Ho-Phuong-Thuy Ngo,^{a,‡}
Seung-Hye Hong,^{b,‡} Myoung-Ki
Hong,^a Tan-Viet Pham,^c
Deok-Kun Oh^{b,*} and
Lin-Woo Kang^{a,*}

^aDepartment of Biological Sciences, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea, ^bDepartment of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea, and ^cDepartment of Advanced Technology Fusion, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea

‡ These authors contributed equally to this work.

Correspondence e-mail:
deokkun@konkuk.ac.kr, lkang@konkuk.ac.kr

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Expression, crystallization and preliminary X-ray crystallographic analysis of alcohol dehydrogenase (ADH) from *Kangiella koreensis*

Alcohol dehydrogenases (ADHs) are a group of dehydrogenase enzymes that facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of NAD⁺ to NADH. In bacteria, some alcohol dehydrogenases catalyze the opposite reaction as part of fermentation to ensure a constant supply of NAD⁺. The *adh* gene from *Kangiella koreensis* was cloned and the protein (KkADH) was expressed, purified and crystallized. A KkADH crystal diffracted to 2.5 Å resolution and belonged to the monoclinic space group *P*2₁, with unit-cell parameters *a* = 94.1, *b* = 80.9, *c* = 115.6 Å, β = 111.9°. Four monomers were present in the asymmetric unit, with a corresponding *V*_M of 2.55 Å³ Da⁻¹ and a solvent content of 51.8%.

1. Introduction

Alcohol dehydrogenases (ADHs; EC 1.1.1.1) are a group of dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of NAD⁺ to NADH (Nosova *et al.*, 1997). In bacteria, some alcohol dehydrogenases catalyze the opposite reaction as part of fermentation to ensure a constant supply of NAD⁺ (Wales & Fewson, 1994). ADHs exist as dimers that comprise 40 kDa subunits and contain zinc in their catalytic site.

ADHs have been widely investigated in various organisms. Unlike alcohol dehydrogenases from eukaryotes, only a few structures of prokaryotic ADHs have been identified to date. Among the reported ADHs, an ADH from a frog (ADH8) is the only alcohol dehydrogenase that has specificity towards NADP(H) instead of NAD(H) (Peralba *et al.*, 1999). A *BLASTP* search at NCBI using ADH8 as a template was performed to search for a bacterial homologue of ADH8 that may share similar activity with ADH8. The *BLASTP* results showed that ADH from *Kangiella koreensis* shared the highest sequence identity (48.9%) with ADH8.

K. koreensis is a Gram-negative, nonmotile, non-spore-forming bacterium isolated from tidal flat sediments at Daepo Beach, Yellow Sea, Korea (Han *et al.*, 2009). Its phylogeny is of interest because of the very isolated location of the genus *Kangiella* in the gamma-proteobacterial order Oceanospirillales (Han *et al.*, 2009). In order to obtain a better understanding of the specific function of *K. koreensis* ADH, the *adh* gene was cloned into *Escherichia coli* ER2566 using the pRSFDuet-1 vector. This study describes the expression, purification, crystallization and preliminary X-ray crystallographic studies of *K. koreensis* ADH.

2. Materials and methods

2.1. Cloning

The genomic DNA from *K. koreensis* DSM 16069 was extracted using a genomic DNA extraction kit (Qiagen, Hilden, Germany). The *Kkadh* gene (1123 bp) encoding the alcohol dehydrogenase was amplified by PCR using the genomic DNA isolated from *K. koreensis* as a template. The sequences of the oligonucleotide primers used

for gene cloning were based on the DNA sequence of *K. koreensis* alcohol dehydrogenase (GenBank accession No. NC_013166). Forward (5'-**GGATCCAATGTGCAACGAAGTGATTAATG**-3') and reverse (5'-**CTCGAGATAATGAATCACGCTACGAATA**-3') primers were designed to introduce *Bam*HI and *Xho*I restriction sites (bold), respectively. The PCR-amplified DNA fragments were purified using a QIAquick gel-extraction kit (Qiagen, Hilden, Germany), inserted into the pRSFDuet-1 vector digested with the same restriction enzymes and ligated into the *Bam*HI and *Xho*I sites of pRSFDuet-1, yielding the recombinant clone pRSFDuet-1-KkADH. The pRSFDuet-1-KkADH plasmid was transformed into *E. coli* ER2566 and plated on Luria-Bertani (LB) agar containing 20 $\mu\text{g ml}^{-1}$ kanamycin. A kanamycin-resistant colony was selected and plasmid DNA from the transformant was isolated using a plasmid purification kit (Promega, Madison, Wisconsin, USA). DNA sequencing was carried out at the Macrogen facility (Seoul, Republic of Korea).

