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Expression, purification, crystallization and preliminary X-ray analysis of carbonyl reductase S1 from *Candida magnoliae*

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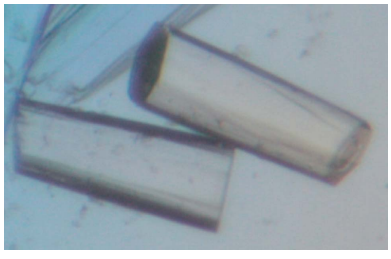

The NADPH-dependent carbonyl reductase S1 from *Candida magnoliae* stereoselectively catalyzes the reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate (CHBE), which is a chiral compound valuable as a building block for pharmaceuticals. Carbonyl reductase S1 was expressed in *Escherichia coli* and purified by Ni-affinity, ion-exchange and size-exclusion chromatography. Crystals of carbonyl reductase S1 were obtained by the sitting-drop vapour-diffusion method using PEG 400 as a precipitant. X-ray diffraction data were collected to 1.90 Å resolution using a synchrotron-radiation source. The crystals belonged to space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 77.7$, $c = 307.5$ Å. The asymmetric unit contained two molecules of the protein, with a solvent content of 44.2%.

1. Introduction

Recently, the demand for chiral compounds as useful building blocks for the synthesis of medicines has increased in the pharmaceutical industry. For example, alkyl (*R*)-3-hydroxybutanoate and (*R*)-3-quinuclidinol are important intermediates in the synthesis of angiotensin-converting enzyme inhibitors (Hasegawa & Nagashima, 2000) and bronchodilatory agents (Prat *et al.*, 2009), respectively. Optical purity is the most important consideration in the use of these chiral compounds as building blocks, because in certain cases compounds with the opposite stereoconfiguration can have undesirable and/or harmful effects on the human body (Smith, 2009). Additionally, the production efficiency of chiral compounds is another important factor in terms of industrial production. Therefore, production technologies have been studied in order to obtain optically pure chiral compounds with high efficiency.

Ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] is the starting chiral compound for the synthesis of HMG-CoA reductase inhibitor (Patel *et al.*, 1992), which is widely used as a cholesterol-lowering drug. (*S*)-CHBE can be synthesized by chemical syntheses (Pai *et al.*, 2000, 2002; Jeulin *et al.*, 2004), but these syntheses require the troublesome optical resolution of a racemic mixture of products (Shimizu *et al.*, 1998). Furthermore, this process generates a large amount of liquid waste. Biocatalytic processes are an attractive way of producing chiral compounds with less liquid waste and high enantioselectivity. Therefore, research into and development of asymmetric bioreduction has been conducted in order to synthesize (*S*)-CHBE using microbial cells or enzymes (Shimizu *et al.*, 1998; Kita *et al.*, 1999).

In this context, carbonyl reductase S1 has been isolated from *Candida magnoliae* AKU4643 as a novel enzyme that produces (*S*)-CHBE (Wada *et al.*, 1998). The enzyme reduces ethyl 4-chloro-3-oxobutanoate (COBE) to (*S*)-CHBE with an enantiomeric excess of 100%. It was identified as a member of the short-chain dehydrogenase/reductase (SDR) family and showed strict NADPH dependence (Wada *et al.*, 1998). To reveal the molecular mechanism underlying the extremely high stereoselectivity of the reaction, we

