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Crystallization and preliminary characterization of dihydropteridine reductase from *Dictyostelium discoideum*

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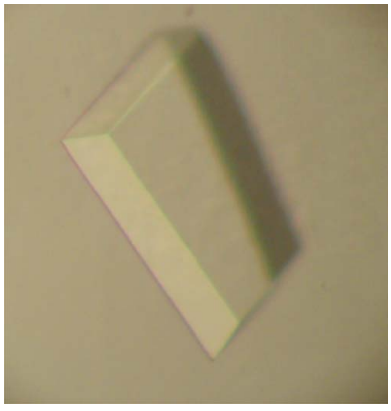
Dihydropteridine reductase from *Dictyostelium discoideum* (dicDHPR) can produce D-*threo*-BH_4 [6*R*-(1′*R*,2′*R*)-5,6,7,8-tetrahydrobiopterin], a stereoisomer of L-*erythro*-BH_4, in the last step of tetrahydrobiopterin (BH_4) recycling. In this reaction, DHPR uses NADH as a cofactor to reduce quinonoid dihydrobiopterin back to BH_4. To date, the enzyme has been purified to homogeneity from many sources. In this report, the dicDHPR–NAD complex has been crystallized using the hanging-drop vapour-diffusion method with PEG 3350 as a precipitant. Rectangular-shaped crystals were obtained. Crystals grew to maximum dimensions of 0.4 × 0.6 × 0.1 mm. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 49.81$, $b = 129.90$, $c = 78.76$ Å, $\beta = 100.00^\circ$, and contained four molecules in the asymmetric unit, forming two closely interacting dicDHPR–NAD dimers. Diffraction data were collected to 2.16 Å resolution using synchrotron radiation. The crystal structure has been determined using the molecular-replacement method.

1. Introduction

Dihydropteridine reductase (DHPR; EC 1.5.1.34) is involved in the last step of tetrahydrobiopterin (BH_4) recycling in higher eukaryotes (Varughese *et al.*, 1994). BH_4 is ubiquitous in nature and is a multifaceted molecule. It is an essential cofactor for the hydroxylation of three aromatic amino hydroxylases that have important biochemical and physiological functions in the biosynthesis of dopamine, norepinephrine and serotonin (Thöny *et al.*, 2000). In addition, BH_4 serves as a cofactor for nitric oxide synthase (NOS; Marletta, 1993) and glyceryl-ether monooxygenase (Kaufman *et al.*, 1990; Taguchi & Armarego, 1998).

In the last step of BH_4 recycling, tetrahydrobiopterin is oxidized to quinonoid dihydrobiopterin (q-BH_2). In this reaction, DHPR uses NADH as a cofactor to reduce q-BH_2 back to BH_4 (Fig. 1). There are four possible BH_4 stereoisomers: L-*erythro*-BH_4, L-*threo*-BH_4, D-*erythro*-BH_4 and D-*threo*-BH_4 (DH_4). While L-*erythro*-BH_4 is common, other BH_4 isomers have also been found in nature: DH_4 [6*R*-(1′*R*,2′*R*)-5,6,7,8-BH_4] in *Dictyostelium discoideum* (Klein *et al.*, 1990) and L-*threo*-BH_4-glycoside in *Chlorobium tepidum* (Cho *et al.*, 1998).

Interestingly, *D. discoideum* (Klein *et al.*, 1990) produces two isomers, DH_4 and BH_4, and both were found to function as a cofactor. Therefore, it was of interest to determine whether *D. discoideum*



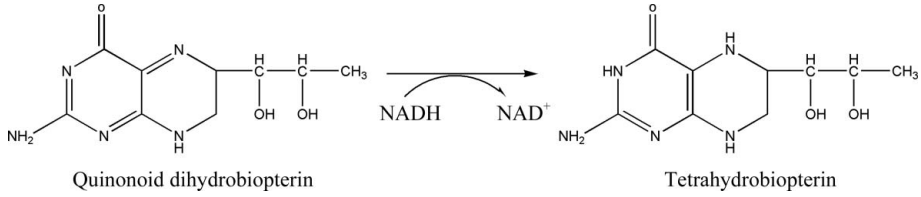


Figure 1
Enzymatic reaction catalyzed by dihydropteridine reductase.

