

Isotopic labeling of the heme cofactor in cytochrome p450 and other heme proteins.

By: David Bryson, Pek-Lang Lim, Alin Lawson, Sandhya Manjunath, and Gregory M. Raner

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Abstract:

A recombinant bacterial expression system that generates ^{13}C -labeled heme or ^{15}N -labeled heme in functional cytochrome P450 enzymes and other heme-containing systems is reported here using a mutant strain of *Escherichia coli* (HU227) in which the *HemA* gene is inactive. By synthesizing several isotopomers of aminolevulinic acid with ^{13}C or ^{15}N at different locations, isotopes have been incorporated with high abundance into the heme cofactor of five different cytochrome P450 isoforms, along with one peroxidase. Confirmed both ^{13}C - and ^{15}N -incorporation; spectral and catalytic assays show the labeled enzymes produced in this system are functional.

Keywords: Aminolevulinic acid | Carbon-13 | Cytochrome P450 | Heme | Isotopic labeling | Nitrogen-15 | biochemistry | biotechnology

Article:

Introduction

The ability to generate hemoprotein samples in which specific positions on the cofactor have been isotopically labeled has found utility in recent studies. Paramagnetic NMR and resonance Raman spectroscopy are two techniques that have taken advantage of this ability, as shown by Alontaga et al. (2006), Caignan et al. (2003) and Balakrishnan et al. (2009). Strategies for labeling the heme cofactor with ^{13}C at specific positions have been developed for several hemoprotein systems, based on the well-characterized heme biosynthetic pathway shown in Fig. 1, which is employed by most bacteria, including *E. coli*. For example, by controlling growth conditions and expression of outer membrane (OM) cytochrome b5 in *E. coli*, in the presence of ^{13}C -labeled aminolevulinic acid (a biosynthetic heme precursor), efficient labeling of the prosthetic group (>85%) at specific locations has been achieved (Rivera and Walker 1995). An

alternative approach was used by Behr et al. (1998) in which the Hema gene coding for 5-aminolevulinic synthase (the gene responsible for synthesis of aminolevulinic acid (ALA) in the cell) was deleted from a strain of *Paracoccus denitrificans*, and the media was supplemented with ^{13}C -labeled aminolevulinic acid. Both of these approaches are useful in specific situations, but have limitations, such as the need for reconstitution.

