Crystallographic analysis of FAD-dependent glucose dehydrogenase

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An FAD-dependent glucose dehydrogenase (GDH) from *Aspergillus terreus* was purified and crystallized at 293 K using the sitting-drop vapour-diffusion method. A data set was collected to a resolution of 1.6 Å from a single crystal at 100 K using a rotating-anode X-ray source. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 56.56$, $b = 135.74$, $c = 74.13$ Å, $\beta = 90.37^\circ$. The asymmetric unit contained two molecules of GDH. The Matthews coefficient was calculated to be $2.2 \text{ Å}^3 \text{ Da}^{-1}$ and the solvent content was estimated to be 44%.

1. Introduction

FAD-dependent glucose dehydrogenase (FAD-GDH; EC 1.1.5.9) is a glucose oxidoreductase that catalyses the oxidation of D-glucose with the reduction of a redox mediator and does not utilize O_2 as an electron acceptor. Recently, fungal extracellular FAD-GDH has been widely used in blood glucose-monitoring systems, which depend on the high substrate specificity of the enzyme for D-glucose (Tsumimura *et al.*, 2006). FAD-GDH and glucose oxidase (GOX; EC 1.1.3.4) belong to the family of GMC (glucose-methanol-choline) oxidoreductases; however, these enzymes are distinguished by properties including oxygen utilization and substrate specificity. Therefore, determining the three-dimensional structure of FAD-GDH would contribute to our understanding of the reaction mechanism towards the development of enzymes with altered properties.

Crystal structures of GOX from *Aspergillus niger* and *Penicillium amagasakiense* have been determined by X-ray crystallography (Hecht *et al.*, 1993; Wohlfahrt *et al.*, 1999). They were homodimeric glycoproteins with one tightly but noncovalently bound FAD. FAD-GDH is considered to be a monomeric protein (Sygmund *et al.*, 2011). Based on the crystal structures of GOX, GOX-substrate (β -D-glucose) complexes were also modelled. However, no structural information is currently available for FAD-GDH. To elucidate the mechanism of the high substrate specificity of FAD-GDH and the structural differences between GDH and GOX, it is necessary to determine X-ray crystal structures of FAD-GDH. In this paper, we report the purification and crystallization of FAD-GDH from *A. terreus* and describe the results of a preliminary X-ray crystallographic analysis.

2. Materials and methods

2.1. Macromolecule production

The gene encoding full-length FAD-GDH was amplified by PCR from *A. terreus* genomic DNA and cloned into a modified expression vector derived from pNENG142F (Tsuboi *et al.*, 2005). The plasmid was introduced into *A. oryzae* NS4 and FAD-GDH was expressed. The cells were grown for 5 d at 303 K in 3.5 l culture medium containing 1% peptone, 2% sucrose, 0.5% dipotassium hydrogen phosphate and 0.05% magnesium sulfate. After culturing, the culture solution was filtered with a filter cloth to harvest the filtrate. The filtrate was centrifuged (7000g, 30 min) to harvest the supernatant, which was then subjected to suction filtration using a membrane filter (Advantech Co. Ltd; 10 µm) to obtain the filtrate. This filtrate was applied onto a Toyopearl Butyl-650C (Tosoh Corp.) column (3.00 cm diameter × 20.0 cm) pre-equilibrated in advance with buffer solution A (50 mM potassium phosphate buffer solution, 50% saturated ammonium sulfate pH 6.0). The ammonium sulfate concentration that led to precipitation was greater than 60% saturated ammonium sulfate. After the column had been washed with buffer solution A, the protein was eluted with a linear gradient of buffer solution B (50 mM potassium phosphate buffer solution pH 6.0) in buffer solution A. The active protein fraction was concentrated, dialyzed against buffer solution C (1 mM potassium phosphate buffer solution pH 6.0) and then passed through a DEAE Cellufine A-500m (JNC Corp.) column (2.10 cm diameter × 22.0 cm) pre-equilibrated with buffer solution C. FAD-GDH was eluted with a linear gradient from buffer C to buffer B. The fractions containing FAD-GDH were collected and concentrated by ultrafiltration. Macromolecule-production information is summarized in Table 1.

