

## 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-*

*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  $\text{NAD}^+$  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  $\text{NAD}^+$  (13,19).

Other types of soluble DHODs exist. For instance, a class 1B-like DHOD able to use molecular oxygen, but not  $\text{NAD}^+$ , 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

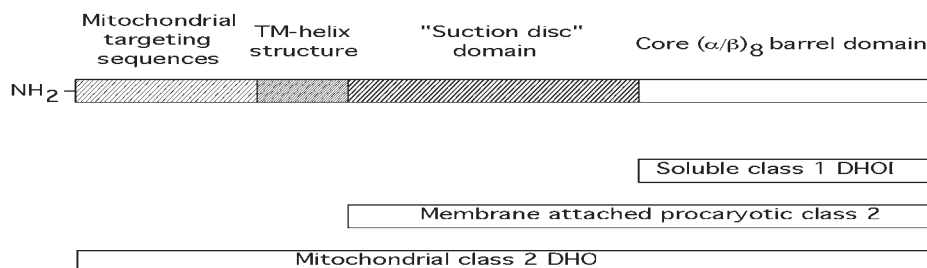


Fig. 1. Schematic representation of the functional roles of sequence elements in the polypeptide chains of different dihydroorotate dehydrogenases. *See* main text for further explanation.

domain enables DHODC to use respiratory quinones as electron acceptors (menaquinone appears to be the physiological electron acceptor of DHODC [31]) and is essential for the association of the enzyme to the membrane by a mechanism that essentially is unknown (28). We call the domain “a suction disk”, but do not know if the quinones, which bind to this domain, are involved in membrane association through their long hydrophobic tails.

The mitochondrial class 2 DHODs share the “suction disk domain” with the enzymes of prokaryotic origin (29), but the N-terminal extensions of the mitochondrial enzymes are longer than their prokaryotic counterparts, as they contain a short segment of 16–20 amino acid residues which (from the sequence) is predicted to form a transmembrane helix just upstream of the “suction disk structure” and carry sequences that target the proteins for import in mitochondria (25) (*see* Fig. 1).

Our current procedure for overexpression, purification, and crystallization dihydroorotate dehydrogenase from *E. coli* consists of the following major steps:

- Growth of cells and over-production of DHOD from a plasmid encoded, inducible gene.
- Disruption of cells by ultrasonic treatment and release of the enzyme from membranes by Triton X-100 in the crude extracts.
- Chromatography on a DE-52 anion exchange column in the presence of Triton X-100.
- Hydrophobic interaction chromatography on a column of Phenyl Sepharose and elution with Triton X-100.
- Chromatography on a second anion exchange column to remove the detergent.
- Crystallization with sodium formate as precipitant.

The procedure yields about 20 mg DHODC per liter bacterial culture (27). The crystal structure was published by Nørager et al. (28).

## 2. Materials

1. *The expression plasmid*: The expression vector pAG1 (27) is a derivative of the ampicillin resistance plasmid pUHE23-2 (32). It carries the 336 codons reading frame of the *E. coli pyrD* gene, encoding DHODC, cloned behind the strong T7<sub>A1/04/03</sub> promoter, which is a synthetic derivative of the T7<sub>A1</sub> early promoter and contains two operator sites for binding the LacI repressor.
2. *Bacterial strains*: The *E. coli* strain SØ6645 (*araD139Δ(ara-leu)7679 galU galKΔ(lac)174ΔpyrD(BssHII-MluI::Km<sup>r</sup>) [F' proAB lacI<sup>q</sup>ZΔM15Tn10]* overproduces the LacI repressor from the *lacI<sup>q</sup>* gene on the episome and is deleted for the promoter proximal part of the chromosomal *pyrD* gene (7). Strain SØ6735 (*rph-1 metA recA56 srl::Tn10*) [*F' proAB lacI<sup>q</sup>Z::Tn5*] (28) is derivative of the methionine requiring strain DL41 previously used for production of selenomethionine substituted proteins (33).
3. Preswollen diethyl aminoethyl cellulose (DE-52) is available from Whatman Ltd. (Maidstone, England).
4. Phenyl Sepharose® CL-4B is available from Pharmacia LKB (Uppsala, Sweden).
5. LB-broth: 10 g Bacto® Tryptone (Difco, Detroit, MI), 5 g yeast extract (Oxoid Ltd., Basington, UK) and 5 g NaCl per liter of ion exchanged water. If needed, the pH was adjusted to 7.0 by addition of NaOH before autoclaving (34).
6. Solution A: 20 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 75 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 30 g KH<sub>2</sub>PO<sub>4</sub>, and 30 g NaCl per liter.
7. Solution B: 20 mL of 1 M MgCl<sub>2</sub>·6H<sub>2</sub>O, 2 mL of 0.5 M CaCl<sub>2</sub>·2H<sub>2</sub>O, and 3 mL of 10 mM FeCl<sub>3</sub>·6H<sub>2</sub>O per 9 L (35).
8. (A + B) basal salt medium: Mix together autoclaved Solution A and Solution B, one part A to nine parts B.
9. Supplements are added from sterile solutions that had been autoclaved separately at 100°C, glucose as a 20% solution, amino acids at a concentration of 5 mg/mL and uracil 2 mg/mL. Ampicillin (Sigma, St. Louis, MI) and isopropyl-β-D-thiogalactoside (IPTG; Bingswood Industrial Estate, Whaley Bridge, UK) are added as solid material.
10. Buffer A: 5 mM sodium phosphate pH 7.0 containing 0.25 mM ethylenediamine tetraacetic acid (EDTA) (see Note 1).
11. Buffer B: 5 mM sodium phosphate pH 7.0, 0.25 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.1% Triton X-100 (Sigma).
12. Buffer C: 50 mM sodium phosphate, pH 6.2, containing 0.1 mM EDTA, and 0.1% Triton X-100.
13. Buffer D: 50 mM sodium phosphate pH 7.0 containing 0.1 mM EDTA.
14. Centrifugation spin columns (Amicon, Centriprep®).

