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Crystallization and preliminary X-ray study of alkaline alanine racemase from *Bacillus pseudofirmus* OF4

Jiansong Ju,[a]‡ Jianxun Qi,[b]‡ Shujing Xu,[c] Kouhei Ohnishi,[d] Michael J. Benedik,[e] Yanfen Xue[a] and Yanhe Ma[a]*

[a]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China, [b]Institute of Physics, Chinese Academy of Sciences, Beijing 100049, People's Republic of China, [c]Life College of Science and Engineering, Shaanxi University of Science and Technology, Xi'an 710021, People's Republic of China, [d]Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi 783-8502, Japan, and [e]Department of Biology, Texas A&M University, College Station, TX 77843-3258, USA

‡ These authors contributed equally to this work.

Correspondence e-mail: mayanhe@im.ac.cn

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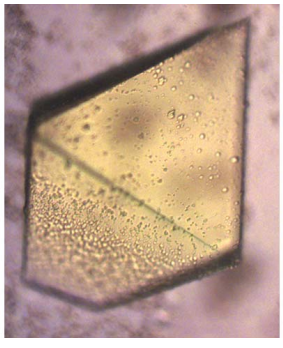

Alanine racemase ($DadX_{OF4}$), a dimeric endogenous PLP-dependent alkaline enzyme from alkaliphilic *Bacillus pseudofirmus* OF4, was expressed in *Escherichia coli* and purified with a His_6 tag in a form suitable for X-ray crystallographic analysis. Crystals were grown by the hanging-drop vapour-diffusion method at 291 K using a solution containing 1.4 *M* sodium/potassium phosphate pH 8.2. The protein crystallized in space group $P2_12_12_1$, with two protein molecules in the asymmetric unit.

1. Introduction

D-Alanine is an essential component of bacterial peptidoglycans and is produced through the racemization of naturally occurring L-alanine catalyzed by alanine racemase (EC 5.1.1.1). Alanine racemase is a pyridoxal 5′-phosphate (PLP) dependent enzyme (Walsh, 1989; Uo *et al.*, 2001); a lysine residue connected to the PLP cofactor by an internal aldimine bond acts as a base for the conversion of D-alanine to L-alanine, while a nearby tyrosine from a second monomer acts as a base for the abstraction of a hydrogen from L-alanine (Strych *et al.*, 2007; Watanabe *et al.*, 1999).

Alanine racemase is ubiquitous in bacteria and only a few similar enzymes have been found in eukaryotes such as yeast (Uo *et al.*, 2001), black tiger prawn (Yoshikawa *et al.*, 2002) and alfalfa seedlings (Ono *et al.*, 2006). Therefore, alanine racemase has attracted much interest as a possible target for antibacterial drugs. Not only is D-alanine a vital component of the bacterial cell wall, but recent studies have also indicated that alanine racemase, which is accessible in the exosporium, plays a key role in the inhibition of germination in *Bacillus* spores (Yan *et al.*, 2007). L-Alanine is an effective germination-promoting compound in *B. cereus* and D-alanine is an effective inhibitor of L-alanine-induced germination (Preston & Douthit, 1988).

Alkaliphilic *B. pseudofirmus* OF4 are obligately aerobic spore-forming Gram-positive motile rods with an optimal pH of 10.5 and a temperature optimum of 303 K (Takami & Krulwich, 2000; Guffanti *et al.*, 1986). Using the partially sequenced genome sequence as a guide, alanine racemase ($DadX_{OF4}$; EU751624) from *B. pseudofirmus* OF4 has been cloned, expressed and characterized (Ju *et al.*, 2009). The enzyme is a strongly endogenous PLP-dependent dimeric enzyme with a high optimal pH value of 10.5 and high catalytic activity: the kinetic parameters K_m and V_{max} at 313 K were 41.79 m*M* and 10 500 units mg^{-1} for L-alanine, and 14.91 m*M* and 3708 units mg^{-1} for D-alanine, respectively.

In order to elucidate the structural basis of the high catalytic activity, high optimal pH value and strong binding of endogenous PLP, crystals of $DadX_{OF4}$ have been obtained. Here, we report the crystallization and preliminary X-ray diffraction data analysis of recombinant $DadX_{OF4}$ protein with a His_6 tag. The three-dimensional structure of this protein is currently being determined.

