PARA-AMINOBENZOIC ACID IS A PRECURSOR IN COENZYME Q₆

BIOSYNTHESIS IN Saccharomyces cerevisiae

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Running Title: pABA is a Coenzyme Q Biosynthetic Precursor in Yeast

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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 ¹³C₆-isotope of pABA (p-amino[aromatic-¹³C₆]benzoic acid (¹³C₆-pABA), is prenylated in either wild-type or abz1 mutant yeast to form prenyl-¹³C₆-pABA. We demonstrate by HPLC and mass spectrometry that yeast incubated with either ¹³C₆-pABA or ¹³C₆-4HB generate both ¹³C₆-demethoxy-Q, a late stage Q biosynthetic intermediate, as well as the final product ¹³C₆-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-DMQ₆. 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 DMQ₆ 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.

Q is an essential polypreneated benzoquinone lipid in cellular energy metabolism. The prenyl tail anchors Q in cellular membranes while 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 (QH₂, or hydroquinone) states.

Cells rely on de novo synthesis for an adequate supply of Q. Studies in Escherichia coli, Schizosaccharomyces pombe and S. 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-coq9) lack Q₆ and are unable to grow on media containing nonfermentable 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 Q₆, 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-hydroxy-phenylpyruvate to 4HB in animal cells are not yet characterized (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 in order 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, as they are able to utilize exogenously added tyrosine (Fig. 1). The steps producing 4HB from tyrosine have not been identified, though the pathway may be similar to that described for the catalysis 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 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 make the 4-aminodeoxychorismate 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). Immuno-gold 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 $^{11}$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 innoculated into 18x100 mm culture tubes containing 5 ml of Drop Out Galactose (Dogo media): 2% galactose, 0.1% dextrose, and 6.8 g/l 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 (mg/l): 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 (Doge; made as above, except galactose was replaced with 3% glucose, 2% ethanol). Solid plate media were made by adding 2.5 g/l of Gelrite (Sigma). When noted media were supplemented with: folic acid, 0.4 μg/ml; 4-hydroxybenzoic acid (4HB), 2μg/ml; para-aminobenzoic acid (pABA; Sigma); or sulfanilamide (Sigma), 100 μg/ml.

**Radioactive and stable isotope labeling**— Radioactive compounds included *p*-hydroxy[1-$^{14}$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/ml (Moravek Biochemicals, Brea CA). *p*-amino[aromatic-\(^{13}\)C]benzoic acid (\(^{13}\)C\(_6\)-pABA) or *p*-hydroxy[aromatic-\(^{13}\)C]benzoic acid (\(^{13}\)C\(_8\)-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-aromatic ring precursors were also examined for purity by HPLC. Yeast cells were grown as described to deplete pABA and folate. Optical density (OD) measurements (600 nm) were recorded for each sample at time of harvest, and the total OD\(_{600\text{nm}}\) 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 OD\(_{600\text{nm}}\) of 0.6, then collected by centrifugation (1000 \(\times\) g, 5 min) and resuspended in small volumes of fresh media (about 2 ml), containing 4 \(\mu\)Ci, of the designated \(^{13}\)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\(_6\)-pABA or \(^{13}\)C\(_8\)-4HB were weighed out and dissolved in DMSO (Sigma), and added to a final concentration of 2–10 \(\mu\)g/ml.

**Competition and pulse experiments–** Time course and competition experiments with normal and stable isotope forms of precursors were conducted in 18x100 mm culture tubes. Cells were collected from a larger volume culture, and equivalent optical density amounts of yeast cells (between 20 and 100 OD\(_{600\text{nm}}\)) were re-suspended in 5 ml fresh media as described, that contained either the DMSO (vehicle control), \(^{13}\)C\(_6\)-pABA, or \(^{13}\)C\(_8\)-4HB at 10 \(\mu\)g/ml, or each of these plus an additional 10 \(\mu\)g/ml of the competing unlabeled precursor 4HB or pABA. These samples were prepared in duplicate or triplicate, and analyzed via mass spectrometry two to three times (n = 4–9). For pulse analyses cells were grown in large volume as described above, re-suspended in prewarmed 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\(_6\)-pABA was added (final concentration 10 \(\mu\)g/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 about one min and three min, respectively.

