Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction analysis of ydjA, a minimal nitroreductase from *Escherichia coli* K12

Ji-Woo Choi, Jieun Lee, Nishi Kosuke, Che-Hun Jung and Jeong-Sun Kim*

Department of Chemistry and Institute of Basic Sciences, Chonnam National University, Gwangju 500-757, Republic of Korea

Correspondence e-mail: jsunkim@chonnam.ac.kr

Received 24 September 2007
Accepted 10 November 2007

Nitroreductases that reduce hazardous nitroaromatic compounds are of interest because of their central role in nitroaromatic toxicity, their potential use in bioremediation and their utility in activating prodrugs in directed anticancer therapies. To provide the molecular background to the enzymatic mechanism of the ydjA nitroreductase, which is one of the smallest nitroreductases, the *ydjA* gene from *Escherichia coli* K12 was cloned and expressed and the expressed protein Ec_ydjA was purified. Ec_ydjA was crystallized from 20%(*w*/*v*) polyethylene glycol 1000, 0.2 *M* lithium sulfate and 0.1 *M* phosphate–citrate pH 4.2. Diffraction data were collected to 2.00 Å resolution using synchrotron radiation. The crystal belongs to the monoclinic space group *C*2, with unit-cell parameters $a = 87.55$, $b = 129.28$, $c = 36.88$ Å, $\alpha = 90$, $\beta = 103.8$, $\gamma = 90°$. With two Ec_ydjA molecules in the asymmetric unit, the Matthews coefficient was 2.43 Å^3 Da^{-1} and the solvent content was 48.33%.

1. Introduction

Nitroaromatic compounds such as nitrotoluenes, nitrofurans and nitroimidazoles have been widely used as pharmaceuticals, agrochemicals, explosives and precursors in the manufacture of plastics and dyes (Spain, 1995; Esteve-Núñez *et al.*, 2001), which can produce large amounts of hazardous compounds. Therefore, their conversion into less hazardous compounds is of importance and is accomplished by nitroreductases.

Nitroreductases reduce nitrobenzenes to the corresponding hydroxylamines and derive reducing equivalents from reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) or other nicotinamides by means of a flavin mononucleotide cofactor (FMN; Bryant & DeLuca, 1991; Bryant *et al.*, 1991).

Recent structural studies of nitroreductases have provided valuable information on FMN binding, the redox state of the FMN ring and substrate binding (Lovering *et al.*, 2001; Haynes *et al.*, 2002). The *Si* side of the bound flavin-ring system is occupied by a rigid β-strand without the space to accommodate another molecule between the protein and the FMN cofactor. In contrast, the *Re* side has sufficient space for the substrate next to the cofactor-binding site. Based on this structural feature, nitroreductases have been suggested to follow bi-bi kinetics or a ping-pong mechanism (Anlezark *et al.*, 1992; Koder & Miller, 1998).

The *ydjA* gene codes for fewer than 190 amino acids and encodes a putative nitroreductase (Blattner *et al.*, 1997); it is conserved in various microorganisms. Thus, it might be one of the smallest nitroreductases as judged by its amino-acid composition. Sequence analysis (http://www.sanger.ac.uk/Software/Pfam) shows that ydjA is similar to the nitroreductase family, with the highest sequence similarity being with a putative nitroreductase from *Haemophilus influenza* (HI1542; Fleischmann *et al.*, 1995).

Despite the annotation of ydjA as a nitroreductase, it shows low sequence similarity to the well characterized nitroreductases (Lovering *et al.*, 2001; Haynes *et al.*, 2002). In order to understand the enzymatic mechanism of ydjA, we have carried out the crystallization and preliminary X-ray crystallographic analysis of ydjA from *Escherichia coli* (Ed_ydjA).

