Purification, crystallization and preliminary X-ray diffraction analysis of NADP-dependent glutamate dehydrogenase from *Aspergillus niger*

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Glutamate dehydrogenase (GDH) catalyzes the NAD-dependent or NADP-dependent oxidative deamination of L-glutamate to 2-oxoglutarate and ammonia. This important reversible reaction establishes the link between carbon and nitrogen metabolism. In this study, *Aspergillus niger* NADP-GDH (AnGDH) has been overexpressed and purified. Purified AnGDH, with a high specific activity of 631.1 units per milligram of protein, was crystallized and the crystal diffracted to 2.9 Å resolution using a home X-ray source. Preliminary analysis of the X-ray diffraction data showed that the crystal belonged to space group *R*32, with unit-cell parameters $a = b = 173.8$, $c = 241.5$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The crystals exhibited an unusually high solvent content (83.0%) and had only one molecule in the asymmetric unit. Initial phases were obtained by molecular replacement, and model building and structure refinement of AnGDH are in progress.

1. Introduction

Assimilation and dissimilation of nitrogen occur in almost all living organisms. Nitrogen metabolism is involved in the synthesis of amino acids, proteins and nucleotides, which in turn are essential for all cellular processes. Glutamate dehydrogenase (GDH) is an oxidoreductase that is involved in the reversible oxidation of L-glutamate to 2-oxoglutarate (Fig. 1) and establishes a link between carbon and nitrogen metabolism (Hudson & Daniel, 1993; Li *et al.*, 2014). There are three types of glutamate dehydrogenases that are grouped into NADP-dependent (EC 1.4.1.4), NAD-dependent (EC 1.4.1.2) and NAD(P)-dependent (or dual-specificity) glutamate dehydrogenases (EC 1.4.1.3) (Choudhury & Puneekar, 2007; Bhuiya *et al.*, 2005). NADP-dependent GDHs are generally involved in ammonium assimilation, whereas the NAD-specific enzymes are linked to glutamate catabolism (Werner *et al.*, 2005). The dual-specific mammalian GDHs can use both NAD as well as NADP with comparable efficiency and are allosterically regulated by a number of small molecules and coenzymes (Banerjee *et al.*, 2003).

Several GDHs from different organisms have been isolated and the sequences of the enzymes are also available. Based on the oligomeric states of the protein, GDHs can be divided into two subfamilies. The NADP-dependent bacterial/fungal GDHs and dual-coenzyme-specific mammalian GDHs are hexameric, with a subunit molecular weight of between 48 and 55 kDa (Sharkey *et al.*, 2013). The NAD-dependent bacterial/fungal enzymes are either homo-hexamers with a subunit molecular weight of 48 kDa (Stillman *et al.*, 1993) or tetramers consisting of four identical 115 kDa subunits (Veronese *et al.*, 1974; Britton *et al.*, 1992). Extensive structural studies of glutamate dehydrogenase from several organisms have been performed. To date, crystal structures have been determined of the *Escherichia coli* (Bilokapic & Schwartz, 2012; Sharkey *et al.*, 2013), *Clostridium symbiosum* (Stillman *et al.*, 1993), *Plasmodium falciparum* (Werner *et al.*, 2005), *Thermotoga maritima* (Knapp *et al.*, 1997), *Thermococcus profundus* (Nakasako *et al.*, 2001), *Thermococcus litoralis* (Britton *et al.*, 1999), *Pyrobaculum islandicum* (Bhuiya *et al.*, 2005), *Peptinophilus saccharolyticus* (Oliveira *et al.*, 2012), *Bos taurus* (Smith *et al.*, 2001) and *Homo sapiens* (Smith *et al.*, 2002) enzymes. Crystal structures of GDHs complexed with

substrates and coenzymes have also been determined, to aid understanding of the catalytic mechanism of the enzyme (Stillman *et al.*, 1993; Bhuiya *et al.*, 2005; Smith *et al.*, 2001). The reported mammalian and bacterial GDH monomers consist of two domains: a substrate-binding domain (domain I) and an NAD/NADP-binding domain (domain II). Domain I is involved in intersubunit contacts to form a hexamer. The substrate-binding pocket is present in the deep junction between domain I and domain II. Structural studies also showed that binding of coenzyme or substrate induces domain closure in both bacterial and mammalian GDHs. Despite the overall structural similarity with bacterial GDHs, mammalian GDHs have an extra antenna domain that is involved in regulating the catalytic activity of the enzyme (Li *et al.*, 2009). Although several structural studies have been reported with bacterial and mammalian GDHs, no structure is available for a fungal enzyme.

