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Overexpression, crystallization and preliminary X-ray crystallographic analysis of dihydrofolate reductase from bacteriophage T4

Yun Kyung Oh, Jinho Moon, Jae Young Lee, Sang Woo Cho, Whanchul Shin and Se Won Suh*

Department of Chemistry, College of Natural Sciences, Division of Chemistry and Molecular Engineering, Seoul National University, Seoul 151-742, South Korea

Correspondence e-mail: sewonsuh@snu.ac.kr

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Dihydrofolate reductase (DHFR) from bacteriophage T4 is a homodimer consisting of 193-residue subunits. It has been crystallized in the presence of the cofactor (NADPH) and an inhibitor (aminopterin) at 296 K using sodium chloride as precipitant. The crystals are tetragonal, belonging to the space group $P4_122$ (or $P4_322$), with unit-cell parameters $a = b = 61.14$, $c = 123.23$ Å under cryogenic conditions. The asymmetric unit contains a single subunit, with a corresponding V_m of 2.65 Å^3 Da^{-1} and a solvent content of 53.6%. Native data have been collected from a crystal to 1.9 Å resolution using synchrotron X-rays.

1. Introduction

Dihydrofolate reductase (DHFR; E.C. 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) using NADPH as a cofactor. DHF is the product of thymidylate synthase and must be recycled to the metabolic pool of THF. After reduction of DHF, THF receives one carbon unit and acts as a one-carbon donor in the biosynthesis of purines/pyrimidines and in the interconversion of amino acids. Because DHFR is required to complete the cycle, blockade of the reduction of DHF to THF leads to cell death (Roth, 1986; Schweitzer *et al.*, 1990; Polshakov *et al.*, 1999). Hence, DHFR has long been recognized as a drug target for inhibiting DNA synthesis in rapidly proliferating cells such as cancer cells (Huennekens, 1994) or bacterial or malarial infections (Roth & Stammers, 1992). Three-dimensional structures of DHFRs from *Escherichia coli*, *Lactobacillus casei*, *Leishmania major*, *Pneumocystis carinii*, *Candida albicans*, chicken, mouse and human have been characterized (Sawaya & Kraut, 1997 and references therein).

DHFR from bacteriophage T4 is a homodimer of 193-residue subunits (M_r 21 713 × 2). The structure of T4 DHFR is of interest for several reasons. Firstly, it is a component of a multienzyme complex for deoxyribonucleoside triphosphate (dNTP) synthesis in which at least eight T4 phage-coded enzymes and two enzymes of host origin are found (Wheeler *et al.*, 1996). Secondly, T4 DHFR is distinctive in being a homodimer whereas most other DHFRs are monomeric (Mosher *et al.*, 1977; Purohit *et al.*, 1981). Thirdly, its sequence identity to other DHFRs is very low (below ~25%), particularly toward the carboxyl terminus. The number of identical residues is 43, 46, 43 and 42 with *E. coli*, *L. casei*, chicken and human DHFRs, respectively. The identities are clustered in the amino-terminal residues that participate in binding the cofactor and the substrate or inhibitors (Purohit & Mathews, 1984). Therefore, structural determination of T4 DHFR has been initiated in this study. It has been successfully overexpressed in *E. coli*, purified and crystallized. In this paper, the crystallization conditions and preliminary X-ray data are reported.

2. Experimental

2.1. Protein expression and purification

The T4 *frd* gene coding for dihydrofolate reductase (DHFR) was inserted into *Nde*I/*Xho*I-digested pET22b under the control of the T7 promotor. Transformation was performed by the modified Hanahan method (Hanahan, 1983), using *E. coli* strain C41(DE3) (Miroux & Walker, 1996) for overexpression. C41(DE3)/pET22b-*frd* cells were grown at 310 K in LB medium containing 100 µg ml^{-1} ampicillin. When cultures reached an OD_{600} of 0.6–0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.5 m*M* to induce expression of the T4 *frd* gene. Cultures were grown for an additional ~4 h after IPTG induction. The cells were then harvested by centrifugation at 6 000 rev min^{-1} (Hanil Supra 21K rotor) for 10 min at 281 K. The cell pellet was suspended in 15 volumes of ice-cold lysis buffer (50 m*M* Tris pH 7.5, 0.1 m*M* EDTA) and then homogenized by sonication. The crude lysate was centrifuged at 18 000 rev min^{-1} (Hanil Supra 21K rotor) for 30 min at 281 K and the supernatant fraction was collected. The expression level of T4 DHFR was very high (~50% of

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Table 1
Data-collection statistics.

	Native I	Native II
X-ray wavelength (Å)	1.5418 (Cu $K\alpha$)	1.000 (BL-6A)
Temperature (K)	293	100
Space group	$P4_122$ (or $P4_322$)	$P4_122$ (or $P4_322$)
Unit-cell parameters (Å)	$a = b = 62.00$ (3), $c = 125.76$ (8)	$a = b = 61.14$ (12), $c = 123.23$ (29)
Resolution range (Å)	20–2.2	50–1.9
No. of measured reflections	104888	76830
No. of unique reflections	12530	17645
Completeness (%)	96.9	90.6
$R_{\rm merge}$† (%)	6.3	10.7

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

total proteins) and most of it (~90%) was in the soluble fraction. The supernatant fraction was brought to 35% saturation with solid ammonium sulfate and left for about 2 h at 281 K. The slurry was centrifuged at 18 000 rev min^{-1} (Hanil Supra 21K rotor) for 30 min at 281 K and the precipitate was collected. The pellet was then dissolved in buffer *A* (50 m*M* Tris pH 7.5, 0.1 m*M* EDTA, 200 m*M* NaCl) and dialyzed overnight against 100 volumes of the same buffer. T4 DHFR was purified by three chromatographic steps on an ion-exchange column (Q-Sepharose HiLoad 26/10, Pharmacia), an NADPH affinity column (Red-Sepharose CL-6B, Pharmacia) and a gel-filtration column (Superdex-75 HiLoad 16/60, Pharmacia). The purified T4 DHFR was homogeneous (~99%) as judged by polyacrylamide-gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate. This procedure yielded approximately 60 mg per litre of culture. The purified T4 DHFR was concentrated by ultrafiltration with a YM10 membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm using the calculated extinction coefficient of 1.317 ml mg^{-1} cm^{-1}.

