeast generate aromatic precursors for Q biosynthesis by at least two pathways. One branches from chorismate to produce *p*ABA, a new aromatic ring precursor in Q biosynthesis. The other pathway also branches from chorismate to produce the typical aromatic ring precursor 4HB, via unknown gene products from tyrosine or 4-hydroxyphenylpyruvate. Both 4HB and *p*ABA are prenylated by Coq2. Yeast lack a homolog of *E. coli* UbiC (chorismate pyruvate lyase), which directly forms 4HB from chorismate (*dotted line*). Animals lack the ability to produce shikimate, and rely on Tyr (or Phe) for production of 4HB. The steps involved in converting 4-hdroxyphenylpyruvate to 4HB are not known in yeast or animal cells.

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

Strain	Genotype	Source
W303-1A	MAT**a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303ΔCOQ2	MAT**a** *ade2-1 his3-1,15 leu2-3,112 trp 1-1 ura3-1 coq2::HIS3*	(49)
W303ΔABZ1	MAT**a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 abz1::KANMX4*	This work
NM101	MAT**a** *leu2-3,112, ura3-52, coq7-1*	(29)
E2–249	MAT**a** *met6, coq3*	(50)
BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(51)[b]
BY4741Δabz1	MAT**a** *abz1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(52)[b]

[a] Dept. of Human Genetics, Columbia University, New York, NY.
[b] European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF), available on-line.

replaced with 3% glycerol, 2% ethanol). Solid plate media were made by adding 2.5 g/liter Gelrite (Sigma). When noted media were supplemented with folinic acid, 0.4 μg/ml; 4HB, 2 μg/ml; or *p*ABA (Sigma) 2 μg/ml.

Radioactive and Stable Isotope Labeling—Radioactive compounds included *p*-hydroxy[U-^{14}C]benzoic acid (450 mCi/mmol, 0.1 mCi/ml, American Radiolabeled Chemicals, Inc., St. Louis, MO), and *p*-amino[*aromatic*-^{14}C]benzoic acid (57 mCi/mmol, 0.1 mCi/ml, Moravek Biochemicals, Brea CA). *p*-Amino[*aromatic*-$^{13}C_6$]benzoic acid ([$^{13}C_6$]*p*ABA) or *p*-hydroxy-[*aromatic*-$^{13}C_6$]benzoic acid ([$^{13}C_6$]4HB) were obtained from Cambridge Isotope Laboratories (Andover, MA). The manufacturers' analyses of the pure ^{13}C-labeled aromatic ring compounds by GC-MS and NMR verified a better than 98% chemical purity with 99% isotopic enrichment. $^{13}C_6$- and ^{14}C-labeled aromatic ring precursors were also examined for purity by HPLC. Yeast cells were grown as described to deplete *p*ABA and folate. Optical density (*A*) measurements (600 nm) were recorded for each sample at time of harvest, and the total $A_{600\,nm}$ values were used to normalize lipid content. For radioactive labeling studies, the cells were suspended in 300–600 ml of fresh media as above (Dogal or Doge) and grown to an $A_{600\,nm}$ of 0.6, then collected by centrifugation (1000 × *g*, 5 min) and resuspended in small volumes of fresh media (~2 ml), containing 4 μCi, of the designated ^{14}C-labeled ring precursor. Cells were collected by centrifugation as before, and pellets were stored at −20 °C. Stable isotopic labeling was essentially as above except that individual amounts of [$^{13}C_6$]*p*ABA or [$^{13}C_6$]4HB were weighed and dissolved in DMSO (Sigma), and then added to a final concentration of 2–10 μg/ml.

Competition and Pulse Experiments—Time course and competition experiments with normal and stable isotope forms of precursors were conducted in 18- × 100-mm culture tubes. Cells were collected from a larger volume culture, and equivalent optical density amounts of yeast cells (between 20 and 100 $A_{600\,nm}$) were re-suspended in 5 ml of fresh media as described, which contained either the DMSO (vehicle control), [$^{13}C_6$]*p*ABA, or [$^{13}C_6$]4HB at 10 μg/ml, or each of these plus an additional 10 μg/ml of the competing unlabeled precursor 4HB or *p*ABA. These samples were prepared in duplicate or triplicate, and analyzed via mass spectrometry two or three times ($n = 4–9$). For pulse analyses cells were grown in large volume as described above, re-suspended in pre-warmed media (30 °C) to a total volume of 16 ml in a 125-ml flask, and incubated with shaking (250 rpm, 30 °C). Prior to addition of labeled ring precursors, two (1 ml) aliquots were removed, to represent a "no-label" control. [$^{13}C_6$]*p*ABA was added (final concentration, 10 μg/ml), and further 1-ml aliquots were collected in duplicate at the time points as described. The time zero point was defined by removing two aliquots prior to addition of labeled precursors. The first time point (termed 2 min) represented the addition of label with mixing and immediate removal of two sequential samples, effectively allowing the label to be present for ~1 min and 3 min, respectively.

Lipid Extraction—Yeast cell pellets were thawed on ice, and solvents were added as follows: 100 μl of H_2O, 1 ml of methanol, and 2 ml of petroleum ether. Samples were vortexed for 30 s each. This was repeated, and then samples were centrifuged at 1000 × *g* to separate layers. The upper layer was moved to a new tube, 2 ml of petroleum ether was added to the lower phase, and the sample was vortexed. This upper phase was added to the previous upper organic phase, and the solvent was evaporated under N_2 gas. Samples were routinely analyzed immediately after extraction. When Q or other intermediates were quantified, Q_4 (Sigma) was added in a known amount (expected final concentration, 1 pmol/μl upon analysis) as internal standard to all samples, and to a simultaneously prepared and extracted calibration curve. Typical standard curve final concentrations were 0.2 fmol/μl, 1 fmol/μl, 25 fmol/μl, 200 fmol/μl, 1 pmol/μl, and 5 pmol/μl. The petroleum ether extracts were dried under nitrogen gas and resuspended in 200 μl of ethanol (USP, Aaper Alcohol and Chemical Co., Shelbyville, KY), in sample vials compatible for use with HPLC. Lipid extractions for the pulse experiments were similar, except that the cells were collected onto glass microfiber filter disks (Whatman) placed on a vacuum apparatus, and the collected cells and disks were immersed in ice-cold methanol (2 ml), containing 125 μl of 0.1% bromcresol green. Samples were stored in methanol at −20 °C. Q_4 was added as an internal standard as described above, and samples were kept on ice during the extraction. Re-extraction with petroleum ether (3 ml) was repeated two times. For all quantitative analyses, the standard curve was prepared and analyzed along side the samples, with freshly prepared internal standard and analytes. Routinely, the low end of the standard curve was monitored for recovery of the analyte by comparison of these concentrations a non-extracted standard curve. To ensure that analytes in low concentration gave a reproducible response, a signal to noise threshold ratio was set at 5. If measured analytes did not meet these criteria, or if the standard integrations did not identify the peak of interest accurately, the data were not used.

RP-HPLC and Detection by Scintillation Counting—Detection of radioactive coenzyme Q and intermediates used a B-ram model 2 (IN/US Systems, Inc., Tampa, FL) with a 500-μl flow cell. The column eluate was combined with Safety Solve (Research Products International Corp., Mount Prospect, IL) at a 2:1 ratio with a dwell setting of 1. Data from the device were collected into the Chem Station software supplied with the Agilent 1090 HPLC system. Simultaneous UV data were collected from the intrinsic diode array detector, 274 ± 4 nm, and 250 ± 4 nm. The system contained a Peltier cooled sample chamber maintained at 4 °C and a column oven set to 40 °C. A binary HPLC solvent delivery system was used with a phenyl-hexyl column (Luna 5μ, 100 × 4.60 mm, 5 μm, Phenomenex). The mobile phase consisted of Solvent A (methanol:isopropanol, 95:5, with 2.5 mM ammonium formate) to Solvent B (isopropanol, 2.5 mM ammonium formate) beginning at 100% Solvent A and over 8 min decreasing to 95%. The total run time was 11 min. Sample volumes for injection ranged from 40 to 70 μl, and the software was set to respond at 1 mV = 1 cpm.