## 3. Methods

### 3.1. Growth and Harvest of Cells

Strain SØ6645 transformed with the expression plasmid pAG1 was grown at 37°C in LB-broth medium containing ampicillin (100 mg/L). To ensure a high production of DHODC we always use freshly transformed cells (see Note 2). A preculture (100 mL) is inoculated in the morning with 4–5 single colonies from

a fresh transformation agar plate and grown to an  $OD_{436}$  of circa 0.5, when it is cooled in an ice bath. The preculture is stored at 4°C overnight and diluted into 2 L of prewarmed medium on the morning of the next day (*see Note 3*).

The culture is grown with vigorous aeration by shaking. IPTG (0.5 mM) is added at  $OD_{436} = 0.7$ –1.0 to induce expression of the *pyrD* gene and growth is continued overnight, while the culture reaches stationary phase at an  $OD_{436}$  about 5 (*see Note 4*). Cells are harvested by centrifugation, washed with 0.9% sodium chloride and frozen at –20°C. The cell-pellet is strongly yellow because of the content of FMN in DHODC.

### 3.2. Extraction and Purification

All operations during purification are carried out at 4°C.

#### 3.2.1. Extraction

1. Frozen cells from 2 L culture (circa 16 g) are resuspended in 80 mL of buffer A and disrupted by ultrasonic treatment.
2. Add  $MgCl_2$  to a final concentration of 5 mM, and Triton X-100 to a final concentration of 0.1% to dissolve the membranes.
3. The extract is cleared by centrifugation in an SS-34 rotor (Sorvall) at 13,000 rpm (20,000g) for 1 h (*see Note 5*).

#### 3.2.2. First Chromatography on DE-52

1. The clear yellow extract is pumped (flow 1 mL/min) onto a column of DE-52 (1.6 × 25 cm) equilibrated with buffer B. The enzyme binds in a narrow zone at the top of the column.
2. After application of the sample, the column is washed first with 50 mL of buffer B and then with 50 mL of buffer C.
3. The enzyme is eluted with a linear gradient (400 mL) from 0 to 0.25 M sodium chloride in buffer C, while 10 mL fractions are collected. The enzyme appears from the column with a peak around 0.15 M NaCl.

#### 3.2.3. Hydrophobic Interaction Column Chromatography

1. The active, yellow fractions from the DE-52 column are pooled and solid ammonium sulfate is dissolved in the liquid at a final concentration of 1.1 M.
2. A turbidity that forms after the addition of ammonium sulfate is removed by centrifugation (10 min at 12,000g). The pellet is colorless.
3. The clear supernatant is pumped (flow 0.5 mL/min) onto a column of Phenyl Sepharose (1.6 × 20 cm) which has been equilibrated with buffer D containing 1.1 M ammonium sulfate. The enzyme binds in a highly concentrated zone at the top of the column.
4. After application of the sample, the column is washed with a linear gradient (160 mL) from 1.1 M to 0 M ammonium sulfate in buffer D followed by 100 mL of

buffer D. The washing removes a substantial amount of contaminating protein, but DHODC remains bound although it spreads a little on the column during the wash.

5. The enzyme is eluted as a sharp peak by pumping buffer D containing 1% Triton X-100 through the column. This high concentration Triton X-100 gradually replaces the enzyme from the column, and the column material changes appearance to a more white and nontransparent texture above moving yellow zone of DHODC (*see Note 6*).

