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Expression and Purification of the Amphipathic Form of Rabbit Cytochrome b_5 in *Escherichia coli*

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1. Introduction

Cytochrome b_5 (cyt b_5) is an electron transfer protein that exists in a membrane-bound form in the endoplasmic reticulum where it is anchored to the membrane via a carboxyl-terminal transmembrane α-helix ***(1–3)***. The membrane-bound form of cyt b_5 provides reducing equivalents for the biosynthesis of a variety of lipids including unsaturated fatty acids, plasmalogens, and cholesterol. In addition, it facilitates the cytochrome P450 catalyzed oxidation of selected substrates ***(2)***. The membrane domain is linked to the amino-terminal catalytic heme-containing domain via an 11 amino acid linker. The mammalian cyts b_5 are typically greater than 90% similar in sequence and may be interchangeable in some systems ***(4)***. Nevertheless, our laboratory uses rabbit cytochrome P450 2B4 and cytochrome P450 reductase and has elected to use the rabbit cyt b_5 so all proteins are from a single species. Cyt b_5 also exists in a soluble form in red blood cells where it functions to maintain hemoglobin in its ferrous oxygen-carrying form ***(5)***.

2. Materials

2.1. *Escherichia coli* (E. coli) *Strains, Media, and Equipment*

1. The key to the marked and reproducible overexpression of the membrane bound form of cyt b_5 is use of the *E. coli* strain C41, a derivative of *E. coli* DE3 (Avidis SA, Saint Beauzire, FR) ***(6)***. This strain moderates the expression of genes downstream from a T7 promoter, thereby, decreasing the toxicity of the large amount of mRNA generated ***(7)*** (Life Technologies-Gibco BRL) (*see* **Note 1**).

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2. LB Agar, 32 g/L water. Autoclave at 121°C for 15 min. Cool and pour into plates. Add carbenicillin to a final concentration of 100 μg/mL (Life Technologies-Gibco RBL).
3. Luria Bertani (LB) medium: 10 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract, 1 mL 1N NaOH.
4. Terrific broth (TB) medium: prepare by dissolving 12 g of bacto-tryptone, 24 g bacto-yeast extract and 4 mL glycerol in 900 mL water. Sterilize for 20 min and allow to cool. Immediately before use, add 100 mL of a sterile solution of 0.17 *M* KH_2PO_4 and 0.72 *M* K_2HPO_4. If the TB medium is autoclaved in the presence of phosphate buffer, a precipitate will occur.
5. Sterile filtered stock solution of 100 mg/mL carbenicillin made fresh prior to use.
6. 1 *M* isopropyl-1-thio-β-D galactopyranoside (IPTG) in water. Store at –20°C.
7. 200 mM Δ-aminolevulinic acid (Δ-ALA). Store at –20°C.
8. Equipment includes 2.8-L Fernbach flasks, Beckman JA10 rotor, Beckman J2-21 centrifuge (or equivalent), Vibra Cell sonicator (Sonic Materials) 3 mm and 1 cm diameter probe, spectrophotometer (Cary 1, Cary 300 Bio or equivalent). Innova Incubator Shaker 4430 (New Brunswick Scientific, Edison, NJ) or equivalent shaker and autoclave.

2.2. Reagents, Chromatography Resins, and Equipment for Cyt b_5 Purification

1. A 1 m*M* solution of heme is prepared by adding hemin chloride to a solution of 50% ethanol in water and 0.1*N* NaOH. After the hemin chloride dissolves, filter the solution through a 0.2 μm filter. Store at 4°C.
2. Detergents: 10% (v/v) Tergitol NP-10 (Sigma). Store at 4°C. Solid Na deoxycholate (Fisher Biotech).
3. Sodium hydrosulfite (sodium dithionite, [Sigma]).
4. BCA protein assay (Pierce).
5. Mini-Protease inhibitor tablets (Boehringer Mannheim).
6. All buffers should be filter sterilized using a 0.2 μm filter.
7. Buffer A: 10 m*M* phosphate buffer, 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.0.
8. Buffer B: 10 m*M* phosphate buffer, 1 m*M* EDTA, pH 7.0, 1% Tergitol NP-10.
9. Buffer C: 20 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 at 25°C, 0.4% Na deoxycholate. 20 m*M* phosphate buffer can be used instead of Tris-HCl.
10. Buffer D: Buffer C, plus 0.4 *M* NaCl.
11. Buffer E: 20 m*M* phosphate buffer, pH 8.0, 1 m*M* EDTA, 0.4% Na deoxycholate.
12. Buffer F: 50 m*M* Tris-acetate, pH 8.1 at 22°, 1 m*M* EDTA.
13. Resins: DEAE Sepharose Fast Flow (Sigma), Superdex-75 prep grade (Amersham Pharmacia Biotech), Sephadex G-25 optional (Sigma).
14. Equipment includes chromatography columns from Bio-Rad, Foxy Jr., or Foxy 200 fraction collector (Isco, Lincoln, NE) or equivalent, Cary spectrophotometer.