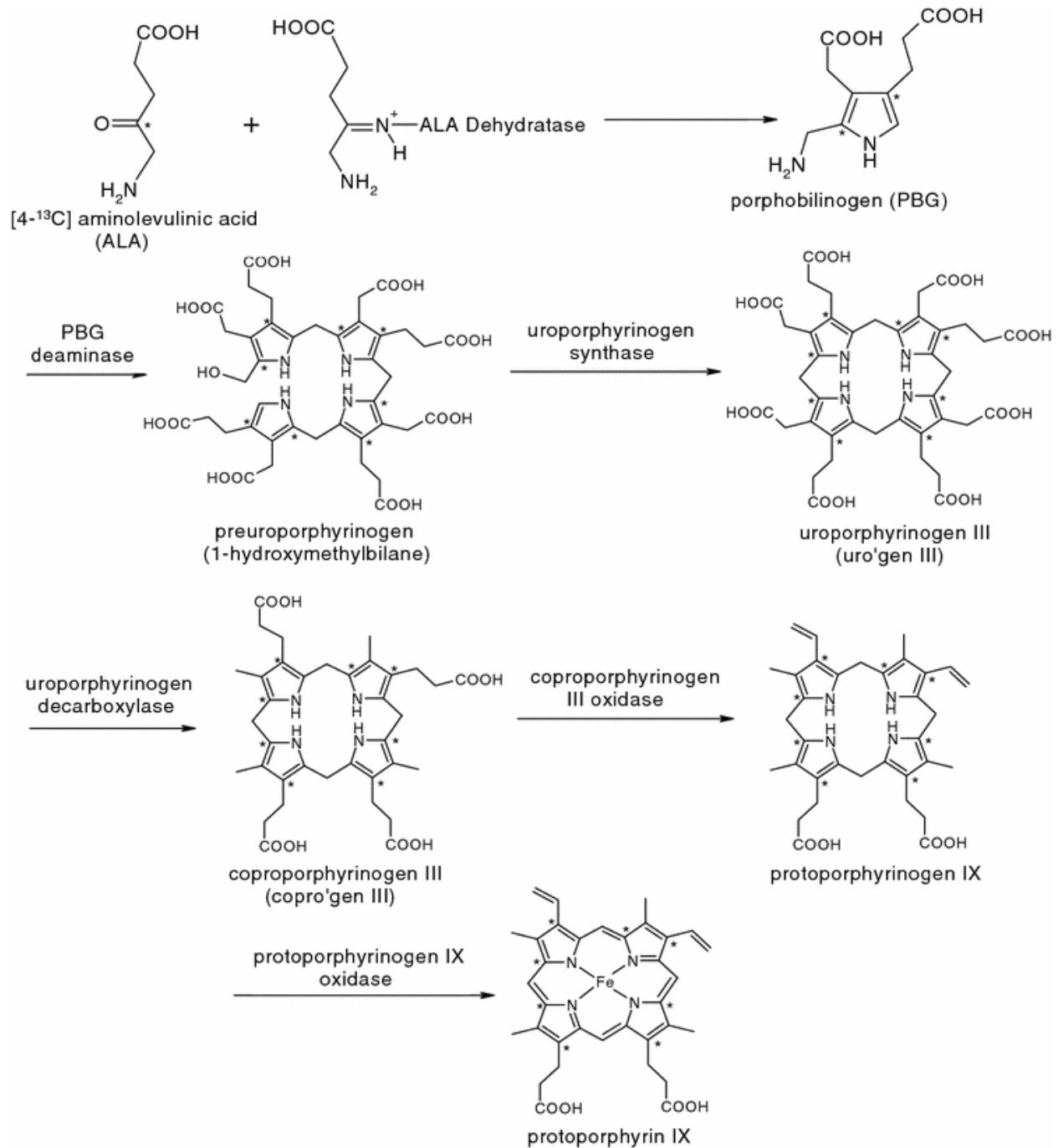


Fig. 1: The biosynthetic pathway of heme starting from [4- ^{13}C] aminolevulinic acid. The labeled atom represents ^{13}C . In *E. coli*, aminolevulinic acid is produced via the C-5 pathway starting

with glutamate. The HemA gene product is a glutamyl-tRNA reductase, which catalyzes the rate-limiting step in aminolevulinic acid synthesis

Cytochrome P450s are very difficult to reconstitute, apparently due to the thiolate ligation. Although methods have been reported (Wagner et al. 1981), these protocols generally require very harsh treatment of the enzyme, which may or may not have adverse irreversible effects on the reconstituted sample. Additionally, certain P450 enzymes (and other heme proteins) have covalently bound heme cofactors (LeBrun et al. 2002), which precludes the use of techniques involving reconstitution. Woodward et al. (2007) developed an *E. coli* system in which modified hemes may be incorporated through media supplementation, however aminolevulinic acid cannot be used as a source of heme precursor, as the inactive gene product in this strain is downstream from ALA synthesis in the biosynthetic pathway (Rivera and Caignan 2004). In addition, cultures must be grown anaerobically, which necessitates the use of more sophisticated culture facilities. Our objective was to develop a simple more universal method for efficient incorporation of ^{13}C or ^{15}N into the heme cofactor of any heme containing enzyme that can be expressed in *E. coli*, using a variant strain of *E. coli* (HU227) that cannot produce the heme precursor aminolevulinic acid (Li et al. 2003).

Materials and methods

Materials

[4- ^{13}C]-Aminolevulinic acid, [1- ^{13}C]-, [2- ^{13}C]- and ^{15}N -glycine, were from Cambridge Isotope Laboratories as a standard. Subsequently, ^{15}N -, [4- ^{13}C] and [5- ^{13}C]-aminolevulinic acid were synthesized according to the method of Wang and Scott (1997) with minor variations, as described below. Plasmids for bacterial expression of P450_{2E1} ($\Delta 3-29$), P450_{2B4} ($\Delta 2-27$), P450_{BM3}, P450_{BM3}-F87G, dehaloperoxidase (DHP) were obtained from Dr. Jud Coon [The University of Michigan, P450_{2E1}($\Delta 3-29$), P450_{2B4}($\Delta 2-27$)], Dr. David Mullen (Tulane University, P450_{BM3}, P450_{BM3}-F87G) and Dr John Dawson (The University of South Carolina, DHP). CYP102A2 was cloned and expressed in our lab according to the method of Gustafsson et al. (2004). The *E. coli* strain HU227 (*F*-, *hemA41*, *relA1*, *spoT1*, *metB1*, *rrnB-2*, *mcrB1*, *creC510*, *Hem-p*) was provided by Dr. Jeremy LaLean and cultures of this strain were initially grown out in LB media in the presence of a minimum of 10–20 mg aminolevulinic acid per liter.

Synthesis of ^{13}C - and ^{15}N -aminolevulinic acid

The procedure used is modified slightly from that of Wang and Scott (1997) and is summarized in Fig. 2. The mole values, reaction yields and NMR data given in this section, are for reagents and products in the synthesis of [5- ^{13}C]ALA, however the same procedure was used for generation of [4- ^{13}C] and ^{15}N -ALA as well. [2- ^{13}C]Glycine (99 atom.% ^{13}C , 1.010 g, 13.28 mmol) was fused with phthalic anhydride (1.992 g, 13.45 mmol) under argon. The

resulting off-white solid was re-crystallized from water, yielding [2-¹³C] phthalylglycine as white needles (2.470 g, 90.2%); ¹H-NMR (DMSO-d₆) δ: 4.28 (d, 2H), 7.88 (m, 4H).

