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Crystallization and preliminary X-ray analysis of the NADPH-dependent 3-quinuclidinone reductase from *Rhodotorula rubra*

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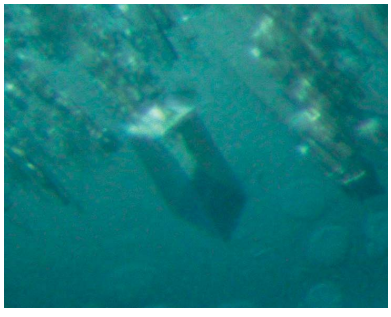

(*R*)-3-Quinuclidinol is a useful compound that is applicable to the synthesis of various pharmaceuticals. The NADPH-dependent carbonyl reductase 3-quinuclidinone reductase from *Rhodotorula rubra* catalyzes the stereospecific reduction of 3-quinuclidinone to (*R*)-3-quinuclidinol and is expected to be utilized in industrial production of this alcohol. 3-Quinuclidinone reductase from *R. rubra* was expressed in *Escherichia coli* and purified using Ni-affinity and ion-exchange column chromatography. Crystals of the protein were obtained by the sitting-drop vapour-diffusion method using PEG 8000 as the precipitant. The crystals belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 91.3$, $c = 265.4$ Å, and diffracted X-rays to 2.2 Å resolution. The asymmetric unit contained four molecules of the protein and the solvent content was 48.4%.

1. Introduction

Chiral compounds are valuable building blocks for pharmaceuticals and chemicals including chemical catalysts, liquid crystals, flavours, agrochemicals and drugs. In most cases, only one of the optical isomers shows the desired activity or efficacy and therefore it is necessary to develop methods to produce optically pure compounds. Enzymatic asymmetric reduction of prochiral carbonyl compounds is a promising method for the production of chiral alcohols because enzymes have the advantage of performing chemical reactions under mild conditions and with specific chemoselectivity, regioselectivity and stereoselectivity (Hummel, 1997; Kataoka *et al.*, 2003; Goldberg *et al.*, 2007). Structural analysis of enzymes used as biocatalysts, including reductases, may provide information that might be valuable for the introduction of mutations to create new enzymes that exhibit high activity, enhanced stability and altered coenzyme specificity, as well as for understanding their reaction mechanisms and substrate-binding specificities.

The chiral alcohol (*R*)-3-quinuclidinol is used as a starting material for the synthesis of various pharmaceuticals such as talsaclidine and revatropate (Rzeszotarski *et al.*, 1988; Bietti *et al.*, 1990; Cross & Stobie, 1993; Takeuchi *et al.*, 1996; Alabaster, 1997; Ward *et al.*, 1998; Leusch *et al.*, 2000; Ishihara *et al.*, 2004). Recently, 3-quinuclidinone reductase was isolated from *Rhodotorula rubra* and used in the stereoselective synthesis of (*R*)-3-quinuclidinol (Uzura *et al.*, 2009). The enzyme purified from *R. rubra* requires NADPH as a cofactor in the stereospecific reduction of 3-quinuclidinone to produce the (*R*)-enantiomer of 3-quinuclidinol. Furthermore, co-expression of 3-quinuclidinone reductase and glucose dehydrogenase in *Escherichia coli* has been developed to produce (*R*)-3-quinuclidinol on a large scale (Uzura *et al.*, 2009). Based on amino-acid sequence similarity, 3-quinuclidinone reductase belongs to the short-chain dehydrogenase/reductase family, which is part of the NAD(P)-binding Rossmann-fold superfamily. Although this family of proteins typically have low sequence identity (about 15–30%), their structures generally contain similar α/β Rossmann folds (Jörnvall *et al.*, 1995; Oppermann *et al.*, 2003). The NAD(P)-binding Rossmann-fold superfamily exhibit diverse substrate specificities that indicate they are likely to have structurally different substrate-binding sites.

In order to elucidate the molecular mechanism of the stereospecific reduction of 3-quinuclidinone, we are undertaking structural analysis of the 3-quinuclidinone reductase. Here, we report the expression, purification, crystallization and preliminary X-ray diffraction analysis of the 3-quinuclidinone reductase from *R. rubra*.

