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Crystallization and preliminary X-ray crystallographic analysis of the NmrA-like DDB_G0286605 protein from the social amoeba *Dictyostelium discoideum*

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The DDB_G0286605 gene product from *Dictyostelium discoideum*, an NmrA-like protein that belongs to the short-chain dehydrogenase/reductase family, has been crystallized by the hanging-drop vapour-diffusion method at 295 K. A 1.64 Å resolution data set was collected using synchrotron radiation. The DDB_G0286605 protein crystals belonged to space group $P2_1$, with unit-cell parameters a = 67.598, b = 54.935, c = 84.219 Å, β = 109.620°. Assuming the presence of two molecules in the asymmetric unit, the solvent content was estimated to be about 43.25% with 99% probability. Molecular-replacement trials were attempted with three NmrA-like proteins, NmrA, HSCARG and QOR2, as search models, but failed. This may be a consequence of the low sequence identity between the DDB_G0286605 protein and the search models (DDB_G0286605 has a primary-sequence identity of 28, 32 and 19% to NmrA, HCARG and QOR2, respectively).

1. Introduction

The DDB_G0286605 protein from *Dictyostelium discoideum* is a protein of unknown function that consists of 302 amino acids. Primary-sequence and secondary-structure analyses suggest that the DDB_G0286605 protein contains an NmrA-like domain and belongs to the short-chain dehydrogenase/reductase (SDR) superfamily. The members of the SDR superfamily have only low pairwise sequence identity (typically 20–30%), but have homologous three-dimensional structures with a single-domain nucleotide-binding Rossmann fold (Kallberg *et al.*, 2010).

In the Protein Data Bank, the *Aspergillus nidulans* NmrA and human HSCARG proteins show highest sequence homology to the DDB_G0286605 protein (Fig. 1). NmrA is a negative transcriptional regulator that is involved in post-translational modulation of the GATA-type transcription factor AreA, forming part of a system that controls nitrogen-metabolite repression in various fungi (Stammers *et al.*, 2001). The ability of NmrA to discriminate between oxidized and reduced forms of dinucleotides may be linked to a possible role in redox sensing (Lamb *et al.*, 2003).

Human HSCARG has structural similarity to NmrA-like SDR proteins and forms an asymmetric dimer with one subunit bound to an NADP(H) molecule and the other unoccupied; the two subunits have dramatically different conformations (Zheng *et al.*, 2007). HSCARG has a much higher affinity for NADPH than for $NADP^+$ and a decrease in the NADPH/$NADP^+$ ratio induces the redistribution of HSCARG within cells. These results indicate that HSCARG may serve as an NADPH sensor (Zheng *et al.*, 2007; Zhao *et al.*, 2008; Lamb *et al.*, 2008). Even though these proteins have high structural homology, they can discriminate between types of dinucleotides and redox states of dinucleotides, and show various conformational changes in response to dinucleotides (Lamb *et al.*, 2008).

In *D. discoideum*, several genes have been annotated as NmrA-like proteins and one of them, PadA (DDB_G0286385 protein), has been reported to be an essential gene for *Dictyostelium* development. *padA*$^-$ cells show many defects, most notably in the specification of the prestalk A cell population and ammonia sensitivity, and consequently show abnormal growth and development. The predicted three-dimensional structure of PadA is most similar to that of NmrA,