2.2. Overexpression and purification

The recombinant *E. coli* cells containing pRSFDuet-1-KkADH coding for residues 1–374 were cultivated in a 2 l flask containing 500 ml LB medium and 20 $\mu\text{g ml}^{-1}$ kanamycin at 310 K with shaking at 200 rev min^{-1} . When the optical density of the bacteria reached 0.6 at 600 nm, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce expression of KkADH. The culture was incubated with shaking at 150 rev min^{-1} and 288 K for an additional 12 h to obtain the highest expression level. The cells were harvested by centrifugation for 20 min at 6000g and 277 K, washed twice with 0.85% NaCl and then resuspended in 50 mM phosphate buffer containing 300 mM KCl and 10 mM imidazole. The resuspended cells were disrupted on ice using a sonicator (Sonic Vibra Cell, Sonics & Materials Inc.). The unbroken cells and cell debris were removed by centrifugation at 13 000g for 10 min at 277 K and the supernatant was filtered through a 0.45 μm filter.

The filtrate was applied onto an immobilized metal-ion affinity chromatography cartridge (Bio-Rad, Hercules, California, USA) equilibrated with 50 mM phosphate buffer pH 8.0. The cartridge was washed extensively with the same buffer and the bound protein was eluted with a linear gradient from 10 to 250 mM imidazole at a flow rate of 1 ml min^{-1} . The eluate was collected and immediately loaded

onto a Bio-Gel P-6 desalting cartridge (Bio-Rad) equilibrated with 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer pH 7.5. The loaded protein was eluted with 50 mM PIPES buffer pH 7.5 at flow rate of 1 ml min^{-1} and the active fractions were collected. The resulting solution was used as the purified enzyme. The homogeneity of the purified protein was analyzed via SDS-PAGE (Fig. 1). 13 additional residues (GSSHHHHHSQDP) from the pRSF Duet-1 vector remained at the N-terminus of KkADH. For crystallization, purified KkADH was dialyzed for 4 h in buffer A (25 mM Tris pH 7.5, 15 mM NaCl, 3 mM β -mercaptoethanol) and concentrated to a final concentration of 8 mg ml^{-1} .

2.3. Crystallization and X-ray data collection

Initial crystallization was carried out at 287 K by the sitting-drop vapour-diffusion method in 96-well Intelli-Plates (Art Robbins) using a Hydra II eDrop automated pipetting system (Matrix) and screening kits from Hampton Research (Index, Crystal Screen, Crystal Screen Cryo, Crystal Screen Lite, PEGRx 1 and PEGRx 2), Emerald BioSystems (Wizard Classic 1 and 2) and Molecular Dimensions (Morpheus). 0.5 μl protein solution was mixed with 0.5 μl reservoir solution and equilibrated against 70 μl reservoir solution. After 3 d, multiple thin needle-shaped crystals (with the appearance of broom heads) were observed in condition F12 of the Index kit from Hampton Research [0.1 M sodium chloride, 0.1 M HEPES pH 7.5, 25%(w/v) PEG 3350; Fig. 2a]. Crystals were reproduced by the sitting-drop method, in which drops consisting of 0.9 μl protein

Figure 1
Purified *K. koreensis* ADH is shown on a 12% SDS-PAGE gel. Lane M, molecular-mass markers (labelled in kDa); lane P, KkADH.

(a)

(b)

Figure 2
Crystals of *K. koreensis* ADH. (a) Initial crystals obtained after 3 d using a reservoir solution consisting of 0.1 M sodium chloride, 0.1 M HEPES pH 7.5, 25%(w/v) PEG 3350 from the Index kit (Hampton Research). (b) Optimized crystal of KkADH with dimensions of 0.1 $\times$ 0.07 $\times$ 0.008 mm obtained using a condition consisting of 0.1 M sodium chloride, 0.1 M HEPES pH 8.0, 24%(w/v) PEG 3350. The scale bar represents 0.02 mm.