RP-HPLC with Detection by MS—A 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used in combination with an Agilent Technologies 1200 HPLC system consisting of a PAL autosampler with thermostatted tray holders and Stack (LEAP Technologies, Carrboro, NC). Nitrogen was used for all gases in the mass

spectrometer; the nitrogen gas was provided as boil-off from a bulk liquid nitrogen storage tank. Applied Biosystems software, Analyst version 1.4.2, was used for data acquisition and processing. Infusion experiments for tuning and optimization were performed with a model 11 plus syringe infusion pump (Harvard Apparatus, Inc., South Natick, MA). RP-HPLC separation was performed as described above. The 4000QT spectrometer was operated in Turbo electrospray positive mode. Q1 and Q3 were operated in unit resolution. For multiple reaction monitoring detection, the precursor-to-product ion transitions in multiple reaction monitoring mode were used to quantify Q and intermediates (*m*/*z*): 591.4/197 (Q_6), 455.2/197 (Q_4), 561.4/167.0 (DMQ_6), and 546.4/150 (prenyl-*p*ABA). Optimum positive turbospray conditions for coenzyme Q compounds: nebulizer gas, 50 psig; turbo gas, 60 psig; curtain gas, 20 psig; collision gas set to "medium"; nebulizer current, 20; and temperature, 450 °C. Optimal settings for compound-dependent parameters are in volts, and dwell is in milliseconds (data are declustering potential, entrance potential, collision energy, collision cell exit potential, and dwell): Q_4 (75, 10, 29, 12, and 125), Q_6 (111, 10, 37, 10, and 125), DMQ_6 (96, 9, 37, 11, and 125), and prenyl-*p*ABA (96, 9, 33, 11, and 125). The same settings were used for ^{13}C-labeled forms. Settings as described are theoretical and based on differences required for the analyses of farnesylated standards (20), with a compensation for increased isoprene length. For ion trap detection, similar gases as above were used for Q and Q intermediates: nebulizer gas, 45 psig; turbo gas, 55 psig; curtain gas, 25 psig; collision gas, high; nebulizer current, 35; and temperature, 450 °C. The enhanced product ion scan used default dynamic settings for trap filling and other parameters. The mass spectrometer detection conditions also included an enhanced resolution scan with standard parameters, between *m*/*z* 520 and *m*/*z* 620. The injection volume was 10 or 20 μl. Stock solutions of the Q_4 and Q_6 (Sigma) were prepared in hexanes and stored under argon gas at −20 °C. Aliquots added to ethanol and the concentrations were then determined spectrophotometrically with a molar extinction coefficient of $E = 14{,}900$ at 275 nm (21). Integration of peak areas was performed with Analyst software, with a bunching factor of 1 and 3 smoothing events. Area ratios were constructed in Microsoft Excel for the calibration curve and experimental samples. The slope was calculated with a linear curve forced through zero. Standard deviations represent duplicate/triplicate samples, independently extracted with duplicate/triplicate injections ($n = 4–6$).

RESULTS

Prenyl-pABA Is a Naturally Occurring Lipid Component of Yeast Cells—Neutral lipid extracts prepared from wild-type yeast cells cultured in standard rich media contain a lipid that we have identified as prenyl-*p*ABA. The identification of prenyl-*p*ABA was based on the presence of an HPLC peak with an elution similar to that for HHB, a previously characterized yeast Q intermediate (22). The precursor ion $[M+H]^+$ of 546, and predominant tropylium [*m*/*z* = 150] and chromenylium [*m*/*z* = 190] product ions, detected in ion-trap analyses, were consistent with a ring amino replacing the ring hydroxyl present in HHB (Fig. 2*A*). The tropylium-like ion is a transition ion generated from prenylated aromatic and benzoquinone rings and is formed under dissociation conditions by incorporation of a methylene remnant (produced by fragmentation of the prenyl tail after the first carbon) to form a 7-membered ring (23). The chromenylium-like ion is larger in mass by +40 (C_3H_4) under these electrospray ionization conditions and is derived by fragmentation and cyclization to include the first four prenyl tail carbons (23).

To confirm the identity of prenyl-*p*ABA, wild-type yeast (W303-1A) were pre-cultured in Dogal media (minus *p*ABA and folate) to deplete cellular stores of *p*ABA (16). Either dextrose or galactose can be used as a fermentable carbon source in minimal media; galactose is used because it is non-repressing to aerobic respiration (24). Yeast cells were transferred to fresh Dogal medium plus folate, and then cultured in the presence of [$^{13}C_6$]*p*ABA as described under "Experimental Procedures." Product ion analyses from the crude lipids of yeast grown in the presence of [$^{13}C_6$]*p*ABA show that yeast incorporate the ring carbons into prenyl-*p*ABA to generate prenyl-[$^{13}C_6$]*p*ABA ($^{13}C_6{}^{12}C_{31}H_{55}NO_2$) (Fig. 2*B*). Although prenyl-*p*ABA is readily detectable in lipid extracts of wild-type yeast (W303-1A harvested at 1.8 *A* in YPGal media contain 89.0 ± 5.6 fmol/*A*), it is much less abundant than Q_6 (84.6 ± 4.3 pmol) under standard log phase growth conditions in rich media.

Yeast coq3 Mutants Cultured with $^{13}C_6$-4HB Produce $^{13}C_6$-labeled HHB—Previous work has shown that yeast *coq* mutants grown in the presence of 4HB produce HHB (25). However, this intermediate is unstable and difficult to detect without derivatization (22). For purposes of comparison, we wished to generate both the normal and ^{13}C-labeled form of HHB. To do this, a *coq3* yeast mutant was cultured in the presence of [$^{13}C_6$]4HB. The E2–249 *coq3* mutant is Q-deficient and is a member of the G31 complementation group defined by Dieckmann and Tzagoloff (26, 27). As shown in Fig. 3 both the normal isotopic form of HHB and [$^{13}C_6$]HHB are detected in lipid extracts prepared from the *coq3* mutant. Our product ion spectra match that described previously (28); of particular note is the fragmentation pattern showing the shift in mass from the precursor molecule (Fig. 3*A*) with an analogous series of fragments from the same compound in the separated crude lipids of [$^{13}C_6$]4HB-labeled *coq3* point mutant (Fig. 3*B*). Both HHB compounds elute with exactly the same retention time. These results demonstrate that the precursor and product ions of prenyl-*p*ABA are each one mass unit less than for HHB, the intermediate formed via the 4HB pathway; HHB $[M+H]^+$ of 547; tropylium ion [*m*/*z* = 151] (compare Figs. 2*A* and 2*B* with 3*A* and 3*B*).

Yeast Cultured with [U-^{14}C]pABA Produce ^{14}C-Labeled Q_6 and DMQ_6—The identification of prenyl-*p*ABA in yeast neutral lipid extracts led us to investigate whether *p*ABA might serve as a ring precursor in yeast Q biosynthesis. We obtained [^{14}C]4HB and [^{14}C]*p*ABA and determined that [*ring*-^{14}C]*p*ABA is free of detectable 4HB and *vice versa* ("Experimental Procedures"). *S. cerevisiae* wild-type cells (W303-1A), *coq7-1* mutants (NM101), or *coq2* mutants (W303ΔCOQ2), were pre-cultured in Dogal media (minus *p*ABA and folate) to deplete cellular stores of *p*ABA (16). Yeast cells were transferred to fresh Dogal medium plus folate, with the addition of either [^{14}C]4HB or [^{14}C]*p*ABA (in each case the specific activity was