NADP-dependent glutamate dehydrogenase is involved in ammonium assimilation in aspergilli (Cardoza *et al.*, 1998; Choudhury *et al.*, 2008). *Aspergillus niger* NADP-GDH (AnGDH) has been purified and kinetically characterized (Noor & Puneekar, 2005). AnGDH shows sigmoid saturation with 2-oxoglutarate ($K_{0.5} = 4.78$ mM) and is inhibited by isophthalate ($K_i = 6.9$ μ M), 2-methyleneglutarate ($K_i = 9.2$ μ M) and 2,4-pyridinedicarboxylate ($K_i = 202$ μ M). Its homotropic allosteric behaviour is thought to be important in adjusting the 2-oxoglutarate flux between the Krebs cycle and glutamate biosynthesis. Furthermore, AnGDH exhibits selective, storage-dependent loss of forward activity in a buffer containing β -mercaptoethanol while retaining its reverse activity (Walvekar *et al.*, 2014). However, NADP-GDH from *A. terreus* (AtGDH) shows hyperbolic saturation with 2-oxoglutarate ($K_m = 6.00$ mM) and remains unaffected in β -mercaptoethanol-containing buffers (Choudhury *et al.*, 2008; Walvekar *et al.*, 2014). The two GDH proteins are also differently cleaved by chymotrypsin (Choudhury *et al.*, 2008). All these differences are striking considering that AnGDH and AtGDH share 88% amino-acid sequence identity, which hence begs a structural explanation. While no structural data exist for fungal GDHs, NADP-GDH is poorly represented within the PDB. The structural basis of the coenzyme specificity of NADP-dependent GDHs is also not yet well understood. X-ray crystallographic studies of AnGDH will provide the first structural details of a fungal enzyme and will help in understanding the functional contexts. Here, we report the purification, crystallization and preliminary X-ray diffraction analysis of NADP-GDH from *A. niger*.

2. Materials and methods

2.1. Expression and purification of *A. niger* NADP-GDH

The NADP-GDH cDNA from *A. niger* was successfully cloned and overexpressed in *E. coli* BL21(DE3) as described previously (Walvekar *et al.*, 2014). *E. coli* cells containing the expression plasmid were grown overnight at 37°C in 5 ml Luria broth with ampicillin (100 μ g ml⁻¹). These cells were grown in 1000 ml Luria broth with the

same concentration of ampicillin at 37°C. After 3 h of growth (when the OD at 600 nm reached 0.45), protein expression was induced by adding 0.3 mM IPTG and the culture was incubated for a further 12 h at 25°C.