**Lipid extraction–** Yeast cell pellets were thawed on ice and solvents were added as follows: 100 \(\mu\)l H\(_2\)O, 1 ml methanol and 2 ml petroleum ether. Samples were vortexed for 30 s each. This was repeated and then samples were centrifuged at 1000 \(\times\) g to separate layers. The upper layer was moved to a new tube, two ml of petroleum ether were added to the lower phase, and the sample 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 1pmol/\(\mu\)l 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/\(\mu\)l, 1 fmol/\(\mu\)l, 25 fmol/\(\mu\)l, 200 fmol/\(\mu\)l, 1 pmol/\(\mu\)l, and 5 pmol/\(\mu\)l. The petroleum ether extracts were dried under nitrogen gas and resuspended in 200 \(\mu\)l ethanol (USP, Aaper Alcohol and Chemical Co., Shelbyville Kentucky), in sample vials compatible for use with HPLC as described. Lipid extractions for the pulse experiments were similar, except that the cells were collected onto glass microfibre filter disks (Whatman) placed on a vacuum apparatus, and the collected cells and disks immersed in ice-cold methanol (2 ml), containing 125 \(\mu\)l of 0.1% bromocresol green. Samples were stored in methanol at –20 °C. Q\(_4\) was added as an internal standard was added 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 five. 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 Corporation, 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 DAD detector, 274 ± 4 nm, and 250 ± 4 nm. The system contained a Peltier cooled sample chamber maintained at 4 ºC, and a column oven set to 40 ºC. A binary HPLC solvent delivery system was used with a Phenyl Hexyl column (Luna 5µ, 100 x 4.60 mm, 5 micron, 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 percent Solvent A and over 8 minutes decreasing to 95%. The total run time was 11 minutes. Sample volumes for injection ranged from 40–70 µl and the software was set to respond 1 millivolt = 1 cpm.

**RP-HPLC with detection by mass spectrometry**– A 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (AB, Foster City, CA, USA) 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. AB 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, USA). 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. **MRM detection**: The precursor-to-product ion transitions in multiple reaction monitoring (MRM) mode were used to quantify Q and intermediates: m/z 591.4/197 (Q6), 455.2/197 (Q4), 561.4/167.0 (demethoxyQ6) 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 ºC. Optimal settings for compound dependent parameters are in volts, and dwell in milliseconds: DP; declustering potential, EP; entrance potential, CE; collision energy, CXP; collision cell exit potential (DP;EP;CE;CXP;Dwell): Q4, (75;10;29;12;125); Q6, (111;10;37;10;125); demethoxy-Q6, (96;9;37;11;125); and prenyl-pABA, (96;9;33;11;125). The same settings were used for 13C-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. **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 ºC. The EPI 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 Q6 (Sigma) were prepared in hexanes and stored under argon gas at –20 ºC. Aliquots added to ethanol and the concentrations were then determined spectrophotometrically with a molar extinction coefficient $E$=14,900 at 275 nm (21). Integration of peak areas was performed with Analyst software, with bunching factor of 1 and 3 smoothing events. Area ratios was performed with 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 to 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 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. 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 chromenylium-like ion is larger in mass by +40 (C3H4) 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 Dogal media (minus pABA, minus 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 Dogal medium plus folate, and then cultured in the presence of 13C6-pABA as described in Experimental Procedures. Product ion analyses from the crude lipids of yeast grown in the presence of 13C6-pABA show that yeast incorporate the ring carbons into prenyl-pABA to generate prenyl-13C6-pABA (13C6 14C 12C3 1H55NO2) (Fig. 2B). Although prenyl-pABA is readily detectable in lipid extracts of wild-type yeast (W303-1A harvested at 1.8 OD in YPGal media contain 89.0 ± 5.6 fmol/OD), it is much less abundant than Q6 (84.6 pmol ± 4.3) under standard log phase growth conditions in rich media.

