

# Expression and Characterization of *E. coli*-produced Soluble, Functional Human Dihydroorotate Dehydrogenase: a Potential Target for Immunosuppression

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## Abstract

Human dihydroorotate dehydrogenase (huDHODH) is essential for *de novo* biosynthesis of pyrimidines and the target of two immunosuppressive drugs, brequinar and the leflunomide metabolite A77-1726 (Chen *et al.*, 1992; Davis *et al.*, 1996). Using a T7 RNA polymerase expression system, we produced huDHODH as a fusion protein containing an amino-terminal decahistidine tag. *Escherichia coli* growth and expression conditions were optimized to enhance huDHODH solubility and to permit purification of the enzyme in the absence of detergent. Soluble huDHODH, purified by a simple two-step procedure, was catalytically active, monomeric, and contained a flavin mononucleotide (FMN) cofactor in a 1:1 FMN/protein molar ratio. Kinetic analysis showed that huDHODH uses a two site ping-pong mechanism, where DHO is oxidized at one site and the second substrate, ubiquinone, is reduced at the other. This result is consistent with the mechanism proposed for bovine liver DHODH (Hines and Johnston, 1989).

## Introduction

Dihydroorotate dehydrogenase (DHODH, EC number: 1.3.3.1) is essential for the *de novo* synthesis of pyrimidines in both prokaryotic and eukaryotic organisms. The enzyme catalyzes the oxidation of dihydroorotate (DHO) to orotic acid by transferring electrons to ubiquinone through an enzyme-bound redox cofactor (Gero *et al.*, 1985).

Prokaryotic and fungal forms of the enzyme have been shown to be either soluble or associated with the inner membrane (Bjornberg *et al.*, 1997). Mammalian DHODH is associated with the mitochondrial inner membrane (Chen and Jones, 1976; Jones, 1980). Early studies of the mammalian enzyme utilized protein purified from native sources (Miller *et al.*, 1968; Kennedy, 1973; Forman and Kennedy, 1978; Gero and O'Sullivan, 1985; Hines *et al.*, 1986). More recently, recombinant human and rat DHODH enzymes have been produced and studied in both *Escherichia coli* (human only; Copeland *et al.*, 1995) and baculovirus expression systems (human and rat; Knecht *et al.*, 1997; Knecht *et al.*, 1996).

Despite the numerous studies on mammalian DHODH enzymes, native molecular weights (monomeric vs. multimeric), endogenous cofactors (flavins and iron-sulfur centers), and the kinetic mechanism remain in doubt. The structure of a mammalian DHODH has not been solved, although the structure of *Lactococcus lactis* DHODH A was recently reported (Rowland *et al.*, 1997). This enzyme was shown to be a homodimer, each subunit containing a single flavin mononucleotide (FMN) molecule and a putative DHO binding site. When aligned with DHODH enzymes of mitochondrial origin, important differences were observed in some of the active-site residues, implying significant mechanistic differences (Rowland *et al.*, 1997). Therefore the relevance of the *L. lactis* DHODH A structure to mammalian DHODH is difficult to assess.

Human DHODH (huDHODH) is the target of two immunosuppressive drugs that block T-cell proliferation: brequinar (Chen *et al.*, 1992) and the leflunomide metabolite A77-1726 (Davis, *et al.*, 1996). To further study the potential of huDHODH as a drug target for immunosuppression, we sought to produce huDHODH for structural and kinetic studies. Production of recombinant huDHODH in *E. coli* and baculovirus systems has been described previously (Copeland *et al.*, 1995; Knecht *et al.*, 1996). The *E. coli*-produced enzyme, purified from an insoluble *E. coli* fraction, was aggregated and had uncertain flavin content (Copeland *et al.*, 1995). The specific activity of this enzyme was relatively low as compared to huDHODH produced in insect cells, suggesting that this *E. coli*-produced enzyme contained improperly folded material or other contaminants. The baculovirus-produced enzyme, purified from insect cell mitochondrial membrane, was monomeric, contained FMN in an approximate 1:1 FMN/protein molar ratio, and had high specific activity (Knecht *et al.*, 1996). For the sake of convenience, we chose to optimize the production and purification of functional huDHODH in *E. coli* rather than in insect cells. Here we report the production of huDHODH in *E. coli* as a soluble, functional monomer, containing FMN in a 1:1 FMN/protein molar ratio. Purification was in the absence of

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detergent, thereby avoiding potential artifacts introduced by detergent which could affect subsequent characterization of the enzyme. Kinetic analysis of this recombinant huDHODH indicates that it has a two site ping-pong mechanism with DHO and ubiquinone binding at separate sites.

## Results and Discussion

### Optimization of huDHODH Expression in *E. coli* and Subcellular Localization of huDHODH Protein and Activity

Previous studies demonstrated the feasibility of producing functional recombinant huDHODH protein in *E. coli* (Copeland *et al.*, 1995) and insect cells (Knecht *et al.*, 1996). In these systems, huDHODH protein was isolated from *E. coli* membranes or insect cell mitochondrial membranes by detergent extraction. In the present study growth conditions were optimized to enhance soluble huDHODH production. Purification of huDHODH was from the soluble fraction, rather than from the insoluble fraction, thereby avoiding potential artifacts introduced by detergent which could affect subsequent characterization of the enzyme.