### 3.2.4 Second DE-52 Column Chromatography

1. The pooled fractions from the Phenyl-Sepharose column are loaded on a second DE-52 column ( $1.6 \times 25$  mL) equilibrated with buffer D. The flow rate is 1 mL/min.
2. The column is washed thoroughly with about 300 mL of buffer D to remove all Triton X-100, which is monitored by the UV-light absorption at 280 nm.
3. The enzyme is eluted in a somewhat broad peak by a linear gradient (400 mL) from 0 to 0.3 M sodium chloride in buffer D. The chromatography on the DE-52 column in the absence of Triton X-100 results in a loss of about one-third of DHODC, which remains stuck at the top of the column even at very high concentrations of NaCl, but we have accepted this loss of enzyme in order to be able to replace Triton X-100 with other detergents (*see Note 7*).

### 3.2.5. Concentration and Storage

1. The active fractions from the second DE-52 column are pooled and concentrated using centrifugation spin columns (Amicon, Centriprep®).
2. For most purposes, the enzyme was dialyzed against buffer D containing 50% glycerol and stored in liquid form at  $-20^{\circ}\text{C}$  at a concentration around 10 mg/mL.
3. Prior to crystallization the protein sample was dialyzed against a solution of 25 mM of sodium phosphate pH 7.0 containing 0.1 mM EDTA and 10% glycerol and stored in 0.5 mL aliquots at  $-20^{\circ}\text{C}$ .

## 3.3. Crystallization

The crystallization of DHODC has been described previously by Rowland et al. (36). Crystals were obtained by the vapor diffusion technique using 5  $\mu\text{L}$  sitting drops in microbridges placed over a 0.6-mL reservoir solution in the Linbro plates closed with cover slides. The experiments were carried out at room temperature. The drops were made from 2.5  $\mu\text{L}$  protein solution (12–15 mg/mL DHODC) and 2.5  $\mu\text{L}$  of the reservoir solution. Crystals could be obtained with reservoir solutions that have the following composition: 0.1 M sodium acetate, sodium formate in the concentration range 3.9–4.4 M, pH 4.0–5.5, and 25 mM  $\beta$ -n-octyl  $\beta$ -D-glucoside ( $\beta$ -OG). Prior to equilibration the drops had a composition contained 6.0–7.5 mg/mL of DHODC, 12.5 mM sodium phosphate pH 7, 0.05 mM EDTA, 5% glycerol (from the protein solution), 12.5 mM  $\beta$ -OG, 0.05 M sodium acetate, and 1.95–2.2 M sodium formate with a pH of 4.0–5.5, while

the reservoir solutions contained 0.1 *M* sodium acetate, and 3.9–4.4 *M* sodium formate with a pH of 4.0–5.5. With reservoir solutions in the afore mentioned range of sodium formate concentrations and pH, the enzyme crystallized within 1–2 wk as yellow needles of the approximate dimensions  $1.5 \times 0.15 \times 0.15$  mm. The crystals have small whiskers at one end that was cut away to make the crystals suitable for X-ray diffraction experiments. To be able to measure diffraction data from crystals under cryogenic conditions, the crystals had to be soaked for a few seconds in a cryoprotecting reagent containing 4.5 *M* sodium formate, 0.1 *M* sodium acetate at the crystallization pH and 10% glycerol. The X-ray diffraction experiments showed that the crystals are tetragonal. To overcome the phase problem the selenomethionine substituted protein was prepared (*see Note 8*). It could be crystallized under the same conditions as the native enzyme. The structure determination was achieved by the MAD (multiple anomalous dispersion) method based on diffraction data collected with synchrotron radiation at three different wavelength around the Se-absorption edge. Further details are described by Nørager et al. (28).

#### 4. Notes

1. Buffers are prepared using doubly distilled water. They were prepared by dilution of five-times concentrated stock solutions and mixed with NaCl from a 5 *M* NaCl stock solution that was passed through a nitrocellulose filter to remove unwanted particles.
2. Other *E. coli* strains can be used, but the F' *proAB lacI<sup>q</sup>ΔM15 Tn10* episome, which directs the overproduction of the LacI repressor, is needed because the plasmid does not itself carry a *lacI* gene. The overproduction of all types of DHOD is toxic to *E. coli* and transformation with plasmid pAG1 is not possible unless the DHOD expression is kept repressed.
3. It is advisable to use freshly transformed cells and keep the culture exponentially growing until the final culture reaches stationary phase before harvest. If growth of culture is interrupted, it should preferably be done at a low cell density, e.g., at  $OD_{436} \leq 0.5$ . If the preculture has been grown into stationary phase, plasmid-free cells tend to outgrow the plasmid containing cells when the preculture is diluted into fresh medium, because the added ampicillin is rapidly broken down. This behavior is in all likelihood related to the fact that the copy number of plasmid pAG1 (and other relaxed plasmids), and hence the production of  $\beta$ -lactamase, increases dramatically when the culture approaches stationary phase. It is possible to store the transformed cells if an aliquot of the uninduced culture at a low cell density ( $OD_{436} \leq 0.5$ ) is mixed with 20% glycerol and the frozen at  $-20^{\circ}\text{C}$ . However, in that case, it is advisable to spread the cells to single colonies on an LB-agar plate with 0.1 mg/mL of ampicillin and test a few colonies for high-protein production in small cultures.
4. An “autoinduction” of *pyrD* expression from plasmid pAG1 occurs at a cell density about  $OD_{436} = 2$ . The reason is that the concentration of repressor binding sites on pAG1 in cultures approaching stationary phase exceeds the amount of LacI repressor produced from the stringently controlled F'-episome. The production of



