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Crystallization and Preliminary Crystallographic Study of 26 kDa *Clonorchis sinensis* glutathione S-transferase Complexed with Inhibitors

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Abstract

A helminth glutathione S-transfease, 26 kDa isozyme from *Clonorchis sinensis* was cocrystallized with inhibitors by the hanging-drop method with monomethylether 550 as a precipitant. The crystals, grown in the presence of S-methylglutathione or *trans*-4-phenyl-3-buten-2-one, suitable for X-ray analysis, belong to the hexagonal space group $P6_2$ (or $P6_4$), with unit cell parameters a = b = 97.6 Å, c = 117.4 Å. X-ray diffraction data were collected with 2.5 Å resolution.

1. Introduction

Glutathione S-transferases (GSTs) are a family of multifunctional enzymes involved in cellular detoxification of xenobiotics and reactive endogenous compound of oxidative metabolism.[1] They catalyze the nucleophilic addition of glutathione (GSH, γ-Glu-Cys-Gly) to a wide variety of electrophilic compounds including alkyl and lactones, expoxides, arylhalides, and activated alkens. They are also involved in the development of resistance of cells to chemotherapeutic agents, herbicides, pesticides, and radiation.[2,3] Drug resistance appears due to increased glutathione S-transferase activity and can limit the effectiveness of many chemotherapeutic agents. Cytosoic GSTs have been grouped into at least seven classes, alpha, mu, phi, theta, sigma, kappa,[4] and zeta,[5] based on their specificities to substrates and inhibitors, immunologic activity, and amino acid sequence identities. A number of three-dimensional structures of GSTs have been determined by X-ray crystallography and revealed that the monomer of all functional GSTs is composed of two domains, a smaller N-terminal alpha/beta-domain and a larger C-terminal alpha-domain. Although GSTs share such common folding pattern, each structure has characteristic features, particularly concerning the substrate-binding site.

Helminths contain two distinct classes of GSTs from Mr = 26,000 to 28,000 with differing substrate specificity.[6] The GSTs may provide the worms primary defense against electrophilic and oxidative damages.[7] The enzymes are known as potential chemo- and immuno-therapeutic targets.[8,9] So far, crystal structures have been determined for isozymes from *Schistosoma japonicum*[10,11] and *Fasciola hepatica*.[12] We have crystallized a 26 kDa GST from *Clonorchis sinensis* (*Cs*GST) complexed with inhibitors as the first step to determine 3-dimensional structure, and we report here the preliminary X-ray diffraction results for the crystals.

2. Materials and Method

2-1. Purification

*Cs*GST was purified by a GSH-affinity chromatography from *E. coli* BL21 [DE3] pLysS, which contains pET23a(+) expression vector (NOVAGEN, Germany) carrying *Cs*GST gene (Hong *et al.*, unpublished work). The cells were grown in broth medium containing 50 mg/m*l* ampicillin until the $O.D_{600}$ was about 0.7, and then 1 mM IPTG was added. After 4 h incubation at 37 °C, harvested cells were suspended in 10 m*l* of 20 mM potassium phosphate buffer (pH = 7.0) containing 1 mM EDTA and 1 mM PMSF, and were disrupted by ultrasonication.

The supernatant of *E. coli* lysate was applied onto a GSH-affinity column. After the column was washed with 20 mM potassium phosphate buffer containing 1 mM EDTA, the *Cs*GST was eluted with 50 mM Tris-Cl buffer (pH = 9.6) containing 15 mM GSH. Activity of the eluted fractions was assayed by the procedure of Habig *et al.*[13] Protein purity was assessed by densitometry of SDS-PAGE gels loaded with dilutions of the concentrated sample and visualized by Coomasie-Blue staining. Protein concentration was determined by the method of Lowry *et al.*[14]

2-2. Crystallization

The sample was dialyzed against 5 mM potassium phosphate buffer (pH = 7.0) and then concentrated with a Centricon concentrator (Amicon corporation) until the protein concentration reached 15~20 mg/m*l*. Inhibitors were dissolved in water and mixed with the protein solution to a final concentration of about 5 mM. The mixture was then incubated at 20 °C for 3 h and used for subsequent crystallization experiments. Initial crystallization conditions were screened according to sparse-matrix methods[15] using a hanging-drop vapour diffusion method. In order to demonstrate that protein in crystal retains intact molecular size, crystals were carefully washed with the reservoir solution and dissolved in a phosphate buffer. The dissolved crystals were then analyzed by SDS-PAGE.

