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Crystallization and preliminary X-ray studies of membrane-associated *Escherichia coli* dihydroorotate dehydrogenase

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Dihydroorotate dehydrogenases (DHODs) are flavin-containing enzymes which catalyse the conversion of (*S*)-dihydroorotate to orotate, the fourth step in the *de novo* biosynthesis of pyrimidine nucleotides. Two major families of DHODs have now been identified based on their amino-acid sequence similarities. The two families differ in their reaction mechanisms, but structures are only known of enzymes belonging to family 1. DHOD from *Escherichia coli* is a typical member of family 2, which contains the membrane-associated enzymes from Gram-negative bacteria and eukaryotes. Yellow crystals grown of this enzyme belong to the space group $P4_12_12$ or $P4_32_12$. The unit-cell parameters are $a = b = 119.2$, $c = 294.3$ Å. Owing to the rather large *c* axis, the currently available resolution of data is 2.2 Å.

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1. Introduction

Dihydroorotate dehydrogenases (DHODs) catalyse the oxidation of (*S*)-dihydroorotate to orotate in the fourth step of the *de novo* biosynthesis of pyrimidine nucleotides. These enzymes have one cofactor in common, a flavin mononucleotide (FMN), which is involved in the transfer of a hydride ion from the substrate to the electron acceptor, the nature of which varies between enzymes of different biological origin. Two major families of DHODs have by now been identified based on amino-acid sequence similarity (Björnberg *et al.*, 1997). The enzymes in family 1 are cytosolic, while all known enzymes of family 2 are membrane-associated. Family 1 contains enzymes from Gram-positive bacteria, while DHODs from eukaryotes and Gram-negative bacteria belong to family 2.

Family 1 can be further subdivided into two groups, 1A and 1B. The organism *Lactococcus lactis* has been shown to contain two different DHODs, which are representative of the two subgroups 1A (DHODA) and 1B (DHODB) (Andersen *et al.*, 1994). DHODA is a homodimer with two subunits composed of 311 amino acids and a FMN group. This enzyme is able to use fumarate and quinones as the final electron acceptors during catalysis (Björnberg *et al.*, 1999). The crystal structure of the enzyme has been reported in both the native form (Rowland *et al.*, 1997) and as a complex with the product orotate (Rowland *et al.*, 1998). The proposed mechanism for this enzyme involves an active-site cysteine (Cys130) as a catalytic base. The second enzyme from *L. lactis*, DHODB, is a heterotetramer (Andersen *et al.*, 1996; Nielsen *et al.*, 1996). The tetramer consists of two pyrD subunits analogous to the DHODA subunits, with a sequence identity of 30% between the two enzymes, and two pyrK subunits each of 262 amino acids. The smaller subunits contain a flavin-adenine dinucleotide (FAD) and a [2Fe–2S] cluster. These subunits enable DHODB to use different electron acceptors to the DHODA enzyme, in particular NAD^+ (Nielsen *et al.*, 1996).

The DHODs found in family 2 have very little similarity (less than 20% sequence identity) to the enzymes in family 1, indicating a major split in the evolutionary tree of the DHODs (Björnberg *et al.*, 1997). Within family 2, the sequence identity between the enzymes is 40% or higher. One member of this family, the *E. coli* DHOD, is attached to the cytoplasmic membrane (Karibian, 1978; Larsen & Jensen, 1985), while the eukaryotic enzymes are localized in the inner mitochondrial membrane (Knecht *et al.*, 1996). All the family 2 enzymes have N-terminal extensions not present in the family 1 enzymes, although these extensions are considerably longer in the mitochondrial enzymes than in the enzymes from Gram-negative bacteria. It has been proposed that these extensions contain mitochondrial targeting sequences and are involved in the binding to the membrane (Rawls *et al.*, 1993). *E. coli* DHOD is closely related to the human enzyme, with a sequence identity of 44% (Knecht *et al.*, 1996). Human DHOD is the rate-limiting enzyme in the *de novo* pyrimidine biosynthetic pathway. Its inhibition by quinone analogues (*e.g.* leflunoamide) is currently being investigated as a potential means of treating immune-based diseases such as rheumatoid arthritis (Davis *et al.*, 1996; Williamson *et al.*, 1995; Löffler *et al.*, 1998).

Table 1
Data-collection statistics for the native crystals of *E. coli* DHOD.

Values in parentheses represent the highest resolution range.

λ (Å)	0.84690
Resolution (Å)	2.2 (2.3–2.2)
Total number of reflections	1786813
Unique reflections	108640
$R_{\rm merge}$† (%)	10.5 (55.4)
Completeness (2.3–2.2 Å) (%)	99.3 (97.7)
Mosaicity (°)	0.4
Redundancy	16.4
$I/\sigma(I)$	11.3 (3.32)
$B_{\rm Wilson}$ (Å^2)	19.7

† $R_{\rm merge} = \sum_{hkl}\sum_i |I(hkl)_i - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I(hkl)_i$.