According to the results of native PAGE, the pre-purified FAD-GDH was modified by an oligosaccharide; therefore, the pre-purified FAD-GDH sample was treated with endoglycosidase H (Roche Diagnostics) for 4 h at 310 K to remove the oligosaccharide. For further purification, the treated solution was loaded onto a Q-Sepharose HP (GE Healthcare) anion-exchange column and eluted with a linear gradient of 0–500 mM sodium chloride in 20 mM Tris–HCl buffer pH 8.5. Fractions containing FAD-GDH were pooled and concentrated to 36 mg ml^{−1} by ultrafiltration using a YM-10 membrane (Millipore). The protein concentration was determined using a kit for measuring the protein concentration (Bio-Rad Protein Assay, Bio-Rad Laboratories Inc.) in accordance with the instruction manual and was calculated with a standard curve determined using bovine serum albumin (BSA; Wako Pure Chemical Industries Ltd) as a standard.

2.2. Crystallization

Before starting the crystallization, the stability of the protein to several precipitants was tested. Because each FAD-GDH protein molecule contained one FAD molecule, the FAD-GDH solution was yellow. After the precipitant had been added to the protein solution, white precipitation was observed for some precipitants. In particular, a single addition

Table 1

Macromolecule-production information.

Source organism	<i>A. terreus</i>
DNA source	FERM BP-08578
DDBJ accession code for gene	DD412265
Forward primer†	5'-GCGTCGAC (TGACCAATTCGCGAGCTCGTCA-AA) ATGTTGGGAAAGCTCTCCTTCCTC-3'
Reverse primer‡	5'-CATGCATGCCCTAACGACGACGATCGGCC-TTGATGAGATCCGCCGCCG-3'
Expression vector	Cloning vector, pNENG142F
Expression host	<i>A. oryzae</i> NS4
Complete amino-acid sequence of the construct produced	SNSTSAKYDYIVIGGTSGLAVANRLSEDPNVNV-LILEAGGSVWNNPNVTNVDGYGLAFGSDIDWQ-YQSVNPQYGGNLSQVLRAGKALGGTSTINGMA-YTRAEDVQIDAWETIGNTGWTWKNLFPYRKS-ENFTVPTKSQTSGLASYEAGAHGHEGLDVAFTQIESNNLTYYLNRTFQGMGLPWTEDVNGGKMRGFLNPSTVNLEEVREDAARAYWPYKSRPNLHVLLNTFANRIVWDGEAHGHITASGVEITSRNGTVRVINAEKEVIVSAGALKSPAILELSG-IGNPSVLDKHNIPVKVNLPTVGENLQDQVNSHMDASGNTSISGKAVSYPDVYDVFGEAESVAKQIRANLKQYAADTAKANGNIMKAADLERLFEVQYDLIFKGRVPIAEVLNYPGSATSVFAEFWALLPFARGSVHIGSSNPAEFVNPINPNYFMLDWD-AKSYVAVAKYIRRSFESYPLSSIVKESTPGYDVIPRNASEQSWKEWVFDKNYRSNFHPVGTAAAMPREIGGVVDERLNVYGTNNRVVDASVLPFQVCCHLVSTLYAVAERAADLIKADAGRR

† The transcription-enhancing factor is shown in parentheses. The restriction-enzyme site (SalI) is underlined. ‡ The restriction-enzyme site (SphI) is underlined.