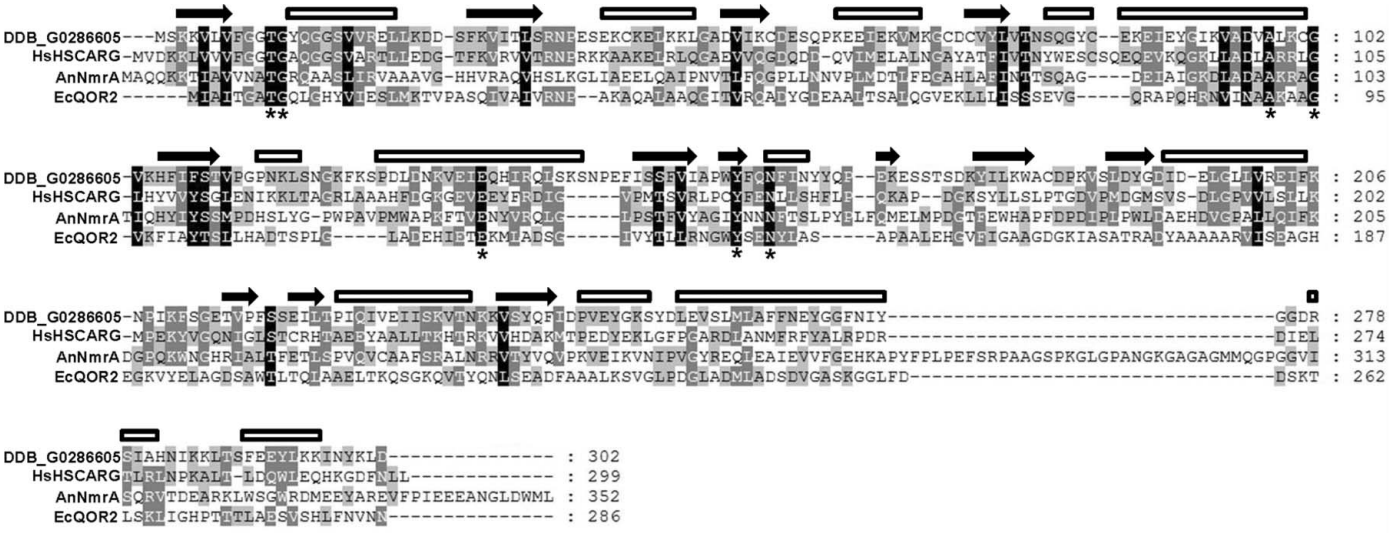

Figure 1
Amino-acid sequence alignment of DDB_G0286605 protein with HSCARG, NmrA and QOR2. The predicted secondary structures (Cole *et al.*, 2008) of the DDB_G0286605 protein are indicated by filled arrows (β-sheets) and empty boxes (α-helices). Asterisks indicate the consensus sequence among the four proteins.

but there is no other evidence that PadA acts as a transcription regulator or an NAD(P)(H)-sensing protein (Núñez-Corcuera *et al.*, 2008). To investigate the structural and functional role of the NmrA-like DDB_G0286605 protein from *D. discoideum*, we report its overexpression, crystallization and preliminary X-ray crystallographic analysis as a first step towards structure determination.

2. Materials and methods

2.1. Expression and purification of DDB_G0286605 protein

The DDB_G0286605 gene was amplified by polymerase chain reaction (PCR) using *D. discoideum* cDNA, which was amplified by reverse transcriptase PCR with total RNA extract from the cells (Han & Kang, 1998). The PCR product was digested with *Nde*I and *Bam*HI and inserted downstream of the T7 promoter of the expression plasmid pET-3a (Novagen). The resulting construct expresses residues 1–302 of the DDB_G0286605 protein without any additional residues. After verifying the DNA sequence, the plasmid DNA was transformed into *Escherichia coli* strain BL21 (DE3). The cells were grown to an OD_{600} of approximately 0.6 in Luria–Bertani medium containing 0.1 mg ml^{-1} ampicillin (Duchefa) at 310 K and expression was induced using 1 m*M* isopropyl β-D-1-thiogalactopyranoside (Duchefa). After 12 h induction at 295 K, the cells were harvested and resuspended in 50 m*M* potassium phosphate (Fluka) pH 7.5 containing 0.1 m*M* ethylenediaminetetraacetic acid (EDTA; Fluka). The cells were disrupted by sonication and the cell debris was discarded by centrifugation at 20 000*g* for 30 min. Ammonium sulfate (Fluka) was added to the supernatant to 55% saturation. After stirring the solution for 1 h, the precipitate was discarded by centrifugation at 20 000*g* for 30 min. The protein solution was loaded onto a Superdex 75 HR 16/60 column (GE Healthcare) pre-equilibrated with 25 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl. The fractions containing an overexpressed 35 kDa band on SDS–PAGE (Fig. 2) were pooled and concentrated. The proteins were loaded onto a Mono-Q Sepharose column (Amersham Biosciences) and the DDB_G0286605 protein was eluted with washing buffer (25 m*M* Tris–HCl buffer pH 7.5). The purified proteins were dialyzed against 25 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl and then concentrated to approximately 30 mg ml^{-1} for crystallization trials.