Although mammalian DHODH enzymes are naturally associated with the mitochondrial inner membrane, the nature of this association remains unclear (Chen and Jones, 1976; Jones, 1980). Analysis of the amino acid sequence of the closely related rat DHODH protein predicts hydrophobic amino acid stretches at both the amino- and carboxy-termini (Knecht, *et al.*, 1997; Rotgeri and Loffler, 1995). The analogous N-terminal stretch is absent from our his<sub>10</sub>-huDHODH construct. The hydrophobic stretches in rat DHODH have been hypothesized to contribute to membrane association of the enzyme. It is possible that the absence of the N-terminal hydrophobic region in our construct contributed to the solubility of his<sub>10</sub>-huDHODH in *E. coli*. Differences observed between our huDHODH and the huDHODH produced by Copeland *et al.*, (1995) may be attributed to different expression and purification conditions (see below).

We cloned the structural gene encoding huDHODH protein into the T7-RNA polymerase expression vectors pET-11a and pET-19b. Expression of the *huDHODH* gene from either vector enabled *E. coli* strain BL21(DE3)*pyrD* to grow in minimal medium in the absence of exogenous pyrimidines, demonstrating that huDHODH was functional in *E. coli* (data not shown). Experiments to optimize production of enzyme in cells containing pET-11a-huDHODH were carried out by growing 50 ml shake flask cultures at various temperatures, with and without induction. Our results indicated that cultures grown at lower temperatures and without induction generally produced greater **total** amounts of DHODH activity (Table 1). Moreover, the majority of total DHODH activity remained soluble in **uninduced** cells grown at lower temperatures (Table 1). Under similar growth conditions, the same subcellular distribution of DHODH activity in pET-19b-huDHODH cells was observed, suggesting that the N-terminal his<sub>10</sub>-tag had no deleterious effect on his<sub>10</sub>-huDHODH solubility (Table 1).

In agreement with our earlier studies of pET vector expression in uninduced cells (Grossman, *et al.*, 1998), the onset of total his<sub>10</sub>-huDHODH protein production in

Table 1. The Effects of Temperature and Induction on Subcellular Localization of huDHODH Activity in *E. coli*

<i>pET-11a-huDHODH cells</i>		
Growth Condition <sup>#</sup>	Fraction <sup>+</sup>	Total activity <sup>**</sup>
16 °C	soluble	3.0
	pellet	0.6
25 °C	soluble	1.2
	pellet	0.3
30 °C	soluble	5.5
	pellet	2.9
37 °C	soluble	2.1
	pellet	0.9
37 °C/25 °C+IPTG	soluble	2.1
	pellet	0.9
37 °C/37 °C+IPTG	soluble	0.7
	pellet	1.6

<i>pET-19b-huDHODH cells</i>		
Growth Temp.#	Fraction <sup>+</sup>	Total activity <sup>**</sup>
25 °C	soluble	15.0
	pellet	2.7
30 °C	soluble	3.3
	pellet	0.4
37 °C	soluble	2.3
	pellet	0.2

<sup>#</sup> 50 ml cultures were grown in 4xYT+Amp with vigorous aeration at either 16 °C, 25 °C, 30 °C or 37 °C. Induced cultures were grown at 37 °C, either during late log phase and grown at either 25 °C or 37 °C (see Experimental Procedure).

<sup>+</sup> Cells were lysed by sonication in 1/2 the original cell culture volume, centrifuged to pellet insoluble material and unbroken cells, and the pellet was resuspended in buffer containing 5% Triton X-100 at 1/4 the original cell volume (see Experimental Procedure). Soluble and detergent-soluble pellet fractions were assayed for DHODH activity. Assays were done in duplicate. Results are from a representative experiment.

<sup>\*\*</sup> Total activity (μM DCIP reduced /minute) per soluble or pellet fraction of cells from 10 ml of culture.

uninduced cells occurred during late log/early stationary phase and steadily increased to high levels in overnight cultures (not shown). However, most of the active protein was produced by early stationary phase. We hypothesized that production of active his<sub>10</sub>-huDHODH may be limited by insufficient flavin biosynthesis in *E. coli*. Therefore, growth at lower temperatures without induction, may enhance recombinant protein production by slowing it down, thereby allowing for better flavin incorporation, resulting in increased amounts of active his<sub>10</sub>-huDHODH. Insufficient flavin biosynthesis could also explain why enzyme produced in late stationary phase was not active. Attempts to supplement growth medium with exogenous riboflavin or FMN did not increase functional his<sub>10</sub>-huDHODH levels, possibly due to limits in cellular uptake or metabolism.

### Purification and Characterization of Soluble his<sub>10</sub>-huDHODH Protein

For ease of purification, we chose to isolate his<sub>10</sub>-huDHODH from the soluble fraction of *E. coli*. Guided by our expression optimization studies we grew strain

Table 2. Purification of Soluble his<sub>10</sub>-huDHODH from *E. coli*

Fraction	Specific Activity <sup>#</sup> U/mg	Total Protein* mg	Total Activity U	Yield Activity %	Purification Fold
Whole lysate <sup>+</sup>	0.55	11720	6399	100	1.0
Supernatant	0.85	8000	6800	106	1.5
Talon IMAC	45.5	31	1410	22	83
Cation IEX	62.6	7.4	483	7.6	119

<sup>#</sup> μM DCIP reduced/minute/mg protein.