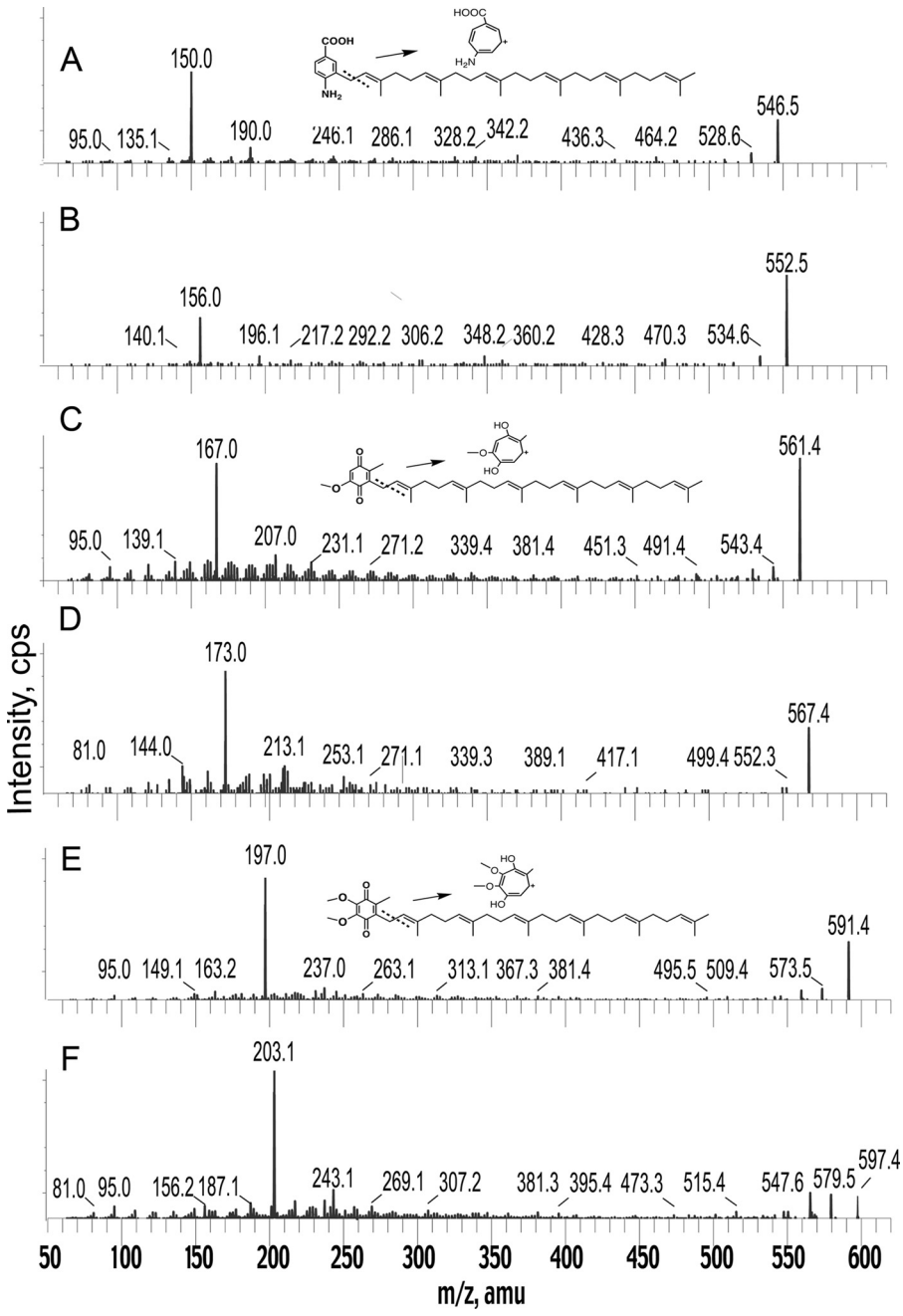

FIGURE 2. **Detection of prenyl-*p*ABA, DMQ_6, and Q_6 in lipid extracts of wild-type yeast cultured in the absence or presence of $^{13}C_6$-*p*ABA.** Wild-type yeast (W303-1A) were pre-cultured in Dogal media (minus *p*ABA and folate) as described under "Experimental Procedures." Cells were harvested during mid-log from large cultures (300–600 ml of media; $A_{600\,nm}$ = 0.6), resuspended in 2 ml of fresh media or fresh media containing 2–10 μg/ml [$^{13}C_6$]*p*ABA (final concentration), incubated at 30 °C and harvested after 16 h. Lipid extracts were prepared and quinones and prenylated intermediates were subjected to RP-HPLC-ESI-MS/MS as described. *A–F* show product ion spectra: *A*, prenyl-*p*ABA $[M+H]^+$ precursor ion ($C_{37}H_{55}NO_2^+$; exact mass, 546.4) and the prenyl-*p*ABA tropylium ion $[M]^+$ ($C_8H_8N_1O_2^+$; exact mass, 150.0); *B*, prenyl-[$^{13}C_6$]*p*ABA $[M+H]^+$ precursor ion ($^{13}C_6{}^{12}C_{31}H_{55}NO_2^+$; exact mass, 552.4) and the $^{13}C_6$-prenyl-*p*ABA tropylium ion $[M]^+$ ($^{13}C_6{}^{12}C_2H_8NO_2^+$; exact mass, 156.0); *C*, DMQ_6 $[M+H]^+$ precursor ion ($C_{38}H_{56}O_3^+$; exact mass, 561.4) and the DMQ_6 tropylium ion $[M]^+$ ($C_9H_{11}O_3^+$; exact mass, 167.1); *D*, [$^{13}C_6$]DMQ_6 $[M+H]^+$ precursor ion ($^{13}C_6{}^{12}C_{32}H_{56}O_3^+$; exact mass, 567.4) and the [$^{13}C_6$]DMQ_6 tropylium ion $[M]^+$ ($^{13}C_6{}^{12}C_3H_{11}O_3^+$; exact mass, 173.1); *E*, Q_6 $[M+H]^+$ precursor ion ($C_{39}H_{58}O_4^+$; exact mass, 591.4) and the Q_6 tropylium ion $[M]^+$ ($C_{10}H_{13}O_4^+$; exact mass, 197.1); *F*, [$^{13}C_6$]Q_6 $[M+H]^+$ precursor ion ($^{13}C_6{}^{12}C_{33}H_{58}O_4^+$; exact mass, 597.4) and the [$^{13}C_6$]Q_6 tropylium ion $[M]^+$ ($^{13}C_6{}^{12}C_4H_{13}O_4^+$; exact mass, 203.1). Q_6 (Sigma) and the lipid extract prepared from NM101 (*coq7-1*) yeast strains were used to establish the retention times of Q_6 and DMQ_6, respectively (29). *A*, *C*, and *E* show the structures of the compounds detected and indicate the identity of the predicted tropylium-like ion of the base peak (tallest peak) formed under collision associated dissociative conditions.

adjusted to 50 Ci/mmol; 800 nM final concentration). Cells were incubated 24 h, lipid extracts were prepared and subjected to RP-HPLC, and the radioactivity was detected as described under "Experimental Procedures." Yeast lipid extracts prepared from wild-type cells contained two peaks of ^{14}C-radiolabeled material, consistent with the elution of Q_6 and Q_6H_2 (the hydroquinone) (Fig. 4). Similarly, the yeast *coq7-1* mutant NM101, when incubated with either [^{14}C]4HB or [^{14}C]*p*ABA, produced radiolabeled material slightly more polar than Q_6, previously identified as DMQ_6 (29). The synthesis of [^{14}C]Q_6 under both experimental conditions requires the yeast *COQ2* gene product (Fig. 4). These results indicate that DMQ_6 and Q_6 may be synthesized in yeast from either *p*ABA or 4HB aromatic ring precursors, and both ring precursors require Coq2p for prenyl tail attachment.

Yeast Cultured with [$^{13}C_6$]pABA produce $^{13}C_6$-labeled Prenyl-pABA, DMQ_6, and Q_6—The results obtained with the ^{14}C-labeled precursors strongly suggest that *p*ABA functions as a ring precursor in yeast Q_6 biosynthesis. Metabolic labeling studies with stable isotopes provide a definitive test, because the ring carbons can be detected in both precursor and product ions by mass spectrometry. Wild-type yeast were first depleted of *p*ABA and folate as described above and then cultured in the presence of [$^{13}C_6$]*p*ABA as described under "Experimental Procedures." The identification of hexaprenylated compounds in the crude lipids of wild-type yeast is represented by the spectra in Fig. 2. The normal (unlabeled) precursor ions monitored included: prenyl-*p*ABA (Fig. 2*A*), demethoxy-Q_6 (Fig. 2*C*), and Q_6 (Fig. 2*E*). Product ion analyses from the crude lipids of yeast grown in the presence of [$^{13}C_6$]*p*ABA show that the ring carbons of this compound are incorporated into demethoxy-Q_6 and Q_6 and alter the average isotopic masses of the tro-

