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Cloning, expression, purification, crystallization and preliminary X-ray analysis of human liver glyceraldehyde-3-phosphate dehydrogenase

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Human liver glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purified and crystallized using PEG 3350 as a precipitant. However, the crystals were extremely fragile towards osmotic shock. A 1% change in PEG 3350 content causes destruction of the crystals. After many trials for freezing the crystals, X-ray diffraction data from a native crystal were collected at 2.8 Å resolution using as a cryoprotectant a mixture consisting of paraffin oil and Paratone-N in a 3:1 ratio and a cryoloop covered with Formver film. Crystals belong to space group $P2_1$, with unit-cell parameters a = 63.23, b = 97.84, c = 84.23 Å, β = 104.1°. Molecular replacement with a starting model consisting of a homology model based on the low-resolution structure of human skeletal muscle GAPDH, which has 90% identical residues with the liver protein, led to a solution. Most of the current model was assigned properly in the electron-density map, but the map corresponding to some important regions containing the phosphate-binding loop was ambiguous. It is planned to crystallize human liver GAPDH in the presence of phosphate ions and/or some kind of inhibitor in order to fix the flexible region.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is an enzyme essential for glycolysis in both prokaryotes and eukaryotes. This enzyme functions as a homotetramer with a molecular weight of approximately 150 kDa and catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate (BPG) using the cofactor NAD^+. The first reaction step involves the formation of a hemiacetal intermediate between GAP and a cysteine residue; this hemiacetal intermediate is then oxidized to a thioester, with concomitant reduction of NAD^+ to NADH. The reduced NADH is then exchanged with the second NAD^+ and the thioester is attacked by a nucleophilic inorganic phosphate to produce BPG (Didierjean *et al.*, 2003).

The crystal structures of NAD^+-bound GAPDHs from various organisms have been determined at various resolutions, including those from the American lobster (Moras *et al.*, 1975), the Chinese lobster (Song *et al.*, 1998, 1999), human skeletal muscle (Mercer *et al.*, 1976), *Bacillus stearothermophilus* (Skarzynski *et al.*, 1987), *Thermotoga maritima* (Korndorfer *et al.*, 1995), *Thermus aquaticus* (Tanner *et al.*, 1996), *Escherichia coli* (Duée *et al.*, 1996; Yun *et al.*, 2000), *Leishmania mexicana* (Kim *et al.*, 1995; Kim & Hol, 1998), *Trypanosoma brucei* (Vellieux *et al.*, 1993, 1995) and *Trypanosoma cruzi* (Souza *et al.*, 1998). The active-site cavity is very large in order to tolerate binding substrates, glyceraldehyde 3-phosphate and phosphate ions and the cofactor NAD^+. The active centres, including the active cysteine residue and the nicotinamide moiety of NAD^+, are completely conserved in the three-dimensional structures of all GAPDHs as well as in terms of amino-acid sequence. On the other hand, structures around the adenine-binding site and the phosphate-binding site vary. In fact, using the differences around the adenine-binding site, a trypanosomal inhibitor with selectivity relative to humans (skeletal) has successfully been obtained (Verlinde *et al.*, 1994). However, selectivity against human liver may also be important in avoiding side effects and/or liver toxicity. A single mutation occurs between human skeletal and liver GAPDH around the adenine-binding site; the loop around the phosphate-binding site is quite different in terms of amino-acid sequence. Therefore, structural study of the mutation sites in human liver GAPDH is also important in obtaining selectivity between human and other organisms, such as *Trypanosoma*. We report here the crystallization and preliminary crystallographic study of human liver GAPDH as the first step towards the discovery of safe drugs.

2. Methods

2.1. Cloning and expression

GAPDH was PCR-amplified using human liver Marathon-Ready cDNA (Clontech) and

cloned into the pTrcHis B vector (Invitrogen) by the *Xho*I and *Eco*RI enzymes. Sequencing of the recombinant DNA, performed with a PRISM310 genetic analyser (Applied Biosystem), confirmed the integrity of the cloned DNA. Luria broth medium containing 50 mg ml^{-1} ampicillin was inoculated with a preculture of the DH5α strain (Toyobo) containing the GAPDH construct. Bacterial growth was performed for 1.5 h at 310 K. Expression was induced with 1 m*M* isopropyl-1-β-D-1-thiogalactopyranoside for 4 h at 310 K. The cells were harvested by centrifugation and the pellet was stored frozen at 213 K. At the time of purification, the cell pellet was thawed and cells were resuspended in buffer *A* (25 m*M* Tris–HCl, 20 m*M* 2-mercaptoethanol, 0.5% Tween 20 and 0.5 mg ml^{-1} lysozyme pH 7.4). The suspension was sonicated and cell debris was removed by centrifugation.