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2. Methods

2.1. Cloning, expression and purification of Ec_ydjA

The *E. coli* gene coding for ydjA (residues 1–183) was amplified from *E. coli* chromosomal DNA by the polymerase chain reaction (PCR). The PCR product was then cloned into pET21a (Invitrogen), which expresses eight extra amino acids, LEHHHHHH, at the C-terminus. The expression construct was transformed into *E. coli* B834(DE3) and grown in LB medium containing 100 μg ml^{-1} ampicillin at 310 K. After induction with 1.0 m*M* IPTG for a further 8 h at 310 K, the culture was harvested by centrifugation at 5000*g* at 277 K. The cell pellet was resuspended in ice-cold buffer *A* (20 m*M* Tris–HCl pH 8.0 and 500 m*M* NaCl) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 11 000*g* for 1 h. The Ec_ydjA fusion protein was purified using a 5 ml HisTrap chelating column (GE Healthcare, Uppsala, Sweden) and the bound protein was eluted with a linear gradient from 0 to 500 m*M* imidazole in buffer *A*. After removal of salts, the protein was purified using a 5 ml HiTrapQ anion-exchange column (GE Healthcare, Uppsala, Sweden). For further purification, size-exclusion chromatography using Sephacryl S-300 HR (GE Healthcare, Uppsala, Sweden) was performed in a buffer consisting of 20 m*M* Tris–HCl pH 8.0 and 200 m*M* NaCl. The purified protein was >95% pure as judged by Coomassie Blue-stained SDS–PAGE.

2.2. Crystallization

For crystallization, the purified Ec_ydjA protein was concentrated to 25 mg ml^{-1} in a buffer consisting of 20 m*M* Tris–HCl pH 7.5 and 300 m*M* NaCl. The initial crystallization conditions of Ec_ydjA were obtained by sparse-matrix screening (Jancarik & Kim, 1991) and included precipitant 1 [20%(*w*/*v*) polyethylene glycol 1000, 0.2 *M* lithium sulfate and 0.1 *M* phosphate–citrate pH 4.2], precipitant 2 [30%(*w*/*v*) polyethylene glycol 8000, 0.2 *M* NaCl and 0.1 *M* imidazole pH 8.0] and precipitant 3 [10%(*w*/*v*) polyethylene glycol 1000, 0.2 *M* NaCl and 0.1 *M* phosphate–citrate pH 4.2]. In order to obtain crystals suitable for X-ray diffraction, the precipitant and protein concentrations, buffer pH and temperature were changed systematically and various equilibriation strategies, for example the hanging-drop and sitting-drop vapour-diffusion methods, were tried.

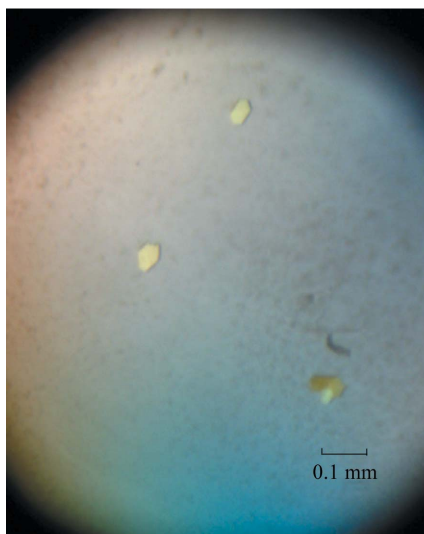

Figure 1
Crystals of Ec_ydjA. The crystals grew at 291 K within two weeks with maximum dimensions of approximately 0.1 × 0.1 × 0.1 mm.

Table 1
Data-collection statistics for Ec_ydjA.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.24
Space group	*C*2
Unit-cell parameters (Å, °)	*a* = 87.55, *b* = 129.28, *c* = 36.88, β = 103.8
Resolution range (Å)	50.0–2.00 (2.07–2.00)
Measured reflections	109943
Unique reflections	20675
Multiplicity	4.2
Temperature (K)	100
Matthews coefficient (Å^3 Da^{-1})	2.43
Solvent content (%)	48.33
No. of molecules in ASU	2
Completeness (%)	97.3 (84.2)
Mean $I/\sigma(I)$	5.4 (1.4)
$R_{\rm merge}$† (%)	9.1 (51.1)
Wilson *B* factor (Å^2)	24.2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of an individual reflection and $\overline{I(hkl)}$ is the mean intensity of that reflection.