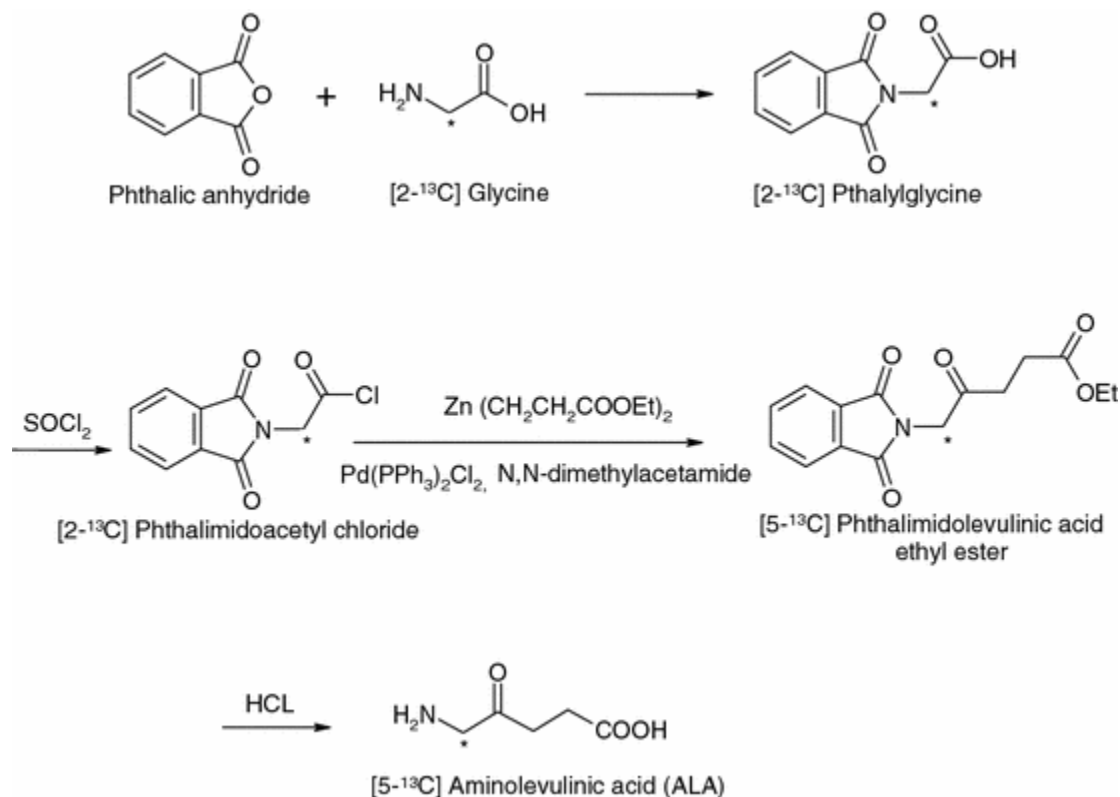


Fig. 2

Synthesis scheme for the preparation of isotopically labeled aminolevulinic acid

Dried, [2-¹³C]phthalylglycine (2.388 g, 11.58 mmol) was refluxed for 18 h in thionyl chloride (12 l, 165.2 mmol) under argon at 80°C. Thionyl chloride was removed with a rotary evaporator leaving [2-¹³C]phthalimidoacetyl chloride as a yellow powder (2.557 g, 98.3%); ¹H-NMR (CDCl₃) δ: 4.82 (d, 2H), 7.85 (m, 4H). The acid chloride was stored at room temperature for up to 2 weeks.

To produce zinc homoenolate, zinc chloride (2.046 g, 15 mmol) was fused under vacuum and dissolved in dry diethyl ether (30 ml). Once a clear homogenous mixture was obtained, dry [(1-ethoxycyclopropyl)-oxy]trimethylsilane (6 ml, 30 mmol) was added to the reaction drop wise with a syringe over 5-min to give a cloudy solution. Stirring continued at room temperature for 1 h followed by refluxing in a hot oil bath for 30 min. Tetrakis(triphenylphosphine)palladium(0) (148 mg, 0.128 mmol) and [2-¹³C]phthalimidoacetyl chloride (2.413 g, 10.7 mmol) were added to the stirring solution on ice. Once a homogenous mixture was obtained, *N,N*-dimethylacetamide (3 ml, 32 mmol) was added slowly via syringe and allowed to stir at 0°C under argon for 1 h followed by 2 h at room temperature. The solution was washed with water:dichloromethane (1:2

v/v) and the organic layer was collected and washed with saturated NaCl. The clear orange solution was evaporated under vacuum and [5-¹³C]phthalimidolevulinic acid ethyl ester was re-crystallized from ethanol (2.706 g, 90.5%); ¹H-NMR (CDCl₃) δ: 1.25 (t, 3H), 2.65 (t, 2H), 2.84 (t, 2H), 4.14 (q, 2H), 4.55 (d, 2H), 7.80 (m, 4H).

[5-¹³C]Phthalimidolevulinic acid ethyl ester (2.662 g, 9.57 mmol) was refluxed overnight in hydrochloric acid:acetic acid (1:1 v/v) at 120°C. The resulting deep yellow solution was evaporated and the residue washed with water to remove excess acid and subsequently with ethyl acetate to remove phthalic acid. The aqueous fraction was concentrated under vacuum, and the aminolevulinic acid (ALA) was re-crystallized from ethanol and diethyl ether. (1.506 g, 93.3%) ¹H-NMR (D₂O) δ: 2.53 (t, 2H), 2.73 (t, 2H), 3.95 (d, 2H). Isotopomers of ALA were stored separately at -20°C in brown glass vials. The overall yield was 67%.

