**para-Aminobenzoic Acid Is a Precursor in Coenzyme Q$_6$ Biosynthesis in Saccharomyces cerevisiae**

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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 (pABA), a well known precursor of folate, also functions as a precursor for Q biosynthesis. A hexaprenylated form of pABA (prenyl-pABA) is normally present in wild-type yeast crude lipid extracts but is absent in yeast abz1 mutants starved for pABA. A stable $^{13}$C$_6$-isotope of pABA (p$^{13}$C$_6$-pABA) is prenylated in either wild-type or abz1 mutant yeast to form prenyl-$^{13}$C$_6$-pABA. We demonstrate by HPLC and mass spectrometry that yeast incubated with either $^{13}$C$_6$-pABA 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-pABA occurs within minutes and precedes the synthesis of Q. Yeast utilizing pABA 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 pABA. We suggest a mechanism where Schiff base-mediated deamination forms DMQ$_6$, quinone, thereby eliminating the nitrogen contributed by pABA. This scheme results in the convergence of the 4HB and pABA pathways in eukaryotic Q biosynthesis and has implications regarding the action of pABA-based antifolates.

Coenzyme Q (Q)$^2$ is an essential polyprenylated benzoquinine 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 ubeI 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 aroIC 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-commarurate to 4HB in Acinetobacter baylgi (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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question: how do yeast utilize chorismate to produce a ring precursor of Q?

Here we describe our surprising findings that para-aminobenzoic acid (pABA), a known precursor of folates, is also an aromatic precursor for Q biosynthesis, via the synthesis of 3-hexaprenyl-4-aminobenzoic acid (prenyl-pABA). These pathways are described in Fig. 1. The biosynthetic steps in yeast necessary for the production of pABA are catalyzed by the ABZ1 and ABZ2 gene products. Abz1 amidates chorismate to the 4-amino-deoxychorismate intermediate (14, 15), and the Abz2 lyase forms free pABA (16). Import of pABA 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 pABA and prenyl-pABA as Q biosynthetic precursors (18). These authors identified pABA 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 pABA is a novel coenzyme Q precursor, and we show prenyl-pABA 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 pABA under competition conditions with the alternative unlabeled ring precursor. In addition we identify 4-imino-DMQ$_6$ in wild-type yeast and in pABA-supplemented abz1 null mutants. Based on our identification of this intermediate, we suggest a possible mechanism for the removal of the nitrogen donated by pABA, 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 (W303Δabz1) 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 Drop Out Galactose (Dogo media): 2% galactose, 0.1% dextrose, and 6.8 g/liter Bio101 yeast nitrogen base minus pABA 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 pABA and folate. Alternatively, cultures were diluted into Drop Out Glycerol Ethanol media (Dogo; made as above, except galactose was

![Figure 1: Yeast aromatic ring precursors involved in Q biosynthesis.](image)

We propose that yeast generate aromatic precursors for Q biosynthesis by at least two pathways. One branch from chorismate to produce pABA, 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 pABA 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-hydroxyphenylpyruvate to 4HB are not known in yeast or animal cells.

| TABLE 1 |
| Genotypes and sources of S. cerevisiae strains |
| Strain | Genotype | Source |
| W303-1A | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 | R. Rothstein* |
| W303ΔCOQ2 | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 1 ura3-1 coq2::HIS3 | | |
| W303ΔABZ1 | MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 1 ura3-1 abz1-1::KANMX4 | This work |
| NM101 | MATa leu2-3,112, ural3-52, coq7-1 | (29) |
| E2–299 | MATa met6, coq3 | (50) |
| BY4741 | MATa his3Δ1 leu2Δ0 met15Δ0 ural3Δ0 | | |
| BY4741Δabz1 | MATa abz1Δ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ural3Δ0 | (52)* |

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* 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 folic acid, 0.4 μg/ml; 4HB, 2 μg/ml; or pABA (Sigma) 2 μg/ml.

