

# Crystallization and preliminary X-ray crystallographic analysis of deoxycytidylate hydroxymethylase from bacteriophage T4

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Deoxycytidylate hydroxymethylase from bacteriophage T4 is a homodimeric enzyme in which each polypeptide chain consists of 246 amino-acid residues. It has been crystallized in the presence of its substrate, deoxycytidine monophosphate, at room temperature using sodium citrate as precipitant. The crystals are monoclinic, belonging to space group *C*2, with unit-cell parameters  $a = 174.22$ ,  $b = 53.12$ ,  $c = 75.17$  Å,  $\beta = 115.29^\circ$ . The asymmetric unit contains one homodimer, with a corresponding  $V_m$  of  $2.65$  Å<sup>3</sup> Da<sup>-1</sup> and solvent content of 54%. Native diffraction data to 1.6 Å resolution have been collected from two crystals using synchrotron radiation.

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## 1. Introduction

Bacteriophage T4 modifies deoxycytidine monophosphate (dCMP) into 5-hydroxymethyl-dCMP (Hm-dCMP) using a virus-encoded enzyme, deoxycytidylate hydroxymethylase (CH; E.C. 2.1.2.8), for its own protection against a restriction system of its host *Escherichia coli*. Hydroxymethyl-deoxycytidine triphosphate (Hm-dCTP), synthesized from Hm-dCMP by the action of T4 deoxynucleoside monophosphate kinase and *E. coli* nucleoside diphosphate kinase, is incorporated into the viral DNA by T4 DNA polymerase. Hydroxymethyl-dCMP residues of the T4 DNA are modified at the second level by glucosylation (Carlson *et al.*, 1994; Greenberg *et al.*, 1994).

T4 CH is a homodimer in which each subunit comprises 246 amino-acid residues (subunit  $M_r = 28450$ ). It is a component of the multienzyme complex known as deoxynucleoside triphosphate (dNTP) synthetase. It has been shown to interact with a number of proteins involved in deoxynucleoside triphosphate (dNTP) biosynthesis and in T4 DNA replication (Wheeler *et al.*, 1992). Gene 42 of T4, encoding CH, has been sequenced (Lamm *et al.*, 1988; Thylen, 1988) and overexpressed (Lamm *et al.*, 1988). T4 CH shows a very low amino-acid sequence identity (19–21%) with thymidylate synthases from T4 and *E. coli*. It also shows a limited sequence similarity with the N-terminal two-thirds of deoxyuridylate hydroxymethylase from bacteriophage SPO1 of *Bacillus subtilis* (Lamm *et al.*, 1987, 1988; Wilhelm & Rüger, 1992). The catalytic mechanism has been proposed for T4 CH by analogy with that for thymidylate synthases (Graves *et al.*, 1992). <sup>18</sup>O-exchange experiments have identified the final exocyclic methylene intermediate formed during the

proposed mechanism (Butler *et al.*, 1994). Roles in cofactor binding and in catalysis of residues Glu60, Cys148, and Asp179 of T4 CH have been examined by site-directed mutagenesis (Graves *et al.*, 1992; Hardy *et al.*, 1995).

T4 CH is an interesting enzyme for structure elucidation owing to its distinct substrate specificity and catalytic activity. To date, no three-dimensional structure of either dCMP or dUMP hydroxymethylase has been reported, although bacteriophage SPO1 dUMP hydroxymethylase has been crystallized (Schellenberger *et al.*, 1995). In order to provide insight into the high substrate specificity, the catalytic mechanism and the possible interactions with other components of the dNTP-synthesizing complex, we have initiated the structure determination of T4 CH. As the first step, crystals diffracting to at least 1.6 Å resolution have been produced. Here, we report the crystallization and preliminary X-ray crystallographic studies of T4 CH.