DHPR recognizes both equally. *D. discoideum* DHPR (dicDHPR) has high protein-sequence similarities to mammalian DHPRs, including rat DHPR (58% similarity) and human DHPR (57% similarity). Although structures of the rat DHPR–NAD complex (ratDHPR; Matthews *et al.*, 1986; Varughese *et al.*, 1992), the human DHPR–NAD complex (huDHPR; Su *et al.*, 1993) and an apo form of DHPR from *Caenorhabditis elegans* (Symersky *et al.*, 2003) have been determined, no primary structure involved in the production of a tetrahydrobiopterin other than the common BH_4 in lower eukaryotes has been published. To elucidate the mechanism of DH_4 regeneration by DHPR, we have expressed soluble dicDHPR in *Escherichia coli* and purified and crystallized it. Knowledge of the three-dimensional structure of the active site of dicDHPR will provide a deeper understanding of the catalytic mechanism of the enzyme activity by comparison with the ratDHPR and huDHPR structures.

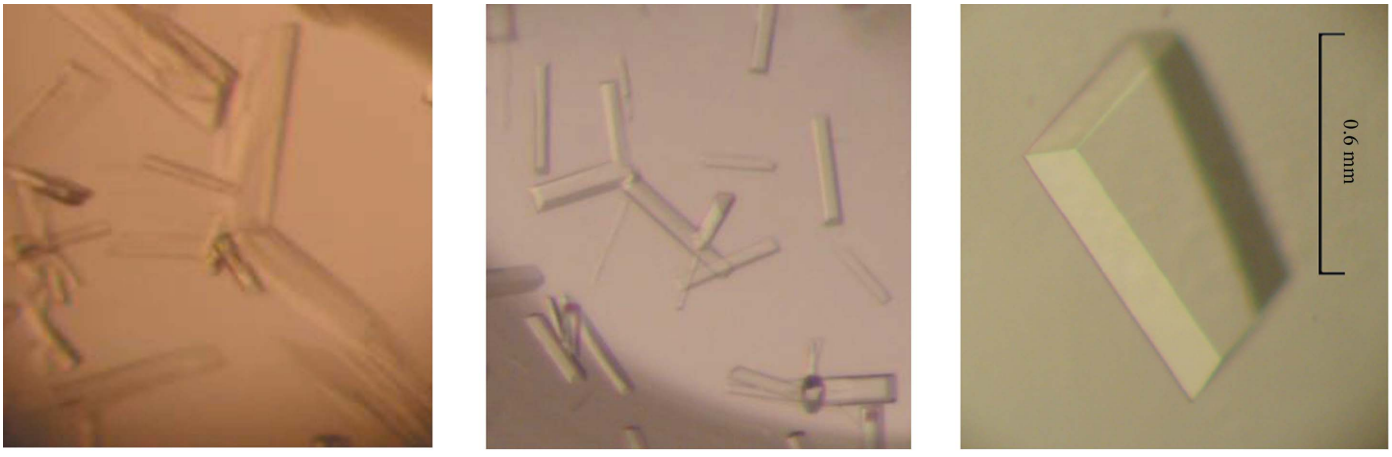
2. Experimental

2.1. Protein expression and purification

The open reading frame encoding DHPR (GenBank accession No. XP_644903) was amplified from *D. discoideum*. The dicDHPR gene (residues 1–231) was cloned into a pET-28b (Novagen, Madison, Wisconsin, USA) expression plasmid between *Nde*I and *Bam*HI to produce recombinant dicDHPR with a hexahistidine tag and a thrombin cleavage site at the N-terminus (MGSSHHHHHHSSGLVPRGSHMSKNIL). The plasmid was transformed into *E. coli* strain BL21 (DE3) for protein expression. 100 ml aliquots of an overnight culture were subcultured into 1000 ml fresh LB (Luria–Bertani) medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl per litre of solution) containing kanamycin (100 µg ml^{-1}) and allowed to grow to an OD_{600} of 0.6 at 310 K. To obtain the maximum protein expression, we tested several different isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations and found that dicDHPR expression was highest at 0.0625 m*M* IPTG. Therefore, protein expression was induced for 4 h with 0.0625 m*M* IPTG and cells were harvested by centrifugation (6 min, 277 K, 6250*g*). The harvested cells were washed with PBS (8 g NaCl, 0.02 g KCl, 1.45 g Na_2HPO_4, 0.25 g KH_2PO_4 per litre, pH 7.4), resuspended in lysis buffer (50 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl and 5 m*M* imidazole) and disrupted by sonication. After centrifugation (1 h, 13 040*g*) at 277 K, the clear supernatant was filtered (0.45 µm pore diameter; Sartorius, Göttingen, Germany) and applied onto a column of nickel–NTA beads (Quiagen, Hilden, Germany) pre-equilibrated with lysis buffer. The column was washed first with 50 column volumes of lysis buffer and then with 15 column volumes of washing buffer (50 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl and 30 m*M* imidazole). The recombinant dicDHPR was eluted with elution buffer (50 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl and 300 m*M* imidazole). Fractions containing dicDHPR were pooled, concentrated and exchanged into 50 m*M* sodium phosphate pH 6.8 by ultrafiltration (Centriprep YM-10; Millipore Corporation, Bedford, Massachusetts, USA). The dicDHPR was further purified by cation-exchange chromatography on an HS20 column (Perseptive Biosystems, Foster City, California, USA). The protein was eluted with a salt gradient and