Radioactive and Stable Isotope Labeling—Radioactive compounds included p-hydroxy[1-14C]benzoic acid (450 mCi/mmol, 0.1 mCi/ml, American Radiolabeled Chemicals, Inc., St. Louis, MO), and p-aminoad[1-14C]benzoic acid (57 mCi/mmol, 0.1 mCi/ml, Moravek Biochemicals, Brea CA). p-Aminoad[1-13C6]benzoic acid ([13C6]pABA) or p-hydroxy-[1-13C6]benzoic acid ([13C6]4HB) were obtained from Cambridge Isotope Laboratories (Andover, MA). The manufacturers’ analyses of the pure 13C-labeled aromatic ring compounds by GC-MS and NMR verified a better than 98% chemical purity with 99% isotopic enrichment. 13C6- and 14C-labeled aromatic ring precursors were also examined for purity by HPLC. Yeast cells were grown as described to deplete aromatic ring precursors were also examined for purity by HPLC. Yeast cells were grown as described to deplete 13C6- and 14C-labeled ring precursors.

Aromatic ring precursors were also examined for purity by HPLC. Yeast cells were grown as described to deplete 13C6- and 14C-labeled ring precursors.
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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, the precursor-to-product ion transitions in multiple reaction monitoring mode were used to quantify Q and intermediates \((m/z): 591.4/197 (Q_4), 455.2/197 (Q_5), 561.4/167.0 (DMQ_6), \) and \(546.4/150 \) (prenyl-pABA). 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). Q₄ (75, 10, 29, 12, and 125), Q₅ (111, 10, 37, 10, and 125), DMQ₆ (96, 9, 37, 11, and 125), and prenyl-pABA (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 Q₄ and Q₅ (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-pABA. The identification of prenyl-pABA 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 chromenylinium \([m/z = 190]\) product ions, detected in ion-trap analyses, were consistent with a ring amino replacing the ring hydroxyl present in HHB (Fig. 2A). 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 chromenylinium-like ion is larger in mass by +40 \((C_{3}H_{4})\) 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-pABA, wild-type yeast \((W303-1A)\) were pre-cultured in Dgal medium (minus pABA and folate) to deplete cellular stores of pABA (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 Dgal medium plus folate, and then cultured in the presence of \(^{[13]C_6]pABA\) as described under “Experimental Procedures.” Product ion analyses from the crude lipids of yeast grown in the presence of \(^{[13]C_6]pABA\) show that yeast incorporate the carbon atoms into prenyl-pABA to generate prenyl-\(^{[13]C_6]pABA\) \((^{[13]C_6}C_{31}H_{55}NO_2)\) (Fig. 2B). Although prenyl-pABA is readily detectable in lipid extracts of wild-type yeast \((W303-1A)\) harvested at 1.8 A in YPGal media contain \(89.0 \pm 5.6\) mol/m³, it is much less abundant than \(Q_6\) \((84.6 \pm 4.3\) 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 HHB 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 \(^{[13]C_6]HHB\). 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. 3A) with an analogous series of fragments from the same compound in the separated crude lipids of \(^{[13]C_6]HHB\)-labeled \(coq3\) point mutant (Fig. 3B). Both HHB compounds elute with exactly the same retention time. These results demonstrate that the precursor and product ions of prenyl-pABA 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. 2A and 2B with 3A and 3B).