2-3. Preliminary X-ray diffraction analysis

Diffraction data were collected at room temperature on a DIP-2000 imaging plate detector (MAC Science Co.) with Cu Kα radiation from a MAC Science M06XHF rotating anode X-ray generator operated at 50 kV and 90 mA. A total of 90 data frames were measured, with an exposure of 15 minutes and an oscillation angle of 1.0 degree per frame. Crystal-to-detector distance was set to 120 mm. The raw data were processed to 2.5 Å by DENZO and scaled by SCALEPACK.[16]

3. Results and Discussion

Large single crystals of *Cs*GST complexed with S-methylglutathione or *trans*-4-phenyl-3-buten-2-one were obtained from a 2.0 M ammonium sulfate in 0.05 M Tris-Cl buffer (pH = 8.5) by the hanging-drop vapor diffusion method. The pencillike crystals appeared within a day and grew up to 0.5 × 0.5 × 0.7 mm in about 4 days (Fig. 1). SDS-PAGE analysis (Fig. 2) of the protein sample used for crystallization and the dissolved crystals showed a strong band on the SDS-PAGE gel at approximately 26 kDa, indicating that crystals contain fully intact *Cs*GST.

(a)

(b)

Fig. 1. (a) A crystal of *Cs*GST/S-methylglutathione complex; (b) A crystal of *Cs*GST/*trans*-4-phenyl-3-buten-2-one complex.

The crystals diffracted to at least 2.5 Å resolution as observed in 15-minute-oscillation images recorded on a DIP2000 imaging plate system with Cu Kα radiation (4.5 kW). X-ray diffraction data showed that the crystals belong to the hexagonal space group $P6_2$ (or $P6_4$) with cell parameters a = 97.6 Å, and c = 117.4 Å. Assuming one dimer molecule of *Cs*GST (molecular mass 25.5 kDa) per crystallo-

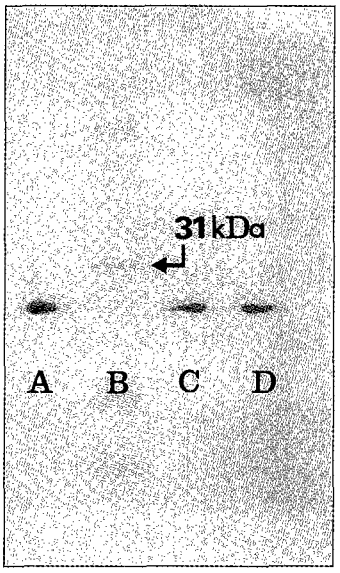

Fig. 2. Coomasie-blue stained SDS-PAGE gel of dissolved crystals and protein samples used for obtaining the crystals. Lane A: CsGST used for crystallization; Lane B: Marker proteins; Lane C: Dissolved crystal of *Cs*GST/S-methylglutathione complex; Lane D: Dissolved crystal of *Cs*GST/*trans*-4-phenyl-3-buten-2-one complex.

graphic asymmetric unit and a partial specific volume of 0.74 cm^3/g, the crystal volume per protein mass, V_m,[17] is 2.74 $Å^3$/dalton, which is within the normal range for protein crystals, and the solvent content of the crystal was estimated to be about 54.6%.

Recently, orthorhombic crystals of *Cs*GST and its fusion proteins with peptides of different lengths were grown from a monomethylether 550 solution containing zinc sulfate (Han *et al.*, submitted for publication). We proposed that the crystallization system may be generally applicable to obtain crystals of small peptides. Therefore, the new crystal form of the *Cs*GST/inhibitor complex is expected to provide an alternative way to crystallize small peptide via *Cs*GST-fusion proteins.

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