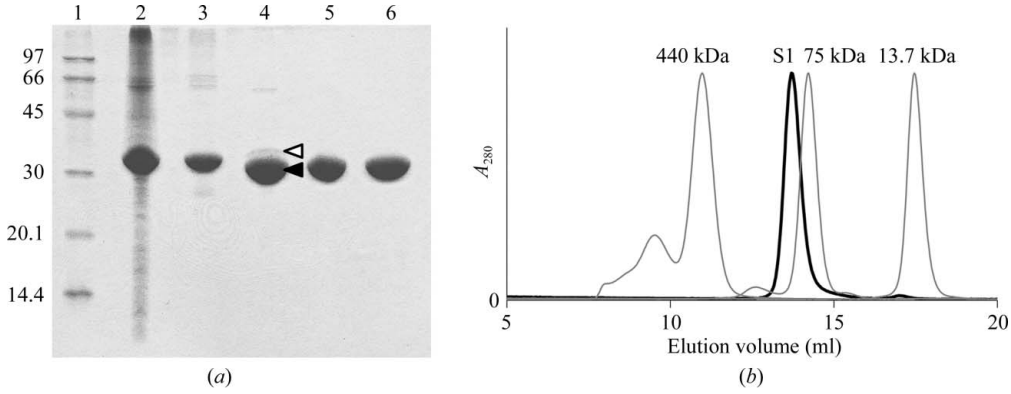

Figure 1
Results of the purification and quaternary-structure analysis of carbonyl reductase S1. (*a*) SDS–PAGE of the purification procedure. Lane 1, molecular-marker proteins (labelled on the left in kDa); lane 2, soluble fraction; lane 3, eluate from Ni-affinity chromatography; lane 4, after anion-exchange chromatography; lane 5, after gel-filtration chromatography. Open and filled arrowheads represent carbonyl reductase S1 with and without 6×His tag, respectively. (*b*) Chromatograms of the gel-filtration analyses. The thick black curve and thin grey curves show the absorbance at 280 nm of carbonyl reductase S1 and the molecular standards, respectively. The peak heights were adjusted to be equal for the sake of comparison.

are currently attempting to solve the crystal structure of carbonyl reductase S1. Here, we report the expression, purification, crystallization and preliminary X-ray analysis of this enzyme.

2. Methods and results

2.1. Protein expression and purification

The gene encoding carbonyl reductase S1 (Yasohara *et al.*, 2000) was subcloned into pET28a vector (Novagen) using the *Nde*I and *Bam*HI restriction sites. This construct contains an additional N-terminal hexahistidine (6×His) tag followed by a thrombin protease cleavage site. The recombinant carbonyl reductase S1 was designed to contain a residual Gly-Ser-His sequence after thrombin protease digestion. *Escherichia coli* Rosetta (DE3) (Novagen) cells were transformed with the expression vector constructed above and the recombinant cells were cultured in 1 l LB medium containing 10 µg ml^{-1} kanamycin at 310 K until the OD_{600} reached 0.6. Protein expression was induced by the addition of 0.1 m*M* isopropyl *β*-D-1-thiogalactopyranoside. Cell growth was continued at 298 K for 20 h after initiating IPTG induction.

The cells were harvested by centrifugation at 5000*g* for 10 min and were resuspended in 50 m*M* Tris–HCl buffer pH 8.0 containing 100 m*M* NaCl and 1 m*M* phenylmethylsulfonyl fluoride. The protein was extracted from the cells by sonication and was separated from insoluble materials by centrifugation at 40 000*g* for 30 min. The supernatant containing 6×His-tagged carbonyl reductase S1 was loaded onto an Ni-Sepharose 6 Fast Flow column (GE Healthcare). After washing the resin with 100 m*M* Tris–HCl buffer pH 7.5 containing 1 *M* NaCl and 40 m*M* imidazole, carbonyl reductase S1 was eluted in a single step with 50 m*M* Tris–HCl buffer pH 7.5 containing 500 m*M* NaCl and 500 m*M* imidazole. The eluate was dialyzed against 20 m*M* Tris–HCl buffer pH 8.0 and the 6×His-tagged carbonyl reductase S1 was digested by thrombin protease (3 U per milligram of protein; GE Healthcare) at 277 K for 18 h. More than 95% of the protein was digested. After centrifugation at 23 000*g* for 10 min, the supernatant was applied onto a Resource Q column (GE Healthcare) and eluted with a linear gradient of 0–400 m*M* NaCl in 20 m*M* Tris–HCl buffer pH 8.0. Two major peaks were obtained in the anion-exchange chromatography at around 150 m*M* NaCl. The first peak contained carbonyl reductase S1 with and without 6×His tag (theoretical pI of 6.95 and 6.54, respectively) and the second peak exclusively contained the protein without 6×His tag. The fractions containing carbonyl reductase S1 exclusively without 6×His tag were applied onto a Superdex 200 column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl buffer pH 8.0 containing 100 m*M* NaCl and eluted as a single peak. The chromatographic steps described above were sufficient to purify the protein (Fig. 1*a*). Finally, the protein solution was concentrated to 23 mg ml^{-1} in 20 m*M* Tris–HCl buffer pH 8.0 containing 100 m*M* NaCl using a Vivaspin 20 centrifugal concentrator (10 kDa molecular-weight cutoff; Sartorius). The purified protein solution was cooled in liquid nitrogen and stored at 193 K. Samples were analyzed by SDS–PAGE followed by Coomassie Brilliant Blue R250 staining. Protein concentrations were calculated from the absorbance at 280 nm using a molar extinction coefficient of 39 880 M^{-1} cm^{-1} (Pace *et al.*, 1995).