3. Methods

3.1. Expression of cyt b_5

1. C41 cells were transformed with the plasmid pLW01-$b_5$mem using standard procedures ***(8,9)***. The transformed cells were plated from a 15% glycerol stock solution onto a LB plate containing 100 μg/mL carbenicillin and incubated overnight at 37°C.
2. A single colony was picked and inoculated into a 2.8 L Fernbach flask containing 500 mL of TB medium supplemented with 0.5 m*M* **Δ**-aminolevulinic acid and 250 μg/mL carbenicillin.
3. The cultures were incubated at 37°C on an Innova Incubator Shaker 4430 (or equivalent) with shaking at 140 rpm.
4. When the OD of the cultures was 0.35 at 600 nm, IPTG was added to a final concentration of 10 μ*M* and the cells were incubated with shaking for an additional 16–20 h.
5. Remove 2 mL of the culture and set aside for determination of cyt b_5 content (*see* later).
6. The remainder of the cell culture was chilled, poured into 500-mL plastic bottles and centrifuged at 11,000*g* for 10 min at 4°C to pellet the whole cells. The cell culture was centrifuged in a JA10 rotor at 8000 rpm for 30 min at 4°C in a Beckman J2-21 centrifuge.
7. Discard the supernatant and resuspend the cells in ≅ 25 mL of cold Buffer A.
8. Repellet the cells under the same conditions. An average of 8.8 g bright pink cell paste is recovered from 500 mL cell culture (*see* **Note 2**).

3.2. Determination of the Amount of Apocyt b_5 in the Cell Culture and Reconstitution of Holocyt b_5 with Heme

1. Because most of the cyt b_5 is expressed as the apoprotein without the heme, it must be reconstituted with heme prior to purification (*see* **Note 3**). Centrifuge the 2 mL aliquot of the cell culture at 10,000*g* for 1 min at room temperature.
2. Discard the supernatant and resuspend the pink cell pellet in 2 mL Buffer B.
3. Sonicate the cells using a Vibra Cell sonicator (Sonic Materials) with two 30 s pulses at 40% power at 50 W. Immerse the cell suspension in a ice/water slush to keep the temperature below 9.5°C. Cool to 4°C between each pulse. Be sure to sonicate vigorously enough to lyse all the cells. The heme cannot penetrate the bacterial cell membrane and will not be able to reconstitute the apocyt b_5 within the cell. Incomplete reconstitution of the cyt b_5 will result in a poor yield.
4. Dilute the sonicated cells 20-fold with Buffer B and record the absorbance spectrum between 350–650 nm.
5. Add 2 μL aliquots of a ≅ 1 m*M* heme solution to the sonicated cells and record the spectrum after each addition. The difference spectrum (final spectrum-initial spectrum) should resemble the spectrum of cyt b_5 as long as the added heme is forming holocyt b_5 ***(7)***. When the difference spectrum caused by addition of the heme begins to resemble that of the heme and not cyt b_5, the apocyt b_5 has been com-

pletely converted to holocyt b_5. An alternative procedure is to titrate the sonicated cells with the 2 μL aliquots of heme and plot the increase in absorbance at 412 nm. When the apocyt b_5 is completely reconstituted, the absorbance increase at 412 nm produced by a 2-μL heme aliquot will decrease, i.e., the slope of the line found by plotting ΔA at 412 vs heme added will decrease. The point at which the change in slope occurs indicates that the apocyt b_5 has been saturated with heme. Once the amount of heme required to reconstitute the apocyt b_5 in a 2-mL sample is known, the amount of heme necessary to reconstitute the apocyt b_5 in the 500 mL cell culture is readily determined. Reconstitute holocyt b_5 with no more than a ≅ 10% molar excess of heme. If too much excess heme is added, it will be difficult to remove and will interfere with quantitation of cyt b_5.