2.2. Purification

After the addition of 2.5 *M* NaCl, the supernatant was loaded onto an Ni–NTA column (Amersham Pharmacia) and washed with five column volumes of buffer *B* (25 m*M* Tris–HCl, 250 m*M* NaCl and 20 m*M* 2-mercaptethanol pH 7.4). The proteins were eluted by three column volumes of buffer *B* with 200 m*M* imidazole. After dialysis against buffer *C* (25 m*M* Tris–HCl and 5 m*M* dithiothreitol pH 7.4), the N-terminal His-tag portion was removed by the EK Max (Invitrogen) enzyme. GAPDH protein was concentrated and loaded onto a Superdex 200 HR (Amersham Pharmacia) size-exclusion column. Homogeneous protein was purified from this column by isocratic elution with buffer *D* (25 m*M* Tris–HCl, 150 m*M* NaCl and 5 m*M* dithiothreitol pH 8.0). The protein was identified by SDS–PAGE and enzymatic activity analyses (Willson *et al.*, 1994).

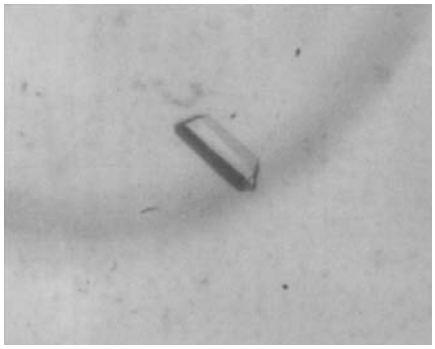

Figure 1
Crystals of human liver GAPDH.

Table 1
X-ray data-collection characteristics and statistics for human liver GAPDH.

Values in parentheses are for the highest resolution shell.

X-ray source	SPring-8 BL32B2
Wavelength (Å)	1.000
Space group	$P2_1$
Unit-cell parameters	
a (Å)	63.23
b (Å)	97.84
c (Å)	84.23
β (°)	104.1
Resolution range (Å)	30.0–2.8
$R_{\rm merge}$ (%)	11.4 (33.2)
Mean $I/\sigma(I)$	7.1 (2.8)
No. observations	84449
No. unique reflections	23174
Completeness (%)	97.9 (100)
Crystal mosaicity (°)	0.81

2.3. Crystallization

Initial screening was performed by the hanging-drop vapour-diffusion method using an Index Screen HT kit (Hampton Research). Crystals containing a variety of polyethylene glycols (PEGs) were obtained under many conditions. The most favourable crystals were obtained under condition D6 of the kit, which was then refined using the sitting-drop method. The best crystals were obtained using 4 µl of protein solution at 10 mg ml^{-1} mixed with 4 µl mother liquor.

2.4. X-ray diffraction data collection

A single crystal was mounted in a nylon loop (Hampton Research) and cooled to 100 K in an N_2-gas stream (Rigaku). The diffraction data set was collected at beamline 32B2 at SPring-8 using an R-AXIS V imaging-plate detector (Rigaku). A wavelength of 1.00 Å and a crystal-to-detector distance of 200 mm were used. Data integration and scaling were performed with *CrystalClear* (Molecular Structure Corporation).

3. Results and discussion

Rod-shaped crystals of GAPDH (Fig. 1) grew to 0.2 mm in the largest dimension and were obtained using 0.1 *M* bis-Tris–HCl buffer pH 5.5 and 20–23% PEG 3350. These crystals took approximately two months to reach their full size. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 63.23, b = 97.84, c = 84.23 Å, β = 104.1°. The presence of one tetramer in the asymmetric unit gives a crystal volume per protein weight, $V_{\rm M}$, of 1.9 Å^3 Da^{-1}, which lies within the range normally found for protein crystals (Matthews, 1968). In spite of these good characteristics, the crystals were extremely fragile towards osmotic shock. A 1% change in the content of PEG 3350 as the main precipitant causes destruction of the crystals. As a result of many trials for freezing the crystals, X-ray diffraction data of the native crystal were collected at 2.8 Å resolution using as a cryoprotectant a mixture consisting of paraffin oil and Paratone-N (Hampton Research) in a 3:1 ratio and a cryoloop covered with Formver film (Fluka). Table 1 summarizes the data-collection statistics.

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