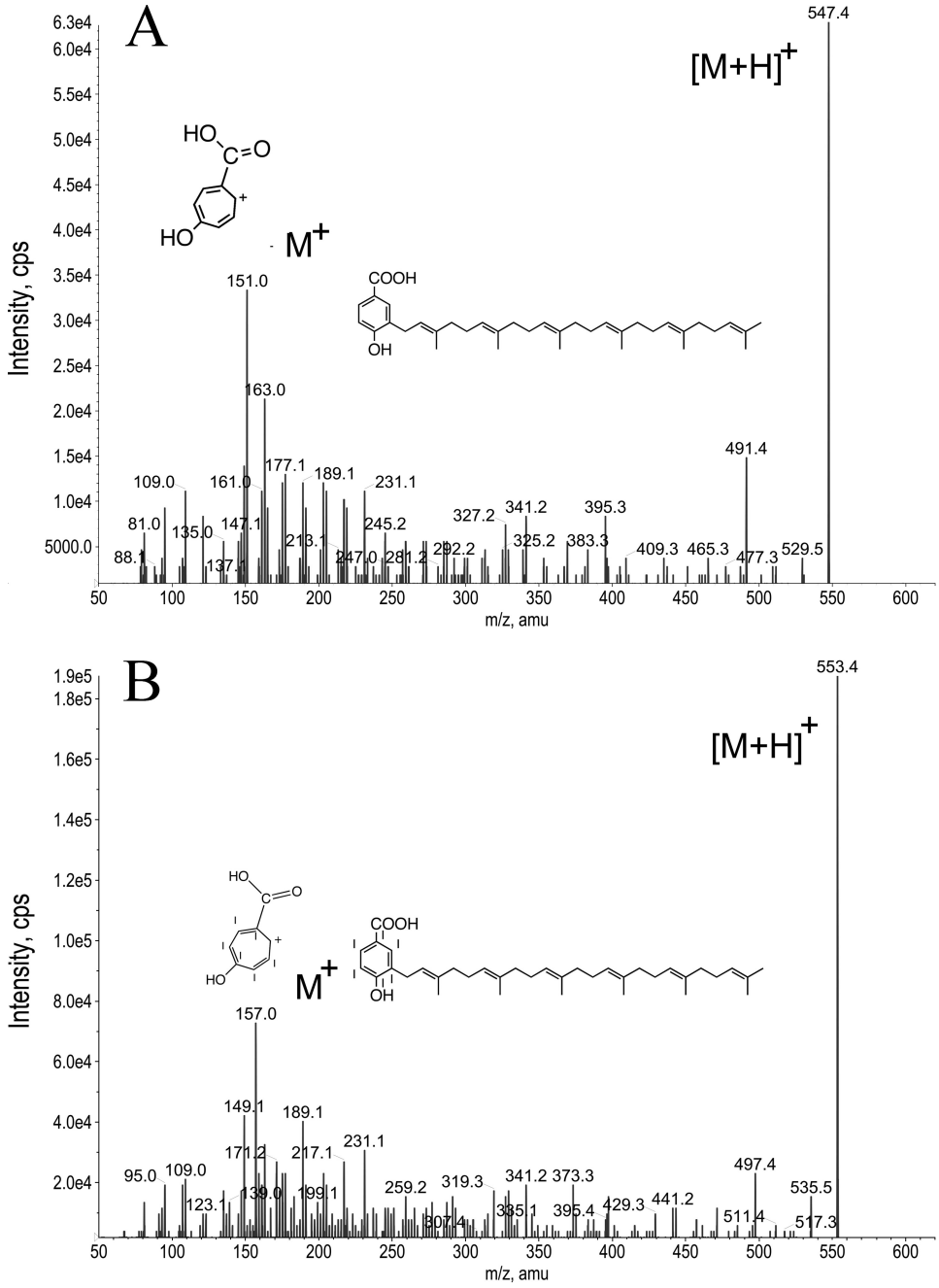

FIGURE 3. **Detection of HHB in lipid extracts of a yeast *coq3* mutant cultured in the absence (*A*) or presence (*B*) of $^{13}C_6$-4HB.** The yeast *coq3* mutant E2-249 was pre-cultured in Dogal media as described previously, harvested, and incubated in fresh media or fresh media with 10 μg/ml [$^{13}C_6$]4HB as described in Fig. 2. Lipid extracts were prepared and subjected to RP-HPLC-ESI-MS/MS as described. Product ion spectra are shown: *A*, HHB [M+ H]$^+$ precursor ion ($C_{37}H_{54}O_3^+$; exact mass, 547.4) and the HHB tropylium ion [M]$^+$ ($C_8H_7O_3^+$; exact mass, 151.0); *B*, [$^{13}C_6$]HHB [M+H]$^+$ precursor ion ($^{13}C_6{}^{12}C_{31}H_{54}O_3^+$; exact mass, 553.4) and the [$^{13}C_6$]HHB tropylium ion ($^{13}C_6{}^{12}C_2H_7O_3^+$; exact mass, 157.0).

pylium-like ion and molecular ion by 6 mass units. This results in the following $^{13}C_6$-labeled compounds in Fig. 2: prenyl-[$^{13}C_6$]*p*ABA ($^{13}C_6{}^{12}C_{31}H_{55}NO_2$) (Fig. 2*B*); $^{13}C_6$-demethoxy-Q_6 ($^{13}C_6{}^{12}C_{32}H_{56}O_3$) (Fig. 2*D*); and [$^{13}C_6$]$Q_6$ ($^{13}C_6{}^{12}C_{33}H_{58}O_4$) (Fig. 2*F*). The incorporation of [$^{13}C_6$]*p*ABA into the penultimate Q intermediate, DMQ_6, was readily detected in wild-type yeast cell lipid extracts (Fig. 2*D*). Wild-type yeast cultured in the presence of [$^{13}C_6$]4HB also generate the expected +6 isotopically labeled forms of Q_6 and DMQ_6, however, under these labeling conditions, it was difficult to detect the +6 form of HHB (data not shown).

Prenyl-pABA Is Absent in Yeast abz1 Mutants Starved for pABA, Yet Content of Q Is Similar to That of Wild-type Yeast—Yeast produce *p*ABA from chorismate by a two-step process that requires the *ABZ1* and *ABZ2* gene products (14, 16). Thus, it seemed likely that the production of prenyl-*p*ABA would depend on the supply of *p*ABA from this biosynthetic pathway, or from the *p*ABA supplied in the media. To test this idea, wild-type yeast, or *abz1* yeast mutants were serially cultured under conditions where the exogenous supply of *p*ABA was eliminated (16). As shown in Fig. 5, *abz1* mutants starved for *p*ABA show dramatic decline in the content of prenyl-*p*ABA, whereas the content of prenyl-*p*ABA in wild-type cells remains unchanged or increased. These results indicate that a combination of the *abz1* deletion and nutritional depletion of *p*ABA results in the depletion of prenyl-*p*ABA. Under these *p*ABA-depleted conditions, the *abz1* mutants are still able to produce DMQ_6 and Q_6. This is consistent with the presence of at least two pathways in yeast able to supply aromatic ring precursors for Q biosynthesis (Fig. 1).

What Are the Relative Contributions of the pABA and 4HB Ring Precursors to Q Biosynthesis?—Once we recognized that [$^{13}C_6$]*p*ABA could serve as a precursor to [$^{13}C_6$]Q_6, we decided to investigate whether cells demonstrated a preference for *p*ABA or 4HB as an aromatic ring precursor for Q. As expected, incubation of wild-type or *abz1* mutant cells labeled for 3.5 h with a single designated precursor show that either [$^{13}C_6$]*p*ABA or [$^{13}C_6$]4HB serve as ring precursors in biosynthesis of DMQ_6 (Fig. 6*A*) and Q_6 (Fig. 6*B*); the *darkly shaded* lower section of each column designates the amount of the [^{12}C]quinone, and the *upper light gray bar* shows the amount of [$^{13}C_6$]quinone. We then performed competition experiments to examine the ability of the normal isotopic ^{12}C form of the alternative precursor to diminish the incorporation of the ^{13}C-form into [$^{13}C_6$]DMQ_6 or [$^{13}C_6$]Q_6 (Fig. 6). For these competitor experi-

