Expression, Purification, Crystallization, and Preliminary X-Ray Analysis of the Human UDP-Glucose Dehydrogenase

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Abstract: UDP-glucose dehydrogenase (UGDH) catalyzes the synthesis of UDP-glucuronic acid from UDP-glucose resulting in the formation of proteoglycans that are involved in promoting normal cellular growth and migration. Overproduction of proteoglycans has been implicated in the progression of certain epithelial cancers. Here, human UGDH (hUGDH) was purified and crystallized from a solution of 0.2 M ammonium sulfate, 0.1 M Na cacodylate, pH 6.5, and 21% PEG 8000. Diffraction data were collected to a resolution of 2.8 Å. The crystal belongs to the orthorhombic space group P2_12_1, with unit-cell parameters a = 173.25, b = 191.16, c = 225.94 Å, and α = β = γ = 90.0°. Based on preliminary analysis of the diffraction data, we propose that the biological unit of hUGDH is a tetramer.

Keywords: UGDH, GMDH, crystallization, UDP-glucose, UDP-glucuronic acid.

INTRODUCTION

UDP-glucose dehydrogenase (UGDH; EC 1.1.1.22) catalyzes the synthesis of UDP-glucuronic acid from UDP-glucose [1]. UDP-glucuronic acid is an important intermediate in mammalian carbohydrate metabolism. In connective tissues it is primarily converted to proteoglycans such as hyaluronan, which is an extracellular matrix component that is directly involved in promoting normal cellular growth and migration. Excessive production of proteoglycans has been implicated in the progression of epithelial cancers such as breast [2], colon [3], and prostate [4]. Inhibition of hyaluronan synthesis has been shown to diminish tumor angiogenesis and thereby impede the growth of human prostate tumors in vivo [5]. Restriction of the availability of the hyaluronan precursor by inhibition of hUGDH is a potential strategy for regulating the production of hyaluronan in tumors. Hence, hUGDH represents an attractive therapeutic target for the development of new anticancer agents.

Bovine liver UGDH has been the most widely studied form of this protein. UGDH is a member of a small group of NAD^+-dependent four-electron-transfer dehydrogenases and has been proposed to function as a "trimer of dimers" [6, 7]. Recently, human UGDH (hUGDH) was cloned, expressed, and affinity-purified from Escherichia coli [8]. The hUGDH cDNA encodes a 494-amino acid protein that has 98% identity to bovine UGDH. The enzyme is active as a hexamer of 57-kDa subunits, and initial evidence suggests that, like the bovine enzyme, it assembles as a trimer of dimers.

The 1.8-Å resolution structure of Streptococcus pyogenes UGDH in complex with NAD^+ as a cofactor and UDP-xylose as a substrate has been reported [9]. This S. pyogenes ortholog shows 33% overall identity to hUGDH, although the NAD^+ binding and catalytic sites share 100% and 60% identity, respectively [10]. The biological unit of this bacterial UGDH is a homodimer, with a single chain comprising the asymmetric unit of the crystal. No other structure of UGDH has been reported, however the structure of GDP-mannose dehydrogenase (GMDH) from Pseudomonas aeruginosa has been solved at 1.55 Å resolution [11]. GMDH is another member of the NAD^+-dependent four-electron-transfer dehydrogenase family and shares 32% identity with hUGDH. GMDH and UGDH are thought to utilize similar mechanisms to catalyze the two-step conversion of an alcohol to the corresponding acid via a thiohemiacetal intermediate. The P. aeruginosa enzyme exists as a homotetramer in the crystal asymmetric unit, although the biological unit remains unclear. This enzyme shows both allosteric and cooperative behavior, implying that its biological activity is dependent on oligomerization [12].

We have recently reported the chemical synthesis of a gene encoding hUGDH and its overexpression in E. coli as a soluble protein. We also identified the NAD^+ and UDP-glucose binding sites by photoaffinity labeling and cassette mutagenesis [13, 14]. We intend to further investigate the structural basis of the catalytic mechanism with a view to anticancer drug design. Here, we report the first step in this process with the crystallization and preliminary crystallographic analysis of hUGDH.