AnGDH was purified as described previously (Noor & Puneekar, 2005; Walvekar *et al.*, 2014) but with some modifications. Cells expressing the protein were harvested by centrifugation at 6000 rev min⁻¹ for 20 min. The cell pellet was collected and suspended in buffer A (100 mM potassium phosphate buffer pH 7.5, 1.0 mM EDTA and 1 $\times$ protease-inhibitor cocktail). All protein extraction and purification steps were carried out at 4°C. Cell disruption was performed by ultrasonication, the extract was centrifuged at 12 000 rev min⁻¹ and the supernatant was collected. The ammonium sulfate precipitation was performed in two steps: the first 0–30% ammonium sulfate saturation pellet was removed by centrifugation at 12 000 rev min⁻¹ for 15 min and the supernatant was then subjected to 30–70% ammonium sulfate saturation. The pellet was collected and dissolved in 6 ml buffer B (20 mM potassium phosphate buffer pH 7.5, 1 mM EDTA). The protein sample was loaded onto a 60 ml Sephadex G-25 column for desalting. The desalted AnGDH sample was loaded onto a 50 ml CR-12 dye-affinity column (Novacron Red LS-BL coupled through an epoxy spacer arm to Sepharose). Bound AnGDH was eluted with a 100 ml linear gradient of 0–2 M potassium chloride and fractions were collected. Active AnGDH fractions were pooled and further purified by anion-exchange chromatography. The AnGDH sample was loaded onto a 5 ml HiTrap DEAE Sepharose column using FPLC (ÄKTAprime Plus, GE Healthcare). Elution of bound AnGDH was performed with a 100 ml linear gradient of 0–1 M potassium chloride (in buffer B). The enriched AnGDH was concentrated to 2.5 ml using an Amicon ultrafiltration concentrator. The concentrated AnGDH sample was further loaded onto a Superdex 200 16/60 (GE Healthcare) gel-filtration column (120 ml bed volume) pre-equilibrated with buffer B. The protein was eluted with a flow rate of 0.3 ml min⁻¹. Peak fractions were collected and concentrated. The purity of AnGDH at different stages of purification was monitored by 12% SDS-PAGE. Fig. 2 shows the SDS-PAGE analysis of the purified AnGDH obtained after the gel-filtration step. Quantification of protein was performed by the method of Bradford (1976) using bovine serum albumin as a standard (Genel). Purified AnGDH was concentrated to ~12.0 mg ml⁻¹ and stored at 4°C for crystallization.

2.2. Enzymatic assay of *A. niger* NADP-GDH

NADP-glutamate dehydrogenase was assayed using the procedure described previously (Noor & Puneekar, 2005; Walvekar *et al.*, 2014). The forward reaction of the enzyme (the reductive amination of 2-oxoglutarate to glutamate) was carried out in a reaction mixture (1 ml) consisting of 10 mM 2-oxoglutarate, 100 mM Tris buffer pH 8.0, 10 mM ammonium chloride, 0.1 mM NADPH. The decrease in absorbance at 340 nm was monitored. The initial rate of NADPH disappearance was used to measure the enzyme activity. One activity

Figure 1
The reaction catalyzed by NADP-dependent *A. niger* glutamate dehydrogenase.

Table 1
Purification profile of AnGDH at different stages of purification.

Purification step	Total protein (mg)	Specific activity (units mg ⁻¹)	Yield (%)
Crude extract preparation	214.2	56.7	100
Ammonium sulfate (30–70%) fractionation	94.8	151.3	96.9
CR-12 dye-affinity chromatography	24.3	325.9	64.3
DEAE anion-exchange chromatography	14.7	451.7	53.8
Gel-filtration (Superdex 200) chromatography	7.2	631.1	36.9

unit corresponds to the amount of enzyme required to oxidize 1 μmol of NADPH per minute under standard assay conditions. The specific activity of the enzyme was calculated after estimation of protein amount and expressed as units per milligram of protein (Table 1).

2.3. Crystallization of *A. niger* NADP-GDH

Concentrated AnGDH sample (~12.0 mg ml⁻¹) was used for crystallization. Crystals of AnGDH complexed with 2-oxoglutarate and NADPH were grown by the hanging-drop vapour-diffusion method at 22°C using flat-bottom 24-well polystyrene plates from Nest Biotech. Co. Ltd. The enzyme–substrate complex was prepared by incubating pure AnGDH with 0.6 mM NADPH and 0.6 mM 2-oxoglutarate for 30 min at 25°C. Subsequently, this mixture was used for crystallization trials. Crystallization screenings were performed with the JCSG Core I Suite (Qiagen) and the PEGs Suite (Qiagen) by mixing 1 μl AnGDH complex solution and 1 μl mother liquor. The crystallization drops were equilibrated against 300 μl mother liquor. Nucleation of small crystals of AnGDH complex was observed within one week in a condition (The JCSG Core I Suite condition No. 68) consisting of 0.1 M sodium citrate pH 5.5, 20% (w/v) PEG 3000. Initially the crystals were small but they grew to their maximum size (0.2 × 0.15 × 0.15 mm; Fig. 3) within one week of their first appearance in the drop.