\* Protein concentration was determined by the bicinchoninic acid assay using BSA as standard (Materials and Methods).

<sup>+</sup> Lysate of cells from a 4.5 liter fermentation.

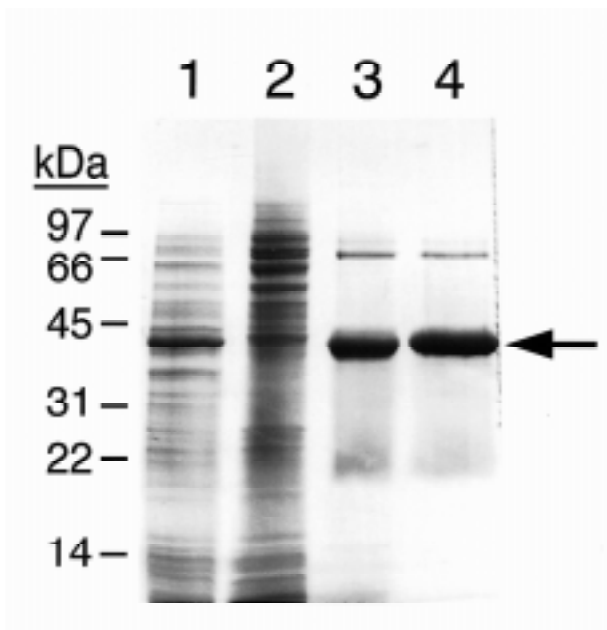


Figure 1. Purification of his<sub>10</sub>-huDHODH Protein  
Cells from a 5 L fermentation were lysed and purified as described in Materials and Methods. Samples taken during each purification step were analyzed by SDS-PAGE. Lane 1, whole cell lysate; lane 2, lysate supernatant; lane 3, metal resin-eluate; lane 4, cation exchange eluate. Arrow indicates his<sub>10</sub>-huDHODH protein band.

BL21(DE3)*pyrD* (pET-19b-huDHODH) to saturation at 25 °C in a 5 L fermentor vessel without induction. Cells were lysed and his<sub>10</sub>-huDHODH was purified from the soluble fraction. Consistent with earlier observations, the soluble fraction contained only a small fraction of the total his<sub>10</sub>-huDHODH protein produced (Figure 1, lane 2), but the majority of active enzyme (Table 2). This finding suggests that much of the huDHODH protein was inactive and insoluble, possibly associated with the membrane fraction or inclusion bodies.

His<sub>10</sub>-huDHODH was purified from the soluble fraction by metal-chelate chromatography, the peak of DHODH activity eluting at greater than 100 mM imidazole. Additional purification by cation exchange chromatography yielded pure and highly active (specific activity = 62.6 U/mg), soluble his<sub>10</sub>-huDHODH protein (Figure 1, Table 2). The apparent molecular weight of purified his<sub>10</sub>-huDHODH by SDS-PAGE analysis was ~42-43 kD, as predicted from its amino acid sequence (Figure 1). Analysis of purified his<sub>10</sub>-huDHODH by analytical gel filtration chromatography showed that the peak of DHODH activity co-eluted with a single protein peak at ca. 30 kD, implying that native his<sub>10</sub>-huDHODH is monomeric (Figure 2). This experiment also suggested that the faint higher molecular weight band observed by SDS-PAGE analysis of purified huDHODH (Figure 1, lanes 3 and 4) may be an artifact of SDS-PAGE sample preparation since we observed no corresponding peak by analytical gel filtration under native conditions in Figure 2. These data are in agreement with findings by Knecht, *et al.*, (1996) which showed that recombinant huDHODH produced by baculovirus and purified from insect cell mitochondria was also monomeric.

Analysis by MALDI-TOF mass spectrometry confirmed a molecular weight of 42,945 daltons (data not shown),

consistent with the predicted molecular weight of full length his<sub>10</sub>-huDHODH protein plus one molecule of FMN. Although attempts to determine the N-terminal sequence of purified his<sub>10</sub>-huDHODH were unsuccessful, presumably due to the long extension of histidines, the enzyme was reactive in a western blot with an anti-polyhistidine monoclonal antibody (not shown), confirming the presence of N-terminal histidines. The high molecular weight band observed in Figure 1 was also immunoreactive with the anti-polyhistidine monoclonal antibody (not shown), consistent with the idea that this protein band may be an enzyme aggregate that formed during SDS-PAGE sample preparation.

Purified his<sub>10</sub>-huDHODH solutions were characteristically yellow and the ultraviolet-visible absorption spectrum resembled a typical spectrum of an oxidized flavin with maxima at ca. 270 nm, 375 nm, and 450 nm (Figure 3; Knecht *et al.*, 1997; Larsen *et al.*, 1985). Upon addition of 2 mM dihydroorotate, the yellow color and the absorption peaks at ca. 375 nm and 450 nm disappeared (Figure 3), indicating reduction of the flavin cofactor upon addition of the enzyme substrate. The associated flavin was identified by fluorescence as FMN and was present in a ratio of 1 mol FMN / mol cation-exchange purified protein (see Experimental Procedures).