Yeast coq3 mutants cultured with 13C6-4HB produce 13C6-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 13C-labeled form of HHB. To do this, a coq3 yeast mutant was cultured in the presence of 13C6-4HB. 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 13C6-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 13C6-4HB 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 Fig. 2A and B with Fig. 3A and B).

Yeast cultured with [U-14C]-pABA produce 14C-labeled Q6 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 14C-4HB and 14C-pABA and determined that [ring-14C]-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 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 14C-4HB or 14C-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 in Experimental Procedures. Yeast lipid extracts prepared from wild-type cells contained two peaks of 14C-radiolabeled material, consistent with the elution of Q6 and Q6H2 (the hydroquinone) (Fig. 4). Similarly the yeast coq7-1 mutant NM101, when incubated with either 14C-4HB or 14C-pABA, produced radiolabeled material slightly more polar than Q6, previously identified as DMQ6 (29). The synthesis of 14C-Q6 under both experimental conditions requires the yeast coq2 gene product (Fig. 4). These results indicate that DMQ6 and Q6 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 13C6-pABA produce 13C6-labeled prenyl-pABA, DMQ6 and Q6. The results obtained with the 14C-labeled precursors strongly
suggest that pAB A functions as a ring precursor in yeast Q, 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 13C6-pABA as described in 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-Q6 (Fig. 2C) and Q6 (Fig. 2E). Product ion analyses from the crude lipids of yeast grown in the presence of 13C6-pABA show that the ring carbons of this compound are incorporated into demethoxy-Q6 and Q6, and alter the average isotopic masses of the tropylium-like ion and molecular ion by 6 mass units. This results in the following 13C6-labeled compounds in Fig. 2: prenyl-13C6-pABA (13C612C613C31H55NO2) (Fig. 2B); 13C6-demethoxy-Q6 (13C612C62H56O3) (Fig. 2D); and 13C6-Q6 (12C612C63H58O4) (Fig. 2F).

The incorporation of 13C6-pABA into the penultimate Q-intermediate, DMQ6 was readily detected in wild-type yeast cell lipid extracts (Fig. 2D). Wild-type yeast cultured in the presence of 13C6-pABA also generate the expected +6 isotopically labeled forms of Q6 and DMQ6, however, under labeling conditions, it was difficult to detect the +6 form of HHB.

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, while 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 DMQ6 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 13C6-pABA could serve as a precursor to 13C6-Q6, 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 13C6-pABA or 13C6-4HB serve as ring precursors in biosynthesis of DMQ6 (Fig. 6A) and Q6 (Fig. 6B); the darkly shaded lower section of each column designate the amount of the 12C-quinone, and the upper light gray bar shows the amount 13C6-quinone. We then performed competition experiments in order to examine the ability of the normal isotopic 12C form of the alternative precursor to diminish the incorporation of the 13C-form into 13C6-DMQ6 or 13C6-Q6 (Fig. 6). For these competitor experiments, 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 13C6-Q6 content. Indeed, total levels of Q6 were slightly higher in wild-type cells incubated with both aromatic ring precursors concurrently. In abz1 mutant cells, the total content of Q6 was increased (relative to the no-addition control) by addition of any combination of aromatic ring precursors, and the presence of added 13C6-competitor significantly decreased the amount of 13C6-Q6 produced from the 13C6-aromatic ring precursor. It seems likely that the presence of both ring precursors better satisfies the requirement for both folate and Q.