In order to estimate the quaternary structure of the purified recombinant carbonyl reductase S1, we performed gel-filtration chromatography using a Superdex 200 HR 10/30 column (GE Healthcare) calibrated with ferritin (440 kDa), conalbumin (75 kDa) and ribonuclease A (13.7 kDa) (Fig. 1*b*). The molecular weight of the recombinant carbonyl reductase S1 was estimated to be 101 kDa, while its theoretical molecular weight was 30.7 kDa, suggesting that

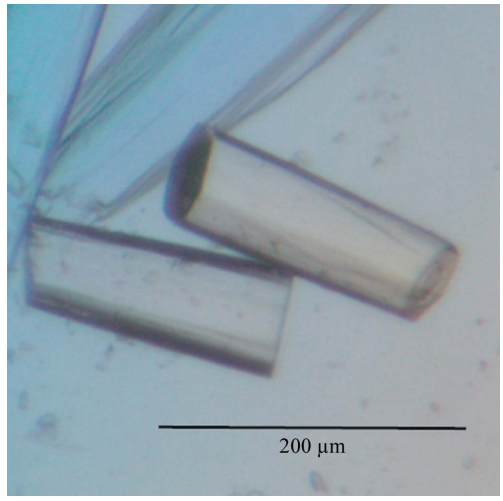

Figure 2
Typical crystals of carbonyl reductase S1 at 293 K obtained using the optimized crystallization conditions. The maximum length of the crystals was approximately 0.2 mm.

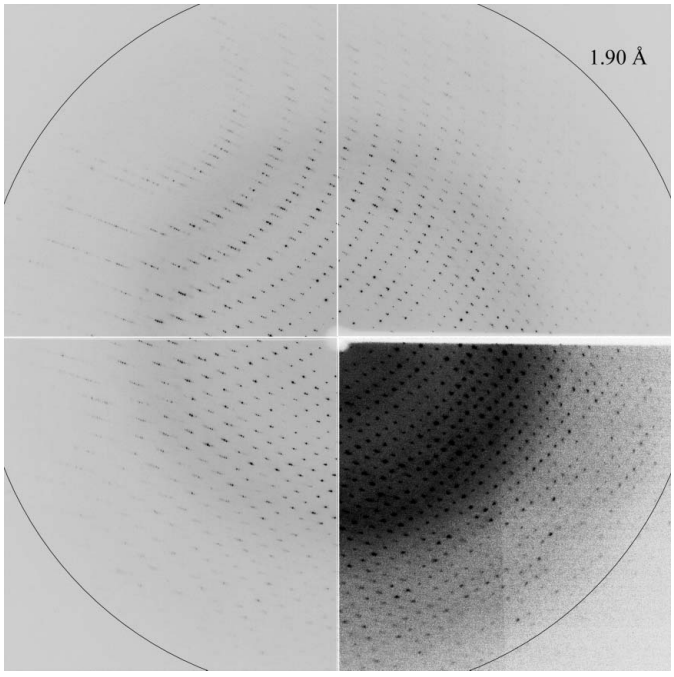

Figure 3
An X-ray diffraction image (0.2° oscillation) of a carbonyl reductase S1 crystal. The circle indicates a resolution of 1.90 Å.

the purified protein formed a tetramer, like some other short-chain dehydrogenase/reductase family proteins.