2. Materials and results

2.1. Protein expression and purification

The gene coding for the 3-quinuclidinone reductase from *R. rubra* (NCBI accession No. DJ045765) was cloned into pET28a vector (Novagen) in-frame with the vector-derived N-terminal His tag. *E. coli* Rosetta (DE3) cells were transformed with the recombinant plasmid. The *E. coli* cells were grown in Luria–Bertani broth containing kanamycin (30 µg ml^{-1}) until mid-log phase and protein expression was then induced by the addition of 1.0 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 298 K. The cells were harvested by centrifugation at 5000*g* for 15 min and frozen at 253 K.

The frozen cells were resuspended in lysis buffer containing 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 1.0 m*M* dithiothreitol (DTT) and 10 m*M* imidazole. The cells were then disrupted by sonication and insoluble materials were removed by centrifugation at 40 000*g* for 60 min. The supernatant containing the protein was loaded onto an Ni–NTA agarose column (Qiagen). The column was washed with 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 1.0 m*M* DTT and 20 m*M* imidazole and the fusion protein was then cleaved on the column by incubation with thrombin protease (GE Healthcare) for 15 h. The cleaved protein was eluted with the same buffer as used for washing. The fraction containing the protein was dialyzed against 20 m*M* Tris–HCl pH 8.0 and 1.0 m*M* DTT. The solution was loaded onto a Resource Q anion-exchange column (GE Healthcare) and eluted with a linear gradient of 0–1.0 *M* NaCl. The protein began to elute at 50 m*M* NaCl. The fraction containing the protein was dialyzed against 20 m*M* Tris–HCl pH 8.0 and 1.0 m*M* DTT and then concentrated to 26 mg ml^{-1} using an Amicon ultrafiltration concentrator (Millipore). The protein concentration was determined using the Bradford Protein Assay (Bio-Rad) with BSA as a standard.

2.2. Crystallization

The purified protein was mixed with 1.0 m*M* NADPH and the solution was used for crystallization experiments. The sparse-matrix screening kits Crystal Screen and Crystal Screen 2 (98 conditions; Hampton Research) and Wizard I and II (96 conditions; Emerald Biostructures) were used for initial crystallization trials using the sitting-drop vapour-diffusion method. Drops containing equal volumes of protein and reservoir solution were equilibrated against reservoir solution at 278 K.

Three-dimensional crystals were obtained with reservoir solutions containing 100 m*M* CHES pH 9.5 and 20% PEG 8000 (Wizard I solution No. 1) and 100 m*M* Tris–HCl pH 8.5 and 8% PEG 8000 (Crystal Screen solution No. 36). The crystals obtained using Wizard I solution No. 1 diffracted X-rays better than those obtained using Crystal Screen solution No. 36 and thus further optimization was carried out based on Wizard I solution No. 1. Reservoir solutions with varying pH and precipitant concentration (24 conditions) were tested and the Additive Screen kit (96 solutions; Hampton Research) was used to improve the crystal quality. Finally, the best crystals were obtained with reservoir solution containing 100 m*M* CHES pH 10.0,

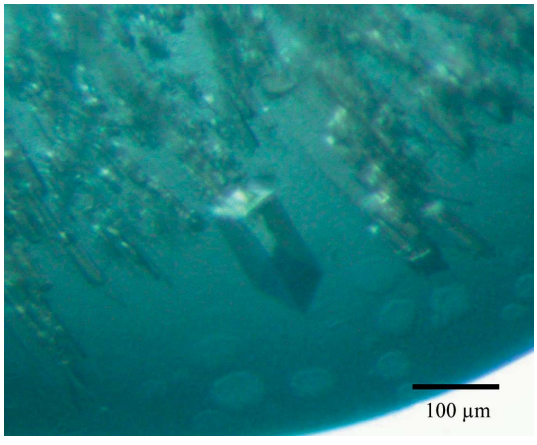

Figure 1
A crystal of 3-quinuclidinone reductase with approximate dimensions of 80 × 80 × 150 µm.