separated at ∼0.15 *M* NaCl at pH 6.8. The fractions containing dicDHPR were concentrated by ultrafiltration (Centriprep YM-10; Millipore Corporation, Bedford, Massachusetts, USA). The dicDHPR was finally purified by gel-filtration chromatography with a Superdex 200 column (GE Healthcare, Piscataway, New Jersey, USA) in 50 m*M* Tris–HCl pH 8.0 and 150 m*M* NaCl. The protein eluted at 16 ml, corresponding to a molecular weight of ∼50 kDa (dimer size). The fractions were concentrated to a final concentration of 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 by ultrafiltration (Microcon YM-10; Millipore Corporation, Bedford, Massachusetts, USA). The final protein yield was 768 mg per litre. The protein purity was examined by SDS–PAGE and native PAGE and the protein concentration was determined by Bradford assay (Bradford, 1976; Zor & Selinger, 1996). The N-terminal His tag was not removed for crystallization.

2.2. Crystallization and data collection

To prepare the dicDHPR–NAD complex, dicDHPR was incubated with 1 m*M* β-NAD for 30 min. The dicDHPR–NAD complex in 20 m*M* Tris–HCl pH 8.0 was used for all crystallization trials. Initial crystallization was carried out with Crystal Screens I and II and Index Screen from Hampton Research (California, USA) and Wizard Screens I, II, Cryo I and Cryo II from Emerald Biostructures (Bainbridge Island, Washington, USA) using the microbatch crystallization method at 291 K. Drops containing equal volumes (1 µl) of dicDHPR–NAD complex (10 mg ml^{-1} protein) and screening solution were equilibrated under Al's oil in a 72-well microbatch plate.



(*a*) (*b*) (*c*)

Figure 2
Crystals of dihydropteridine reductase from *D. discoideum* (dicDHPR). (*a*) Large plate-shaped crystals obtained from initial screening. (*b*) Rod-shaped crystals obtained after optimization. (*c*) The best large single crystals obtained after controlling the amount of nucleation and the growth rate. The crystal has approximate dimensions of 0.4 × 0.6 × 0.1 mm.

Table 1
Data statistics for dicDHPR crystals.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 49.81, b = 129.90, c = 78.76, β = 100.00
No. of molecules in ASU	4
V_M (Å^3 Da^{-1})	2.24
Wavelength (Å)	1.0000
Resolution (Å)	50–2.16 (2.24–2.16)
Unique reflections	50235 (4669)
Completeness (%)	95.1 (88.1)
R_{merge}† (%)	6.9 (42.3)
Redundancy	6.9 (6.5)
$\langle I/\sigma(I)\rangle$	14.9 (5.7)
Solvent content (%)	45.1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

Screening solution No. 43 (0.1 *M* bis-tris pH 6.5, 25% PEG 3350) from Index Screen produced rod-shaped microcrystals (Fig. 2*a*). Further screenings to find optimal crystallization conditions for crystal growth were accomplished by hanging-drop vapour-diffusion trials varying the salt and precipitant concentrations and the volume of the drop. The volume of reagent in the reservoir was 0.5 ml and the ratio of protein to precipitant solution was 1:1. The most promising condition was found to be 0.1 *M* bis-tris pH 6.5, 17% PEG 3350 (Fig. 2*b*). The best large single crystals were produced in 4 µl drops (2.0 µl protein solution and 2.0 µl well solution; Fig. 2*c*). Crystals grew to maximum dimensions of 0.4 × 0.6 × 0.1 mm in one week. Crystals were flash-frozen in liquid nitrogen for data collection after soaking for 10 min in reservoir solution with 20% ethylene glycol.