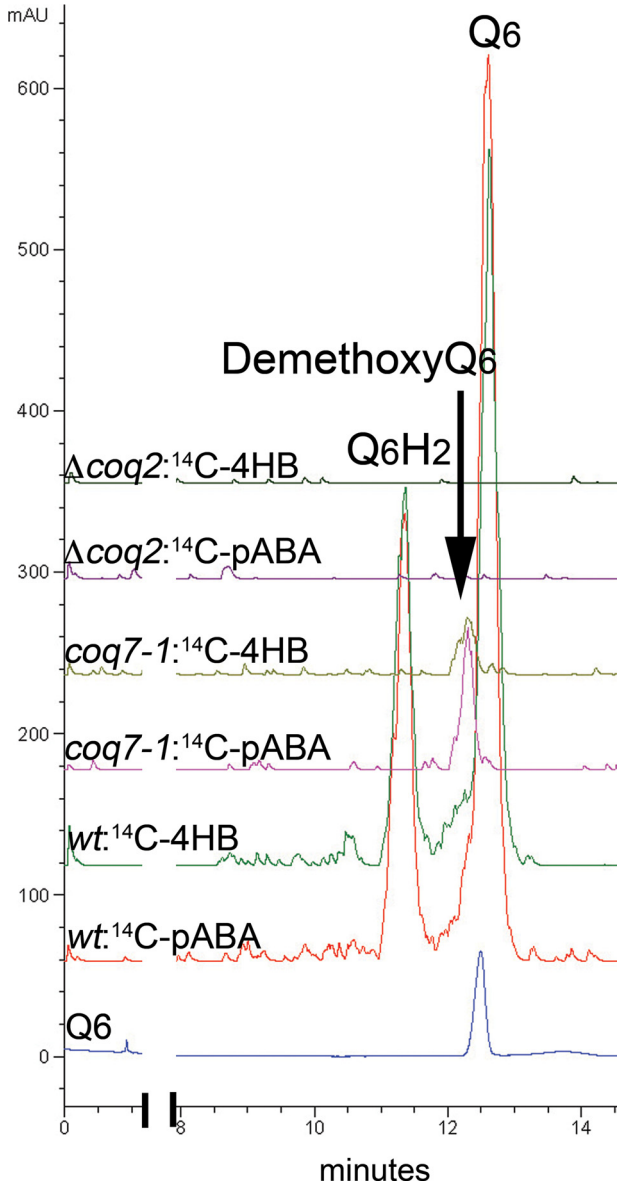

FIGURE 4. **Yeast cultured with ^{14}C-*p*ABA or ^{14}C-4HB produce ^{14}C-labeled Q_6 and DMQ_6.** Wild-type yeast (W303-1A), *coq7-1* mutants (NM101), or *coq2* mutants (W303ΔCOQ2), were pre-cultured in Dogal media (minus *p*ABA, minus folate) to deplete cellular stores of *p*ABA (16). Yeast cells were transferred to fresh Dogal medium plus folate, with the addition of either [^{14}C]4HB or [^{14}C]*p*ABA (in each case specific activity was adjusted to 50 Ci/mmol; 800 nM final concentration). Cells were incubated 24 h, and lipid extracts were prepared and subjected to RP-HPLC and the radioactivity detected as described under "Experimental Procedures." The *bottom blue* trace indicates the Q_6 standard (12.2 min, 274 nm). *Green* and *red* traces show elution of ^{14}C-radiolabeled material present in lipid extracts of wild-type yeast cultured (24 h) with either [^{14}C]4HB or [^{14}C]*p*ABA, as indicated. The elution of [^{14}C]Q_6 at 12.35 min includes a time delay of 0.15 min between the UV detector and the BetaRam (Model 2 in-line scintillation) detector. Reduced [^{14}C]Q_6H_2 eluted 2 min earlier at 11.3 min. *Olive* and *Pink* traces identify ^{14}C-radiolabeled material eluting at 12.1 min as DMQ_6, because the *coq7-1* yeast mutant lacks Q_6 and contains DMQ_6 (29). The *top two traces* (*dark green* and *purple*) indicate the lack of incorporation of ^{14}C precursors into *coq2* null cells. The *arrow* designates the ^{14}C-material co-migrating with DMQ_6, present in the NM101 extracts. Chromatograms are not normalized; the amounts of [^{14}C]DMQ_6 produced in NM101 is 5- to 20-fold lower than the amount of [^{14}C]Q_6 present in wild-type cells.

ments, cells were incubated (3.5 h) in the presence of equivalent concentrations of the alternative ring precursor (10 μg/ml). In wild-type cells, the presence of the competitor did not substantially affect the amount of [$^{13}C_6$]Q_6 content. Indeed, total levels of Q_6 were slightly higher in wild-type cells incubated with both aromatic ring precursors concurrently. In *abz1* mutant cells, the total content of Q_6 was increased (relative to the no-addition control) by addition of any combination of aromatic ring precursors, and the presence of added ^{12}C competitor significantly decreased the amount of [$^{13}C_6$]Q_6 produced from the ^{13}C-aromatic ring precursor. It seems likely that the presence of both ring precursors better satisfies the requirement for both folate and Q.

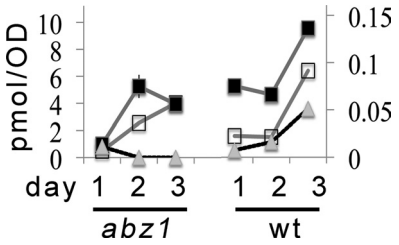

FIGURE 5. **Nutritional and genetic depletion of *p*ABA eliminate the formation of prenyl-*p*ABA.** BY4741 wild-type yeast and BY4741Δ*abz1* yeast mutants were grown overnight in YPD, and then diluted 1:100 (v/v) into fresh Dogal media minus *p*ABA and folate and incubated for 1 day. The *day 1* culture was used to inoculate fresh Dogal media (minus *p*ABA and folate), and the process was repeated to generate the *day2* and *day 3* cultures. The serial dilution into *p*ABA minus media exhausts endogenous stores of *p*ABA (16). Cells were harvested, and lipid extracts were examined for content of Q_6 (*filled squares*; *left y axis*), DMQ_6 (*open squares*; *right y axis*), and prenyl-*p*ABA (*triangles*; *right y axis*).

Although the different ring precursors had only modest effects on the amount of Q_6 formed, they had dramatic effects on the content of DMQ_6. For example, both wild-type yeast and *abz1* mutants had increased content of DMQ_6 when incubated with *p*ABA as compared with 4HB. In fact *abz1* mutants incubated with [$^{13}C_6$]*p*ABA produced a high content of DMQ_6 of which almost all is [$^{13}C_6$]DMQ_6 (Fig. 6*A*). We speculate that prenyl-*p*ABA, or more likely a subsequent intermediate derived from prenyl-*p*ABA, might act to inhibit the hydroxylation of DMQ.

Prenylation of pABA Precedes Biosynthesis of DMQ and Q from pABA—A pulse-labeling experiment was conducted to determine whether the incorporation of $^{13}C_6$-ring carbons into $^{13}C_6$-prenyl-*p*ABA preceded the formation of [$^{13}C_6$]DMQ_6 and [$^{13}C_6$]Q_6. Wild-type cells and *abz1* yeast mutants were pre-cultured as described in Fig. 6 and incubated with [$^{13}C_6$]*p*ABA over a time course from 0 to 30 min, as described in Fig. 7 and "Experimental Procedures." Samples were removed from the incubation at stated times, collected by filtration, and quenched, and lipid extracts were analyzed to determine the amounts of prenyl-*p*ABA, DMQ_6, and Q_6 by HPLC-MS/MS and multiple reaction monitoring. In both wild-type and *abz1* mutant yeast, prenyl-[$^{13}C_6$]*p*ABA is detectable within a minute of label addition (see *inset*, Fig. 7), and its *de novo* synthesis precedes that of [$^{13}C_6$]DMQ_6 and [$^{13}C_6$]Q_6, consistent with the notion of a precursor-product relationship.

pABA-replete Yeast Produce 4-Imino-DMQ_6—In the crude lipid extracts of wild-type cells grown in media supplemented with [$^{13}C_6$]*p*ABA, we identify what appears to be a nitrogen containing form of 4-imino-DMQ_6 (Fig. 8). The tropylium, chromenylium, and molecular ion are all shifted in accordance with M+6 (*m*/*z*), as is detected for DMQ_6 (Fig. 2*D*);

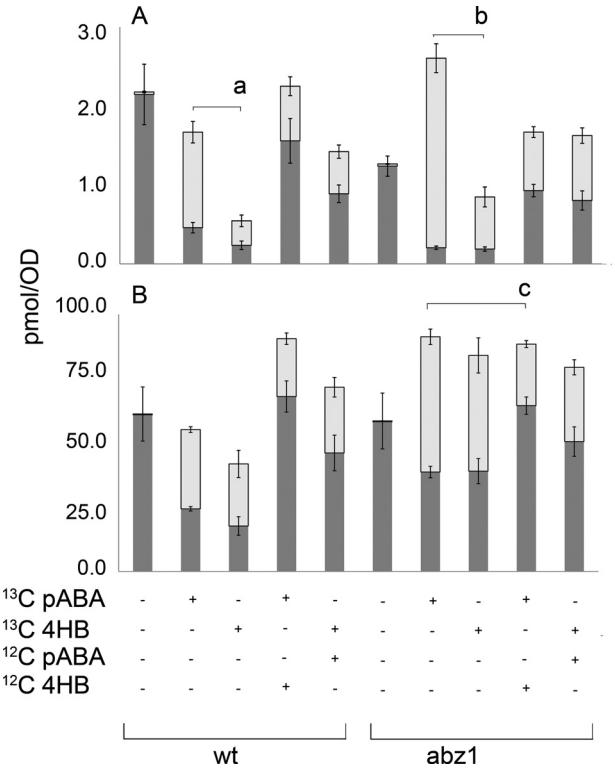

FIGURE 6. **4HB and *p*ABA are mutually incorporated into Q_6, but differentially incorporated into DMQ_6.** Wild-type (W303-1A) or *abz1* null (W303ΔABZ1) cells were serially cultured to deplete them of *p*ABA and folinic acid as described. The depleted cells were harvested and used to inoculate a 4-ml culture. This was divided to supply duplicate samples of cells to five different media conditions. The media contained either no addition or 10 μg/ml of the following: [$^{13}C_6$]*p*ABA, [$^{13}C_6$]4HB, or similar cultures in the presence of equivalent concentrations of the alternative unlabeled (^{12}C) ring precursor (10 μg/ml). Independent replicates were made of each condition. The amount of the competing ^{12}C form of precursor (ratio of ^{13}C:^{12}C) was 1:1. After incubation for 3.5 h, cells were harvested, and lipid extracts were subjected to analyses by RP-HPLC-ESI/MS-MS. *A*, DMQ_6 content; *B*, Q_6 content. The content of [^{12}C]DMQ_6 or [^{12}C]Q_6 is designated by the *darkly shaded lower section* of each *bar* (in *A* and *B*, respectively), and [$^{13}C_6$]DMQ_6 and [$^{13}C_6$]Q_6 by the *upper light gray bar* (in *A* and *B*, respectively). Each condition was cultured as an independent replicate, and each lipid extract was injected twice ($n = 4$). This experiment is representative of three others, conducted independently. The *brackets* with *letters* denote the values being compared: Content of [$^{13}C_6$]DMQ_6 is significantly different in wild-type cells cultured in [^{13}C]*p*ABA as compared with [^{13}C]4HB (*a*, $p = 0.0002$); content of [$^{13}C_6$]DMQ_6 is significantly different in *abz1* mutant cells cultured in [^{13}C]*p*ABA as compared with [^{13}C]4HB (*b*, $p = 0.0002$); and content of [$^{13}C_6$]Q_6 is significantly different in in *abz1* mutant cells cultured in [^{13}C]*p*ABA as compared with [^{13}C]*p*ABA plus unlabeled 4HB (*c*, $p = 0.0001$).