#### Kinetic Mechanism of the huDHODH Reaction

The Lineweaver-Burk plot of the initial velocity data for the reaction of DHO and coenzyme Q<sub>0</sub> displayed a parallel line pattern suggesting a ping-pong mechanism for huDHODH. Figure 4 shows a representative experiment. A parallel line pattern has also been observed for bovine liver DHODH (Hines and Johnston, 1989). The values of K<sub>m</sub> for DHO and coenzyme Q<sub>0</sub> are 12 ± 2 μM and 110 ± 20 μM, respectively, and the value of k<sub>cat</sub> is 15 ± 1 s<sup>-1</sup>. The kinetic values are the average of at least two independent

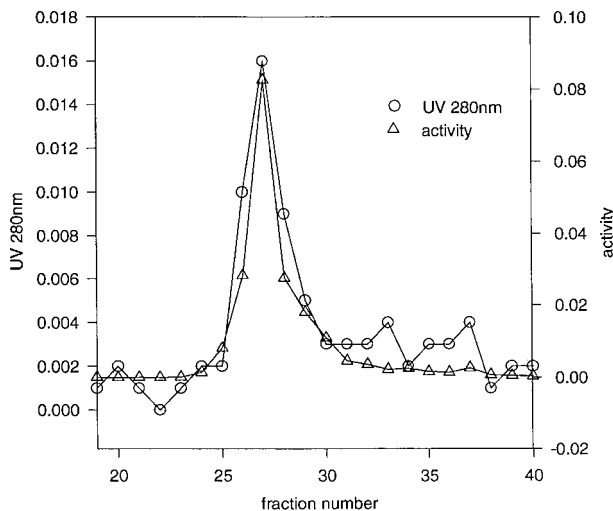


Figure 2. Analytical Gel Filtration of Native his<sub>10</sub>-huDHODH  
Pure his<sub>10</sub>-huDHODH (17 μg), from cation exchange chromatography, was analyzed by gel filtration chromatography as described in Experimental Procedures. Fractions were tested for UV absorbance at 280 nm as well as DHODH activity (orotate measured as the change in absorbance per minute at 287 nm). Gel filtration standards for this column assign a molecular weight ca. 30 kDa to the A<sub>280</sub> peak. Open triangles denote DHODH activity and open circles denote UV absorbance.

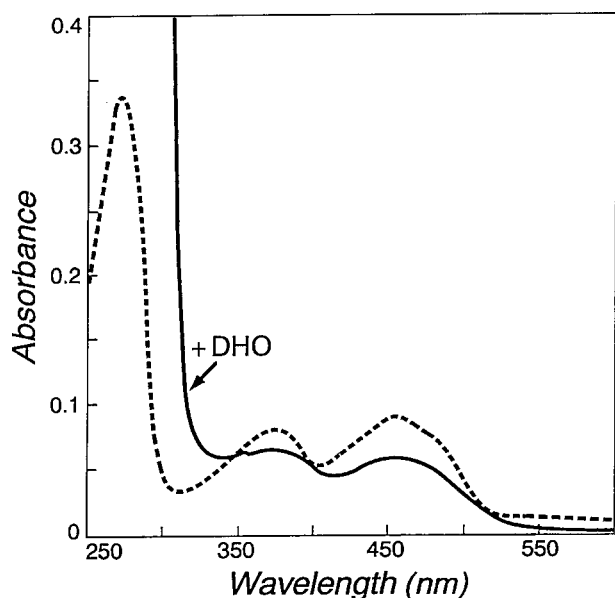


Figure 3. Ultraviolet-visible Absorption Spectrum of his<sub>10</sub>-huDHODH Protein. The absorption spectrum was recorded for pure enzyme (0.3 mg/ml) obtained from cation exchange chromatography in the absence (dashed line) or presence of 2 mM dihydroorotate (" +DHO", solid line).

experiments. These values are consistent with preliminary kinetic analysis of truncated versions of huDHODH (Copeland *et al.*, 1995; Knecht *et al.*, 1996) and similar to those reported for bovine DHODH (Hines and Johnston, 1989).

#### Product and Dead End Inhibition

A two site ping-pong mechanism has been proposed for bovine liver DHODH (Hines and Johnston, 1989). We employed inhibition studies to determine if huDHODH uses a similar mechanism. In a classical ping-pong mechanism,

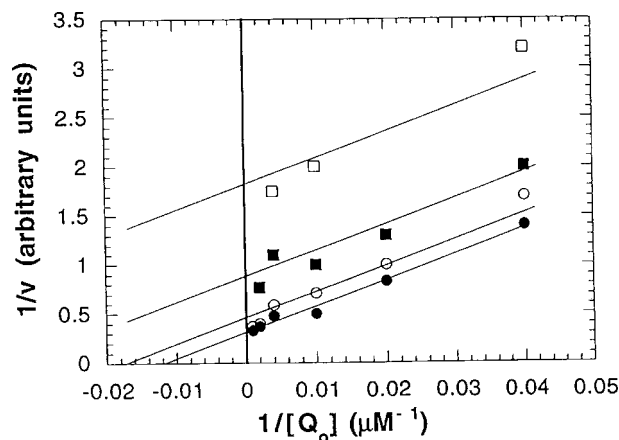


Figure 4. Initial Velocity Data for the huDHODH Reaction. Assays were performed at 25 °C containing 150 mM KCl, 50 mM Tris, pH 8.0, 9 nM enzyme and varying amounts of DHO (2.5 - 200 μM) and Q<sub>o</sub> (25 - 1000 μM). (open squares), 2.5 μM DHO; (closed squares), 5.0 μM DHO; (open circles), 25 μM DHO; (closed circles), 50 μM DHO.

