Crystallization and preliminary crystallographic analysis of acetophenone reductase from *Geotrichum candidum* NBRC 4597

Yosuke Sugiyama,^a Miki Senda,^b Toshiya Senda^b and Tomoko Matsuda^{a*}

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^aDepartment of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan, and ^bStructural Biology Research Center, Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK), 1-1 Oho, Tsukuba, Ibaraki 305-0801, Japan.

*Correspondence e-mail: tmatsuda@bio.titech.ac.jp

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Acetophenone reductase (APRD) from *Geotrichum candidum* NBRC 4597 was crystallized by the hanging-drop vapour-diffusion method using PEG 3350 as a precipitant. The crystal belonged to space group $P6_522$, with unit-cell parameters $a = b = 104.5$, $c = 273.7$ Å, and diffracted to 2.6 Å resolution. Phasing using the single-wavelength anomalous diffraction method was successful. Model building and crystallographic refinement are in progress.

1. Introduction

The discovery and development of asymmetric organic synthesis using biocatalysts has been indispensable for the production of pharmaceuticals and agrochemicals (Bornscheuer *et al.*, 2012; Nestl *et al.*, 2014). Among biocatalysts for this process, alcohol dehydrogenase (EC 1.1.1.1), which catalyzes both the reduction of ketones and the oxidation of alcohols, has been considered to be particularly valuable. For example, an alcohol dehydrogenase with extremely high enantioselectivity and high stability is necessary for the efficient and 'green' industrial production of chiral intermediates. We have been studying alcohol dehydrogenases from *Geotrichum candidum* because they have excellent enantioselectivities for a wide variety of substrates and can be used in organic solvents, supercritical carbon dioxide and ionic liquids (Nakamura & Matsuda, 1998; Matsuda *et al.*, 2003, 2006, 2008; Matsuda, Harada, Nakajima *et al.*, 2000; Matsuda, Harada & Nakamura, 2000; Tanaka *et al.*, 2009; Cao *et al.*, 2013). Recently, we purified one of the alcohol dehydrogenases, acetophenone reductase, from *G. candidum* NBRC 4597 (APRD; Nakata *et al.*, 2010) and overexpressed the enzyme in *Escherichia coli* (Yamamoto *et al.*, 2013). This enzyme is characterized by its robustness: it can catalyze enantioselective reduction of the substrate as well as the reduction of NAD⁺ with 2-propanol for recycling with a high concentration of 2-propanol and the substrate (Fig. 1). From the deduced amino-acid sequences, APRD is classified as a member of the zinc-containing medium-chain dehydrogenases/reductases (MDR) family. Multiple sequence-alignment analysis of MDR family proteins identified a zinc-binding motif in APRD (Yamamoto *et al.*, 2013). While several crystal structures of APRD homologues have been determined [PDB entries 4c4o (Man *et al.*, 2014), 3wnq, 3wlf and 3wle (Wang, Nie, Xu *et al.*, 2014; Wang, Nie, Yan *et al.*, 2014); approximately 45% amino-acid sequence identity to APRD], the crystal structures of these homologous enzymes are not sufficient to elucidate the mechanism of the

Table 1
Macromolecule-production information.

Source organism	<i>G. candidum</i> NBRC 4597
DNA source	Total cDNA
Forward primer	5'-ATGGGNAAGTNCNGARAC-3'
Reverse primer	5'-CTAGATGTCATCGAAATCAG-3'
Cloning vector	pCR4-TOPO-APRD
Expression vector	pET-21b(+)-APRD
Expression host	<i>E. coli</i> Rosetta(DE3)pLysS
Complete amino-acid sequence of the construct produced†	MGKVPETHKGYVFTSGSSRLTLKDVPYTKPGPGE-VLLKLEASGVCHSDHLILQGSFPIPSNSVLGH-EITGTVVAYGLGVDPKTYPEGQLYAAHGNPC-GSCRECRSGKDNLCNAENRTNYGLGYGGYQQY-TLAKVHNLIKVPDVGAAIAAVTTDAVLTPYH-AFKKADINGLSKILIIIGLGGGINAVQIAKAM-GAHVTAYDLKESRQLARQFGADVLESRLTD-DASKEYDFVADIVSIQSTFDLALKQVKSNGLV-IPLGLGSPKLTDFDNDLLVREIRILGSFWGTS-LDQAEVFDLVKSGAFKPVETGKFKDLNEILE-KLEKGQIKSRLVLTDFDDIMASMTGGQQMGRD-PNSSVDKLAALAEHHHHHH

† The sequence from pET-21b(+) is underlined.

excellent enantioselectivity as well as to clarify the reason for the extreme stability towards a high concentration of organic compounds and high temperature. The crystal structure of APRD itself is therefore required. Here, we report the crystallization and preliminary X-ray crystallographic data of APRD.