**Yeast Cultured with \([U-^{14}C]\)pABA Produce \(^{14}C_labeled\) \(Q_6\) and DMQ₆—The identification of prenyl-pABA in yeast neutral lipid extracts led us to investigate whether pABA might serve as a ring precursor in yeast Q biosynthesis. We obtained \(^{[14]C]4HB\) and \(^{[14]C]pABA\) and determined that \([ring]^{[14]C]pABA\) 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 Dgal media (minus pABA and folate) to deplete cellular stores of pABA (16). Yeast cells were transferred to fresh Dgal medium plus folate, with the addition of either \(^{[14]C]4HB\) or \(^{[14]C]pABA\) (in each case the specific activity was...
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Wild-type yeast (W303-1A) were pre-cultured in Dogal media (minus pABA and folate) as described under “Experimental Procedures.” Cells were harvested after 16 h. Lipid extracts were prepared and subjected to RP-HPLC-ESI-MS/MS as described in the Procedures. The identification of hexaprenylated compounds in the crude lipids of wild-type yeast is shown in Figure 2. The normal (unlabeled) precursor ions monitored included: A, prenyl-pABA (M+H)^+ precursor ion (C_{39}H_{58}O_{4})~; exact mass, 546.4) and the prenyl-pABA tropylium ion [M]^+ (C_{39}H_{56}O_{4})~; exact mass, 546.4); B, prenyl-pABA (M+H)^+ precursor ion (C_{39}H_{58}O_{4})~; exact mass, 552.4) and the prenyl-pABA tropylium ion [M]^+ (C_{39}H_{56}O_{4})~; exact mass, 552.4); C, DMQ\textsubscript{6} (M+H)^+ precursor ion (C_{37}H_{55}NO_{2})~; exact mass, 561.4) and the DMQ\textsubscript{6} tropylium ion [M]^+ (C_{37}H_{53}O_{2})~; exact mass, 561.4). D, [13C\textsubscript{6}]DMQ\textsubscript{6} (M+H)^+ precursor ion (C_{39}H_{58}O_{4})~; exact mass, 575.5) and the [13C\textsubscript{6}]DMQ\textsubscript{6} tropylium ion [M]^+ (C_{39}H_{56}O_{4})~; exact mass, 575.5); E, [13C\textsubscript{6}]Q\textsubscript{6} (M+H)^+ precursor ion (C_{40}H_{63}O_{4})~; exact mass, 597.4) and the [13C\textsubscript{6}]Q\textsubscript{6} tropylium ion [M]^+ (C_{40}H_{61}O_{2})~; exact mass, 597.4) and the [13C\textsubscript{6}]Q\textsubscript{6} tropylium ion [M]^+ (C_{40}H_{61}O_{2})~; exact mass, 597.4). Q\textsubscript{6} (Sigma) and the lipid extract prepared from NM101 (coq7-1) yeast strains were used to establish the retention times of Q\textsubscript{6} and Q\textsubscript{6}H\textsubscript{2} (the hydroquinone) (Fig. 4). Similarly, the yeast coq7-1 mutant NM101, when incubated with either [1\textsuperscript{3}C\textsubscript{6}]4HB or [1\textsuperscript{3}C\textsubscript{6}]pABA, produced radiolabeled material slightly more polar than Q\textsubscript{6}, previously identified as DMQ\textsubscript{6} (29). The synthesis of [1\textsuperscript{3}C\textsubscript{6}]Q\textsubscript{6} under both experimental conditions requires the yeast COQ2 gene product (Fig. 4). These results indicate that DMQ\textsubscript{6} and Q\textsubscript{6} may be synthesized in yeast from either pABA or 4HB aromatic ring precursors, and both ring precursors require Coq2p for prenyl tail attachment.

**Yeast Cultured with [1\textsuperscript{3}C\textsubscript{6}]pABA produce [1\textsuperscript{3}C\textsubscript{6}]-labeled Prenyl-pABA, DMQ\textsubscript{6} and Q\textsubscript{6}**