Cell growth and enzyme expression

Escherichia coli HU227 cells were made competent using CaCl₂ treatment and batches were transformed using 1 ng of each plasmid described above using standard transformation protocols. All P450 isoforms were grown in TB media containing 0.02 g ¹³C-ALA/l at room temperature and induced after 24–48 h growth using 0.3 g IPTG/l. DHP cultures were grown in LB media containing 0.02 g ¹³C-ALA/l at 37°C overnight and induced by addition of 0.15 g IPTG/l. After induction, all cell cultures were grown for an additional 24 h at room temperature. To monitor cytochrome P450_{2E1} (Δ3-29) and P450_{2B4} (Δ2-27) activities, an auxiliary enzyme, NADPH cytochrome P450-reductase was also required. A plasmid containing the cDNA encoding the rat enzyme was acquired from Dr. Charles Kasper, University of Wisconsin and used to transform *E. coli* BL-21. This enzyme was expressed according to published procedures (Porter et al. 1987).

Protein purifications

Purifications of P450_{BM3}, P450_{BM3}-F87G and DHP were carried out according to published protocols (Raner et al. 2002 and Osborne et al. 2006). For the two mammalian P450s, 2B4(Δ2-27) and 2E1(Δ3-29), and the NADPH P450 reductase, enzymes were not purified to homogeneity, rather crude *E. coli* membrane samples were prepared via cell lysis, centrifugation to remove the cytosol, and re-suspension of 0.5 g cell membranes in 2 ml 100 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 15% (v/v) glycerol.

Assays for P450_{BM3}, P450_{BM3}-F87G, P450_{2B4}(Δ2-27), P450_{2E1} (Δ3-29), CYP102A2 and DHP catalytic activity

Cytochrome P450_{BM3} and P450_{BM3}-F87G activities were monitored with p-nitrophenoxy dodecanoic acid as substrate as described in the literature Schwaneberg et al. (1999) using a Varian Cary 100 Bio UV–visible spectrophotometer. This compound was synthesized according to the published protocol. For P450_{2E1}(Δ3-29) and P450_{2B4}(Δ2-27) assay, 10 μl reductase

membranes and 10 μ l P450 membranes were initially combined and pre-incubated for 5 min at 37°C. Additional reaction components, including the substrate p-nitrophenol and NADPH, were then added and the product nitrocatechol was monitored by HPLC as described by Larson et al. (1991). For P4502E1(Δ 3-29) and P4502B4(Δ 2-27), 50 μ M p-nitrophenol was used. Product analysis was performed on a Shimadzu LC 20A Series HPLC system consisting of an SPD-20A UV/Vis detector, LC 20AT solvent delivery, and a Sil 20A autosampler, all controlled using the Shimadzu EZStart version 7.3 SP1 software package. DHP activity was measured as described (Osborne et al. 2004) except the column was at 20°C rather than 4°C.

Mass spectral analysis

Samples of isotopically labeled heme were obtained individually from purified samples of DHP. Initially, 20 μ l of 100 μ M purified DHP sample was added to 180 μ l acetonitrile (containing 0.5% acetic acid). The sample was centrifuged and the supernatant subjected to HPLC analysis on a 150 \times 4.6 cm C18 column (Stellar Phases Inc., Longhorne PA). The eluent was monitored at 398 nm. The mobile phase used was acetonitrile:water (3:2 v/v) with 0.5% acetic acid at 1 ml/min. The largest peak, eluting between 8 and 10 min, was collected. Samples were lyophilized and frozen at -20°C. Dry heme samples were diluted to 100 μ l with methanol and used to make 1:10 and 1:100 dilutions in methanol for spotting. The matrix used was a saturated solution of α -cyano-4-hydroxycinnamic acid in acetonitrile with 0.1% trifluoroacetic acid. Spotting solutions were prepared by adding 1 μ l sample to 12 μ l matrix diluent, and finally 12 μ l matrix solution was added. A MALDI plate was spotted with 0.5 μ l of this sample for analysis on an Applied Biosystems 4700 Proteomics Analyzer in reflector positive mode.

LC-MS analysis of purified heme isotopomers

Dried heme samples that had been HPLC purified were taken up in 200 μ l HPLC mobile phase. Samples were centrifuged and injected onto a HPLC equipped with a 5 cm Prevail C-18 column (Alltech). MS analysis was performed on an LCQ Advantage Thermo Finnigan coupled LC-MS with electrospray ionization. The mass range analyzed was from 350 to 800 Da, and the flow rate used during analysis was 0.2 ml/min.