MATERIALS AND METHODS

Protein Purification and Enzyme Assay

The wild-type hUGDH was purified as described previously [13]. Briefly, fresh overnight cultures of the trans-
formed cells were used to inoculate 1 L of Luria-Bertani medium containing 100 µg ml⁻¹ ampicillin. The cells were then grown at 310 K until the OD₆₀₀ reached 1.0. The culture was treated for 3 h at 310 K with 1 mM isopropyl β-D-thiogalactopyranoside to induce expression of the protein. Cell pellets were collected and suspended in 100 ml of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 5 mM dithiothreitol and then sonicated. The lysates were centrifuged at 25,000 _ g for 20 min at 277 K, and the supernatant was loaded onto a Blue Sepharose CL-6B column equilibrated with 20 mM Tris-HCl (pH 6.5), 5 mM MgCl₂, and 10 mM β-mercaptoethanol. The enzyme was eluted by a gradient of 0 to 500 mM NaCl. The fractions containing hUGDH were pooled and applied to a FPLC Resource-Q column equilibrated with 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 10 mM β-mercaptoethanol. The enzyme was then eluted using a linear gradient of NaCl (0–300 mM) at 3 ml/min.

The purified enzyme was analyzed by SDS-PAGE, followed by Western blotting using monoclonal antibodies against the hUGDH. The activity of hUGDH was determined spectrophotometrically by measuring the reduction of NAD⁺ in the presence of UDP-glucose at 340 nm [13]. Reactions were performed in a total volume of 1 ml with hUGDH adjusted to give a measured rate of less than 0.04 absorbance units/min. One unit of hUGDH activity was defined as the amount of enzyme required to reduce 2 mM of NAD⁺ min⁻¹ at pH 8.7 and room temperature. The highly active, highly purified fractions were pooled and concentrated for crystallization trials.

Crystallization and Data Collection

Initial screening for protein crystallization was carried out using the sitting-drop vapor-diffusion method in 96-well intelli-plates (Art Robbins Instruments). A HYDRA96 (Robbins Scientific) automated dispenser was used to mix equal volumes (0.2 µl) of the protein and reservoir solutions. Each sitting drop was placed over 0.1 ml of reservoir solution. The following crystallization screening kits were used (number of buffer conditions in parentheses): Index (96), SaltRx (96), PEG/Ion (48), crystal screen I (48), crystal screen II (48), crystal screen lite (48), grid screen (48), grid screen ammonium sulfate (24), Grid screen PEG 6000 (24), Grid screen MPD (24), Grid screen PEG/LCl (24), Grid screen NaCl (24), Grid screen Na malonate (24), Quik screen (24), Natrix (48), MembFac (48) (Hampton Research), Precipitant Synergy Primary 64 crystallization screen (64), Precipitant Synergy Expanded with 33% of Primary (64), Precipitant Synergy Expanded with 67% of Primary (64), Wizard I (48), Wizard II (48), Wizard cryo I (48), Wizard cryo II (48) (Emerald Biosystems), 3D-1 (24), and 3D-2 (24) (Molecular Dimensions). All crystallization trials were performed at room temperature (295 ± 1 K).

Construction of the Deletion Mutant

Plasmid DNA was digested with BamHI and HindIII deleting a 31-base fragment encoding amino acids 488–494, and the vector DNA was purified by electrophoresis through a 1% low-melting point agarose gel. The 31-base BamHI/HindIII fragment was replaced with a 10-base synthetic DNA duplex to generate the deletion mutant. Mutagenized oligonucleotides were annealed, ligated into the pUC18 vector, and transformed into DH5α cells, and the deletion mutant was identified and verified by DNA sequencing. The plasmid encoding the truncated protein was then transformed into E. coli strain BL21(DE3) for protein production.

RESULTS AND DISCUSSIONS

Crystals of wild-type hUGDH were obtained in several conditions as shown in (Fig. 1). Crystals formed from the precipitate after 5 days and grew to a largest dimension of 0.1 mm. 25% (v/v) ethylene glycol was added to the crystallization mixture before flash freezing in liquid nitrogen. The crystals were placed in an X-ray beam at the 4A Macromolecular Crystallography Wiggler Beamline of the Pohang Accelerator Laboratory (Pohang, Korea). These wild-type hUGDH crystals did not diffract to a suitable resolution for structure determination.