2.4. X-ray diffraction study

Diffraction data were collected under liquid-nitrogen cryoconditions at –173°C in mother liquor containing 30% glycerol as a

Figure 2
SDS-PAGE showing the final purity of AnGDH after gel-filtration chromatography. Lane 1, molecular-weight marker (labelled in kDa); lane 2, purified AnGDH.

Table 2
Space group and data-collection statistics of an AnGDH crystal.

Values in parentheses are for the highest resolution shell.	
Temperature (°C)	–173
Wavelength (Å)	1.5418
Space group	<i>R</i> 32
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 173.8, <i>c</i> = 241.5, α = β = 90, γ = 120
Resolution (Å)	40.0–2.9 (3.0–2.9)
Mosaicity (°)	0.3
Observed reflections	164550 (15791)
Unique reflections	31011 (3001)
Multiplicity	5.3 (5.3)
Mean <i>I</i> /σ(<i>I</i>)	11.66 (3.04)
Completeness (%)	99.1 (99.8)
<i>R</i> _{merge} [†] (%)	15.9 (62.7)
<i>R</i> _{meas} (%)	17.7 (69.7)
<i>CC</i> _{1/2}	0.98 (0.80)

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$.

cryoprotectant (0.1 M sodium citrate pH 5.5, 20% PEG 3000, 30% glycerol). Crystals were flash-cooled by rapidly moving them into the cold nitrogen stream. A data set was collected by the rotation method with 0.5° rotation per frame at a wavelength of 1.5418 Å using Cu *K*α X-ray radiation generated by a Bruker Microstar diffractometer equipped with a MAR 345 detector. Fig. 4 shows a diffraction pattern obtained from one of these crystals. The image frames of this data set were indexed and integrated using *XDS* (Kabsch, 2010). The intensities were converted to structure factors with *F2MTZ* and *CAD* from *CCP4* (Winn *et al.*, 2011). Initial phases were obtained by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the structure of *E. coli* glutamate dehydrogenase (EcGDH; PDB entry 4bht, Sharkey *et al.*, 2013) as a model. The properties and data-collection statistics of this crystal form of AnGDH are summarized in Table 2.

3. Results and discussion

A. niger NADP-dependent glutamate dehydrogenase (AnGDH) was overexpressed in *E. coli* BL21(DE3) cells. The expressed AnGDH is a 460-residue polypeptide with an expected theoretical molecular weight of 49.39 kDa. A highly pure AnGDH sample was obtained after ammonium sulfate precipitation, CR-12 dye-affinity chromato-

Figure 3
Crystals of NADP-dependent glutamate dehydrogenase from *A. niger*. The approximate dimensions of the crystal used for data collection were 0.2 × 0.15 × 0.15 mm. The scale bar represents 0.1 mm.

graphy and DEAE anion-exchange chromatography followed by a final step involving gel-filtration chromatography. As expected, SDS-PAGE of the purified enzyme showed a single band with a monomeric molecular weight of 49.4 kDa (Fig. 2). However, the protein eluted as a hexamer from a gel-filtration column (data not shown) calibrated with standard molecular-weight proteins. The pure AnGDH sample obtained after the final purification step was highly active with a specific activity of 631.1 units per milligram of protein (Table 1). The yield and purity of this preparation were adequate for crystallization studies.

Commercially available screening solutions were used for several rounds of crystallization trials for AnGDH. The crystallization was performed using the hanging-drop vapour-diffusion method at 22°C. Crystals of AnGDH complexed with NADPH and 2-oxoglutarate appeared in a condition with mother liquor consisting of 0.1 M sodium citrate pH 5.5, 20% (w/v) PEG 3000. The crystals were initially small but grew to their maximum size within one week (Fig. 3). Different concentrations (5, 10, 15, 20 and 30%) of glycerol in the mother liquor were tried to freeze AnGDH crystals. Mother liquor containing 30% glycerol as a cryoprotectant was the best for freezing the crystals and hence was used for data collection.