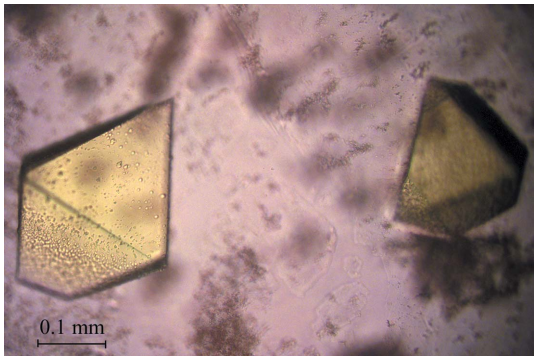

Figure 1
Typical crystals of $DadX_{OF4}$.

2. Materials and methods

2.1. Expression, purification and crystallization

The gene cloning, expression and purification of the protein to homogeneity by His_6-tag affinity-column, gel-filtration and ion-exchange chromatography have been described elsewhere (Ju *et al.*, 2009). Purified $DadX_{OF4}$ protein (EU751624; 369 residues) with eight additional residues at the C-terminus (LEHHHHHH) was used for crystallization screening. Crystallization screening was carried out using Crystal Screens I and II, Index, SaltRx and PEG/Ion from Hampton Research at 291 K with the hanging-drop method in 16-well plates. The protein concentration was between 5 and 20 mg ml^{-1} in 10 m*M* phosphate buffer pH 8.0 with 10 µ*M* PLP; this solution was also used to dilute the protein for crystallization studies. 2 µl protein solution mixed with an equal volume of reservoir solution was equilibrated against 300 µl reservoir solution.

2.2. X-ray data collection and processing

For X-ray diffraction measurements, the $DadX_{OF4}$ crystals were first soaked in reservoir solution containing 30%(*v*/*v*) glycerol for about 30 s. The soaked crystals were then mounted in nylon loops and flash-cooled in a stream of gaseous nitrogen (Parkin & Hope, 1998) at 100 K.

Table 1
Diffraction data statistics.

Values in parentheses are for the outer shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
a	97.21
b	97.44
c	112.54
Resolution range (Å)	40.62–1.95 (2.02–1.95)
Total No. of reflections	514047
No. of unique reflections	77216
Average redundancy	6.66 (6.53)
Completeness (%)	98.4 (97.7)
R_{merge}† (%)	6.7 (30)
Average $I/\sigma(I)$	14.3 (5.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean intensity of all symmetry-related reflections.

Diffraction data were collected using an in-house X-ray source (Rigaku MicroMax007 desktop rotating-anode X-ray generator with a Cu target operated at 40 kV and 30 mA) and an R-AXIS IV++ imaging-plate detector at a wavelength of 1.5418 Å. The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results

Recombinant $DadX_{OF4}$ protein was successfully expressed and purified to apparent homogeneity. $DadX_{OF4}$ with a His_6 tag was confirmed as a functional alanine racemase in the D-alanine auxotrophic strain MB2795 (Ju *et al.*, 2009). Its molecular mass was estimated to be 4 kDa on the basis of relative mobility on the SDS–PAGE gel, which is in good agreement with the predicted molecular mass of 41 387 Da.

Crystal Screens I and II, Index, SaltRx and PEG/Ion kits from Hampton Research were used for crystallization screening using the hanging-drop vapour-diffusion technique. After 6–90 d incubation at 291 K, crystals with different colours and shapes were obtained using condition No. 21 of SaltRx (deep yellow small cubes), condition No. 69 of SaltRx (deep yellow irregular tetrahedra), condition No. 8