We then used molecular biology to generate a truncated enzyme from the wild-type synthetic hUGDH gene which lacked the last 7 amino acids (488–494) that comprise a flexible region in the bacterial UGDH crystal structure [9]. Purification and crystallization screening of the truncated hUGDH were performed using the same procedure as the wild-type hUGDH. Crystals of the mutant hUGDH were obtained under three conditions: (i) 0.2 M ammonium sulfate, 0.1 M Na cacodylate pH 6.5, and 30% PEG 8000; (ii) 0.2 M ammonium sulfate, 0.1 M HEPES pH 7.5, and 25% PEG 3350; and (iii) 0.2 M lithium sulfate, 0.1 M HEPES pH 7.5, and 25% PEG 3350.

Optimization of the crystallization was conducted and best crystals of the truncated hUGDH were grown by equilibrating a mixture containing a 1:µl protein solution (10 mg ml⁻¹ protein in 50 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 10 mM β-mercaptoethanol, 5 mM UDP-glucose, 2 mM NAD⁺, and 7% glycerol) and 1 µl reservoir solution (0.2 M ammonium sulfate, 0.1 M Na cacodylate [pH 6.5], and 21% PEG 8000) against 0.5 ml of reservoir solution as shown in (Fig. 2). A data set to 2.8 Å was collected at the 4A Macromolecular Crystallography Wiggler Beamline of the Pohang Accelerator Laboratory (Pohang, Korea) using the X-ray beam at a single wavelength (1.00 Å ) and 1° oscillations. The data set was indexed and processed with the HKL-2000 suite of programs [15]. Data collection statistics are given in Table 1.

The crystals are of the space group P2₁2₁2₁, with unit-cell parameters a = 173.25, b = 191.16, c = 225.94 Å, and α = β = γ = 90.0°. These dimensions are sufficiently large that a 50% level of occupation by protein atoms is consistent with two homohexamers being present in the asymmetric unit. Initial attempts to solve the structure of hUGDH by molecular replacement using template structure of bacterial UGDH homodimer produced two clearly placed dimers. These two molecules are related by a translation of 1/2 along the x-axis and are supported by a strong corresponding peak in the native Patterson function. Crystallographic symmetry reproduces the tetramer arrangement observed in GMDH for both molecules. The self rotation function only shows peaks consistent with two fold symmetry, which in turn is consistent with the tetrameric structure observed for GMDH and not
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**Figure 1.** Crystals of the wild-type hUGDH. A. 20% PEG1000 and 0.1 M Tris, pH 8.5. B. 0.2 M magnesium acetate and 20% PEG3350, pH 7.7. C. 0.2 M ammonium sulfate and 20% PEG3350, pH 6.0. D. 15% PEG4000, 0.1 M Tris, pH 8.5, and 0.2 M sodium acetate. These crystals did not provide diffraction that was of sufficient quality for structural analysis.

**Figure 2.** A crystal of the truncated hUGDH grown in a solution containing 0.2 M ammonium sulfate, 0.1 M Na cacodylate, pH 6.5, and 21% PEG 8000. This crystal diffracted to 2.8 Å.

consistent with a hexameric arrangement. We have not managed to locate the positions of the outstanding molecules in the asymmetric unit and are now preparing selenium me-thionine hUGDH to adopt a MAD approach. However, based on this initial analysis we assert that hUGDH is likely to be functional as a tetramer rather than as a hexamer. Furthermore, as the tetrameric arrangement of GMDH is repeated in hUGDH, we propose that this tetrameric form represents the biological unit of both families of the enzymes.

### Table 1. Data-Collection Statistics

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<td>$I/σ(I)$</td>
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$R_{int} = \frac{\sum_h|I(h) - \langle I(h)\rangle|}{\sum_h I(h)}$, where $I$ is the intensity of reflection $h$, $\Sigma_h$ is the sum over all reflections, and $\Sigma_i$ is the sum over $i$ measurements of reflection $h$. Values in parentheses refer to data in the highest resolution shell.
ACKNOWLEDGEMENTS

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ABBREVIATIONS

GMDH = GDP-mannose dehydrogenase
hUGDH = Human UDP-glucose dehydrogenase
UGDH = UDP-glucose dehydrogenase

REFERENCES