A single AnGDH crystal was used for diffraction (Fig. 4) data collection using a home X-ray source. The good-quality diffraction data could be processed to 2.9 Å resolution (Table 2). Processing of the diffraction data using *XDS* showed that the crystal belonged to space group *R*32, with unit-cell parameters $a = b = 173.8$, $c = 241.5$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The Matthews coefficient (V_M ; Matthews, 1968) and solvent content were calculated to be $2.4 \text{ Å}^3 \text{ Da}^{-1}$ and 48%, respectively, assuming the presence of three molecules in an asymmetric unit. Surprisingly, a structure-solution trial by *Phaser* (McCoy *et al.*, 2007) using the *E. coli* glutamate dehydrogenase (EcGDH) structure (PDB entry 4bht, 53% sequence identity) correctly placed only one molecule in the asymmetric unit. The substrate-binding

domain (domain I) and coenzyme-binding domain (domain II) of the EcGDH structure were used independently as search models. The correctness of the solution from *Phaser* was clearly indicated by highest *Z*-scores and log-likelihood gain values for domain I (RFZ = 3.8, TFZ = 12.5, LLG = 168) and domain II (RFZ = 4.3, TFZ = 13.1, LLG = 393). After finding the correct orientation of the model, the model was further refined using *REFMAC* (Murshudov *et al.*, 2011). The first five cycles of restrained refinement produced a decrease in the R_{factor} and R_{free} values of 10.2% (final R_{factor} 36.6%) and 8.8% (final R_{free} 38.4%), respectively, with a final figure of merit (FOM) of 0.63. One molecule of AnGDH in an asymmetric unit corresponds to a Matthews coefficient and solvent content of $7.1 \text{ Å}^3 \text{ Da}^{-1}$ and 83%, respectively. Such a high solvent content is not commonly observed in protein crystals. Recent analysis (Kantardjieff & Rupp, 2003) showed that generally the value of V_M lies between 1.5 and $6.0 \text{ Å}^3 \text{ Da}^{-1}$, with a mean of around $2.69 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of ~47%. The same study also reported that the frequency of distribution of V_M is broader compared with the original distribution observed by Matthews (1968) and only a small fraction of the available protein crystals have exceptionally high V_M values (above $7.0 \text{ Å}^3 \text{ Da}^{-1}$) as well as solvent contents (above 80%) that do not follow the general distribution. For example, crystals of β_2 -glycoprotein (PDB entry 1qub; solvent content 86%; Bouma *et al.*, 1999), dihydrolipoyl acetyltransferase (PDB entry 1b5s; solvent content 89%; Izard *et al.*, 1999) and chloramphenicol phosphotransferase (PDB entry 1qhx; solvent content 86%; Izard & Ellis, 2000) have been reported to have very high Matthews coefficient values of 8.5, 11.5 and $8.8 \text{ Å}^3 \text{ Da}^{-1}$, respectively. An initial prediction of three molecules per asymmetric unit was made assuming that the AnGDH crystal followed the general distribution of V_M values. The correct solution by *Phaser*, the good initial refinement statistics and the well resolved electron-density map disproved the prediction and clearly showed the presence of a single molecule in the asymmetric unit. The high Matthews coefficient observed for the AnGDH crystal is yet another exception to the general distribution of V_M . This unusual, very high solvent content of the AnGDH crystal was also revealed by crystal packing analysis, which showed the presence of a hexameric biological assembly of AnGDH in the crystal along with large solvent cavities of 104 Å diameter. Further optimization of crystallization conditions will be attempted to obtain better quality crystals and high-resolution diffraction data using a synchrotron-radiation source. Model building and refinement of the AnGDH structure using the available crystallographic data are in progress.

Data were collected using a home X-ray source at the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India. We would like to thank Dr Ashok K. Varma and Ulka U. Sawant for providing us with access to the X-ray diffractometer at ACTREC. The work was supported by a Ramalingaswami Re-entry Fellowship (BT/RLF/Re-entry/42/2011) from the Department of Biotechnology, Ministry of Science and Technology, India and a seed grant (11IRCCSG020) from Indian Institute of Technology Bombay, Powai, India.

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Figure 4
Diffraction pattern of an AnGDH crystal recorded using the home X-ray source at the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India. A 2 min exposure time was used to collect each diffraction image. Resolution rings are shown as dotted circles.

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