E. coli DHOD has previously been described as a homodimer with a subunit size of 37 kDa, comprising 336 amino acids and one FMN molecule per subunit (Larsen & Jensen, 1985). In solution, it probably interacts with quinones of the respiratory chain (Karibian, 1978). As with all currently sequenced family 2 enzymes, *E. coli* DHOD lacks the active-site cysteine conserved in all family 1 enzymes. Instead, it contains a serine in the corresponding position and this serine is conserved in all known family 2 enzymes.

Here, we report the crystallization and preliminary X-ray diffraction analysis of the native *E. coli* DHOD. The availability of structures from both families of DHODs will enable a detailed structural comparison and analysis of the reaction mechanisms.

2. Methods and results

2.1. Purification

The native protein was purified and characterized as previously described (Björnberg *et al.*, 1999). The protein was stored in a buffer containing 50 m*M* NaH_2PO_4 pH 7.0, 100 m*M* EDTA and 50% glycerol. Prior to crystallization, the native protein was dialysed against a solution containing 25 m*M* NaH_2PO_4 pH 7.0, 100 m*M* EDTA and 10% glycerol. After dialysis, the protein was stored in aliquots at 253 K.

Figure 1
Crystals of the *E. coli* dihydroorotate dehydrogenase. The crystals grow as long yellow needles, with the unit-cell *c* axis corresponding to the needle axis. Prior to use, the crystals were cut to separate the whiskers from the regular needle.

2.2. Crystallization

An initial search for crystallization conditions was carried out using vapour-diffusion experiments with the standard sparse-matrix crystal screen solutions (Jancarik & Kim, 1991) from Hampton Research (Crystal Screen I and Crystal Screen II). The crystallization drops comprised 2.5 µl protein (12 mg ml^{-1}) and 2.5 µl reservoir solution and were suspended over 1 ml reservoir solution. Since the protein *in vivo* is membrane-associated, it was decided to add a detergent, *n*-octyl-β-D-glucoside (β-OG), to the protein to help the crystallization process. The addition of detergent decreases the surface tension of the drops, resulting in the necessity of using the sitting-drop technique. The protein was mixed with the detergent prior to setting up the crystallization trials in order to obtain a concentration of 25 m*M* β-OG in the crystallization drop prior to equilibration of the drop but after mixing with the reservoir liquid. This concentration is close to the critical micelle concentration for β-OG (Ducruix & Giegé, 1992). The crystallization trials were conducted at room temperature.

After about three months, a cluster of tiny yellow needles had appeared in one of the drops containing 4.0 *M* sodium formate. Subsequent optimizations produced further star-shaped clusters of needles and occasionally some larger needles. Increasing the sodium formate concentration to 4.3–4.4 *M* led to the growth of many single crystals in most of the drops and in some cases resulted in larger crystals. In these primary conditions no buffer was added, so in order to ensure better stability of the crystallization pH, different buffers at various pH values were tried. Reproducible crystals were finally obtained from the following optimized conditions: 3.9–4.4 *M* sodium formate, 0.1 *M* sodium acetate pH 4–5.5, 25 m*M* β-OG prior to equilibration after mixing and 12–15 mg ml^{-1} protein. The amount of reservoir solution used was either 600 µl or 1 ml, with 2.5 + 2.5 µl sitting drops. Under these conditions, crystals appear in 1–2 weeks as yellow needles of approximate dimensions 1.5 × 0.15 × 0.15 mm. Fig. 1 shows a drop containing some of the crystals grown under the optimized conditions.

In order to collect data under cryogenic conditions, the crystals were soaked for a few seconds in a cryoprotectant containing 4.5 *M* sodium formate, 0.1 *M* sodium acetate at the crystallization pH and 10% glycerol.

3. X-ray diffraction analysis

X-ray diffraction data have been collected both in-house and using synchrotron radiation; the latter was required to improve the quality of the data.

A native data set was collected under cryogenic conditions (T = 120 K) at the BW7B beamline at DESY in Hamburg, Germany. In order to separate the individual spots, a crystal-to-detector distance of 420 mm was necessary owing to the long *c* axis, limiting the resolution to 2.19 Å.

Two different experimental settings were used to collect the data in order to ensure the optimal measurements of both the high- and low-resolution data. For the high-resolution data, the 345 mm plate of the MAR 345 image-plate detector was used and 191 images were collected with an oscillation of 0.25° per image. The low-resolution data to 4 Å was collected on the 180 mm plate. The same angular range was covered as for the high resolution, but in steps of 1° per image.

The data were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and structure factors were derived from the reflection intensities using the program *TRUNCATE* (Collaborative Computational Project, Number 4, 1994). The data-collection statistics are given in Table 1.

The symmetry of the diffraction pattern (4/*mmm*) and the conditions for the reflections ($h00$, $h = 2n$; $00l$, $l = 4n$) established the space group of the crystals to be either $P4_12_12$ or $P4_32_12$, with four to eight molecules in the asymmetric unit. The unit-cell parameters are $a = b = 119.2$, $c = 294.3$ Å. The needle axis corresponds to the *c* axis. A self-rotation search carried out on the data using *AMoRe* (Navaza, 1994) strongly suggests the presence of only four molecules in the asymmetric unit, related by twofold axes. With four molecules in the asymmetric unit, the solvent content and corresponding Matthews coefficient, V_m (Matthews, 1968), would be 64% and 3.85 Å^3 Da^{-1}, respectively.

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