Hui-Min Qin,^a Takuya Miyakawa,^a Akira Nakamura,^a You-Lin Xue,^a Takashi Kawashima,^b Takuya Kasahara,^b Makoto Hibi,^c Jun Ogawa^b and Masaru Tanokura^{a*}

^aDepartment of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, ^bDivision of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan, and ^cLaboratory of Industrial Microbiology, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

Correspondence e-mail:
amtanok@mail.ecc.u-tokyo.ac.jp

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Expression, purification, crystallization and preliminary X-ray analysis of a novel N-substituted branched-chain L-amino-acid dioxygenase from *Burkholderia ambifaria* AMMD

Ferrous ion- and α -ketoglutarate-dependent dioxygenase from *Burkholderia ambifaria* AMMD (SadA) catalyzes the C3-hydroxylation of N-substituted branched-chain L-amino acids, especially N-succinyl-L-leucine, coupled to the conversion of α -ketoglutarate to succinate and CO₂. SadA was expressed in *Escherichia coli*, purified and crystallized using the sitting-drop vapour-diffusion method at 293 K. Crystals of selenomethionine-substituted SadA were obtained using a reservoir solution containing PEG 3000 as the precipitant at pH 9.5 and diffracted X-rays to 2.4 Å resolution. The crystal belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 49.3, *b* = 70.9, *c* = 148.2 Å. The calculated Matthews coefficient (*V*_M = 2.1 Å³ Da⁻¹, 41% solvent content) suggested that the crystal contains two molecules per asymmetric unit.

1. Introduction

The ferrous ion- and α -ketoglutarate-dependent dioxygenases can catalyze the hydroxylation of proteins, nucleic acids, lipids and small molecules (McDonough *et al.*, 2010). These enzymes participate in a vast array of protein side-chain modifications, repair of alkylated DNA/RNA, biosynthesis of antibiotics and plant products and so on (Hausinger, 2004). The enzymatic hydroxylation of prolyl and lysyl residues is a well known post-translational modification (Prockop & Kivirikko, 1995) and the related human dioxygenases have been proposed as targets in cancer treatment (Shi, 2007). Asymmetric synthesis is increasingly being targeted for drug synthesis, which can benefit greatly from the selectivity gains associated with enzymatic catalysis (Matsuda *et al.*, 2009; Savile *et al.*, 2010). For example, L-isoleucine dioxygenase from *Bacillus thuringiensis* can stereoselectively hydroxylate L-Ile to (2*S*,3*R*,4*S*)-4-hydroxyisoleucine, a natural nonproteinogenic amino acid which has insulinotropic and anti-obesity effects and appears to be a potential drug for diabetes (Kodera *et al.*, 2009).

Hydroxylation reactions as catalyzed by dioxygenases require dioxygen as well as ferrous ion (Fe^{II}) and α -ketoglutarate (α -KG). One of the O atoms is incorporated into the substrate to form a hydroxyl amino acid, while the other O atom is used to oxidatively break down α -KG into succinate and CO₂. Structural analyses have revealed that this family of enzymes possess a common protein fold called the double-stranded β -helix (DSBH) fold as the core of the structure and an HXD/EX_nH motif in the active site that coordinates the Fe^{II} cofactor (Chowdhury *et al.*, 2009; Clifton *et al.*, 2006; McDonough *et al.*, 2010; Strieker *et al.*, 2007). However, there is much greater variation in the secondary substrate-binding sites, which define the substrate specificity and stereoselectivity of the hydroxylation reaction.

SadA is a member of the dioxygenase family from *Burkholderia ambifaria* AMMD and consists of 273 amino-acid residues (30 664 Da). This enzyme catalyzes (*R*)-selective hydroxylation at the C3 position of N-substituted branched-chain L-amino acids, especially N-succinyl-L-leucine, from which N-succinyl-(*R*)-3-hydroxy-L-leucine is produced with >99% diastereoselectivity (Hibi *et al.*, unpublished data). (*R*)-3-Hydroxy-L-leucine in particular is a promising target material for the preparation of certain cyclic depsipeptides (Taniuchi *et al.*, 2003; Tymiak *et al.*, 1989). SadA is the first enzyme that has been shown to catalyze the C3-hydroxylation of aliphatic amino-

acid-related substances. Sequence alignment shows that SadA has low sequence identity to other members of the family (12% sequence identity to PHD2; PDB entry 3hqr; Chowdhury *et al.*, 2009). However, the structure of SadA has not been determined. Therefore, high-resolution crystal structure analysis will be required in order to elucidate its stereoselective reaction mechanism.

Here, we report the expression, purification, crystallization and preliminary X-ray analysis of SadA in order to elucidate the structural basis of its stereoselective hydroxylation and substrate specificity. Determination of the structure of SadA may help us to better understand the relationship between its structure and function. Furthermore, SadA may be utilized as an industrial enzyme for the manufacture of pharmaceuticals on a commercial scale in the future.