solution mixed with 0.9 μ l reservoir solution were equilibrated against 1 ml reservoir solution. Optimization was achieved by varying the concentration of PEG 3350, the pH and the incubation temperature. Crystals with adequate dimensions were obtained after 5 d at 303 K using a reservoir solution consisting of 0.1 M sodium chloride, 0.1 M HEPES pH 8.0, 24%(w/v) PEG 3350 (Fig. 2*b*). The fully grown crystals (0.1 $\times$ 0.07 $\times$ 0.008 mm) were flash-cooled at 100 K in liquid nitrogen using 20%(v/v) glycerol, 0.1 M sodium chloride, 0.1 M HEPES pH 8.0, 24%(w/v) PEG 3350 as a cryoprotectant. X-ray diffraction data were collected from the cryoprotected crystal (at 100 K) on an ADSC Q315r detector using 1° oscillations with a crystal-to-detector distance of 400 mm on beamline 5C SBII at the Pohang Light Source (PLS), Republic of Korea. The crystals diffracted to 2.5 Å resolution. A diffraction image is shown in Fig. 3. Diffraction data were integrated and scaled using the *HKL-2000* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Like other ADHs, KkADH eluted in a dimeric form in size-exclusion chromatography. Initial crystals obtained from sparse-matrix crystallization screening were multiple thin needles with the appearance of broom heads. Crystals with adequate dimensions were obtained after one month by the sitting-drop method at room temperature (Fig. 2*b*). A shorter growth time was achieved (5 d) by changing the incubation temperature from room temperature to 303 K.

The crystal belonged to the monoclinic space group *P*2₁. The unit-cell parameters were $a = 94.1$, $b = 80.9$, $c = 115.6$ Å, $\beta = 111.9^\circ$. The space group was assigned by auto-indexing (Otwinowski & Minor, 1997) and data-collection statistics are provided in Table 1. According to calculation of the Matthews coefficient (Matthews, 1968), there are probably four molecules in the asymmetric unit, with a V_M of 2.55 Å³ Da⁻¹ and a solvent content of 51.8%. Self-rotation functions were calculated at $\chi = 180$, 120, 90 and 60° to detect twofold, threefold, fourfold and sixfold symmetry, respectively. The self-rotation function was calculated using data from 50 to 4 Å resolution

Table 1

Data-collection statistics.

Values in parentheses are for the outer shell.

X-ray source	Beamline 5C SBII, PLS
Wavelength (Å)	0.97951
Unit-cell parameters (Å, °)	$a = 94.1$, $b = 80.9$, $c = 115.6$, $\alpha = \gamma = 90.0$, $\beta = 111.9$
Total rotation (°)	360
Mosaicity (°)	0.6
Multiplicity	7.2 (5.3)
Space group	<i>P</i> 2 ₁
Resolution	50.0–2.5 (2.54–2.50)
Total No. of observations	400525
No. of unique observations	55363
Completeness (%)	99.3 (90.6)
$R_{\text{merge}}^\dagger$ (%)	7.4 (38.8)
$\langle I/\sigma(I) \rangle$	42.8 (5.3)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

in *MOLREP* (Vagin & Teplyakov, 2010). A strong peak was displayed in the $\chi = 180^\circ$ section, indicating the presence of twofold noncrystallographic symmetry (Fig. 4). The presence of pseudo-translation was also detected by *MOLREP*. Molecular replacement (MR) using *Phaser* in the *CCP4* program package (McCoy *et al.*, 2007) with human glutathione-dependent formaldehyde dehydrogenase (PDB entry 2fze; 69.3% sequence identity; Lang *et al.*, 2012) as a search model was successful and showed that four monomers were present in the asymmetric unit. The initial R value from the MR solution was 49.2%. The resulting electron-density maps were clear and fitted the main chain of the model well. No clashes were found between molecules. Rigid-body and restrained refinement were performed in *REFMAC5* (Murshudov *et al.*, 2011), which decreased the R value to 35.7% and R_{free} to 42.7%. The side chains that were outside the electron-density map were re-fitted and the coordinates were again input to *REFMAC5* to run restrained refinement. After several cycles of fitting and refining, the R value decreased to 19.5% and R_{free} to 25.5%. Currently, the structure is

Figure 3
X-ray diffraction image from a crystal of *K. koreensis* ADH.

Figure 4
Self-rotation function at $\chi = 180^\circ$ calculated for the KkADH crystal.

being refined. Final structural details will be described in a separate paper.

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