Although the different ring precursors had only modest effects on the amount of Q6 formed, 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 to 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 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-pABA preceded the formation of $^{13}$C$_6$-DMQ$_6$ and $^{13}$C$_6$-Q$_6$. Wild-type cells and absI yeast mutants were pre-cultured as described in Fig. 6, and incubated with $^{13}$C$_6$-pABA over a time course from zero to 30 minutes, as described in Fig. 7 and Experimental Procedures. Samples were removed from the incubation at stated times, collected by filtration, quenched, and lipid extracts were analyzed to determine the amounts of prenyl-pABA, DMQ$_6$, and Q$_6$ by HPLC-MS/MS and MRM. In both wild type and absI mutant yeast, prenyl-$^{13}$C$_6$-pABA 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$_6$-pABA, we identify what appears to be a nitrogen containing form of 4-imino-DMQ$_6$ (Fig. 8). The tryporium, chomenylium and molecular ion are all shifted in accordance with M+6 (m/z), as is detected for DMQ$_6$ (Fig. 2D), however the masses of these ions are reduced by one dalton relative to the fragment ions masses of DMQ$_6$ consistent with an imino nitrogen rather than a quinone oxygen atom in the para ring position. The chomenylium ion fragment of 4-imino-DMQ$_6$ is abundant as 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$_6$-pABA was performed over a time course of 0 to 60 min, and the content of $^{13}$C$_6$-labeled intermediates (prenyl-$^{13}$C$_6$-pABA, $^{13}$C$_6$-imino-DMQ$_6$ and $^{13}$C$_6$-DMQ$_6$) were determined (Fig. 9). The rate of formation of prenyl-$^{13}$C$_6$-

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 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 (ARHI) (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 labeling studies with ^13^C_6-pABA show that pABA-deficient abz1 null yeast mutants synthesize prenyl-^13^C_6-pABA within one minute of incubation (Fig. 7). This rapid incorporation of the isotope into prenyl-pABA 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 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 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 pre-cultured under conditions to deplete pABA, followed by growth with pABA supplementation, have a higher DMQ_6 content as compared to wild-type yeast or abz1 mutants supplemented with 4HB (Fig.6). In isotopic labeling studies with the abz1 mutant greater than 90\% of the DMQ_6 detected contains the ^13^C_6-aromatic ring from pABA. The preferential incorporation of pABA into DMQ_6 is particularly intriguing in light of our finding that 4-imino-DMQ_6, 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-DMQ_6 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 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 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-DMQ_6H_2 to imino-DMQ_6, and DMQ_6 to DMQ_6H_2.

*Which ring precursor, 4HB or pABA is normally preferred?* Our labeling with ^13^C-aromatic ring precursors 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 Q_6 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 ^13^C-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 ^13^C-Q synthesis. In addition, if ^13^C-pABA was converted into ^13^C-4HB prior to its prenylation by mitochondrial Coq2, then the enhanced accumulation of ^13^C_6-DMQ_6 from the ^13^C-pABA labeling relative to that of ^13^C-pABA (Fig. 6A) would be unlikely. While the abz1 null avidly incorporates and synthesizes ^13^C-demethoxy from ^13^C-pABA, our data do not suggest that pABA 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 pABA into Q_6 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 (pK_a: pABA: pKa 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 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 inhibition by pABA. E. coli have ubiC encoded chorismate lyase, which directly converts chorismate to 4HB (9) (Fig. 1), while 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 better 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 lack an identifiable the phenylalanine ammonia lyase homologue. The Acinetobator 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, since 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 analogue (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 dihydropteroate synthase (DHPS) is the target of the pABA analogue sulfamethoxazole, within the “sulfa” drug class. Previous work by Macreadie’s group on resistance to this drug showed resistance depended on expression of the DHPS homologue 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. While 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 dihydropteroate synthase (DHPS), the enzyme that couples pABA to a pterin moiety during folate synthesis. Although inhibition occurs at micromolar levels of pABA, may also act as uncouplers to the electric field of the media (45).

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 to 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.
REFERENCES