The results obtained with the [1\textsuperscript{3}C\textsubscript{6}]-labeled precursors strongly suggest that pABA functions as a ring precursor in yeast Q\textsubscript{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 pABA and folate as described above and then cultured in the presence of [1\textsuperscript{3}C\textsubscript{6}]pABA 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-pABA (Fig. 2A), demethoxy-Q\textsubscript{6} (Fig. 2C), and Q\textsubscript{6} (Fig. 2E). Product ion analyses from the crude lipids of yeast grown in the presence of [1\textsuperscript{3}C\textsubscript{6}]pABA show that the ring carbons of this compound are incorporated into demethoxy-Q\textsubscript{6} and Q\textsubscript{6} and alter the average isotopic masses of the tro-
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Pylium-like ion and molecular ion by 6 mass units. This results in the following 13C6-labeled compounds in Fig. 2:
Prenyl-\([\text{13C}_6\text{pABA}]\) \(\text{13C}_6\text{12C}_3\text{1H}_5\text{5NO}_2\) \(\text{Fig. 2B}\); 13C6-de-methoxy-\(\text{Q}_6\) \(\text{13C}_6\text{12C}_3\text{1H}_5\text{6O}_3\) \(\text{Fig. 2D}\); and \([\text{13C}_6\text{Q}_6]\) \(\text{13C}_6\text{12C}_3\text{1H}_5\text{6O}_4\) \(\text{Fig. 2F}\). The incorporation of \([\text{13C}_6\text{pABA}]\) into the penultimate Q intermediate, \(\text{DMQ}_6\), was readily detected in wild-type yeast cell lipid extracts (Fig. 2D). Wild-type yeast cultured in the presence of \([\text{13C}_6\text{4HB}]\) also generate the expected +6 isotopically labeled forms of Q6 and \(\text{DMQ}_6\), 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 pABA 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-pABA would depend on the supply of pABA from this biosynthetic pathway, or from the pABA 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 pABA was eliminated (16). As shown in Fig. 5, abz1 mutants starved for pABA show dramatic decline in the content of prenyl-pABA, whereas the content of prenyl-pABA in wild-type cells remains unchanged or increased. These results indicate that a combination of the abz1 deletion and nutritional depletion of pABA results in the depletion of prenyl-pABA. Under these pABA-depleted conditions, the abz1 mutants are still able to produce \(\text{DMQ}_6\) and Q6. 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 \([\text{13C}_6\text{pABA}]\) could serve as a precursor to \([\text{13C}_6\text{Q}_6]\), we decided to investigate whether cells demonstrated a preference for pABA 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 \([\text{13C}_6\text{pABA}]\) or \([\text{13C}_6\text{4HB}]\) serve as ring precursors in biosynthesis of \(\text{DMQ}_6\) (Fig. 6A) and Q6 (Fig. 6B); the darkly shaded lower section of each column designates the amount of the \([\text{13C}_6\text{Q}_6]\) precursor, and the upper light gray bar shows the amount of \([\text{13C}_6\text{Q}_6]\). We then performed competition experiments to examine the ability of the normal isotopic \(\text{12C}\) form of the alternative precursor to diminish the incorporation of the \(\text{13C}\)-form into \([\text{13C}_6\text{DMQ}_6]\) or \([\text{13C}_6\text{Q}_6]\) (Fig. 6). For these competitor experi-

FIGURE 3. Detection of HBB in lipid extracts of a yeast coq3 mutant cultured in the absence (A) or presence (B) of \([\text{13C}_6\text{4HB}]\). 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 \([\text{13C}_6\text{4HB}]\) 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, HBB \([\text{M+H]}^+\) precursor ion \(\text{13C}_6\text{12C}_3\text{1H}_5\text{5NO}_2\); exact mass, 547.4) and the HBB tropylium ion \([\text{M}^+\text{H}^+\text{H}]^+\) precursor ion \(\text{13C}_6\text{12C}_3\text{1H}_5\text{5NO}_2\); exact mass, 553.4) and the \([\text{13C}_6\text{HBB}]^+\) tropylium ion \(\text{13C}_6\text{12C}_3\text{1H}_5\text{5NO}_2\); exact mass, 157.0).
Wild-type yeast (W303-1A), coq7-1 mutants (NM101), or coq2 mutants (W303ΔCOQ2), were pre-cultured in Dogal media (minus pABA, minus folate) to deplete cellular stores of pABA (16). Yeast cells were transferred to fresh Dogal medium plus folate, with the addition of either [13C6]pABA or [13C6]pABA (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 Q6 standard (12.2 min, 274 nm). Green and red traces show elution of 14C-radiolabeled material present in lipid extracts of wild-type yeast cultured (24 h) with either [14C]4HB or [14C]pABA, as indicated. The elution of [14C]Q6, 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 [14C]Q6,H2 eluted 2 min earlier at 11.3 min. Olive and Pink traces identify 14C-radiolabeled material eluting at 12.1 min as DMQ6, because the coq7-1 yeast mutant lacks Q6, and contains DMQ6 (29). The top two traces (dark green and purple) indicate the lack of incorporation of 14C precursors into coq2 null cells. The arrow designates the 14C-material co-migrating with DMQ6 present in the NM101 extracts. Chromatograms are not normalized; the amounts of 14C-DMQ6 produced in NM101 is 5- to 20-fold lower than the amount of 14C-Q6 present in wild-type cells.