Results and discussion

Expression of recombinant heme proteins in *E. coli* Hu227

Standard protocols for making *E. coli* HU227 cells competent using 100 mM CaCl₂, resulted in inconsistent transformations, often yielding no transformants. By eliminating the snap-freezing in DMSO prior to addition of plasmid DNA, the transformations became more consistent, routinely producing >50 transformants from 100 µl competent cells with 1 ng plasmid. Following overnight growth at room temperature and induction using 0.3 g IPTG/l, expression of cytochrome P450 enzyme was monitored in the whole cells using absorption difference spectrophotometry. Figure 3 shows a typical ferrous vs ferrous-Co difference spectrum for the whole cells expressing CYP102A2 in the HU227 system. An ϵ_{450} value of $9.1 \times 10^{-5} \mu\text{M}^{-1} \text{cm}^{-1}$ was used to approximate the yield of cytochrome P450 in the culture (Gustafsson et al. 2004). From this culture we estimated a P450 yield of ~250 nM. Table 1 shows the maximum yield of different cytochrome P450 isoforms obtained. All of the cytochrome P450 constructs examined produced yields in the 70–250 nM range. All of the BM3 plasmid constructs used were T7-based yet expression of the P450 gene was still significant, albeit lower than in the lac-based systems. The DHP was very efficiently expressed in HU227, yielding nearly 800 nM on a consistent basis. Here, the ϵ_{406} used to calculate DHP concentration was 193,000 $\mu\text{M}^{-1} \text{cm}^{-1}$ (Roach et al. 1997).

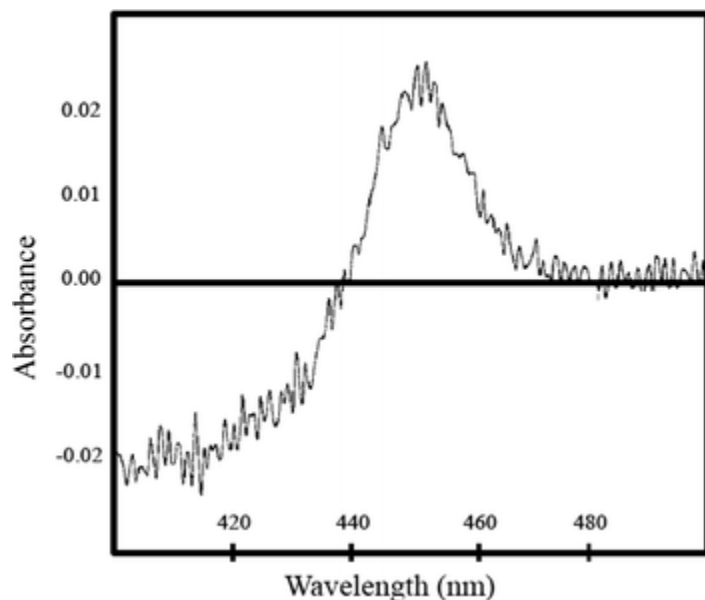


Fig. 3

Reduced versus reduced-CO electronic difference spectrum for expressed cytochrome P450102A2 in whole *E. coli* HU227 cells following IPTG induction. The cells were grown in TB media containing 20 mg [5-¹³C] ALA/l

Table 1

Summary of the expression levels, enzymatic activity and the isotopes incorporated, along with the observed change in m/Z, for each of the Heme-containing enzymes produced.

Heme protein	Maximum expression	Activity	Isotopes
Dehaloperoxidase	850 nmoles/l	Trichlorophenol-dechlorination $282 \pm 20 \text{ min}^{-1}$	^{13}C +8 m/Z ^{15}N +4 m/Z
Cytochrome P450 BM3	200 nmoles/l	<i>p</i> -Nitrophenoxy dodecanoic acid $134 \pm 14 \text{ min}^{-1}$	^{13}C +8 m/Z ^{15}N +4 m/Z
Cytochrome P450 BM3-F87G	150 nmoles/l	<i>p</i> -Nitrophenoxy dodecanoic acid $235 \pm 8 \text{ min}^{-1}$	^{13}C +8 m/Z
Cytochrome P450 102A2	250 nmoles/l	<i>p</i> -Nitrophenoxy dodecanoic acid $9.3 \pm 1 \text{ min}^{-1}$	^{13}C +8 m/Z
Cytochrome P450 2E1(Δ 3–29)	100 nmoles/l	<i>p</i> -Nitrophenol hydroxylation $\sim 10 \text{ nmol/min}^a$	^{13}C +8 m/Z
Cytochrome P450 2B4(Δ 2–28)	70 nmoles/l	<i>p</i> -Nitrophenol hydroxylation $\sim 0.5 \text{ nmol/min}^a$	^{13}C +8 m/Z

^aActivities were reported per mg of crude *E. coli* membranes

Mass spectral analysis of heme cofactor

The metabolic pathway for heme biosynthesis from ALA in microorganisms is well understood and, based on this knowledge, it was expected that the labeling pattern in the heme cofactor of DHP and P450 would follow the pattern shown in Fig. 4. Both enzymes were expressed in the presence of three isotopomers of ALA ([4- ^{13}C]-, [5- ^{13}C]- and ^{15}N -ALA) in HU227 cells, and the proteins were purified to homogeneity. The heme cofactor was extracted and purified by HPLC and subjected to both electrospray ionization and MALDI Mass spectrometry. Figure 5 a is the MALDI mass spectrum acquired for a sample of native heme obtained from a commercial source (Sigma-Aldrich). The observed mass for this sample was 616.2 m/Z as expected. This spectrum