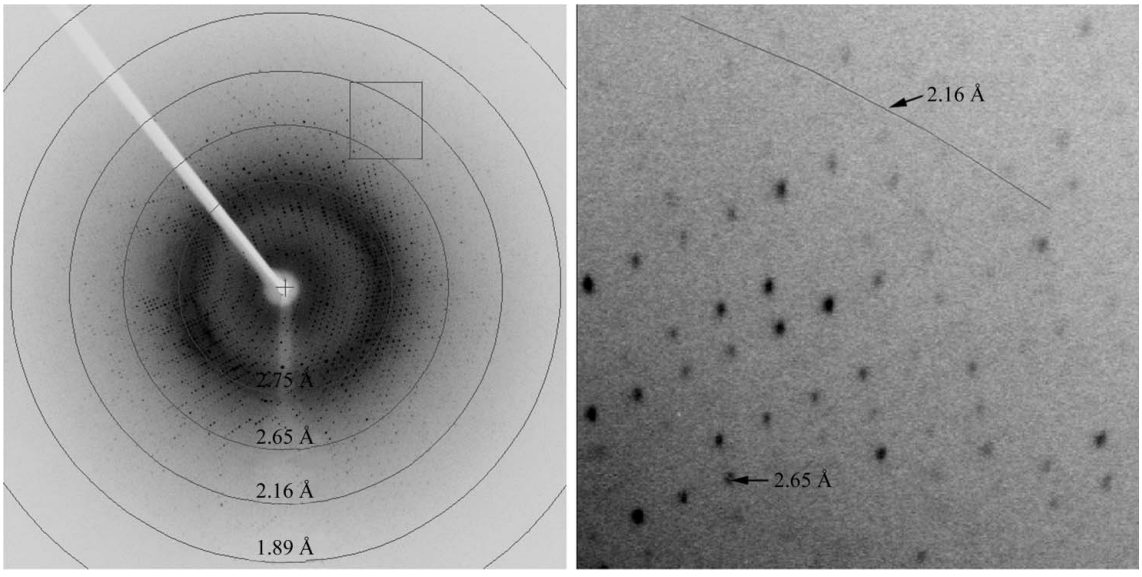

Figure 2
Diffraction pattern of a $DadX_{OF4}$ crystal. The image on the right is an enlargement of the area framed on the left.

of Crystal Screen II (colourless prisms) and condition No. 19 of Index Screen (deep yellow bipyramids). After eight weeks, a well diffracting crystal was obtained using condition No. 19 of Index Screen, which contained 1.4 *M* sodium/potassium phosphate pH 8.2, with a protein concentration of 7.5 mg ml^{-1}. The largest crystals were about 250 × 250 × 265 µm in size (Fig. 1). Further studies revealed that crystals grew with good reproducibility using this condition.

A summary of the data-collection statistics of this crystal is given in Table 1. The data set is 98.4% complete at 1.95 Å resolution (Fig. 2), with an $R_{\rm merge}$ of 6.7%. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 97.21, b = 97.44, c = 112.54 Å, and contains two protein molecules per asymmetric unit (the Matthews coefficient is about 3.22 Å^3 Da^{-1}, corresponding to a solvent content of 61.8%; Matthews, 1968).

The three-dimensional structure of the DadX$_{\rm OF4}$ alanine racemase will provide insights into the biochemical properties of the alanine racemase from *B. pseudofirmus* OF4, including its alkaline adaptation, strongly endogenous PLP-dependency and high catalytic activity.

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References

Guffanti, A. A., Finkelthal, O., Hicks, D. B., Falk, L., Sidhu, A., Garro, A. & Krulwich, T. A. (1986). *J. Bacteriol.* **167**, 766–773.
Ju, J., Xu, S., Wen, J., Li, G., Ohnishi, K., Xue, Y. & Ma, Y. (2009). In the press.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Ono, K., Yanagida, K., Oikawa, T., Ogawa, T. & Soda, K. (2006). *Phytochemistry*, **67**, 856–860.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Parkin, S. & Hope, H. (1998). *J. Appl. Cryst.* **31**, 945–953.
Preston, R. A. & Douthit, H. A. (1988). *J. Gen. Microbiol.* **134**, 3001–3010.
Strych, U., Davlieva, M., Longtin, J. P., Murphy, E. L., Im, H., Benedik, M. J. & Krause, K. L. (2007). *BMC Microbiol.* **7**, 40–46.
Takami, H. & Krulwich, T. A. (2000). *Extremophiles*, **4**, 19–22.
Uo, T., Yoshimura, T., Tanaka, N., Takegawa, K. & Esaki, N. (2001). *J. Bacteriol.* **183**, 2226–2233.
Walsh, C. T. (1989). *J. Biol. Chem.* **264**, 2393–2396.
Watanabe, A., Yoshimura, T., Mikami, B. & Esaki, N. (1999). *J. Biochem.* **125**, 987–990.
Yan, X., Gai, Y., Liang, L., Liu, G. & Tan, H. (2007). *Arch. Microbiol.* **187**, 371–378.
Yoshikawa, N., Dhomae, N., Takio, K. & Abe, H. (2002). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **133**, 445–453.