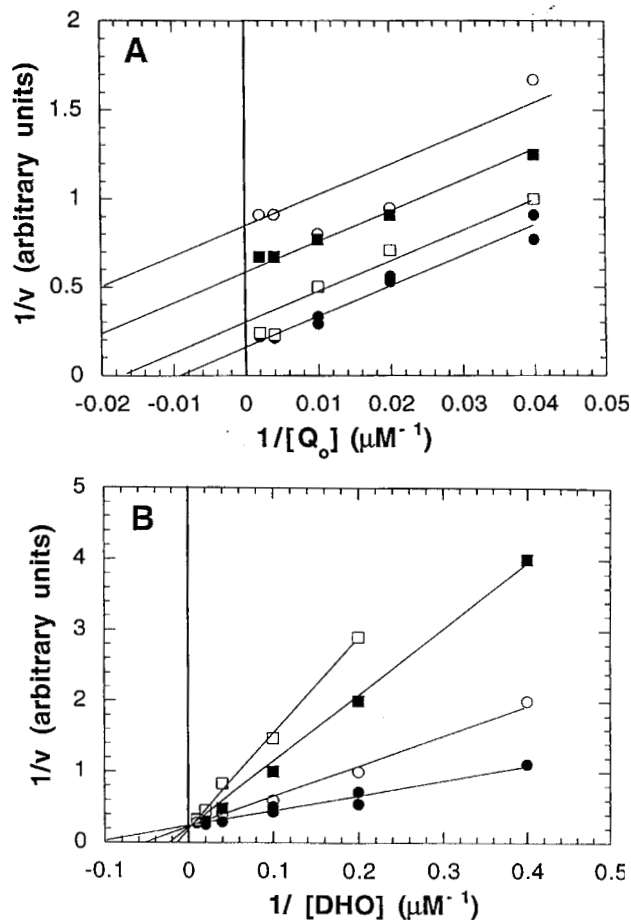


Figure 5. Product Inhibition of huDHODH

Assays were performed as in Figure 4. A. Orotate inhibition with respect to Q<sub>o</sub>. The concentration of DHO was fixed at 25 μM. Closed circles, no orotate; open squares, orotate = 50 μM; closed squares, orotate = 100 μM; open circles, orotate = 200 μM. B. Orotate inhibition with respect to DHO. The concentration of Q<sub>o</sub> was fixed at 100 μM. Closed circles, no orotate; open circles, orotate = 25 μM; closed squares, orotate = 50 μM; open squares, orotate = 100 μM.

the first product is a competitive inhibitor with respect to the second substrate (Cleland, 1977). However, as shown in a representative experiment in Figure 5, orotate was an uncompetitive inhibitor with respect to Q<sub>o</sub> ( $K_{ij} = 60 \pm 10 \mu\text{M}$ ) and a competitive inhibitor with respect to DHO ( $K_{is} = 22 \pm 3 \mu\text{M}$ ). This product inhibition pattern is consistent with a sequential rather than a ping-pong mechanism. However, the ping-pong mechanism can be confirmed by dead end inhibitor studies: barbituric acid was an uncompetitive inhibitor with respect to Q<sub>o</sub> ( $K_{ij} = 250 \pm 60 \mu\text{M}$ ) and a competitive inhibitor with respect to DHO ( $K_{is} = 100 \pm 10 \mu\text{M}$ ). These inhibition patterns are consistent with a ping-pong mechanism. To reconcile these findings, we propose a two site ping-pong mechanism, illustrated in Figure 6, with DHO oxidized at one site and Q<sub>o</sub> reduced at the other. This proposal suggests that different faces of the flavin may interact with each substrate, and that the reduced and oxidized enzyme forms have different conformations. We anticipate that future structural studies of his<sub>10</sub>-huDHODH will help further elucidate the mechanism.

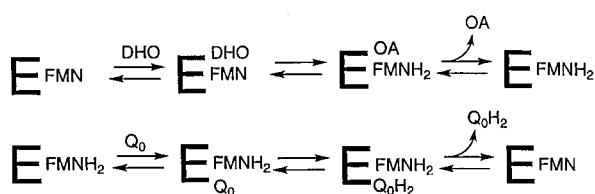


Figure 6. Proposed Kinetic Mechanism for huDHODH. E, huDHODH; FMN, enzyme-bound oxidized flavin; FMNH<sub>2</sub>, enzyme-bound reduced flavin. Substrates and products: DHO, dihydroorotate; OA, orotic acid; Q<sub>0</sub>, coenzyme Q<sub>0</sub>; Q<sub>0</sub>H<sub>2</sub>, reduced coenzyme Q<sub>0</sub>.