3.3. Measurement of Holocyt b_5

Holocyt b_5 is measured as previously described ***(10)***. Briefly,

1. Place 1 mL of sample into both a reference and sample cuvet and record a baseline in the spectrophotometer.
2. Reduce the cyt b_5 in the sample cell by addition of ≅ 1 mg of solid sodium dithionite.
3. Record a difference spectrum by subtracting the oxidized spectrum from the reduced spectrum and determine the change in absorbance between 426 and 409 nm. Upon reduction, cyt b_5 increases its absorbance at 426 nm and decreases its absorbance at 409 nm. An extinction coefficient of 185 mM^{-1} cm^{-1} for the absorbance change at 426 minus 409 nm was used to calculate the amount of cyt b_5 present. When the protein is pure and other interfering compounds are absent, an extinction coefficient of 117 mM^{-1} cm^{-1} at 413 nm was used to calculate the amount of cyt b_5 ***(11)***.

3.4. Lysis of E. coli and Membrane Isolation

All of the following procedures with the exception of the chromatography on DEAE were performed at 4°C (*see* **Note 4**).

1. Defrost the pink cell pellet and resuspend in ≅ 25 mL Buffer A.
2. Add two Mini-Protease inhibitor tablets and dissolve them in the cell paste (*see* **Note 5**).
3. Sonicate the cells using a 1-cm-diameter probe that is immersed 2 cm into the cell suspension. Sonicate the cells with ≅ 6 pulses of 2-min duration at 80% power using a 50-W setting. Immerse the cell paste in an ice/water slush during the sonication and do not let the temperature rise above 9.5°C. Other equivalent methods of cell lysis can be used. Regardless of which method of cell lysis is used, it is important to ensure that all cells have been lysed in order to obtain a good yield.
4. Following cell lysis, reconstitute the apocyt b_5 by adding a 10% molar excess of heme based on the amount of apocyt b_5 present in 2 mL of the lysed cell suspension.
5. Centrifuge the sonicated cells at 3000*g* for 15 min at 4°C to remove any unlysed cells. If the pellet is red, resonicate to lyse any remaining intact cells.
6. Centrifuge the membrane containing supernatant at 100,000*g* for 1 h at 4°C.

7. Discard the supernatant and resuspend the cyt b_5 membrane containing pellet in ≅ 30 mL of Buffer B. The pellet can be resuspended by using either a teflon homogenizer or a brief 30s sonication pulse.
8. After resuspending the cell pellet, dilute to 100 mL with Buffer B and stir at 4°C while determining the protein concentration of the solution using the BCA assay.
9. Dilute the cyt b_5 containing membranes with Buffer B to a volume which will give a protein concentration of 4 mg/mL. The final volume is usually ≅ 150 mL with a detergent: protein (w/w) ratio of 2.5:1.
10. Stir at 4°C for ≅ 3 h to solubilize the cyt b_5.
11. Centrifuge the solubilized cyt b_5 at 100,000*g* for 1 h. The pellet should be almost colorless.
12. Load the dark red supernatant which contains the cyt b_5 onto the DEAE-Sepharose column.

3.5. DEAE-Sepharose Chromatography

1. Equilibrate a 2.5 × 16-cm column of DEAE-Sepharose with ≅ 1 L of Buffer C (*see* **Note 6**). The DEAE-Sepharose chromatography was performed at room temperature in order to prevent deoxycholate gel formation which occurs at 4°C, pH less than 8.0, and high-salt concentration.
2. Load the cyt b_5 containing solution onto the column at a rate of ≅ 3 mL/min. Cyt b_5 will bind to the top one-third of the column.
3. Wash the column with ≅ 300 mL of Buffer C.
4. Elute the cyt b_5 with a linear gradient formed with equal amounts of Buffer C and Buffer D (i.e., Buffer C in 0.4 *M* NaCl).
5. Pool the fractions with an A_{412}/A_{280} ratio greater than 1.6 and then concentrate in a 50-mL Amicon stirred cell using a YM-10 membrane. An equivalent method of concentration such as Centriprep can be used.
6. When the volume has been reduced to ≅ 30 mL, dilute four-fold with 120 mL of Buffer C and reconcentrate to ≅ 30 mL to decrease the salt which can cause gelling of the deoxycholate containing buffer. The sample is now ready to be applied to the sizing column.

3.5. Superdex-75 Chromatography

1. Prepare a 5 × 62-cm column with Superdex-75 prep grade and equilibrate with Buffer E. At a flow rate of 0.25 mL/min it can be equilibrated over the weekend.
2. Apply the concentrated sample carefully to the column, taking care not to disturb the top of the column. The smaller the volume loaded onto the column the better the resolution. Load the sample by gravity flow at a rate of ≅ 0.5 mL/min.
3. Begin eluting the column by gravity flow with Buffer E until the cyt b_5 has entered 4 cm of the column. The rosey pink protein should elute in a regular band for best resolution.
4. At this point, add a pump to elute the column slowly at a rate of 1 mL/min. In 0.4% deoxycholate, cyt b_5 elutes as a dimer of 35 kDa ***(3)***.
5. Combine fractions with an A_{412}/A_{280} nm ratio greater than 2.5.