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**FOOTNOTES**

**Acknowledgements**

*We thank John Greaves, University of California, Irvine, for his insight and advice about the presence of nitrogen in our unknown that was 1 Da less than HHB. We also thank Jennifer Shepherd, Gonzaga University, for discussions regarding aromatic compounds containing nitrogen substituents, and Jane Strouse, Chemistry and Biochemistry at UCLA, for assistance with mass spectrometry. We thank the CF Clarke lab group members for discussions and input. This material is based upon work supported by the National Science Foundation under Grant No. 0919609 (to CFC and BNM), and by NIH GM4592 (to CFC). UCLA mass spectrometry resources were funded by NIH grant for mass spectrometry resources: 4000QT: Grant Number S10RR024605 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Science Foundation.*
The abbreviations used are: 13C6-4HB, p-hydroxy[aromatic-13C6]benzoic acid; 13C6-pABA, p-amino[aromatic-13C6]benzoic acid; DMQ, demethoxy-Q; ESI-MS/MS, electrospray ionization mass spectrometry; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; 4HB, 4-hydroxybenzoic acid; 4-imino-DMQ6, 3-hexaprenyl-4-imino-5-methoxy-2-methyl-cyclohexa-2,5-diene; pABA, 4-aminobenzoic acid; prenyl-pABA, 3-hexaprenyl-4-aminobenzoic acid; Q6, coenzyme Q6 with a tail of 6 isoprene units; Q6H2, reduced or hydroquinone form of Q6; RP-HPLC, reverse phase high performance liquid chromatography.

FIGURE LEGENDS

Fig. 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 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), that 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.

Fig. 2. Detection of prenyl-pABA, DMQ6 and Q6 in lipid extracts of wild-type yeast cultured in the absence or presence of 13C6-pABA. Wild-type yeast (W303-1A) were pre-cultured in Dogal media (minus pABA, minus folate) as described in Experimental Procedures. Cells were harvested during mid-log from large cultures (300-600 ml media; OD600nm = 0.6), resuspended in 2 ml fresh media or fresh media containing 2-10 µg/ml 13C6-pABA (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. Panels A-F show product ion spectra: A, prenyl-pABA, [M+H]+ precursor ion (C37H55NO2+; exact mass, 546.4); and the prenyl-pABA tropylium ion [M]+ (C8H8N1O2+; exact mass, 150.0); B, prenyl-13C6-pABA [M+H]+ precursor ion (13C6C31H55NO2+; exact mass, 552.4) and the 13C6-prenyl-pABA tropylium ion [M]+ (13C6C2H8NO2+; exact mass, 156.0); C, DMQ6 [M+H]+ precursor ion (C38H56O3+; exact mass, 561.4); and the DMQ6 tropylium ion [M]+ (C9H11O3+; exact mass, 167.1); D, 13C6-DMQ6 [M+H]+ precursor ion (13C6C32H56O3+; exact mass, 567.4); and the 13C6-DMQ6 tropylium ion (13C6C3H11O3+; exact mass, 173.1); E, Q6 [M+H]+ precursor ion (C39H58O4+; exact mass, 591.4); and the Q6 tropylium ion [M]+ (C10H13O4+; exact mass, 197.1); F, 13C6-Q6 [M+H]+ precursor ion (13C6C33H58O4+; exact mass, 597.4); and the 13C6-Q6 tropylium ion (13C6C4H13O4+; exact mass, 203.1). Q6 (Sigma) and the lipid extract prepared from NM101 (coq7-1) yeast strains were used to establish the retention times of Q6 and DMQ6, respectively (29). Panels A, C & 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.

Fig. 3. Detection of HHB in lipid extracts of a yeast coq3 mutant cultured in the absence (A) or presence (B) of 13C6-4HB. 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 13C6-4HB 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 (C37H54O3+; exact mass, 547.4) and the HHB tropylium ion [M]+ (C9H13O3+; exact mass, 173.1); B, 13C6-HHB [M+H]+ precursor ion (13C6C2H7O3+; exact mass, 157.0).

Fig. 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 culture medium.
Nutritional and genetic depletion of 4HB and pABA are mutually incorporated into Q6, but differentially incorporated into DMQ6. Time course assays with 13C6-pABA.

Fig. 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 minus folate and incubated for 1 day. The day 1 culture was used to inoculate fresh Dogal media (minus pABA minus folate) and the process 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).