was used for comparison with the heme samples obtained from the recombinant enzymes. All of the heme samples derived from ^{13}C -labeled ALA ($[4\text{-}^{13}\text{C}]\text{-ALA}$ and $[5\text{-}^{13}\text{C}]\text{-ALA}$) displayed a major peak at 624.2 m/Z. Figure 5b shows the MALDI-MS for the DHP heme produced from the $[5\text{-}^{13}\text{C}]\text{ALA}$. The mass of 624 is consistent with the predicted mass for these isotopomers, since it is expected that a total of eight ^{13}C atoms would be incorporated. The mass of heme synthesized from $[^{15}\text{N}]\text{ALA}$ is shown in Fig. 5c. The major peak at 620.2 m/Z is also consistent with the expected mass for this isotopomer, and suggests that four ^{15}N atoms were successfully incorporated into the heme. There were also two additional major peaks observed in each of the MALDI spectra obtained on the isotopically labeled samples at 590 and 634 m/Z. These two additional peaks were of identical mass in each sample, and therefore do not appear to be related to the heme itself, but rather impurities from the heme purification, as they are absent in the native heme sample. Identical results were obtained using the electrospray ionization technique (results not shown).

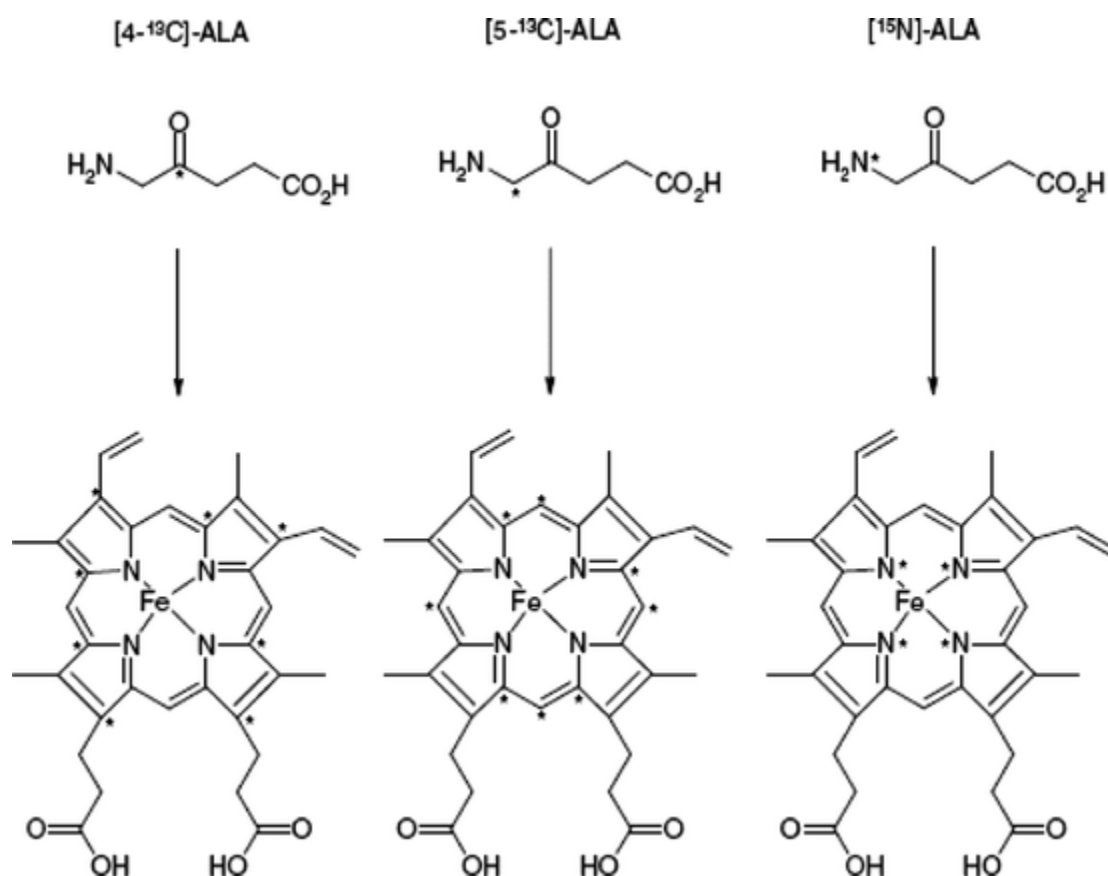


Fig. 4

Expected labeling pattern for the different heme groups based on the use of different aminolevulinic acid isotopomers and known heme biosynthetic pathway in *E. coli*