6. The deoxycholate can be removed either by extensive dialysis against 20 m*M* KPO_4 buffer pH 8.0 and 1 m*M* EDTA or size-exclusion chromatography on a Sephadex G25 column (1×100 cm) preequilibrated with 10 m*M* KPO_4 buffer pH 8.0 and 1 m*M* EDTA.

This procedure should yield approx 120 mg of pure protein from 500 mL of *E. coli* cell culture.

4. Notes

1. Contact information for obtaining C41 from Avidis: isabelemounier@avidis.fr, or fergalhill@avidis.fr, Tel: +33(0)4 7364 4390, Fax +33 (0)4 7364 4393.
2. In previous procedures, our laboratory and other laboratories have reported significant proteolysis of the membrane-bound form of cyt b_5 to the soluble form of cyt b_5 during bacterial cell lysis ***(12,13)***. The simple procedure of washing the cells in buffer removes a significant amount of the proteases found in the cell culture medium and other contaminating proteins. The marked decrease in proteases during cell lysis is one of the factors that allows complete recovery of the membrane bound form of cyt b_5. No soluble cyt b_5 is formed during the purification procedure if appropriate precautions are taken.
3. Only approx 10% of the cyt b_5 expressed under our experimental conditions is holo protein. The remaining 90% is apoprotein which must be reconstituted with heme as soon as possible after cell lysis because the apo form is more susceptible to proteolysis.
4. Because loss of the membrane anchor of cyt b_5 by proteolysis results in inactivation, the purification procedure is performed under "almost" sterile conditions with filtered buffers (0.2-μm filters) to prevent contamination.
5. The protease inhibitors must be present during cell lysis to prevent cleavage of the hydrophobic membrane anchor from the amphipathic form of cyt b_5.
6. Equilibration of the DEAE column is critical. The column is adequately equilibrated when the pH and resistance of the buffer eluting from the column is identical to that being loaded onto the column. Proper equilibration ensures good resolution and reproducibility with the DEAE-Sepharose column.

References

1. Vergeres, G., Ramsdem, J., and Waskell, L. (1995) Interaction of cytochrome b_5 with the microsomal membrane: Insertion topology of the C terminus and function of Pro 115. *J. Biol. Chem.* **270**, 3414–3422.
2. Vergeres, G. and Waskell, L. (1995) Cytochrome b_5, its function, structure and membrane topology. *Biochimie* **77**, 604–620.
3. Spatz, L. and Strittmatter, P. (1971) A form of cytochrome b_5 that contains an additional hydrophobic sequence of 40 amino acid residues. *Proc. Nat. Acad. Sci. USA* **68**, 1041–1046.
4. Ozols, J. (1989) Structure of cytochrome b_5 and its topology in the microsomal membrane. *Biochimica et Biophysica Acta* **997**, 121–130.

5. Hegesh, E., Hegesh, J., and Kaftory A. (1986) Congenital methemoglobinemia with a deficiency of cytochrome b_5. *New Eng. J. Med.* **314**, 757–761.
6. Miroux, B. and Walker, J. E. (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**, 289–298.
7. Mulrooney, S. and Waskell, L. (2000) High-level expression in *Escherichia coli* and purification of the membrane-bound form of cytochrome b_5. *Protein Express. Purif.* **19**, 173–178.
8. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., et al., ed., (1997) *Cur. Protocols Molecular Biol.* Wiley, New York.
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
10. Estabrook, R. W. and Werringloer, J. (1978) The measurement of difference spectra: application to the cytochromes of microsomes. *Meth. Enzymol.* **52**, 212–220.
11. Strittmatter, P. and Velick, S. F. (1956) The isolation and properties of microsomal cytochrome. *J. Biol. Chem.* **221**, 253–264.
12. Holmans, P. L., Shet, M. S., Martin-Wixtrom, C. A., Fisher, C. W., and Estabrook, R. W. (1994) The high-level expression in *Escherichia coli* of the membrane-bound form of human and rat cytochrome b_5 and studies on their mechanism and function. *Arch. Biochem. Biophys.* ***312,*** 554–565.
13. Chudaev, M. V. and Usanov, S. A. (1997) Expression of functionally active cytochrome b_5 in *Escherichia coli*: Isolation, purification, and use of the immobilized recombinant heme protein for affinity chromatography of electron-transfer proteins. *Bio-chemistry (Moscow)* **62**, 401–411.