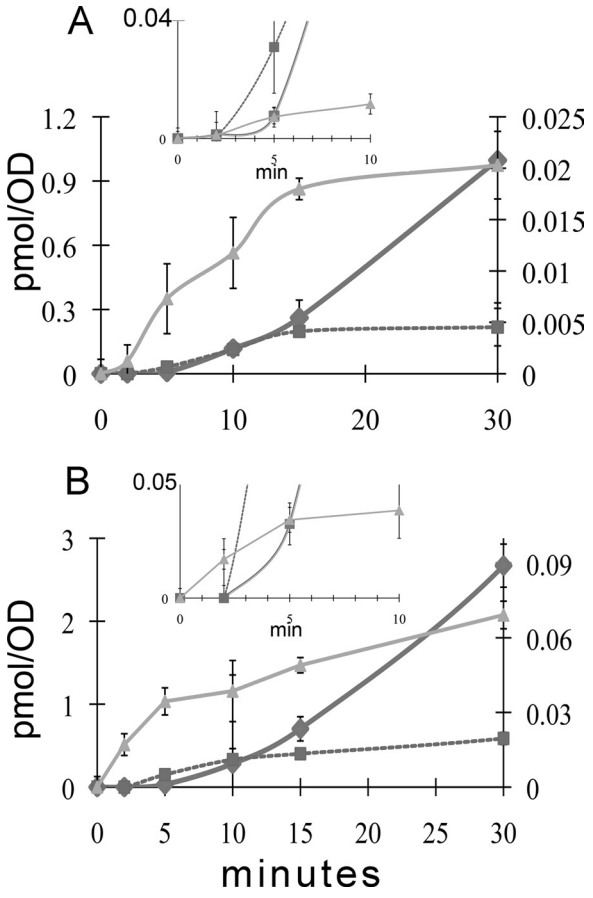

FIGURE 7. **Time course assays with $^{13}C_6$-*p*ABA.** *A*, wild-type (W303-1A) or *B*, *abz1* null (W303ΔABZ1) cells were pre-cultured as described to deplete endogenous *p*ABA, resuspended in pre-warmed Dogal media (30 °C) to a total volume of 16 ml in a 125-ml flask, and incubated with shaking (250 rpm, 30 °C). Prior to addition of ring precursors, two (1 ml) aliquots were removed, to represent a "no-label or zero time" control. [$^{13}C_6$]*p*ABA was added (final concentration, 10 μg/ml), and further 1-ml aliquots were collected in duplicate at the time points indicated on the glass filter disks for lipid extraction: no label control (defines the "zero" time point), 2, 5, 10, 15, or 30 min. Lipid extracts were subjected to analyses by RP-HPLC-ESI/MS-MS as described, and the amount of *de novo* prenyl-[$^{13}C_6$]*p*ABA (*triangles*), [$^{13}C_6$]DMQ_6 (*squares*), and [$^{13}C_6$]Q_6 (*diamonds*) is depicted. The *left y axis* represents the picomoles/*A* (for plots of [$^{13}C_6$]DMQ_6 and [$^{13}C_6$]Q_6), whereas the *right y axis* shows the content in picomoles/*A* for prenyl-[$^{13}C_6$]*p*ABA. Values are plotted as average ± S.D. ($n = 4$, wild type; $n = 6$ *abz1* null). The *insets* in *each panel* depict early time points (in minutes) for all three components plotted on the same scale (pmol/*A*).

however, the masses of these ions are reduced by 1 Da relative to the fragment ion masses of DMQ_6, consistent with an imino nitrogen rather than a quinone oxygen atom in the *para* ring position. The chromenylium ion fragment of 4-imino-DMQ_6 is abundant, because it is likely to be more stable to dissociative conditions due to the interaction of the first four carbons of the prenyl tail with the imino nitrogen forming a second ring (23). The normal isotopic form of 4-imino-DMQ_6 is present in crude lipid extracts of strains grown in YPD (data not shown).

The detection of 4-imino-DMQ_6 led us to test whether it might function as an intermediate in Q_6 biosynthesis. To simplify the precursor-product relationship between 4-imino-DMQ_6 and DMQ_6, we examined the synthesis of both as a function of time in NM101 yeast, a strain known to be blocked in Q biosynthesis and to produce DMQ_6 due to a mutation in *COQ7* (29). The pulse labeling with [$^{13}C_6$]*p*ABA was performed over a time course of 0 to 60 min, and the content of ^{13}C-labeled intermediates (prenyl-[$^{13}C_6$]*p*ABA, [$^{13}C_6$]imino-DMQ_6, and [$^{13}C_6$]DMQ_6) were determined (Fig. 9). The rate of formation of prenyl-[$^{13}C_6$]*p*ABA and [$^{13}C_6$]imino-DMQ_6 were very similar, suggesting that both serve as biosynthetic precursors in the synthesis of DMQ_6. We note that DMQ_6 is relatively unstable in the *coq7-1* mutant, as is evident from the rate of loss of the [^{12}C]DMQ_6. This rate of loss seems to be compensated by the formation of [$^{13}C_6$]DMQ_6. These results suggest that the nitrogen substituent of *p*ABA may be retained up to the step generating 4-imino-DMQ_6, and are consistent with a possible mechanism where oxygen from a water-based hydroxyl could replace the nitrogen imino via Schiff base chemistry (Fig. 10).