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	24-well sitting-drop plate (Hampton Research Cryschem plate)
Temperature (K)	293
Protein concentration (mg ml ^{−1})	36
Buffer composition of protein solution	150 mM NaCl, 20 mM Tris–HCl pH 8.5
Composition of reservoir solution	30% PEG 8000, 0.2 M ammonium sulfate, 0.04% sodium azide, 0.1 M sodium acetate pH 4.5
Volume and ratio of drop (µl)	2:2
Volume of reservoir (µl)	500

of ammonium sulfate produced heavy white precipitation that strongly suggested that the FAD-GDH molecule had been denatured. Precipitants that denatured the proteins were not used in crystallization. However, a combination of polyethylene glycol (PEG) 8000 and ammonium sulfate produced single crystals that were suitable for X-ray diffraction studies. The composition of the optimized reservoir solution was 30% PEG 8000, 0.2 M ammonium sulfate, 0.1 M sodium acetate pH 4.5. Crystallization was carried out using the sitting-drop vapour-diffusion technique at 293 K. Protein droplets were prepared from a mixture of 2 µl protein solution and 2 µl reservoir solution and were equilibrated against 500 µl reservoir solution.

2.3. Data collection and processing

For data collection, the crystal was soaked for a few seconds in reservoir solution containing 10%(v/v) glycerol and flash-cooled in a nitrogen stream at 100 K. X-ray diffraction data

were collected to a resolution of 1.6 Å on an R-Axis imaging-plate camera system (Rigaku Co. Ltd) using a rotating-anode generator operated at 40 kV and 100 mA with a fine-focus filament. Diffraction images covering a total oscillation range of 180° with a step size of 0.5° and an exposure time of 300 s were processed using *d*TREK* (Pflugrath, 1999) and the *CCP4* program suite (Winn *et al.*, 2011).

3. Results and discussion

The yellow plate crystals grew to dimensions of $0.4 \times 0.3 \times 0.1$ mm over about two weeks from drops that were obtained by mixing equal volumes (2 µl) of protein solution and

Figure 1
Photograph of a crystal of FAD-GDH from *A. terreus* obtained using PEG 8000 as a precipitant. The crystal dimensions are $0.4 \times 0.3 \times 0.1$ mm.

Figure 2
Diffraction pattern of a crystal of the FAD-dependent GDH from *A. terreus*. The edge of the detector corresponds to a resolution of 1.6 Å.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Rigaku MicroMax-007 rotating anode
Wavelength (Å)	1.5418
Temperature (K)	100
Detector	R-Axis VII
Crystal-to-detector distance (mm)	90.0
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	300
Space group	<i>P2</i> ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	56.56, 135.75, 74.13
α , β , γ (°)	90.00, 90.37, 90.00
Resolution range (Å)	35.75–1.60 (1.66–1.60)
Total No. of reflections	478874
No. of unique reflections	141637
Completeness (%)	96.5 (93.8)
Multiplicity	3.38 (3.29)
$\langle I/\sigma(I) \rangle$	16.4 (5.5)
<i>R</i> _{int}	0.042 (0.167)
Overall <i>B</i> factor from Wilson plot (Å ²)	13.9

precipitant solution and equilibrated over wells containing 500 µl 30% PEG 8000, 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.5 (Table 2, Fig. 1). Diffraction from these crystals reached a resolution of 1.6 Å (Fig. 2). The detailed data-collection statistics are summarized in Table 3. The crystals belonged to space group *P2*₁. Assuming the presence of two molecules per asymmetric unit, the calculated Matthews coefficient, *V*_M, was 2.2 Å³ Da^{−1} (Matthews, 1968), giving an estimated solvent content of 44%. Structure determination was performed by molecular replacement using GOX from *A. niger* (Tsuboi *et al.*, 2005; PDB entry 1cf3) as a search model. The sequence identity between GOX from *A. niger* and FAD-GDH from *A. terreus* is 32%. The search model was modified with *CHAINS*AW (Stein, 2008) from the *CCP4* software suite (Winn *et al.*, 2011), truncating the nonconserved residues at the C^β atom. Molecular-replacement calculations were performed using *MOLREP* (Vagin & Teplyakov, 2010). The result of molecular replacement showed a clear solution. The *R* factor after an initial round of rigid-body refinement was 50.5%. The crystal contained two monomers per asymmetric unit. Structure analysis and refinement are now in progress.

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