2. Materials and methods

2.1. Macromolecule production

APRD was overexpressed using *E. coli* Rosetta(DE3)pLysS cells with pET-21b(+)-APRD and was purified as described previously (Table 1; Yamamoto *et al.*, 2013). Briefly, the recombinant cells were grown in LB medium and the enzyme was induced with 1 mM IPTG at 293 K. The cell-free extract was prepared by sonication and centrifugation and was loaded onto a HisTrap FF crude column. The His₆-tagged APRD eluted with 500 mM imidazole was applied to SDS-PAGE and it was confirmed that the enzyme was purified to near-homogeneity (Fig. 2*a*). The obtained APRD was utilized for crystallization after concentrating the protein in 20 mM imidazole, 0.3 mM dithiothreitol (DTT), 20 mM HEPES pH 7.4 with Amicon Ultra-15 (10 kDa cutoff; Merck Millipore, Billerica, USA). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

Figure 1
Enantioselective reduction of ketones and recycling of the oxidized form of the coenzyme NAD⁺ with 2-propanol.

2.2. Crystallization

Initial crystallization screening of APRD was performed with NAD⁺ and a substrate. We performed three distinct sets of initial screenings with three different substrates: acetophenone, 2-pentanone and cyclopentanol. Purified APRD was concentrated to 7.7 mg ml⁻¹, NAD⁺ (final concentration of 2.1 mM) and a substrate (final concentration of 2.1 mM) were added and the solution was utilized for crystallization screening. The final concentration of APRD was 7.4 mg ml⁻¹. Crystallization was performed at 293 K by the sitting-drop vapour-diffusion method using the PXS crystallization robot developed in our laboratory (Hiraki *et al.*, 2006). Crystal Screen and Crystal Screen 2 (Hampton Research, Laguna Niguel, California, USA; Jancarik & Kim, 1991), PEG/Ion and PEG/Ion 2 (Hampton Research), Crystal Screen Cryo and Crystal Screen 2 Cryo (Hampton Research), MembFac (Hampton Research), Footprint Screen (Molecular Dimensions) and Wizard I and II (Emerald Bio) were used as reservoir solutions. A sitting drop was prepared by mixing

Figure 2
Purification and crystallization of APRD. (a) SDS-PAGE of purified APRD. Lane 1, cell-free extract; lane M, molecular markers (labelled in kDa); lane 2, HisTrap FF-purified APRD. (b) A crystal of the APRD-NAD⁺ complex.

Table 2
Crystallization of the APRD–NAD⁺ complex.

Method	Hanging-drop vapour diffusion
Plate type	VDX plate
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	8.4
Buffer composition of protein solution	3.0 mM NAD ⁺ , 20 mM imidazole, 0.3 mM DTT, 20 mM HEPES pH 7.4
Composition of reservoir solution	20% (w/v) PEG 3350, 0.2 M trisodium citrate dihydrate pH 7.9
Volume and ratio of drop	1 µl:1 µl protein:crystallization solution
Volume of reservoir (ml)	0.5

Figure 3
Crystallographic data. (a) Anomalous scattering factors f' and f'' around the absorption edge of Zn. The anomalous scattering factors were calculated using *CHOOCH* (Evans & Pettifer, 2001) from the fluorescence spectrum of the APRD–NAD⁺–2-pentanone complex. (b) An X-ray diffraction pattern of the APRD–NAD⁺ crystal. The diffraction data were collected on beamline BL-1A at PF using a PILATUS 2M-F detector.

0.5 µl each of the protein solution and the reservoir solution and was equilibrated against 180 µl reservoir solution.

Crystallization of the APRD–NAD⁺ complex was performed at 293 K by the hanging-drop vapour-diffusion method. Purified APRD was concentrated and NAD⁺ was added (final concentration of 3.0 mM). The final concentration of APRD was 8.4 mg ml⁻¹. To obtain large crystals, the concentration of PEG 3350 and the pH were systematically optimized (Table 2).

2.3. Data collection and processing

Since horse liver alcohol dehydrogenase from the same enzyme family as APRD is a zinc-binding protein, APRD was also expected to be a zinc-binding protein (Plapp *et al.*, 1983). The X-ray absorption spectrum around the absorption edge of Zn was therefore measured for a crystal of the APRD–NAD⁺–2-pentanone complex. Diffraction data were collected from APRD–NAD⁺–2-pentanone, APRD–NAD⁺–acetophenone and APRD–NAD⁺ complex crystals at 95 K using an ADSC CCD 270 detector on beamline NE-3A of Photon Factory Advanced Ring (PF-AR; Photon Factory, Tsukuba, Japan) or using a PILATUS 2M-F detector on beamline BL-1A of Photon Factory (PF) (Table 3). The diffraction data were processed and scaled using *XDS* and *XSCALE*, respectively (Kabsch, 2010).