## 2. Experimental

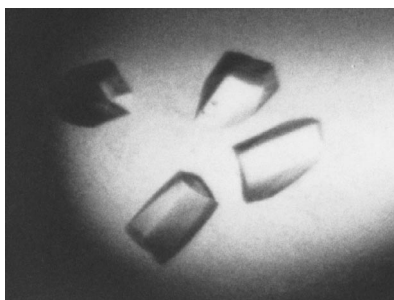
### 2.1. Protein expression and purification

The gene encoding T4 CH was amplified by PCR using T4 genomic DNA as template. The amplified DNA was inserted into *NdeI/XhoI*-digested pET-22b. This vector construction, designated as pET-22b-CH, added six histidine residues to the C-terminus of the gene product to facilitate protein purification. The complete nucleotide sequence of the insert was confirmed by dideoxy-DNA sequencing performed at the Research Center for Microbiology, Seoul National University. The enzyme was highly overexpressed in soluble form in BL21(DE3)pLysS cells upon induction by 0.5 mM IPTG at 310 K. Cells were grown in LB medium for 4 h after IPTG induction and

were harvested by centrifugation at 6000 rev min<sup>-1</sup> (Sorvall GSA rotor) for 7 min at 281 K. The cell pellet was resuspended in ice-cold lysis buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 50 mM imidazole) and was then homogenized by sonication. The crude lysate was centrifuged at 36000g (18000 rev min<sup>-1</sup>, Hanil Supra 21K rotor) for 1 h at 281 K, and the recombinant protein in the supernatant fraction was purified by three chromatography steps. The first step utilized the C-terminal histidine-tag by metal-chelate chromatography on Ni-NTA resin (Qiagen). After buffer exchange by gel filtration, an ion-exchange chromatographic step was performed on Source 15Q resin packed in a HR 10/10 column (Pharmacia), which had previously been equilibrated with buffer A (20 mM sodium phosphate pH 6.7). The protein was eluted with a linear gradient of 0–0.4 M sodium chloride in buffer A. Further purification was achieved by gel filtration on a HiLoad 16/60 Superdex 200 preparation-grade column (Pharmacia), which had previously been equilibrated with buffer A containing 150 mM sodium chloride, 1 mM EDTA and 1 mM β-mercaptoethanol. The purified T4 CH was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate (Laemmli, 1970). This procedure yielded approximately 60 mg of homogeneous T4 CH from a 3 l culture. The protein solution was concentrated using a YM 10 membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing the absorption coefficient 2.28 mg<sup>-1</sup> ml cm<sup>-1</sup>.

## 2.2. Dynamic light-scattering studies

The dynamic light-scattering experiment was performed on a Model DynaPro-801 instrument from Protein Solutions (Charlottesville, Virginia). The data were



**Figure 1**  
Monoclinic crystals of deoxycytidylate hydroxymethylase from bacteriophage T4 grown in the presence of its substrate dCMP. Approximate dimensions of the crystals are 0.7 × 0.4 × 0.3 mm.

measured at room temperature with 1 mg ml<sup>-1</sup> of protein in 20 mM sodium phosphate pH 6.7, 1 mM EDTA, 1 mM β-mercaptoethanol and 150 mM NaCl.

## 2.3. Crystallization

Crystallization was performed by the hanging-drop vapour-diffusion method at room temperature (295–297 K) using 24-well tissue-culture plates (Flow Laboratories). The hanging drop on a siliconized cover slip was prepared by mixing equal volumes (4 μl each) of the protein solution and the reservoir solution. The protein solution was prepared by addition of dCMP to the apoenzyme in tenfold molar excess followed by a 1 h incubation on ice. The protein concentration was 9 mg ml<sup>-1</sup> prior to mixing with the reservoir solution. The hanging drop was placed over 1 ml reservoir solution.