In summary, we have shown that catalytically active, soluble huDHODH can be produced in *E. coli* and purified by a simple two-step procedure. We expect that his<sub>10</sub>-huDHODH will be a valuable tool in the investigation of the mechanism of action of known DHODH inhibitors, such as brequinar and A77-1726, and in the discovery of new DHODH inhibitors for the development of new immunosuppressive drugs.

### Experimental Procedures

#### Isolation and Expression of the Human DHODH Gene (*huDHODH*) and Protein Production

cDNA sequences encoding the *huDHODH* structural gene (Genbank accession no. M94065; Minet *et al.*, 1992) were amplified by PCR from a bacteriophage  $\lambda$  Zap library containing Jurkat T-cell cDNA (Stratagene, La Jolla, CA). Primers for PCR were designed to encode a 5' *NdeI* site (containing the ATG start codon, the first 7 huDHODH amino acids: MATGDER...) and a 3' *BamHI* site (immediately following the native TGA stop codon). For gene expression in *E. coli* the ~1.2 kb *NdeI/BamHI huDHODH* fragment, encoding an N-terminally truncated huDHODH protein minus putative mitochondrial targeting sequences, was cloned into the *NdeI/BamHI* sites of either pET-11a or pET-19b (Novagen, Madison, WI). The latter vector produced huDHODH as an N-terminal ten histidine-fusion protein (his<sub>10</sub>-huDHODH).

*E. coli* expression strain BL21(DE3)*pyrD* was constructed by P1 transduction of the *pyrD* 34 allele from strain CY307 (Coli Genetic Stock Center, New Haven, CT) into BL21(DE3) (Novagen, Madison, WI), as described by Miller (1992). P1 *kc+* was isolated from *E. coli* lysogen KL739 (Coli Genetic Stock Center, New Haven, CT). Tetracycline resistant transductants were screened for the *pyrD* phenotype (inability to grow in minimal medium without uridine (M63 medium; Miller, 1992) and for the absence of DHODH activity in cell lysates using the chromagen-reduction assay (see below).

Plasmids pET-11a-huDHODH and pET-19b-huDHODH were transformed into BL21(DE3)*pyrD* for protein production. For optimization of growth and protein production experiments, 50 ml cultures were grown at various temperatures, shaking at 300 rpm in 250 ml shake-flasks containing 4xYT+Ap medium (per liter: 32 g Bacto tryptone (Difco, Detroit, MI), 20 g Bacto yeast extract (Difco, Detroit, MI), 5 g NaCl, ampicillin, 50  $\mu$ g/ml). For induction, 1 mM IPTG (Sigma, St. Louis, MO) was added to late log phase cultures (OD<sub>600</sub>=0.8-1.0) and induced cultures were analyzed after 3 hours at 37 °C. Production cultures were inoculated with seed stocks derived from early log phase cells (OD<sub>600</sub>=0.1-0.5) frozen in 15% glycerol at -80 °C. Uninduced cells were grown to saturation at 25 °C in either a 2L or 5 L fermentor vessel equipped with 3 Rushton impellers (B. Braun Biotech, Allentown, PA) containing 4xYT+Ap medium (1.75 L or 4.5 L working volumes). Aeration was maintained at 700 rpm and typical final cell densities were OD<sub>600</sub> = 15-18.

#### Subcellular Localization and DHODH Assay

All chemical reagents used in purification and assay procedures were from standard commercial sources unless otherwise stated. Frozen pET-11a-huDHODH cell pellets from 50 ml shake-flask cultures were thawed and suspended to one half of the original cell culture volume in cold lysis buffer containing 50 mM Tris, 1 mM EDTA, 1 mM NaCl pH 7.5. The suspension was sonicated on ice using a Branson Sonifier 250 for 6 x 30 second intervals at 90% output with a three minute break between sonications. The suspension was centrifuged at 23,000 x g for 45 minutes to pellet insoluble material and unbroken cells. The supernatant, containing soluble huDHODH, was assayed for DHODH activity (see below). The insoluble pellet was

resuspended to 1/4 the original cell culture volume in lysis buffer containing 5% Triton X-100 and stirred for 2 hours at 4 °C. Detergent-insoluble material was pelleted by centrifugation at 35,000 x g for 30 minutes and the detergent-soluble fraction was assayed for DHODH activity by the chromagen-reduction assay as previously described (Knecht *et al.*, 1996). The reaction mixture contained 1 mM L-dihydroorotate, 0.04 mM 2,6-dichloroindophenol, 0.1% Triton X-100, and 0.1 mM decylubiquinone in 50 mM Tris HCl, 150 mM KCl, pH 8.0. The assay was initiated by adding L-dihydroorotate, at 25 °C, and loss of absorbance was measured at 600 nm (extinction coefficient=18,800 x mol<sup>-1</sup> x cm<sup>-1</sup> of 2,6-dichloroindophenol).