DHODC from pAG1 is almost as high in “uninduced” stationary cultures as it is in cultures that are induced by addition of IPTG, but because this “autoinduction” may depend on subtle differences in the culture conditions, we have retained the induction with IPTG as described. The “autoinduction” of protein expression in stationary cultures, which we have seen with several plasmids where repression relies upon a *lacI* gene on an F'-episome, may also contribute to the strong tendency of plasmid-loss and low protein production in cultures that are inoculated with outgrown precultures.

5. When DHODC was purified from bacteria that expressed the protein either from the chromosomal *pyrD* gene or from low production plasmids (23) we disrupted the cells by use of a French press and isolated the membranes, which contained near 100% of the enzyme, by centrifugation. The protein was then released from the membranes by addition of Triton X-100 (37). The isolation of membranes prior to release of the enzyme gave a substantial purification ( $\geq 10$ -fold), but with the large overproduction of DHODC, achieved by the use of plasmid pAG1, the majority of DHODC remains in the supernatant, when the membranes are isolated. Therefore, this step is omitted from the purification procedure and the membranes are dissolved by addition of Triton X-100 prior to all fractionation.
6. The Phenyl-Sepharose column can be regenerated by extensive washing with 20% ethanol in water. The removal of Triton X-100 can be followed by monitoring the UV-absorbance.
7. The behavior of DHODC during chromatography on the DE-52 ion column in the absence of detergent is unusual. At low ionic strength, the enzyme appears to bind to the column material primarily by electrostatic forces and be released by a moderate salt concentrations. However, at high-salt concentrations, it sticks to the column material by hydrophobic interactions. In an attempt to elute the protein from the DE-52 column in a more-concentrated manner than obtained by the described salt gradient, we applied a solution of 1 M NaCl in buffer D to the column directly after Triton X-100 had been removed. All of the enzyme remained at the column during the high salt wash, and a part of it (about two-thirds) was eluted by a backward gradient from 1 M to 0 M NaCl in buffer D with a peak about 0.15 M NaCl.
8. Strain SØ6735 transformed with pAG1 was used to produce selenomethionine substituted DHODC for crystallization and structure determination (28). The strain was grown in the phosphate buffered minimal (A+B)-medium (35) supplemented with glucose (0.5%), methionine, leucine, isoleucine, and valine (all at a concentration of 50 mg/L) and with uracil (20 mg/L) and ampicillin (100 mg/L). Uracil was added because the *rph-I* mutation in strain DL41 (a derivative of MG1655) has a polar effect on transcription of the *pyrE* gene, which generates a strong stress in the supply of pyrimidine nucleotides and a reduced growth rate in pyrimidine free media (38). The preculture was grown in a medium supplied with normal L-methionine. At  $OD_{436} = 0.5$  the preculture was cooled in an ice bath. The cells were harvested by centrifugation, washed with basal salt medium, and resuspended at an  $OD_{436} = 0.05$  in 2 L of prewarmed medium, similar to the medium described aforementioned, but with DL-selenomethionine (0.1 g/L) replacing L-methionine.



After a few minutes, the growth rate declined to half of that seen in the preculture, indicating that all L-methionine had been consumed and that the cells were now thriving on selenomethionine. The synthesis of DHODC was induced at  $OD_{436} = 0.5$  and the culture was left to reach stationary phase overnight at an  $OD_{436}$  of 2–3. Harvest of the cells, extraction, and protein purification was performed as aforementioned with the notable exception that 1 mM dithiothreitol (DTT) was included in all the buffers to prevent oxidation. Furthermore, only 0.9 M ammonium sulfate was added to the enzyme solution prior to application on the Phenyl-Sepharose column and the subsequent gradient changed accordingly to go from 0.9 M to 0 M ammonium sulfate. The reduction in the ammonium sulfate concentration was made because the selenomethionine substituted DHODC precipitates in the presence of 1.1 M ammonium sulfate. The yield of DHODC, fully substituted with selenomethionine, was circa 10 mg per liter of medium was.

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