mAU

Q6

DemethoxyQ6

Δcoq2:14C-4HB

Δcoq2:14C-pABA

cog7-1:14C-4HB

coq7-1:14C-pABA

wt:14C-4HB

wt:14C-pABA

minutes

0

10

12

14

12

13

10

600

400

200

0

FIGURE 4. Yeast cultured with 14C-pABA or 14C-4HB produce 14C-labeled Q6 and DMQ6. Wild-type yeast (W303-1A), coq7-1 mutants (NM101), or coq2 mutants (W303ΔCOQ2), were pre-cultured in Dogal media (minus pABA, minus folate) to deplete cellular stores of pABA (16). Yeast cells were transferred to fresh Dogal medium plus folate, with the addition of either [13C6]4HB or [13C6]pABA (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 Q6 standard (12.2 min, 274 nm). Green and red traces show elution of 14C-radiolabeled material present in lipid extracts of wild-type yeast cultured (24 h) with either [14C]4HB or [14C]pABA, as indicated. The elution of [14C]Q6, 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 [14C]Q6,H2 eluted 2 min earlier at 11.3 min. Olive and Pink traces identify 14C-radiolabeled material eluting at 12.1 min as DMQ6, because the coq7-1 yeast mutant lacks Q6, and contains DMQ6 (29). The top two traces (dark green and purple) indicate the lack of incorporation of 14C precursors into coq2 null cells. The arrow designates the 14C-material co-migrating with DMQ6 present in the NM101 extracts. Chromatograms are not normalized; the amounts of 14C-DMQ6 produced in NM101 is 5- to 20-fold lower than the amount of 14C-Q6 present in wild-type cells.

Nutritional and genetic depletion of pABA eliminate the formation of prenyl-pABA. BY4741 wild-type yeast and BY4741Δabz1 yeast mutants were grown overnight in YPD, and then diluted 1:100 (v/v) into fresh Dogal media minus pABA and folate and incubated for 1 day. The day 1 culture was used to inoculate fresh Dogal media (minus pABA and folate), and the process was repeated to generate the day 2 and day 3 cultures. The serial dilution into pABA minus media exhausts endogenous stores of pABA (16). Cells were harvested, and lipid extracts were examined for content of Q6 (filled squares; left y axis), DMQ6 (open squares; right y axis), and prenyl-pABA (triangles; right y axis).

FIGURE 5. Nutritional and genetic depletion of pABA eliminate the formation of prenyl-pABA. BY4741 wild-type yeast and BY4741Δabz1 yeast mutants were grown overnight in YPD, and then diluted 1:100 (v/v) into fresh Dogal media minus pABA and folate and incubated for 1 day. The day 1 culture was used to inoculate fresh Dogal media (minus pABA and folate), and the process was repeated to generate the day 2 and day 3 cultures. The serial dilution into pABA minus media exhausts endogenous stores of pABA (16). Cells were harvested, and lipid extracts were examined for content of Q6 (filled squares; left y axis), DMQ6 (open squares; right y axis), and prenyl-pABA (triangles; right y axis).

Although the different ring precursors had only modest effects on the amount of Q6, they had dramatic effects on the content of DMQ6. For example, both wild-type yeast and abz1 mutants had increased content of DMQ6 when incubated with pABA as compared with 4HB. In fact abz1 mutants incubated with [13C6]pABA produced a high content of DMQ6 of which almost all is [13C6]DMQ6 (Fig. 6A). We speculate that prenyl-pABA, or more likely a subsequent intermediate derived from prenyl-pABA, might act to inhibit the hydroxylation of DMQ6.