#### Isolation and Purification of his<sub>10</sub>-DHODH

Frozen cell paste (~100 gm) from a 4.5 L culture was thawed and suspended in 1-2 liters of lysis buffer containing 20 mM Tris, 300 mM NaCl, 15 mM imidazole, pH 7.6. Cells were disrupted by passage through an M-110T microfluidizer (Microfluidics Corp., Newton, MA) under 10,000 psi pressure. Insoluble material was pelleted by centrifugation at 23,000 x g for 45 min and soluble his<sub>10</sub>-huDHODH was recovered from the supernatant using 25 ml TALON metal affinity resin (Clontech Laboratories, Palo Alto, CA) equilibrated in lysis buffer. After incubation for 1-2 hours at 4 °C, the resin was recovered by low speed centrifugation and washed with 4 x 600 ml lysis buffer, pH 7.0, containing 10% glycerol. A column was prepared with the resin and eluted with an imidazole gradient (15-250 mM) in a minimum of 6 column volumes (CV), DHODH activity eluted at greater than 100 mM imidazole. The eluate was either diluted to 0.1 mg/ml with 50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10% glycerol, pH 6.7 and dialyzed overnight against ion exchange buffer (50 mM HEPES, 50 mM NaCl, 2 mM EDTA, 10% glycerol, pH 6.7) or diluted in 50 mM HEPES, 2 mM EDTA, 10% glycerol pH 6.7 to a final salt concentration of 50 mM NaCl. Cation exchange chromatography was carried out using a BioCAD HPLC workstation (PerSeptive Biosystems, Framingham, MA) with a 4.6 x 100 mm II HS/M column (PerSeptive Biosystems, Framingham, MA) equilibrated in ion exchange buffer. After sample application, the column was washed with 10 CV and eluted with a salt gradient (50-300 mM NaCl) in 200 CV at a rate of 5 ml/min. Active his<sub>10</sub>-huDHODH eluted from the column at approximately 200 mM NaCl.

#### SDS-PAGE Analysis, Protein Determination, Mass Spectrometry

Cell fractions and purified enzyme were analyzed by SDS-PAGE on pre-cast 12% gels (BioRad, Hercules, CA) stained with Coomassie brilliant blue. Protein concentration was determined using the bicinchoninic acid protein assay with a bovine serum albumin standard (Smith *et al.*, 1985). MALDI-TOF mass spectrometry was performed at M-Scan, Inc. (West Chester, PA) using a PerSeptive Biosystems Voyager Elite Biospectrometry Research Station coupled with a Delayed Extraction laser-desorption mass spectrometer. An aliquot of each sample was diluted with 0.1% trifluoroacetic acid and analyzed using a matrix of sinapinic acid.

#### Analytical Gel Filtration

Pure his<sub>10</sub>-huDHODH (17  $\mu$ g), recovered from cation exchange chromatography, was analyzed using a Superose 6 HR 10/30 column on an FPLC chromatography system (Pharmacia, Piscataway, NJ). The sample was eluted with 50 mM potassium phosphate, pH 7.2, and 150 mM potassium chloride at a flow rate of 0.3 ml/min. The column was calibrated with gel filtration standards of the following molecular masses: 94 kDa, 67 kDa, 43 kDa, 30 kDa and 20 kDa. Activity assays for the collected fractions, containing 150 mM KCl, 50 mM Tris, pH 8.0, 5  $\mu$ M DHO and 100  $\mu$ M coenzyme Q<sub>0</sub>, were by examining orotate formation at A<sub>287</sub> (Hines, *et al.*, 1986).

#### Flavin Determination

Ultraviolet-visible spectroscopy was performed on a Lambda 6 spectrometer (Perkin-Elmer, Norwalk, CT) using a 0.3 mg/ml (7  $\mu$ M) sample of purified his<sub>10</sub>-huDHODH recovered from cation exchange chromatography. An extinction coefficient of 1.22 x 10<sup>4</sup> M<sup>-1</sup>cm<sup>-1</sup> at 450 nm was used to calculate the concentration of FMN in the protein sample (Whitby, 1953). Flavin was also identified and quantitated by fluorescence spectroscopy as previously described (Faeder and Siegel, 1973) using a SPF-500C spectrofluorometer (SLM Instruments, Urbana, IL).

#### Determination of Kinetic Parameters

DHODH activity with coenzyme Q<sub>0</sub> (Sigma, St. Louis, MO) as the electron acceptor was measured by monitoring A<sub>287</sub> (Hines *et al.*, 1986). Assays contained 150 mM KCl, 50 mM Tris, pH 8.0, 9 nM enzyme and varying amounts of DHO (2.5 - 200  $\mu$ M) and Q<sub>0</sub> (25 - 1000  $\mu$ M). Inhibition experiments were performed at subsaturating concentrations of the constant substrate (either 25  $\mu$ M DHO or 100  $\mu$ M Q<sub>0</sub>). Orotate concentrations varied between 25 - 100  $\mu$ M and barbituric acid concentrations varied between 50-1000  $\mu$ M. Initial velocity data was fitted to the appropriate equations using KinetAsyst software.

Ping pong mechanism:

$$v = k_{cat}[E][DHO][Q_0]/(K_{DHO}[Q_0] + K_Q[DHO] + [DHO][Q_0])$$

uncompetitive inhibition:

$$v = V_m [S]/\{K_m + [S](1 + [I]/K_{ii})\}$$

competitive inhibition:

$$v = V_m [S]/\{K_m(1 + [I]/K_{is}) + [S]\}$$

where  $v$  is the initial velocity,  $K_{DHO}$ ,  $K_Q$ , and  $K_m$  are the Michaelis constants for DHO,  $Q_0$  and the varied substrate respectively,  $K_{ii}$  and  $K_{is}$  are the slope and intercept inhibition constants respectively.