2.2. Crystallization and X-ray data collection

An initial crystallization condition composed of 30%(*v*/*v*) PEG 400, 0.2 *M* Li_2SO_4, 0.1 *M* sodium cacodylate pH 6.5 was obtained from Wizard I and II (Emerald BioSystems) by the sitting-drop vapour-diffusion method in 96-well plates (Art Robbins Instruments) using purified protein solution supplemented with 5 m*M* *β*-NADPH (Oriental Yeast). The crystallization condition was optimized by varying the precipitant concentration and pH and by using Additive Screen HT (Hampton Research). The final crystallization condition was 31%(*v*/*v*) PEG 400, 0.2 *M* Li_2SO_4, 0.08 *M* sodium potassium tartrate tetrahydrate, 0.1 *M* sodium cacodylate pH 6.5 at 293 K using the sitting-drop vapour-diffusion method in 24-well plates (Hampton Research). The crystallization drops were prepared by mixing 1 µl protein solution supplemented with 5 m*M* *β*-NADPH and 1 µl reservoir solution and were equilibrated against 0.5 ml reservoir solution. The crystals grew to dimensions of 60 × 60 × 200 µm within 3 d. Fig. 2 shows the crystals obtained using the final crystallization condition.

Crystals were soaked in a 7:3 mixture of reservoir solution and 100% ethylene glycol. The crystals were mounted in nylon loops (Hampton Research) and flash-cooled in a stream of nitrogen gas at 95 K. X-ray diffraction data were collected with an ADSC Quantum 210 CCD detector using synchrotron radiation on the AR-NW12 beamline of the Photon Factory, Tsukuba, Japan. A total of 900 diffraction images were collected at a wavelength of 1.0000 Å with a crystal-to-detector distance of 189.7 mm, 0.2° oscillation and 1 s exposure time. Fig. 3 shows an X-ray diffraction image. The diffraction images were indexed, integrated and scaled to 1.90 Å resolution with the program *XDS* (Kabsch, 2010).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Source	NW12A, Photon Factory
Wavelength (Å)	1.00000
Space group	$P6_122$ or $P6_522$
Unit-cell parameters (Å)	$a = b = 77.7$, $c = 307.5$
Resolution (Å)	20.0–1.90 (1.95–1.90)
Observed reflections	878833
Unique reflections	44625
Multiplicity	19.7 (12.0)
Data completeness (%)	99.8 (99.8)
R_{merge}†	0.062 (0.388)
R_{meas}‡	0.063 (0.405)
$\langle I/\sigma(I)\rangle$	37.6 (6.12)
Mosaicity (°)	0.187

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the observations $I_i(hkl)$ of reflection hkl. ‡ $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $N(hkl)$ is the multiplicity of the reflection hkl, $I_i(hkl)$ is the ith intensity measurement of the reflection hkl and $\langle I(hkl)\rangle$ is its average.

The space group was determined to be $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 77.7$, $c = 307.5$ Å. The data-collection statistics are summarized in Table 1. The mosaicity was estimated to be 0.187°, suggesting that the crystal was of good quality. The Matthews coefficient was 2.20 $Å^3$ Da^{-1} (Matthews, 1968) with a solvent content of 44.2%, assuming the presence of two molecules in the asymmetric unit. Structure determination by molecular replacement is currently under way.

The synchrotron-radiation experiments were performed on AR-NW12 at the Photon Factory, Tsukuba, Japan (Proposal No. 2008S2-001). This work was supported by the Targeted Proteins Research Program (TPRP) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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