2.2. Crystallization and X-ray data collection

Initial screening was conducted by the hanging-drop vapour-diffusion method using screening kits from Hampton Research and the Wizard I and II screening solutions from Emerald BioSystems. Droplets composed of 1.5 µl protein solution and an equal volume of crystallization screening solution were equilibrated against 350 µl reservoir solution at 295 K. Several bundles of rod-shaped crystals were produced using a condition consisting of 0.2 *M* sodium thiocyanate, 20% PEG 3350 in two weeks. The crystallization conditions were then optimized by the addition of 5 m*M* dithiothreitol (DTT) to the protein solution, which led to the growth of crystals that were large enough for data collection (Fig. 3). Since the crystals were not separated and formed chain-like bundles, we seperated one node of

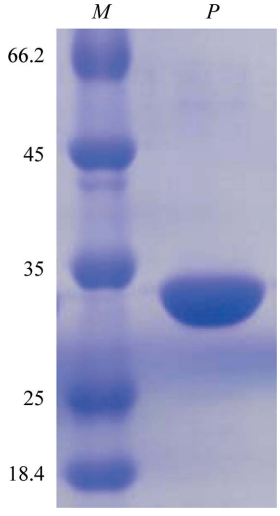

Figure 2
SDS–PAGE analysis of purified DDB_G0286605 protein. Lane *M*, molecular-mass markers (kDa); lane *P*, 10 µg purified recombinant DDB_G0286605 protein.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	Native	NADP(H) soaking
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 67.598, b = 54.935, c = 84.219, β = 109.62	a = 68.474, b = 54.274, c = 86.680, β = 112.77
Wavelength (Å)	0.97925	1.10000
Resolution (Å)	50–1.64 (1.64–1.62)	50–1.86 (1.91–1.86)
Completeness (>0σ) (%)	96.3 (90.7)	98.6 (92.1)
$R_{\rm merge}$† (%)	6.1 (31.1)	8.4 (21.6)
Average $I/\sigma(I)$	38.6 (3.2)	29.7 (3.6)
Unique reflections	68975 (3219)	48534 (2253)
Average multiplicity	5.5 (3.6)	5.4 (3.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of observed reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of symmetry-equivalent reflections.

the crystal bundle using Micro-Tools from Hampton Research for data collection.

Crystals were maintained at ∼100 K during data collection in order to minimize radiation damage. Native data were collected at 100 K using an Area Detector Systems Corporation (ADSC) Quantum 210 charge-coupled device (CCD) area-detector system on BL-6B and BL-6C of the Pohang Light Source (PLS), South Korea (Fig. 4). The diffraction data were processed and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Investigation of systematic absences in the reflections showed that the crystals of the DDB_G0286605 protein belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 67.598, b = 54.935, c = 84.219 Å, β = 109.620°. The crystal volume per unit molecular weight ($V_{\rm M}$) was calculated to be 2.17 Å^3 Da^{-1}, with a solvent content of 43.25% by volume (Matthews, 1968), when the asymmetric unit was assumed to contain two molecules (99% probability). In the self-rotation function, which was calculated with the *GLRF* program (Tong & Rossmann, 1997) using data in the resolution range 15–4 Å and an integration radius of 25 Å, no dominant features were found except in the κ = 180° section. The κ = 180° section revealed two peaks corresponding to twofold axes parallel to the crystallographic b axis (Fig. 5). Isothermal titration calorimetry analysis indicated that the DDB_G0286605 protein interacts with NADP(H) but not with NAD(H) (data not shown). Therefore, we attempted to obtain crystals of a cofactor-bound complex using the same conditions, but crystals did not grow. To solve the structure of the protein–cofactor complex, native crystals were soaked with NADP or NADPH for 5 min and diffraction data were then collected. The data-collection statistics are summarized in Table 1.

Attempts were made to solve the crystal structure of the DDB_G0286605 protein by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) and *Phaser* (McCoy, 2007) within the *CCP*4 software suite (Collaborative Computational Project, Number 4,