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### References

- Bjornberg, O., Rowland, P., Larsen, S., and Jensen, K.F. 1997. Active site of dihydroorotate dehydrogenase A from *Lactococcus lactis* investigated by chemical modification and mutagenesis. *Biochem.* 36: 16197-16205.
- Chen, S.F., Perrella, F.W., Behrens, D.L., and Papp, L.M. 1992. Inhibition of dihydroorotate dehydrogenase activity by brequinar sodium. *Cancer Res.* 52: 3521-3527.
- Chen, J.J., and Jones, M.E. 1976. The cellular location of dihydroorotate dehydrogenase: relation to *de novo* biosynthesis of pyrimidines. *Arch. Biochem. Biophys.* 176: 82-90.
- Cleland, W.W. 1977. Determining the chemical mechanisms of enzyme-catalyzed reactions by kinetic studies. *Adv. Enzymol.* 45: 273-387
- Copeland, R.A., Davis, J.P., Dowling, R.L., Lombardo, D., Murphy, K.B., and Patterson, T.A. 1995. Recombinant human dihydroorotate dehydrogenase: Expression, purification, and characterization of a catalytically functional truncated enzyme. *Arch. Biochem. Biophys.* 323: 79-86.
- Davis, J.P., Cain, G.A., Pitts, W.J., Magolda, R.L., and Copeland, R.A. 1996. The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. *Biochem.* 35: 1270-1273.
- Faeder, E.J., and Siegel, L.M. 1973. A rapid micromethod for determination of FMN and FAD in mixtures. *Anal. Biochem.* 53: 332-336
- Forman, H.J., and Kennedy J. 1978. Mammalian dihydroorotate dehydrogenase: Physical and catalytic properties of the primary enzyme. *Arch. Biochem. Biophys.* 191: 23-31.
- Gero, A.M., and O'Sullivan, W.J. 1985. Human spleen dihydroorotate dehydrogenase: properties and partial purification. *Biochem. Med.* 34: 70-82.
- Grossman, T.H., Kawasaki, E.S., Punreddy, S. R., and Osburne, M.S. 1998. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene.* 209: 95-103.
- Hines, V., and Johnston, M. 1989. Analysis of the kinetic mechanism of the bovine liver mitochondrial dihydroorotate dehydrogenase. *Biochem.* 28: 1222-1226.
- Hines, V., Keys, L.D., and Johnston, M. 1986. Purification and properties of the bovine liver mitochondrial dihydroorotate dehydrogenase. *J. Biol. Chem.* 261: 11386-11392.
- Jones, M.E. 1980. Pyrimidine nucleotide biosynthesis in animals: Genes, enzymes, and regulation of UMP biosynthesis. *Ann. Rev. Biochem.* 49: 253-79.
- Kennedy, J. 1973. Distribution, subcellular localization, and product inhibition of dihydroorotate oxidation in the rat. *Arch. Biochem. Biophys.* 157: 369-373.
- Knecht, W., Altekruze, D., Rotgeri, A., Gonski, S., and Loffler, M. 1997. Rat dihydroorotate dehydrogenase: Isolation of the recombinant enzyme from mitochondria of insect cells. *Protein Exp. Purif.* 10: 89-99.
- Knecht, W., Bergjohann, U., Gonski, S., Kirschbaum, B., and Loffler, M. 1996. Functional expression of a fragment of human dihydroorotate dehydrogenase by means of the baculovirus expression vector system, and kinetic investigation of the purified recombinant enzyme. *Eur. J. Biochem.* 240: 292-301.
- Larsen, J.N., and Jensen, K. F. 1985. Nucleotide sequence of the *pyrD* gene of *Escherichia coli* and characterization of the flavoprotein dihydroorotate dehydrogenase. *Eur. J. Biochem.* 151: 59-65.
- Miller, J.H. 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Miller, R.W. 1975. A high molecular weight dihydro-orotate dehydrogenase of *Neurospora crassa*. Purification and properties of the enzyme. *Can. J. Biochem.* 53: 1288-1300.
- Miller, R.W., Kerr, C.T., and Curry, J.R. 1968. Mammalian dihydroorotate-ubiquinone reductase complex. *Can. J. Biochem.* 46: 1099-1106.
- Minet, M., Dufour, M.E., and Lacroute, F. 1992. Cloning and sequencing of a human cDNA coding for dihydroorotate dehydrogenase by complementation of the corresponding yeast mutant. *Gene.* 121: 393-396.
- Rotgeri, A., and Loffler, M. 1995. Molecular cloning and sequence analyses of rat liver dihydroorotate dehydrogenase. In: Purine and Pyrimidine Metabolism in Man VIII. A. Sahota and M. Taylor, eds. Plenum Press, New York. p. 693-697.
- Rowland, P., Nielsen, F.S., Jensen, K.F., and Larsen, S. 1997. The crystal structure of the flavin containing enzyme dihydroorotate dehydrogenase A from *Lactococcus lactis*. *Structure.* 5: 239-252.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* 150: 76-853.
- Whitby, L.G. 1953. A new method for preparing flavin-adenine dinucleotide. *Biochem. J.* 54: 437-442.