Fig. 6. 4HB and pABA are mutually incorporated into Q6, but differentially incorporated into DMQ6. Wild-type (W303-1A) or abz1 null (W303ΔABZ1) cells were serially cultured to deplete them of pABA and folic acid as described. The depleted cells were harvested and used to inoculate a 600 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: 13C6-pABA, 13C6-4HB, or similar cultures in 13C6-pABA minus folate and incubated for 1 day. The coq7-1 yeast mutant lacks Q6 and contains DMQ6 (29). Reduced 14C-Q6H2 elutes two minutes earlier at 11.3 min. Olive and Pink traces identify 14C-radiolabeled material eluting at 12.1 min as DMQ6, since the coq7-1 yeast mutant lacks Q6 and contains DMQ6. 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. Spectra 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.

Fig. 7. Time course assays with 13C6-pABA. A, Wild-type (W303-1A) or B, abz1 null (W303ΔABZ1) cells were pre-cultured as described to deplete endogenous pABA, resuspended in pre-warmed Dogal media (30ºC) to a total volume of 16 ml in a 125 ml flask and incubated with shaking (250 rpm, 30ºC). Prior to addition of ring precursors, two (1 ml) aliquots were removed, to represent a “no-label or zero time” control. 13C6-pABA was added (final concentration 10 µg/ml), and further 1 ml aliquots were collected in duplicate at the time points indicated on 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 RPHPLC-ESI/MS-MS as described and the amount of de novo prenyl-13C6-pABA (triangles), 13C6-DMQ6 (squares), and 13C6-Q6 (diamonds) is depicted. The left y axis represents the pmol/OD (for plots of 13C6-DMQ6 and 13C6-Q6) while the right y axis shows the content in pmol/OD for prenyl-13C6-pABA. Values...
are plotted as average ± standard deviations (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/OD).

**Fig. 8.** Identification of 4-imino-DMQ6 in lipid extracts of wild-type cells. An imino form of 13C6-DMQ6 is present in wild-type yeast cultured in the presence of 13C6-pABA 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-Q6 (see Fig. 9). 13C6-imino-DMQ6 [M+H]+ precursor ion (13C6-12C32H56NO2+; exact mass, 566.4) and the 13C6-imino-DMQ6 tropylium ion [M]+ (13C6-12C3H11NO2+; exact mass, 172.2) and the 13C6-imino-DMQ6 chromenylium ion [M]+ (13C6-12C6H16NO2+; exact mass, 212.3).

**Fig. 9.** Time course assays with 13C6-pABA examine the formation novel Q-intermediates in NM101 (coq7-1) yeast. NM101 yeast cells were pre-cultured and analyzed by pulse-labeling with 13C6-pABA as described in Fig. 7. Lipid extracts were subjected to analyses by RPHPLC-ESI/MS-MS as described and the amount of de novo prenyl-13C6-pABA (green squares), 13C6-DMQ6 (red circles), and 13C6-imino-DMQ6 (purple cross) is depicted. Also depicted is the decay of non-isotopically labeled DMQ6 (blue open circles). The left y axis represents the pmol/OD (for plots of prenyl-13C6-pABA and 13C6-imino-DMQ6) while the right y axis shows the content in pmol/OD for DMQ6 and 13C6-DMQ6. Values are plotted as average ± standard deviations (n=4).

**Fig. 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, deimination, reduction allow the two pathways to converge at formation of DMQ6H2.
Table 1
Genotypes and Sources of *S. cerevisiae* strains

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<th>Genotype</th>
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<td>R. Rothstein*</td>
</tr>
<tr>
<td>W303ΔCOQ2</td>
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*aDepartment of Human Genetics, Columbia University, New York, NY USA  
bhttp://web.uni-frankfurt.de/fb15/mikro/euroscarf/complete.html*
Figure 1
Page 16
Figure 3
4HB → HHB

Coq2 → Coq3-Coq6, Coq8, Coq9, Yah1, Arh1, unknown oxygenase

DMQH₂ → QH₂

inhibition of Coq7?

Schiff base mediated deamination?

pABA → prenyl-pABA

4-amino-DMQH₂ → 4-imino-DMQ

Figure 10  Page 25
Para-aminobenzoic acid is a precursor in coenzyme Q6 biosynthesis in Saccharomyces cerevisiae
Beth Marbois, Letian X. Xie, Samuel Choi, Kathleen Hirano, Kyle Hyman and Catherine F. Clarke

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