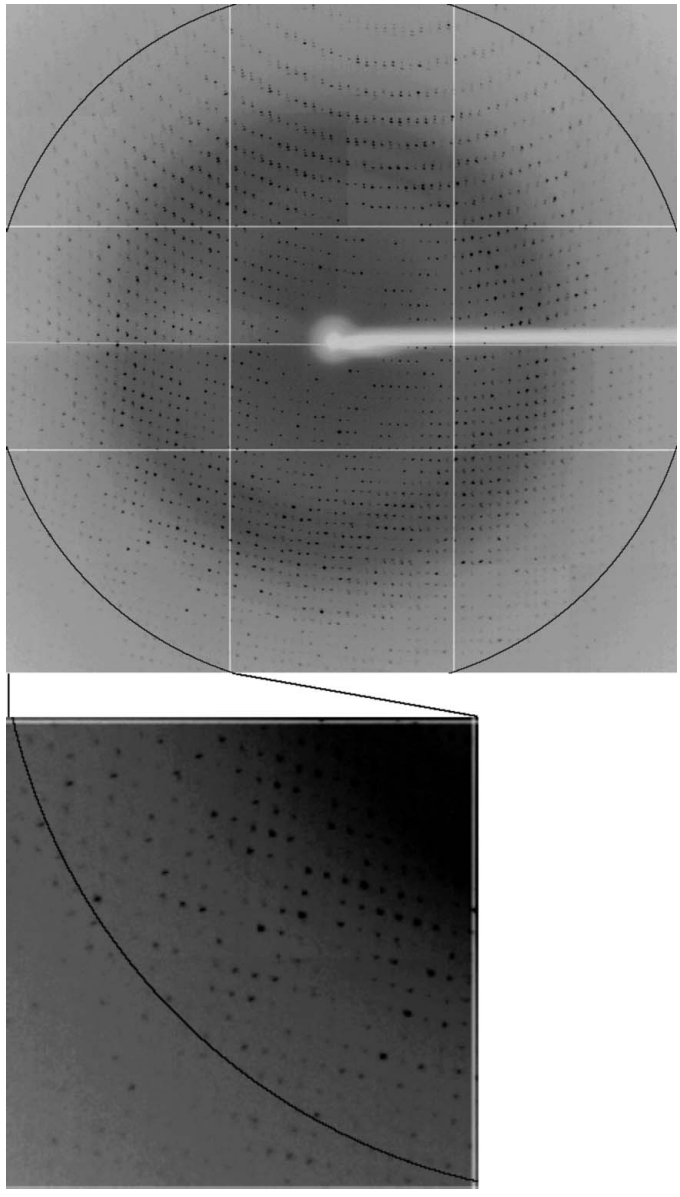

Figure 2
A diffraction image (0.2° oscillation) of a crystal of 3-quinuclidinone reductase. The X-ray diffraction extends to 2.2 Å resolution (indicated by the curved line).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	PF BL-5A
Wavelength (Å)	1.000
Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 91.3$, $c = 265.4$
Resolution range (Å)	20.0–2.20 (2.28–2.20)
Observed reflections	689047
Unique reflections	55949
Completeness (%)	96.5 (81.0)
$R_{\rm merge}$† (%)	9.2 (23.9)
$\langle I\rangle/\langle\sigma(I)\rangle$	55.6 (11.4)

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

30% PEG 8000 and 3% sucrose. The crystals used for data collection had typical dimensions of 80 × 80 × 150 µm (Fig. 1).

2.3. Data collection and processing

X-ray diffraction experiments were performed on beamline BL-5A of the Photon Factory (PF), Tsukuba, Japan. The crystals were cryoprotected by adding a solution consisting of 100 m*M* CHES pH 10.0, 30% PEG 8000 and 30% glycerol to the drop and were flash-cooled in a nitrogen stream at 95 K, which was the sole trial for cryoprotection. The diffraction data were collected using a Quantum 315 CCD X-ray detector (Area Detector Systems Corporation) in 0.2° oscillation steps; the crystal diffracted X-rays to 2.2 Å resolution (Fig. 2). To avoid overlapping spots occurring during data collection, the detector was kept at an optimal distance from the crystal (the crystal-to-detector distance was 352.3 mm). When we analyzed diffraction snapshots at closer distances, the number of overlapping spots was increased. The data set was processed and scaled using the program *HKL*-2000 (Otwinowski & Minor, 1997). The crystal belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 91.3$, $c = 265.4$ Å. The data-collection statistics are shown in Table 1. The asymmetric unit contained four molecules of the protein, with a calculated solvent content of 48.4% and a Matthews coefficient of 2.38 Å^3 Da^{-1} (Matthews, 1968). Determination of the structure of 3-quinuclidinone reductase by molecular replacement using the structure of *Thermotoga maritima* TM0441 (PDB code 1vl8) as a search model is currently under way using the programs *Phaser* (McCoy *et al.*, 2007) and *MOLREP* (Vagin & Teplyakov, 1997) from *CCP*4 (Collaborative Computational Project, Number 4, 1994). The protein structure obtained by X-ray crystallography will reveal the reaction mechanism and substrate specificity at atomic resolution and will be utilized for mutation analysis in order to understand the enzymological properties of 3-quinuclidinone reductase.

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