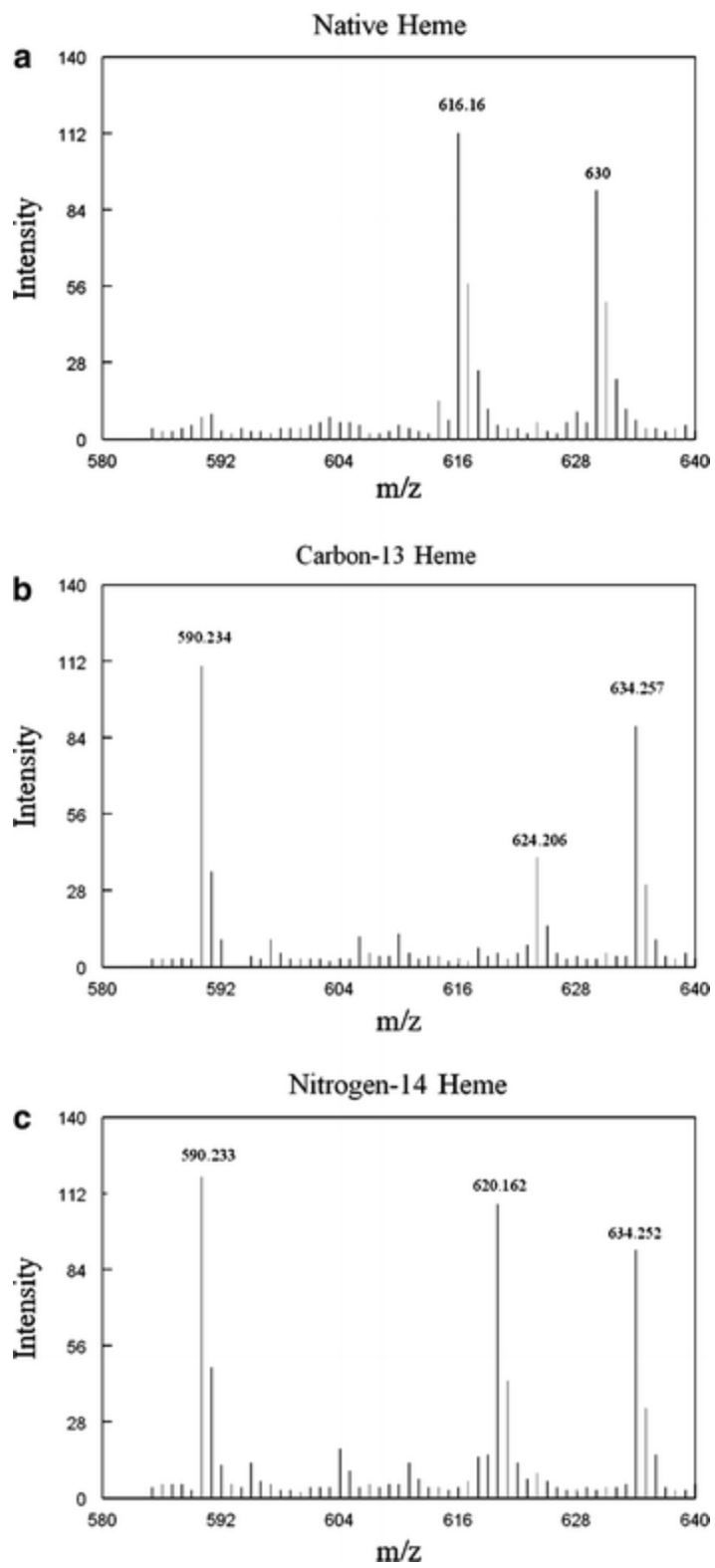


Fig. 5

MALDI mass spectra for (a) native heme (b) heme extracted from dehaloperoxidase grown and expressed in the presence of [5-¹³C] ALA and (c) heme extracted from dehaloperoxidase grown and expressed in the presence of ¹⁵N-ALA

Functional characterization of labeled enzymes

That the expressed proteins in *E. coli* HU227 were in their native state was determined using standard activity assays for each P450 enzyme. Table 1 summarizes the results of these activity assays. For example, P450_{BM3} wild-type and F87G were active in the de-alkylation of *p*-nitrophenoxy dodecanoic acid, with rates of 134 ± 14 and $235 \pm 8 \text{ min}^{-1}$, respectively, using saturating concentrations of substrate. These values are consistent with published k_{cat} values of 120 min^{-1} for the wild-type and our unpublished data with F87G indicating a k_{cat} of $233 \pm 38 \text{ min}^{-1}$ when purified from *E. coli* BL-21 (DE-3). CYP102A2 activity was also measured using *p*-nitrophenoxydodecanoic acid as a substrate. Here a rate of 9.3 min^{-1} was observed compared with 4.3 min^{-1} when this enzyme was expressed in *E. coli* HB101. In the case of P450s 2B4 and 2E1, assays were carried out in the presence of the rat NADPH-cytochrome P450 reductase, which was also expressed in *E. coli*. For these two isoforms, rates of catalysis shown in Table 1 were estimated by measuring *p*-nitrophenol oxidation in crude *E. coli* membranes containing both the P450 and cytochrome P450 reductase. All five P450 isoforms were active in their respective assays, demonstrating the functional properties of the isotopically labeled enzymes.

Given that reconstitution methods involving cytochrome P450 enzymes are very tedious and are carried out under harsh conditions (Wagner et al. 1981), this method represents a simple and effective means to produce active cytochrome P450 enzymes that would be useful for selective isotopic labeling studies. It can also be adapted for use with different classes of heme proteins, as illustrated using DHP. Recombinant DHP produced in HU227 supplemented with 5-¹³C-ALA catalyzed the dechlorination of trichlorophenol at a rate of $282 \pm 20 \text{ min}^{-1}$. This value is higher than the value of 198 min^{-1} reported by Osborne et al. (2004), however their studies were carried out at 4°C whereas in the current study, a temperature of 20°C was used.