X-ray diffraction data for the dicDHPR–NAD complex were collected from a single crystal to 2.16 Å resolution using X-rays of wavelength 1.0000 Å and a Bruker CCD detector at station 4A of the Pohang Accelerator Laboratory, Pohang, Republic of Korea. The crystal-to-detector distance was set to 210.0 mm and a 1° oscillation and 10 s exposure were used per image. All diffraction images were indexed, integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997). Data statistics are shown in Table 1.

3. Results

We have established the expression, purification and crystallization of dicDHPR. A 2.16 Å resolution data set was collected from a crystal of the dicDHPR–NAD complex and processed. The crystal was found to belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 49.81, b = 129. 90, c = 78.76 Å, β = 100.00° (Table 1). The molecular weight of the protein was estimated to be about 50 kDa from gel-filtration chromatography and that of the monomer of dicDHPR to be approximately 25 kDa from SDS–PAGE, which is similar to the theoretical molecular weight of 24.6 kDa. Therefore, the protein exists as a dimer in solution. We solved the structure by molecular replacement using the program *AMoRe* (Navaza & Vernoslova, 1995) with ratDHPR (PDB code 1dhr; Varughese *et al.*, 1992), which shares 58% amino-acid sequence identity, as a search model (correlation coefficient = 72.3, R factor = 55.9% after translation search). There were four molecules in the asymmetric unit, forming two dimers, with a solvent content of 45.1% and a V_M of 2.24 Å^3 Da^{-1} (Matthews, 1968; Table 1). Thus, in the crystal, two dimers were formed by close interactions in the asymmetric unit (Fig. 3). Structure refinement of the dicDHPR complex is in progress.

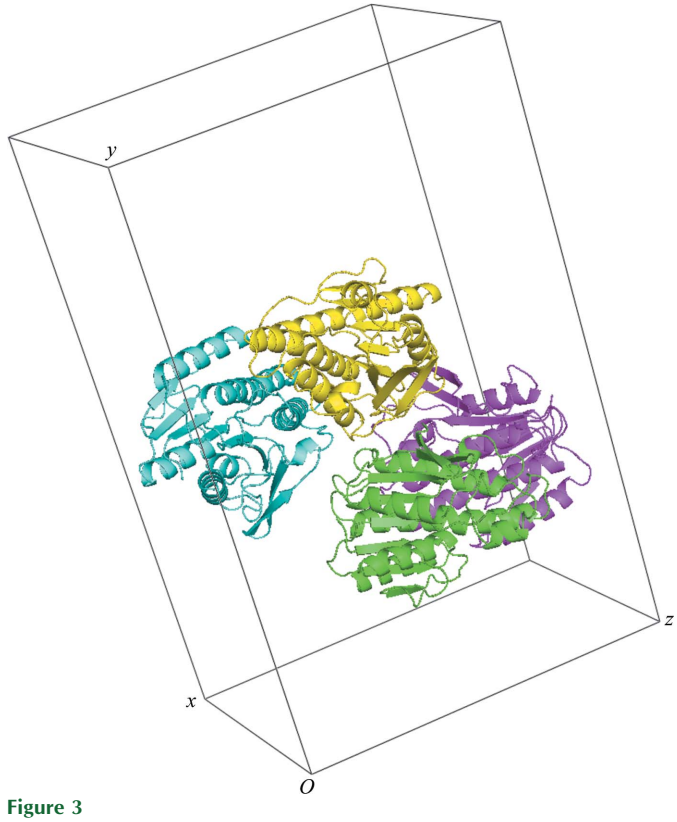


Figure 3
Four molecules of dihydropteridine reductase form two dimers in the asymmetric unit. The molecules are shown in magenta, cyan, yellow and green.

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