3. Results and discussion

Initial crystallization screening gave thin rod-shaped crystals in a week under several conditions that included PEG 3350 as a precipitant. Large rod-shaped crystals of the APRD–NAD⁺–2-pentanone, APRD–NAD⁺–acetophenone and APRD–NAD⁺–cyclopentanol complexes appeared in PEG/Ion Screen 2 condition No. 20 [20% (w/v) PEG 3350, 0.2 M succinic acid], PEG/Ion Screen condition No. 46 [20% (w/v) PEG 3350, 0.2 M trisodium citrate dihydrate] and PEG/Ion Screen 2 condition No. 40 [20% (w/v) PEG 3350, 0.03 M citric acid], respectively. On the basis of the results of the initial crystallization screening, crystallization of the APRD–NAD⁺ complex was performed by the hanging-drop vapour-diffusion method. The pH (5.6–8.0) and concentration of PEG 3350 [15–25% (w/v)] were systematically optimized. The largest rod-shaped crystals of the APRD–NAD⁺ complex were obtained in 20% (w/v) PEG 3350, 0.2 M trisodium citrate dihydrate pH 7.9 (Table 2, Fig. 2b). The crystals obtained were utilized for data collection.

The X-ray absorption spectrum around the absorption edge of Zn suggested that the APRD crystal contained Zn ions (Fig. 3a). Crystals of the APRD–NAD⁺–2-pentanone complex only diffracted to 3.7 Å resolution without cryoprotection at 95 K on NE-3A at PF-AR. The crystals were soaked in a cryoprotectant solution [27% (v/v) glycerol, 18% (w/v) PEG 3350, 0.18 M succinic acid pH 6.5] for 2 min and flash-cooled in an N₂ stream at 95 K. As a result, the crystals diffracted to better than 3.0 Å resolution. Crystals of the APRD–NAD⁺–acetophenone and APRD–NAD⁺ complexes were soaked in

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	APRD–NAD ⁺ –2-pentanone complex (Zn SAD data)	APRD–NAD ⁺ –acetophenone complex	APRD–NAD ⁺ complex
Diffraction source	NE-3A, PF-AR	NE-3A, PF-AR	BL-1A, PF
Wavelength (Å)	1.2827	1.2827	1.1000
Temperature (K)	95	95	95
Detector	ADSC CCD 270	ADSC CCD 270	PILATUS 2M-F
Crystal-to-detector distance (mm)	271.0	248.0	242.2
Rotation range per image (°)	0.5	0.5	0.5
Total rotation range (°)	0–180	0–360	0–180
Exposure time per image (s)	3	3	1
Space group	<i>P</i> ₆ ₅ 22	<i>P</i> ₆ ₅ 22	<i>P</i> ₆ ₅ 22
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 105.0, <i>c</i> = 273.1, α = β = 90, γ = 120	<i>a</i> = <i>b</i> = 104.9, <i>c</i> = 273.1, α = β = 90, γ = 120	<i>a</i> = <i>b</i> = 104.5, <i>c</i> = 273.7, α = β = 90, γ = 120
Mosaicity (°)	0.2	0.2	0.2
Resolution range (Å)	90.89–3.25 (3.43–3.25)	90.84–2.95 (3.11–2.95)	90.51–2.60 (2.74–2.60)
Total No. of reflections	304803 (45968)	805905 (120595)	514554 (74309)
No. of unique reflections	26286 (3887)	35391 (5184)	51299 (7501)
Completeness (%)	99.1 (98.6)	99.9 (99.8)	99.7 (99.8)
Multiplicity	11.5 (11.8)	22.7 (23.2)	10.0 (9.9)
<i>I</i> /σ(<i>I</i>)	27.0 (7.4)	34.2 (9.3)	23.3 (5.9)
<i>R</i> _{int}	0.081 (0.440)	0.085 (0.486)	0.066 (0.465)
Overall <i>B</i> factor from Wilson plot (Å ²)	46.73	53.32	52.93
No. of Zn sites for phasing	4	—	—
FOM values (acentric/centric)	0.370/0.060	—	—

a cryoprotectant solution [27% (v/v) glycerol, 18% (w/v) PEG 3350, 0.18 *M* trisodium citrate dehydrate pH 7.1] for 2 min and flash-cooled in an N₂ stream at 95 K. The crystals of the APRD–NAD⁺–acetophenone and APRD–NAD⁺ complexes diffracted to 2.95 and 2.60 Å resolution, respectively (Table 3, Fig. 3*b*).

All APRD crystals belonged to space group *P*₆₅22 with similar unit-cell parameters (Table 3). Assuming two subunits of APRD (40.3 kDa) per asymmetric unit of the crystal, the Matthews coefficient (*V*_M) was calculated to be 2.70 Å³ Da^{−1}, which corresponds to a solvent content of 54.4% (Matthews, 1968). An anomalous difference Patterson map was calculated using the diffraction data of the APRD–NAD⁺–2-pentanone complex collected with X-rays of wavelength 1.2827 Å and showed clear peaks in a Harker section (Supplementary Fig. S1). This result strongly suggested that the APRD molecules contained Zn²⁺ ions. Single-wavelength anomalous diffraction (SAD) phasing was performed using *autoSHARP*, resulting in a clear electron-density map (Vonnrhein *et al.*, 2007; Supplementary Fig. S2). The SAD phasing revealed the space group of the APRD crystals to be *P*₆₅22. Model building and crystallographic refinement are in progress.

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