2.3. X-ray data collection

For data collection, crystals were briefly immersed into a precipitant solution containing 10%(*w*/*v*) glycerol as a cryoprotectant and were immediately placed in a 100 K nitrogen-gas stream. Native X-ray diffraction data were collected at MAXII6C, Pohang Accelerator Laboratory (PAL, Republic of Korea) using 1° oscillation per image with a crystal-to-detector distance of 150 mm. The crystal was exposed for 10 s per image. A data set was collected to 2.00 Å resolution from a single crystal. The data were indexed and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

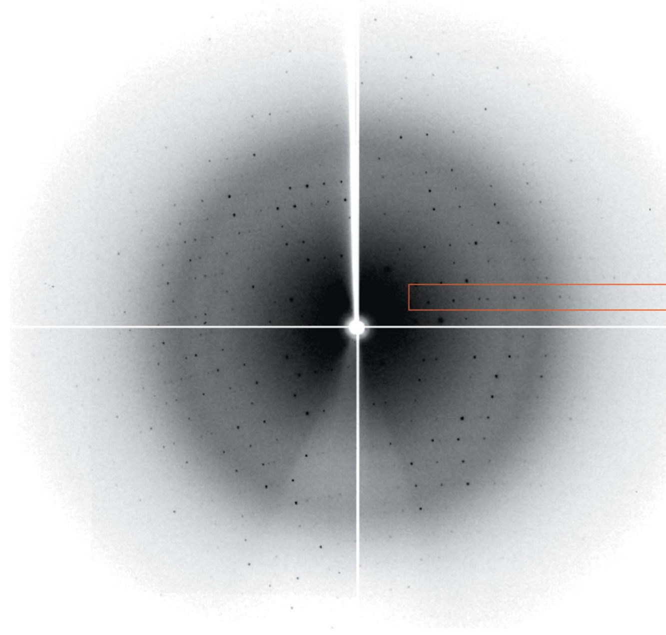

Figure 2
Representative X-ray diffraction image from Ec_ydjA. The crystal was exposed for 10 s with a 1° oscillation range. The edge of the detector corresponds to a resolution of 2.00 Å.

3. Results and discussion

Recombinant Ec_ydjA protein was successfully expressed and homogeneously purified using sequential chromatographic steps. Crystals suitable for diffraction experiments were obtained within 2 d using the hanging-drop vapour-diffusion method at 291 K by mixing 1 µl protein solution and 1 µl reservoir solution and equilibrating against 200 µl reservoir solution, which consisted of 20%(*w*/*v*) polyethylene glycol 1000, 0.2 *M* lithium sulfate and 0.1 *M* phosphate–citrate pH 4.2 (Fig. 1). The dimensions of the crystal used for data collection were approximately 0.1 × 0.1 × 0.1 mm and it diffracted to 2.00 Å resolution (Fig. 2), which should be sufficient for structural study of Ec_ydjA even though $I/\sigma(I)$ is low. This may be the result of suboptimal exposure times and cryoprotection and we anticipate improvements in the data we collect for structure solution and refinement. The crystal belongs to the monoclinic space group *C*2, with unit-cell parameters a = 87.55, b = 129.28, c = 36.88 Å, α = 90, β = 103.8, γ = 90°. The asymmetric unit may contain two Ec_ydjA molecules, resulting in a Matthews coefficient of 2.43 $Å^3\ Da^{-1}$ and a solvent content of 48.33% (Matthews, 1968).

We have attempted molecular-replacement methods for phase determination using the nitroreductase structures that show the highest sequence similarity (PDB code 2i7h, 29% identity, 46% similarity; PDB code 2isj, 27% identity, 40% similarity; Taga *et al.*, 2007) as search models. We have also used other nitroreductase structures [PDB codes 1ds7 (Parkinson *et al.*, 2000), 1kqb (Haynes *et al.*, 2002), 1nec, 1ywq and 1zch (Morokutti *et al.*, 2005)], but have not yet been successful. Therefore, the crystal structure of Ec_ydjA is now being solved by the MAD or SAD method with selenium as the anomalous scatterer.

This work was supported by the CNU Specialization Grant funded by Chonnam National University and by the CNU Special Grant for Administration No. 2007-0250. We thank Dr K. J. Kim, Pohang Light Source for help with data collection.

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