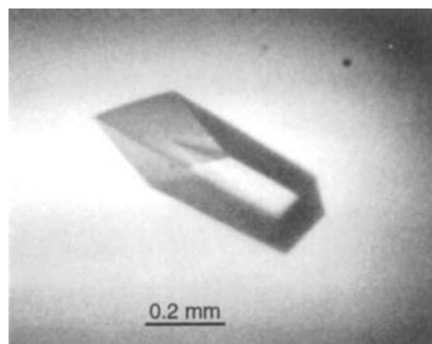

Figure 1
A tetragonal crystal of dihydrofolate reductase from bacteriophage T4. The approximate dimensions of the crystal are 0.2 × 0.2 × 0.6 mm.

2.2. Crystallization

Crystallization was performed by the hanging-drop vapour-diffusion method at 296 K using 24-well tissue-culture plates (Hampton Research). Each hanging drop was prepared on a siliconized cover slip by mixing equal volumes (2–3 µl) of the protein solution and the reservoir solution [100 m*M* Tris–HCl pH 8.1, 2.8 *M* NaCl and 120 m*M* $(NH_4)_2HPO_4$]. The protein solution, at a concentration of 6.5 mg ml^{-1} and containing the cofactor (NADPH) and an inhibitor (aminopterin) in a tenfold molar excess, was incubated for 1 h on ice before mixing with the reservoir solution.

2.3. Data collection

For the first set of native X-ray diffraction data, a crystal was mounted in a thin-walled glass capillary and the capillary was sealed with wax after filling both ends with mother liquor. X-ray experiments were carried out using Cu $K\alpha$ X-rays from a rotating-anode generator with double-mirror focusing optics (Rigaku RU-200BH) running at 50 kV and 50 mA. A set of X-ray diffraction data was collected at 293 K on a MacScience DIP2030 image-plate area-detector system. The data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

The second set of native X-ray diffraction data was collected using the modified Weissenberg camera for macromolecular crystallography at the BL-6A experimental station of the Photon Factory, Tsukuba, Japan (Sakabe *et al.*, 1997). Before flash-freezing the crystal in the nitrogen-gas stream at 100 K, it was dipped for a few seconds into a solution containing 20%(*v*/*v*) glycerol in addition to the reservoir solution. The wavelength of synchrotron X-rays was 1.000 Å and a 0.1 mm collimator was used. One image plate (20 × 40 cm, Fuji BASIII) was placed at a distance of 429.7 mm from the crystal. The oscillation range per frame was 4.5°, with a speed of 1.0° s^{-1} and a coupling constant of 1.0° mm^{-1}. An overlap of 0.5° was allowed between contiguous frames. The number of oscillations per frame was between 12 and 15. The diffraction patterns recorded on the image plates were digitized with an off-line scanner (BAS2000). The raw data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The space group was determined by examining the systematic absences of the X-ray diffraction intensity data.

3. Results

Well diffracting crystals of T4 DHFR were obtained at 296 K when the reservoir solution contained 100 m*M* Tris, 2.8 *M* NaCl and 120 m*M* $(NH_4)_2HPO_4$ at a final pH of 8.2. These crystals grew to approximate dimensions of 0.2 × 0.2 × 0.6 mm within a week (Fig. 1). The crystals diffracted to 2.1 Å resolution with Cu $K\alpha$ X-rays from a rotating-anode source and were very stable in the X-ray beam. They are therefore suitable for structure determination at high resolution. The first set of diffraction data was collected from a native crystal at 293 K using Cu $K\alpha$ radiation. A total of 104 888 reflections were measured, which were merged to 12 530 unique reflections with an $R_{\rm merge}$ (on intensity) of 6.3%. The merged data set is 96.9% complete to 2.20 Å resolution, with the shell completeness between 2.24 and 2.20 Å being 94.3%. The systematic absences indicated that the crystals belong to the tetragonal space group $P4_122$ (or $P4_322$), with unit-cell parameters $a = b = 62.00$ (3), $c = 125.76$ (8) Å.

With synchrotron X-rays, the crystals diffracted to 1.7 Å resolution and a second set of native data extending to 1.9 Å resolution were collected at 100 K. A total of 76 830 reflections were measured, which were merged to 17 645 unique reflections with an $R_{\rm merge}$ (on intensity) of 10.7%. The merged data set is 90.6% complete to 1.9 Å, with the shell completeness between 1.93 and 1.90 Å being 81.2%. Table 1 summarizes the statistics of the data collection. The asymmetric unit contains half a homodimer, giving a crystal volume per protein mass (V_m) of 2.65 Å^3 Da^{-1} and a solvent content of 53.6%. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). Since molecular replacement was not successful, a search for heavy-atom derivatives in order to solve the structure by the multiple isomorphous replacement method is in progress.

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