2. Materials and methods

2.1. Overexpression and purification

The SadA gene (GenBank accession No. YP_777923) was cloned into pQE80 vector (Qiagen) between the *Bam*HI and *Hind*III sites. The expressed SadA protein was fused to a hexahistidine (His₆) tag (MRGSHHHHHHGS) at the N-terminus. *Escherichia coli* Rosetta (DE3) cells (Novagen) were transformed with the plasmid and were grown in lysogeny broth (LB) medium containing 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol at 310 K. When the OD₆₀₀ reached 0.5, the cells were transferred into M9 medium supplemented with 50 µg ml⁻¹ selenomethionine (SeMet). The culture was cooled to 298 K, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture was further incubated at 298 K overnight.

The cells were harvested by centrifugation and disrupted by sonication in resuspension buffer (20 mM Tris-HCl pH 8.0, 10 mM imidazole, 0.5 M NaCl, 1 mM dithiothreitol). The cell debris was removed by centrifugation and filtration. SeMet-substituted SadA (SadA^{SeMet}) in the solution was trapped on Ni-NTA Superflow resin (Qiagen) using the His₆ tag. After washing, the His₆-tagged protein was eluted with resuspension buffer containing 200 mM imidazole. The eluted solution was dialyzed against 20 mM Tris-HCl pH 8.0, 1 mM dithiothreitol, and the SadA^{SeMet} was further purified using Resource Q (GE Healthcare). The solution containing purified

SadA^{SeMet}, from which the His₆ tag had not been cleaved, was concentrated to 15 mg ml⁻¹ using Vivaspin-20 (10 000 molecular-weight cutoff) prior to crystallization trials.

2.2. Crystallization

Crystallization of SadA^{SeMet} was carried out with the sparse-matrix screening kits Crystal Screen HT (Hampton Research), Index HT (Hampton Research) and Wizard I and II (Emerald BioSystems) in 96-well plates using the sitting-drop vapour-diffusion method. For refinement of the crystallization conditions, 1 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 0.5 ml reservoir solution at 293 K in 24-well plates (Hampton Research).

2.3. X-ray data collection and processing

X-ray diffraction data were collected from a SadA^{SeMet} crystal on the BL-17A beamline at Photon Factory, Tsukuba, Japan. The wavelength (0.97894 Å) for data collection was determined from the selenium absorption spectrum. The single-wavelength anomalous diffraction (SAD) data set for the SadA^{SeMet} crystal was composed of 720 images and was collected using a 0.5° oscillation with a crystal-to-detector distance of 361.6 mm and an exposure time of 2 s for each image. All diffraction data were indexed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

SadA^{SeMet} was expressed in *E. coli* and purified by affinity-resin and anion-exchange chromatography. Crystals of SadA^{SeMet} were obtained using a reservoir solution consisting of 30% (w/v) PEG 3000, 0.1 M CHES pH 9.5 at 293 K. Fig. 1 shows a typical crystal 3 d after initiation of crystallization, with approximate dimensions of 0.10 × 0.20 × 0.15 mm. X-ray diffraction data were collected to 2.4 Å resolution. Fig. 2 shows an X-ray diffraction image of the SadA^{SeMet} crystal. The crystal belonged to space group *P*₂₁₂₁₂₁, with unit-cell parameters *a* = 49.3, *b* = 70.9, *c* = 148.2 Å. 20 990 unique reflections were collected in the resolution range 20–2.40 Å with 100% completeness and an *R*_{merge} of 8.7%. The data-collection and

Figure 1
Typical crystal of SadA^{SeMet} grown at 293 K using 30% (w/v) PEG 3000 as the precipitant at pH 9.5. The crystal has approximate dimensions of 0.10 × 0.20 × 0.15 mm.

Figure 2
An X-ray diffraction image (0.5° oscillation) from the SadA^{SeMet} crystal. The edge of the detector corresponds to 2.4 Å resolution (indicated by the circle).

Table 1Data-collection statistics for SadA^{SeMet}.

Values in parentheses are for the highest resolution shell.

Beamline	BL-17A, Photon Factory
Wavelength (Å)	0.97894
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)	<i>a</i> = 49.3, <i>b</i> = 70.9, <i>c</i> = 148.2
Resolution (Å)	20–2.40 (2.44–2.40)
Unique reflections	20990 (1041)
Multiplicity	14.3 (14.2)
Completeness (%)	100 (100)
<i>R</i> _{merge} [†]	0.087 (0.423)
<i>I</i> / <i>σ</i> (<i>I</i>)	64.2 (7.8)

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

processing statistics are summarized in Table 1. Matthews analysis suggested that there were two molecules per asymmetric unit in the SadA crystal (Matthews coefficient of 2.1 Å³ Da^{−1}, 41% solvent content; Matthews, 1968). Determination of the three-dimensional structure will be performed by the SAD method using the data set from the SadA^{SeMet} crystal. The protein structure obtained by X-ray crystallography will be utilized to characterize the substrate-binding site of SadA.

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