Prenylation of pABA Precedes Biosynthesis of DMQ and Q from pABA—A pulse-labeling experiment was conducted to determine whether the incorporation of 13C6-ring carbons into 13C6-prenyl-pABA preceeded the formation of 13C6-DMQ6 and 13C6-Q6. Wild-type cells and abz1 yeast mutants were pre-cultured as described in Fig. 6 and incubated with [13C6]pABA 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-pABA, DMQ6, and Q6 by HPLC-MS/MS and multiple reaction monitoring. In both wild-type and abz1 mutant yeast, prenyl-[13C6]pABA is detectable within a minute of label addition (see inset, Fig. 7), and its de novo synthesis precedes that of [13C6]DMQ6 and [13C6]Q6, consistent with the notion of a precursor-product relationship.

pABA-replete Yeast Produce 4-Imino-DMQ6—In the crude lipid extracts of wild-type cells grown in media supplemented with [13C6]pABA, we identify what appears to be a nitrogen containing form of 4-imino-DMQ6 (Fig. 8). The tryptophyll, chromenyl, and molecular ion are all shifted in accordance with M+6 (m/z), as is detected for DMQ6 (Fig. 2D);
however, the masses of these ions are reduced by 1 Da relative to the fragment ion masses of DMQ6, consistent with a possible mechanism where oxygen from a water-based hydroxyl could replace the nitrogen imino via Schiff base chemistry (Fig. 10).
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 pABA as a ring precursor in Q biosynthesis. This is a surprising finding, because pABA is a crucial intermediate in folate biosynthesis. It is also surprising because the addition of pABA 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 pABA ring competes with 4HB at the ring:polyprenyltransferase step (catalyzed by Coq2), and the product prenyl-pABA 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-pABA, a normal metabolite present in lipid extracts of wild-type yeast, as a Q intermediate. The synthesis of prenyl-pABA depends on Coq2 (Fig. 4), and we show that prenyl-pABA 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-pABA 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 pABA 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-pABA in yeast Q biosynthesis, demonstrating prenyl-pABA is a bona fide biosynthetic precursor. pABA can be prenylated immediately following its addition to cells. Pulse label-
**pABA Is a Coenzyme Q Biosynthetic Precursor in Yeast**

The scheme for the generation of 4-imino-DMQ₆ and loss of nitrogen generating DMQ₆ is particularly intriguing in light of our findings that null yeast mutants synthesize prenyl-[¹³C₆]Q₆ and is consistent with a precursor-product relationship. Yeast abz1 mutants, known to be defective in pABA biosynthesis (15, 16), become depleted in prenyl-pABA when cultured in pABA-free media (Fig. 5). However, under these culture conditions Q is still produced, reflecting use of an alternative precursor (4HB) (Fig. 1). Prenylation of 4HB by Coq2 is the “classic” arm of the pathway. We also identify the first time the underivatized forms of HHB, detected in a coq3 yeast mutant.

**Identification of 4-imino-DMQ₆ and a Model for Nitrogen Loss**—Both wild-type and abz1 yeast null mutants when precultured under conditions to deplete pABA, followed by growth with pABA supplementation, have a higher DMQ₆ 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 DMQ₆ detected contains the [¹³C₆]-aromatic ring from pABA. The preferential incorporation of pABA into DMQ₆ is particularly intriguing in light of our finding that 4-imino-DMQ₆, a lipid derived from prenyl-pABA, is present in the pABA-supplemented abz1 mutant and in normally cultured wild-type cells (Fig. 8). The rate of formation of 4-imino-DMQ₆ is similar to that of prenyl-pABA (Fig. 9), identifying it as a new Q intermediate and indicating that the nitrogen of pABA can potentially be retained up to the step preceding DMQ₆.

If this is the case, what is the fate of 4-imino-DMQ₆? We speculate 4-imino-DMQ₆ is produced from the two-electron oxidation of 4-amino-DMQ₆H₂ (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 Schiße base chemistry. Although we designate the ring nitrogen loss step as immediately preceding DMQ₆, it is possible the loss could occur earlier. However, we favor the depiction in Fig. 10, because DMQ₆ 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 DMQ₆ in cells first starved for and then supplemented with pABA may be due to inhibition of the Coq7 monooxygenase step (Fig. 10). However, the effect may also be indirect, as the influx of pABA 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-DMQ₆H₂ to imino-DMQ₆ and DMQ₆ to DMQ₆H₂.