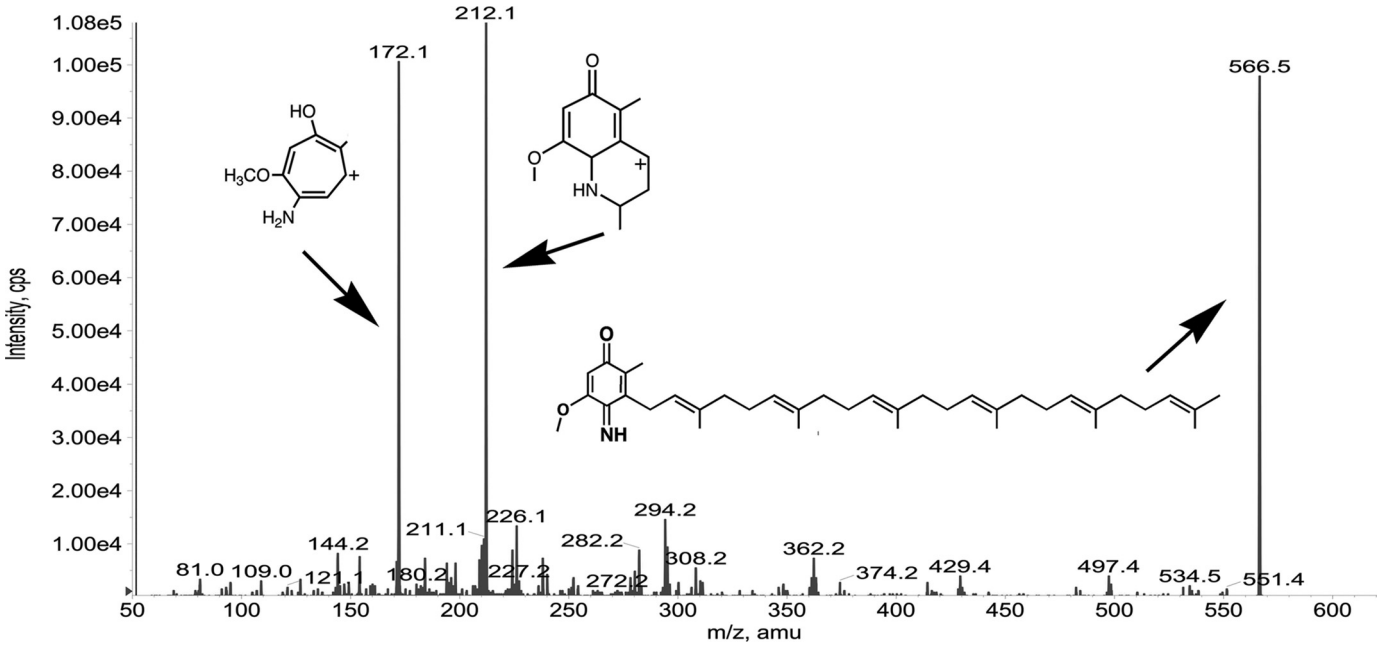

FIGURE 8. **Identification of 4-imino-DMQ$_6$ in lipid extracts of wild-type cells.** An imino form of [$^{13}C_6$]DMQ$_6$ is present in wild-type yeast cultured in the presence of [$^{13}C_6$]*p*ABA and is consistent with the structures as shown. The imino substituent forms a more stable interaction with isoprene proximal to the ring, creating more stable and abundant chromenylium ion upon collision associated dissociation: *m/z* 212. Masses detected at *m/z* 497.4, 429.4, 362.2 and 294.2 are characteristic of the loss of successive isoprene units (*m/z* 68). This compound can be detected in wild-type strains grown in rich media and appears to be a normally accumulating form of demethoxy-Q$_6$ (see Fig. 9). [$^{13}C_6$]imino-DMQ$_6$ [M+H]$^+$ precursor ion ($^{13}C_6{}^{12}C_{32}H_{56}NO_2$+; exact mass, 566.4), the [$^{13}C_6$]imino-DMQ$_6$ tropylium ion [M]$^+$ ($^{13}C_6{}^{12}C_3H_{11}NO_2{}^+$; exact mass, 172.2), and the [$^{13}C_6$]imino-DMQ$_6$ chromenylium ion [M]$^+$ ($^{13}C_6{}^{12}C_6H_{16}NO_2{}^+$; exact mass, 212.3).

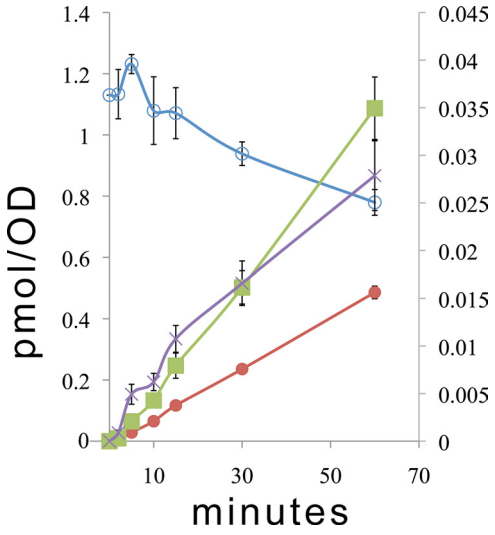

FIGURE 9. **Time course assays with $^{13}C_6$-*p*ABA examine the formation novel Q intermediates in NM101 (*coq7-1*) yeast.** NM101 yeast cells were pre-cultured and analyzed by pulse-labeling with [$^{13}C_6$]*p*ABA as described in Fig. 7. Lipid extracts were subjected to analyses by RP-HPLC-ESI/MS-MS as described, and the amount of *de novo* prenyl-[$^{13}C_6$]*p*ABA (*green squares*), [$^{13}C_6$]DMQ$_6$ (*red circles*), and [$^{13}C_6$]imino-DMQ$_6$ (*purple cross*) is depicted. Also depicted is the decay of non-isotopically labeled DMQ$_6$ (*blue open circles*). The *right y axis* represents the pmol/*A* (for plots of prenyl-[$^{13}C_6$]*p*ABA and [$^{13}C_6$]imino-DMQ$_6$), whereas the *left y axis* shows the content in pmol/*A* for DMQ$_6$ and [$^{13}C_6$]DMQ$_6$. Values are plotted as average ± standard deviations ($n = 4$).

DISCUSSION

Schemes of Q biosynthesis in *E. coli*, yeast, and animals universally depict 4HB as the aromatic ring precursor. In each of these species, isoprenylation of 4HB is thought to represent a committed step in Q biosynthesis. Here we show that *S. cerevisiae* can also utilize *p*ABA as a ring precursor in Q biosynthesis. This is a surprising finding, because *p*ABA is a crucial intermediate in folate biosynthesis. It is also surprising because the addition of *p*ABA to either *E. coli* or human cells causes a concentration-dependent inhibition of Q biosynthesis (30–33). In *E. coli*, rat, and human cells, the *p*ABA ring competes with 4HB at the ring:polyprenyltransferase step (catalyzed by Coq2), and the product prenyl-*p*ABA appears to be a dead-end product. Recently another aromatic ring inhibitor, 4-nitrobenzoic acid, was shown to inhibit Q biosynthesis in mammalian cells through its competition with 4HB for Coq2 (34). Thus it appears that several benzoic acid ring analogs function as competitive inhibitors of Q biosynthesis in mammalian cells (33, 34).

In contrast, our studies identify prenyl-*p*ABA, a normal metabolite present in lipid extracts of wild-type yeast, as a Q intermediate. The synthesis of prenyl-*p*ABA depends on Coq2 (Fig. 4), and we show that prenyl-*p*ABA is a normal metabolite present in lipid extracts of wild-type yeast cultured in standard yeast media (Fig. 2). An interesting independent confirmation of prenyl-*p*ABA in yeast neutral lipid extracts was recently published, found in lipid extracts of yeast with defects in ferredoxin (*YAH1*) and ferredoxin reductase (*ARH1*) (18). These authors discovered the role of *p*ABA in Q biosynthesis through their analyses of iron chaperones required for the activity of Coq7, the Coq diiron enzyme required for the last ring hydroxylation in Q biosynthesis (29, 35).

Our work clearly explores the biochemical relationship of prenyl-*p*ABA in yeast Q biosynthesis, demonstrating prenyl-*p*ABA is a *bona fide* biosynthetic precursor. *p*ABA can be prenylated immediately following its addition to cells. Pulse label-

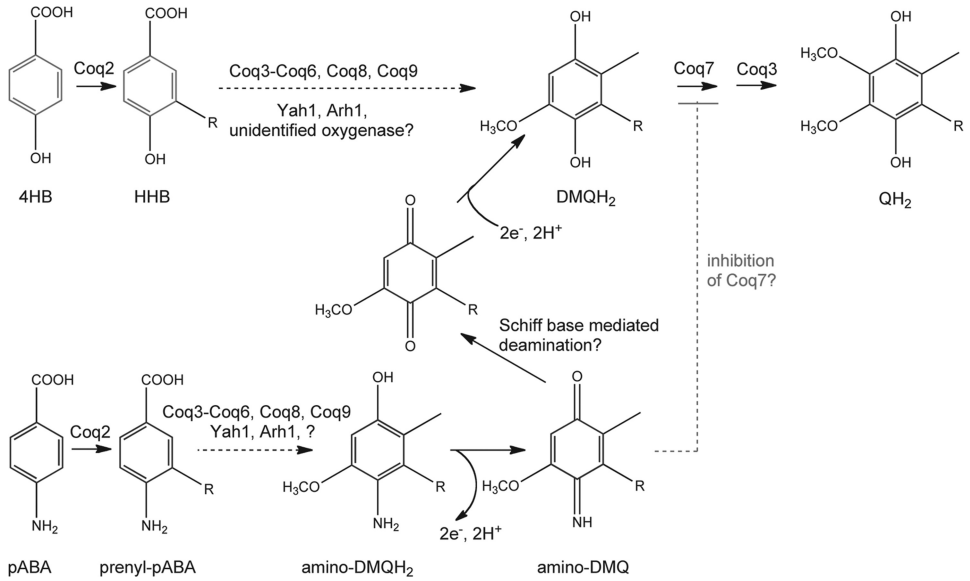

FIGURE 10. **Scheme for generation of 4-imino-DMQ_6 and loss of nitrogen generating DMQ_6.** The pulse-labeling studies in Figs. 7 and 9 suggest that prenyl-*p*ABA and 4-imino-DMQ_6 are *bona fide* precursors in yeast Q synthesis from *p*ABA. A possible mechanism for replacement of the imino nitrogen with a hydroxy substituent via Schiff base chemistry is proposed. The new steps of oxidation, de-imination, and reduction allow the two pathways to converge at formation of DMQ_6H_2.

ing studies with [$^{13}C_6$]*p*ABA show that *p*ABA-deficient *abz1* null yeast mutants synthesize prenyl-[$^{13}C_6$]*p*ABA within 1 min of incubation (Fig. 7). This rapid incorporation of the isotope into prenyl-*p*ABA precedes the formation of [$^{13}C_6$]DMQ_6 and [$^{13}C_6$]Q_6 and is consistent with a precursor-product relationship. Yeast *abz1* mutants, known to be defective in *p*ABA biosynthesis (15, 16), become depleted in prenyl-*p*ABA when cultured in *p*ABA-free media (Fig. 5). However, under these culture conditions Q is still produced, reflecting use of an alternate ring precursor, 4HB (Fig. 1). Prenylation of 4HB by Coq2 is the "classic" arm of the pathway. We also identify for the first time the underivatized forms of HHB, detected in a *coq3* yeast mutant.