In summary, the current study has demonstrated the utility of the HU227 *E. coli* strain in the potential large scale production cytochrome P450 samples with highly enriched heme cofactor. The system generates substantial quantities of functional cytochrome P450 enzymes that can be labeled at specific *C*- and *N*-positions in the heme. The method appears to be universally functional with all P450s tested, and does not suffer from the limitations associated with reconstitution, thus it should prove to be an effective tool in the preparation of samples for use with a variety of spectroscopic methods.

Acknowledgments

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References

- Alontaga AY, Bunce RA, Wilks A, Rivera M (2006) ^{13}C NMR spectroscopy of core heme carbons as a simple tool to elucidate the coordination state of ferric high-spin heme proteins. *Inorg Chem* 45:8876–8881
- Balakrishnan G, Zhao X, Podstawska E, Proniewicz LM, Kincaid JR, Spiro TG (2009) Subunit-selective interrogation of CO recombination in carbonmonoxy hemoglobin by isotope-edited time-resolved resonance Raman spectroscopy. *Biochemistry* 48:3120–3126
- Behr J, Hellwig P, Mäntele W, Michel H (1998) Redox dependent changes at the heme propionates in cytochrome c oxidase from *Paracoccus denitrificans*: direct evidence from FTIR difference spectroscopy in combination with heme propionate ^{13}C labeling. *Biochemistry* 37:7400–7406
- Caignan GA, Deshmukh R, Zeng Y, Wilks A, Bunce RA, Rivera M (2003) The hydroxide complex of *Pseudomonas aeruginosa* heme oxygenase as a model of the low-spin iron(III) hydroperoxide intermediate in heme catabolism: ^{13}C NMR spectroscopic studies suggest the active participation of the heme in macrocycle hydroxylation. *J Am Chem Soc* 125:11842–11852
- Gustafsson MC, Roitel O, Marshall KR, Noble MA, Chapman SK, Pessegueiro A, Fulco AJ, Cheesman MR, von Wachenfeldt C, Munro AW (2004) Expression, purification, and characterization of *Bacillus subtilis* cytochromes P450 CYP102A2 and CYP102A3: flavocytochrome homologues of P450 BM3 from *Bacillus megaterium*. *Biochemistry* 43:5474–5487
- Larson JR, Coon MJ, Porter TD (1991) Purification and properties of a shortened form of cytochrome P-450 2E1: deletion of the NH_2 -terminal membrane-insertion signal peptide does not alter the catalytic activities. *Proc Natl Acad Sci USA* 88:9141–9145
- LeBrun LA, Xu F, Kroetz DL, Ortiz de Montellano PR (2002) Covalent attachment of the heme prosthetic group in the CYP4F cytochrome P450 family. *Biochemistry* 41:5931–5937
- Li J, Deslouches B, Cosloy SD, Russell CS (2003) A heme-deficient strain of *Escherichia coli* has a three-base pair deletion in a “hotspot” in *hemA*. *Biochim Biophys Acta* 1626:102–105
- Osborne RL, Taylor LO, Han KP, Ely B, Dawson JH (2004) Amphitrite dehaloperoxidase: enhanced activity for the catalytically active globin using MCPBA. *Biochem Biophys Res Commun* 324:1194–1198

Osborne RL, Raner GM, Hager LP, Dawson JH (2006) *C. fumago* chloroperoxidase is also a dehaloperoxidase: oxidative dehalogenation of halophenols. *J Am Chem Soc* 128:1036–1037

Porter TD, Wilson TE, Kasper CB (1987) Expression of a functional 78,000 dalton mammalian flavoprotein, NADPH-cytochrome P-450 oxidoreductase, in *Escherichia coli*. *Arch Biochem Biophys* 254:3533–3567

Raner GM, Hatchell JA, Dixon MU, Joy T, Haddy AE, Johnston ER (2002) Regioselective peroxo-dependent heme alkylation in P450BM3-F87G by aromatic aldehydes: Effects of alkylation on catalysis. *Biochemistry* 41:9601–9610

Rivera M, Caignan GA (2004) Recent developments in the ¹³C NMR spectroscopic analysis of paramagnetic hemes and heme proteins. *Anal Bioanal Chem* 378:1464–1483

Rivera M, Walker FA (1995) Biosynthetic preparation of isotopically labeled heme. *Anal Biochem* 230:295–302

Roach MP, Chen YP, Woodin SA, Lincoln DE, Lovell CR, Dawson JH (1997) *Notomastus lobatus* chloroperoxidase and *Amphitrite ornata* dehaloperoxidase both contain histidine as their proximal heme iron ligand. *Biochemistry* 36:2197–2202

Schwaneberg U, Schmidt-Dannert C, Schmitt J, Schmid RD (1999) A continuous spectrophotometric assay for P450 BM-3, a fatty acid hydroxylating enzyme, and its mutant F87A. *Anal Biochem* 269:359–366

Wagner GC, Perez M, Toscano WA Jr, Gunsalus IC (1981) Apoprotein formation and heme reconstitution of cytochrome P-450cam. *J Biol Chem* 256:6262–6265

Wang J, Scott AI (1997) An efficient synthesis of δ -aminolevulinic acid (ALA) and its isotopomers. *Tetrahedron Lett* 38:739–740

Woodward JJ, Martin NI, Marletta MA (2007) An *Escherichia coli* expression-based method for heme substitution. *Nat Methods* 4:43–45