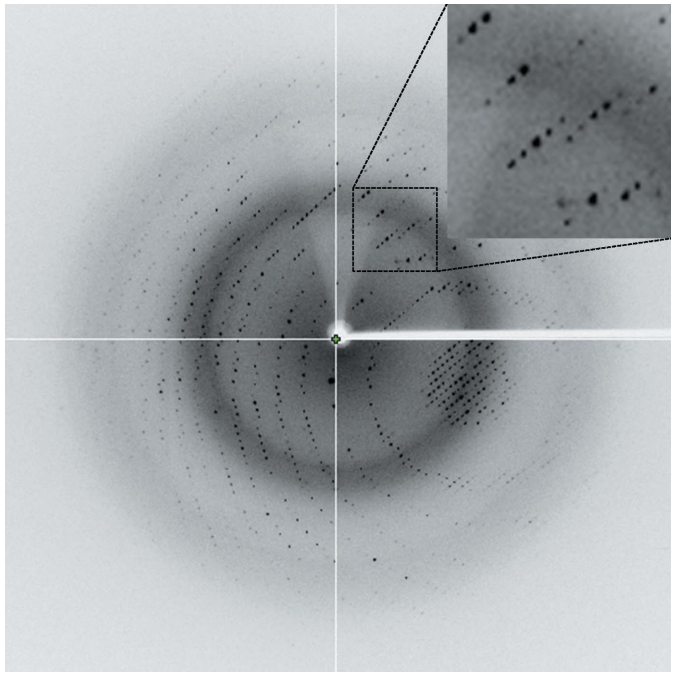

Figure 4
An X-ray diffraction pattern from a crystal of the DDB_G0286605 protein.

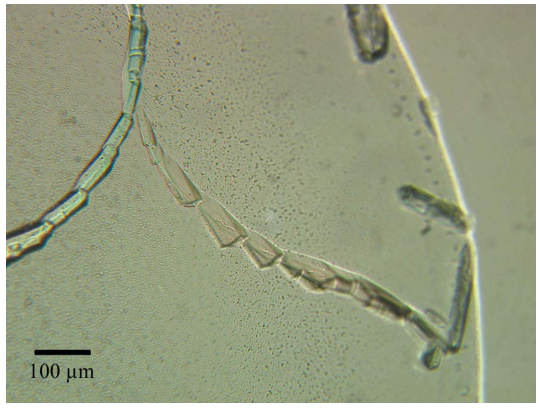

Figure 3
Crystals of the DDB_G0286605 protein.

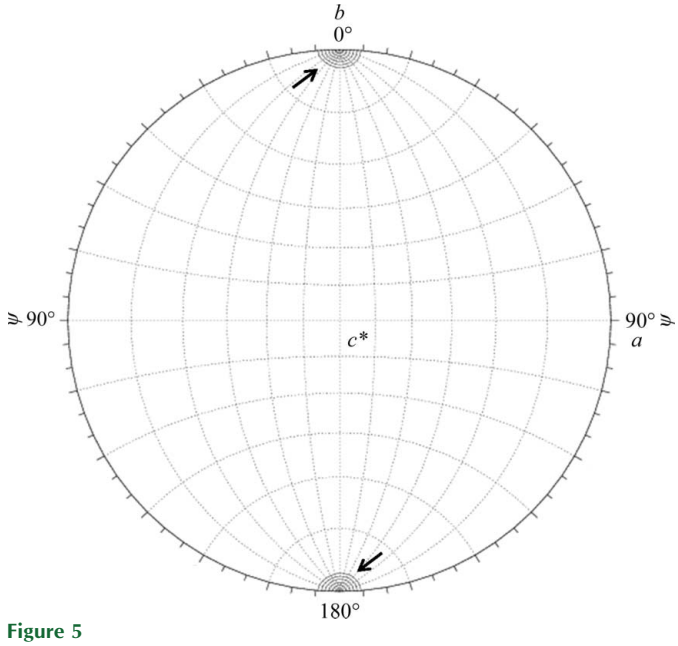

Figure 5
The κ = 180° section of the self-rotation function from the data set of a native crystal. The self-rotation function was calculated using a 25 Å radius of integration and data in the resolution range 15–4 Å.

1994) using the structures of NmrA (PDB code 1k6i; Stammers *et al.*, 2001), HSCARG (PDB code 2exx; Zheng *et al.*, 2007) and QOR2 (PDB code 2zcu; Kim *et al.*, 2008) as search models. However, all of the trials resulted in failure. Since the NmrA-like proteins of the SDR superfamily have well conserved N-terminal dinucleotide-binding Rossmann folds and relatively varied C-terminal substrate-binding domains (Jörnvall *et al.*, 1995), the failure of the MR trials seems to be caused by the low primary-sequence identity and the different topology of the C-terminal domains.

The crystal structures of the NmrA-like DDB_G0286605 protein and of its complex with cofactor will help in understanding how the NmrA-like proteins of *D. discoideum* modulate biological functions in response to the redox state of the cell. Therefore, we are attempting to grow crystals of selenomethionine-substituted DDB_G0286605 protein in order to solve the crystal structure using the multiple-wavelength anomalous dispersion method.

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