**Which Ring Precursor, 4HB or pABA, Is Normally Preferred?**—Our labeling with [¹³C₆]pABA is accomplished by lowering the supply of pABA 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 pABA) for Q₆ production in wild-type cells, under physiological conditions. It remains possible that pABA could be converted to 4HB prior to lipidation in mitochondria. However, the competition experiments (Fig. 6) suggest that, when one [¹³C]-ring precursor is provided to yeast cells in a one to one molar ratio with the other unlabeled ring, pABA and 4HB are indistinguishable for [¹³C]Q synthesis. In addition, if [¹³C₆]pABA was converted into [¹³C₆]4HB prior to its prenylation by mitochondrial Coq2, then the enhanced accumulation of [¹³C₆]DMQ₆ from the [¹³C₆]pABA labeling relative to that of [¹³C₆]4HB (Fig. 6A) would be unlikely. Although the abz1 null avlyde incorporates and synthesizes [¹³C]demethoxy from [¹³C]pABA, our data do not suggest that pABA is a better source for demethoxy Q₆ 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 pABA into Q₆ are valid only if the uptake of 4HB and pABA are identical.

It is likely but uncertain that 4HB and pABA may share some mechanisms of uptake and transport of the free form into mitochondria. pABA and 4HB are weakly ionic compounds (pKₐ: pKₐ, 4.9; 4HB: pKₐ, 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 pABA growth inhibition in *S. cerevisiae* (40), other work has shown pABA uptake cannot be saturated (41). The studies of inhibition of yeast growth by pABA are intriguing (40), and are different from *E. coli* growth inhibi-

![FIGURE 10. Scheme for generation of 4-imino-DMQ₆ and loss of nitrogen generating DMQ₆. The pulse-labeling studies in Figs. 7 and 9 suggest that prenyl-pABA and 4-imino-DMQ₆ are bona fide precursors in yeast Q synthesis from pABA. A possible mechanism for replacement of the imino nitrogen with a hydroxy substituent via Schiff base chemistry is proposed. The new steps of oxidation, deamination, and reduction allow the two pathways to converge at formation of DMQ₆H₂.](image-url)
tion by pABA. 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 pABA, and relief is accomplished by large concentrations of 4HB (31). However, rescue of growth inhibition by pABA 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 Acinetobacter 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 pABA metabolism. Depletion of folate is difficult, because it is recycled as a cofactor and yeast carry reserves of both folate and pABA. Yeast cells must be serially cultured in media depleted of these nutrients to elicit a pABA or folate growth deficiency (16). A pABA deficiency is also accomplished by inclusion of sulfanilamide in the media, because sulfanilamide acts as a competitive pABA analog (14). The discovery of sulfanilamide antibiotics hinges on the ability of this class of compound to interfere with the condensation of pABA to pteroglutamoyl for the synthesis of folate (19). The enzyme dihydropterotate synthase is the target of the pABA 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 dihydropterotate synthase homolog in yeast (FOL1), as long as pABA was supplied in the media (45).

This report describes a novel and physiologically relevant, lipidated form of pABA 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 pABA in microorganisms satisfies the logistics for folate synthesis, just as a glucosylated form of pABA functions in plants (41, 45, 46). It would be undesirable for a crucial metabolic intermediate such as pABA to fall into low supply, but large quantities of the free acid may act as uncouplers to the electrochemical gradient (47). Larger amounts of pABA may also act as a substrate inhibitor of dihydropterotate synthase, the enzyme that couples pABA to a pterin moiety during folate synthesis. Although inhibition occurs at micromolar levels of pABA for the bacterial dihydropterotate synthase homolog (48), its inhibition by high amounts is not complete and it retains much lower but steady activity.

In summary, our analyses document pABA and 4HB as two aromatic rings that serve as precursors for DMQ₆ and Q₆. 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-pABA in a coq7-1 point mutant (Fig. 9), suggest that both are novel Q intermediates. Based on the 4-imino-DMQ₆ intermediate, we suggest a mechanism for the loss of the pABA-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-pABA.

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