Identification of 4-Imino-DMQ_6 and a Model for Nitrogen Loss—Both wild-type and *abz1* yeast null mutants when precultured under conditions to deplete *p*ABA, followed by growth with *p*ABA supplementation, have a higher DMQ_6 content as compared with wild-type yeast or *abz1* mutants supplemented with 4HB (Fig. 6). In isotopic labeling studies with the *abz1* mutant >90% of the DMQ_6 detected contains the $^{13}C_6$-aromatic ring from *p*ABA. The preferential incorporation of *p*ABA into DMQ_6 is particularly intriguing in light of our finding that 4-imino-DMQ_6, a lipid derived from prenyl-*p*ABA, is present in the *p*ABA-supplemented *abz1* mutant and in normally cultured wild-type cells (Fig. 8). The rate of formation of 4-imino-DMQ_6 is similar to that of prenyl-*p*ABA (Fig. 9), identifying it as a new Q intermediate and indicating that the nitrogen of *p*ABA can potentially be retained up to the step preceding DMQ_6.

If this is the case, what is the fate of 4-imino-DMQ_6? We speculate 4-imino-DMQ_6 is produced from the two-electron oxidation of 4-amino-DMQ_6H_2 (Fig. 10). Once the imino-quinone is formed, the potential for loss of the ring nitrogen as ammonia and its replacement by oxygen from a water or hydroxide ion to form the quinone could reasonably occur by Schiff base chemistry. Although we designate the ring nitrogen loss step as immediately preceding DMQ_6, it is possible the loss could occur earlier. However, we favor the depiction in Fig. 10, because DMQ_6 is a relatively abundant Q intermediate, detected even in wild-type cells (36, 37), and is also a component of a Q biosynthetic complex (38). It seems possible that the enhanced accumulation of DMQ_6 in cells first starved for and then supplemented with *p*ABA may be due to inhibition of the Coq7 monooxygenase step (Fig. 10). However, the effect may also be indirect, as the influx of *p*ABA would also replete folate synthesis. We note that the redox state of mitochondria might very well affect the relative rates of oxidation and reduction of amino-DMQ_6H_2 to imino-DMQ_6, and DMQ_6 to DMQ_6H_2.

*Which Ring Precursor, 4HB or p*ABA, *Is Normally Preferred?*—Our labeling with ^{13}C-aromatic ring precursors is accomplished by lowering the supply of *p*ABA and folate to cells with deficient media prior to isotopic incorporation. Thus, at present we cannot differentiate the relative importance of each ring precursor (4HB or *p*ABA) for Q_6 production in wild-type cells, under physiological conditions. It remains possible that *p*ABA could be converted to 4HB prior to lipidation in mitochondria. However, the competition experiments (Fig. 6) suggest that, when one ^{13}C-ring precursor is provided to yeast cells in a one to one molar ratio with the other unlabeled ring, *p*ABA and 4HB are indistinguishable for [^{13}C]Q synthesis. In addition, if [$^{13}C_6$]*p*ABA was converted into [$^{13}C_6$]4HB prior to its prenylation by mitochondrial Coq2, then the enhanced accumulation of [$^{13}C_6$]DMQ_6 from the [$^{13}C_6$]*p*ABA labeling relative to that of [$^{13}C_6$]4HB (Fig. 6*A*) would be unlikely. Although the *abz1* null avidly incorporates and synthesizes [^{13}C]demethoxy from [^{13}C]*p*ABA, our data do not suggest that *p*ABA is a better source for demethoxy Q_6 in normal yeast. Media conditions, for example carbon source and nitrogen source(s), may radically alter ring precursor preference. Finally, we note that our conclusions regarding the incorporation of 4HB and *p*ABA into Q_6 are valid only if the uptake of 4HB and *p*ABA are identical.

It is likely but uncertain that 4HB and *p*ABA may share some mechanisms of uptake and transport of the free form into mitochondria. *p*ABA and 4HB are weakly ionic compounds (*p*ABA: pK_a 4.9; 4HB: pK_a 4.67 (39)), and their uptake and retention has a pH-dependent component. Uptake is favored at low pH, and the formation of the carboxylate anion once imported into the cell favors retention. Although the pH of our media is 6.0, which has been shown to relieve *p*ABA growth inhibition in *S. cerevisiae* (40), other work has shown *p*ABA uptake cannot be saturated (41). The studies of inhibition of yeast growth by *p*ABA are intriguing (40), and are different from *E. coli* growth inhibi-

tion by *p*ABA. *E. coli* have *ubiC* encoded chorismate lyase, which directly converts chorismate to 4HB (9) (Fig. 1), whereas yeast lack this homolog. *E. coli* can be growth inhibited by excess *p*ABA, and relief is accomplished by large concentrations of 4HB (31). However, rescue of growth inhibition by *p*ABA in *S. cerevisiae* requires aromatic amino acids, with phenylalanine and tyrosine in combination to best resume growth (40).

What Are the Pathway(s) to 4HB Production?—Radiolabeled cinnamic and coumaroyl acids produce radiolabeled Q when fed to bakers' yeast (42), with coumarate being the best precursor, and both these compounds are shown to precede 4HB. Other work describes the ability of radiolabeled aromatic amino acids to donate their carbons to Q in yeast (43), however the intermediary compounds have not been described. In other microorganisms the direct precursors for 4HB have been examined more recently and thoroughly. An alternative is described in plants (44), where phenylalanine is a probable precursor, although *S. cerevisiae* lacks an identifiable phenylalanine ammonia lyase homolog. The *Acinetobactor baylyi* system describes the production of 4HB precursors from the catabolism of primarily plant cell wall components into hydroxycinnamate precursors (13).

Do Anti-folates Target Both Folate and Q?—Studies of chorismate synthesis have been stimulated by investigations of drug resistance in microorganisms and *p*ABA metabolism. Depletion of folate is difficult, because it is recycled as a cofactor and yeast carry reserves of both folate and *p*ABA. Yeast cells must be serially cultured in media depleted of these nutrients to elicit a *p*ABA or folate growth deficiency (16). A *p*ABA deficiency is also accomplished by inclusion of sulfanilamide in the media, because sulfanilamide acts as a competitive *p*ABA analog (14). The discovery of sulfanilamide antibiotics hinges on the ability of this class of compound to interfere with the condensation of *p*ABA to pteroglutamoyl for the synthesis of folate (19). The enzyme dihydropteroate synthase is the target of the *p*ABA analog sulfamethoxazole, within the "sulfa" drug class. Previous work by Macreadie's group on resistance to this drug showed resistance depended on expression of the dihydropteroate synthase homolog in yeast (*FOL1*), as long as *p*ABA was supplied in the media (45).

This report describes a novel and physiologically relevant, lipidated form of *p*ABA in yeast, establishing a possible linkage between Q biosynthesis and folate metabolism. Although direct relationships between coenzyme Q biosynthesis and folate metabolism have not been characterized until now, relationships are known to exist between folate synthesis and sulfa drug resistance. A storage form of *p*ABA in microorganisms satisfies the logistics for folate synthesis, just as a glucosylated form of *p*ABA functions in plants (41, 45, 46). It would be undesirable for a crucial metabolic intermediate such as *p*ABA to fall into low supply, but large quantities of the free acid may act as uncouplers to the electrochemical gradient (47). Larger amounts of *p*ABA may also act as a substrate inhibitor of dihydropteroate synthase, the enzyme that couples *p*ABA to a pterin moiety during folate synthesis. Although inhibition occurs at micromolar levels of *p*ABA for the bacterial dihydropteroate synthase homolog (48), its inhibition by high amounts is not complete and it retains much lower but steady activity.

In summary, our analyses document *p*ABA and 4HB as two aromatic rings that serve as precursors for DMQ_6 and Q_6. The observation of a normally produced imino form of DMQ found in the crude lipid extract of wild-type cells (Fig. 8), and its similar rate of formation as compared with prenyl-*p*ABA in a *coq7-1* point mutant (Fig. 9), suggest that both are novel Q intermediates. Based on the 4-imino-DMQ_6 intermediate, we suggest a mechanism for the loss of the *p*ABA-derived nitrogen. Finally the findings presented here suggest an intimate relationship may exist between synthesis of folic acid, necessary for many cellular essential functions and cellular respiration in *S. cerevisiae*, coordinated through the overlapping substrate prenyl-*p*ABA.

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