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Dihydroorotate Dehydrogenase of *Escherichia coli*

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1. Introduction

1.1. Different Types of Dihydroorotate Dehydrogenases (DHODs)

Dihydroorotate dehydrogenase (DHOD) catalyzes the fourth reaction in the pathway for *de novo* synthesis of UMP and forms the 5,6-double bond of the pyrimidine base. In this reaction, two electrons and two protons are transferred from dihydroorotate to an electron acceptor that varies between different types of the enzyme. Sequence alignments have shown that all DHODs contain a polypeptide chain that is encoded by a *pyrD* gene. This polypeptide forms the catalytic core structure, folding into an $(\alpha/\beta)_8$-barrel. The active site, which contains a tightly bound molecule of flavin mononucleotide (FMN), is formed by loops that protrude from the top of the barrel (e.g., **ref.** ***1***). The first half reaction, in which the enzyme is reduced and dihydroorotate is oxidized to orotate, is initiated by binding of dihydroorotate at the *si*-side of the isoalloxazine ring of FMN ***(2)*** and, after abstraction of a proton from the 5'-position of dihydroorotate by a cysteine or a serine residue in the enzyme, a hydride ion is transferred to FMN from the 6-position of the substrate ***(3,4)***. The first half reaction is common to all DHODs, but different types of DHODs deviate from each other in quaternary structure, subcellular location, and use of electron acceptors to reoxidize the reduced enzyme in a second half reaction ***(5)***.

1.1.1. The Soluble Class 1 DHODs

The class 1 DHODs are soluble proteins. Two types have been identified. Class 1A DHODs are dimeric proteins able to use fumarate as electron acceptors. The enzymes are found in milk fermenting bacteria like *Lactococcus lac-*

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tis ***(6,7)*** and *Enterococcus faecalis* ***(8)***, in the anaerobic yeast *Saccharomyces cerevisiae* ***(9,10)*** and in some eukaryotic parasites ***(11,12)***. The enzyme from *L. lactis* (DHODA) has been studied in considerable detail and the crystal structure has been solved of the free enzyme and as a complex with the product orotate ***(1,2)***.

Class 1B DHODs are heterotetrameric enzymes that use NAD^+ as electron acceptor ***(13)***. The occurrence is restricted to Gram positive bacteria. The closely related strains *L. lactis* ***(14)*** and *E. faecalis* ***(15)*** have both a class 1A and a class 1B DHOD ***(6)***, but species of *Bacillus* ***(16,17)*** and *Clostridium* ***(4,18)*** only possess a class 1B enzyme. The protein from *L. lactis* (DHODB) has been studied in detail ***(13)*** and the crystal structure has been solved for the free enzyme and as a complex with the product orotate ***(19)***. Two of the subunits are encoded by the *pyrDb* gene, and together they form a dimeric protein like DHODA. Associated with this catalytic core are two tightly bound electron transfer subunits, which are encoded by the *pyrK* gene and protrude from the catalytic dimer like two moose horns. The PyrK polypeptides belong to the ferredoxin reductase superfamily. They have flavin adenine dinucleotide (FAD) and a [2Fe-2S] cluster as cofactors and are engaged in the channeling of electrons to NAD^+ ***(13,19)***.

Other types of soluble DHODs exist. For instance, a class 1B-like DHOD able to use molecular oxygen, but not NAD^+, has been found in *Lactobacillus* and is devoid of an electron transfer subunit ***(20,21)***. In addition, the archaeon *Sulfolobus solfataricus* has a class 1B-type DHOD associated with an iron-sulfur cluster protein different from PyrK. The electron acceptor preferences of this protein is unknown ***(22)***.

1.1.2. The Membrane Associated Class 2 DHODs

The membrane associated class 2 DHODs use quinones of the respiratory chain as electron acceptors. They are found in Gram negative bacteria like *E. coli* ***(23)*** and *Helicobacter pylori* ***(24)***, where they are associated with the cytoplasmic membrane, and in most eukaryotic organisms, where are anchored in the inner mitochondria membrane ***(25)***. The class 2 enzymes are monomeric proteins with a strong tendency to aggregate ***(26,27)***. The core part of the enzymes, with the active site, forms an $(\alpha/\beta)_8$-barrel structure similar to the structure of the class 1 enzymes ***(28,29)*** although the sequence similarity between the two classes of DHODs is very low, 12–20% identity ***(5,30)***. The polypeptide chains of all class 2 enzymes are extended in the N-terminal relative to the class 1 enzymes (*see* **Fig. 1**). In bacteria this extension sequence is just a little more than 40 amino acid residues. In the *E. coli* enzyme (DHODC) it forms a separate helical domain with a hydrophobic cavity between two of the helices, located at the side of the core domain ***(28)***. The small N-terminal