## 2.4. X-ray diffraction analysis

A crystal was mounted in a thin-walled glass capillary and the capillary was sealed with wax, after filling both ends with the mother liquor. Initial X-ray experiments were carried out using graphite-monochromated Cu Kα radiation from a rotating-anode generator (Rigaku RU-200BH) operating at 40 kV and 70 mA with a 0.3 mm focus cup. The first set of X-ray diffraction data was collected at 293 K on a FAST area-detector system (Enraf-Nonius) using the MADNES software (Messerschmidt & Pflugrath, 1987). The unit-cell parameters were determined using the autoindexing and parameter-refinement procedure of the MADNES software. The reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled by the Fourier scaling program (Weissman, 1982). The second set of native diffraction data was collected at 290 K using a large-format image plate and Weissenberg camera for macromolecular crystallography at the BL-6B experimental station of the Photon Factory, Tsukuba, Japan (Sakabe *et al.*, 1997). The wavelength was 1.000 Å and a 0.1 mm collimator was used. Two large image plates (400 × 800 mm, Fuji BASIII) were placed at a distance of 573 mm from the crystal. The oscillation range per image plate was 5.5°, with a rate of 2.0° s<sup>-1</sup> and a coupling constant of 1.5° mm<sup>-1</sup>. An overlap of 0.5° was allowed between contiguous image plates. The diffraction patterns recorded on the image plates were digitized by the off-line scanner IPR4080. Two sets of data with a total oscillation of 180° from one crystal and a further 50° from another

**Table 1**  
Synchrotron data-collection statistics.

Number of crystals	2
Space group	C2
Unit-cell parameters (Å, °)	$a = 174.22, b = 53.12,$ $c = 75.17,$ $\beta = 115.29$
Number of measured reflections	430634
Number of unique reflections	80033
$R_{\text{merge}}^{\dagger}$ (%)	6.6
Data completeness (%)	
50–1.6 Å	97.3
1.7–1.6 Å	83.9

<sup>†</sup>  $R_{\text{merge}} = \sum_h \sum_i |I(h)_i - \langle I(h) \rangle| / \sum_h \sum_i I(h)_i$ , 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  $h$ .

crystal with a different orientation were merged. The data were processed and scaled using the programs DENZO (Otwinowski, 1993) and SCALEPACK (Otwinowski, 1993). The space group was determined by examining the systematic absences of the intensity data.

## 3. Results

The molecular mass of 74 kDa, as estimated by the dynamic light-scattering analysis, indicates that T4 CH exists as a dimer. The presence of dCMP improved the dynamic light-scattering behavior of T4 CH, resulting in a polydispersity of 28%. Well diffracting crystals were obtained in the presence of the substrate dCMP using citrate as precipitating agent. They grew to dimensions of 0.7 × 0.4 × 0.3 mm within a week (Fig. 1) under the optimized reservoir conditions of 100 mM Tris-HCl and 0.9 M sodium citrate at final pH of 8.73. These crystals grew only when the substrate dCMP was present in the protein hanging drop.

The crystals diffracted to 1.9 Å resolution with Cu Kα X-rays from a rotating-anode source and were very stable in the X-ray beam. Thus, they are suitable for structure determination at high resolution. The first set of diffraction data has been collected from a native crystal using Cu Kα radiation. A total of 76735 reflections were measured, which were merged into 24844 unique reflections with an  $R_{\text{merge}}$  (on intensity) of 3.5%. The merged data set is 85.4% complete to 2.3 Å. The systematic absences indicated that the crystal belongs to the monoclinic space group C2, with unit-cell parameters  $a = 175.27, b = 53.25, c = 75.48$  Å,  $\beta = 115.51^\circ$ .

With synchrotron radiation, a second set of native data extending to 1.6 Å resolution has been collected. A total of 430634 reflections were measured, which were

merged into 80033 unique reflections with an  $R_{\text{merge}}$  (on intensity) of 6.6% (rejecting 2.7% outliers). The merged data set is 97.3% complete to 1.6 Å, with the shell completeness between 1.7 and 1.6 Å being 83.9%. Table 1 summarizes the statistics for the synchrotron data collection. The asymmetric unit contains one molecule of homodimer, giving a crystal volume per protein mass ( $V_m$ ) of 2.65 Å<sup>3</sup> Da<sup>-1</sup> and a solvent content of 54%. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). We are now in the process of solving the three-dimensional structure by multiple isomorphous replacement.

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