Crystallization and preliminary X-ray diffraction analysis of (*R*)-carbonyl reductase from *Candida parapsilosis*

Shanshan Wang,^{a,†} Yao Nie,^{a,†}
Xu Yan,^{a,*} Tzu-Ping Ko,^b
Chun-Hsiang Huang,^c
Hsiu-Chien Chan,^c Rey-Ting
Guo^{c,*} and Rong Xiao^d

^aSchool of Biotechnology and Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, People's Republic of China, ^bInstitute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan, ^cIndustrial Enzymes National Engineering Laboratory, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, People's Republic of China, and ^dCenter for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ 08854, USA

† These two authors contributed equally.

Correspondence e-mail: yxu@jiangnan.edu.cn, guo_rt@tib.cas.cn

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The NADH-dependent (*R*)-carbonyl reductase from *Candida parapsilosis* (RCR) catalyzes the asymmetric reduction of 2-hydroxyacetophenone (HAP) to produce (*R*)-1-phenyl-1,2-ethanediol [(*R*)-PED], which is used as a versatile building block for the synthesis of pharmaceuticals and fine chemicals. To gain insight into the catalytic mechanism, the structures of complexes of RCR with ligands, including the coenzyme, are important. Here, the recombinant RCR protein was expressed and purified in *Escherichia coli* and was crystallized in the presence of NAD⁺. The crystals, which belonged to the orthorhombic space group *P*2₁2₁2₁, with unit-cell parameters *a* = 85.64, *b* = 106.11, *c* = 145.55 Å, were obtained by the sitting-drop vapour-diffusion method and diffracted to 2.15 Å resolution. Initial model building indicates that RCR forms a homotetramer, consistent with previous reports of medium-chain-type alcohol dehydrogenases.

1. Introduction

Optically active alcohols play a rapidly growing role as versatile chiral building blocks for synthetic procedures in the pharmaceutical and fine-chemical industries (Patel, 2008). Among them, enantiopure 1-phenyl-1,2-ethanediol (PED) can serve as a valuable intermediate for the preparation of pharmaceuticals, liquid crystals, agrochemicals and flavour fragrances (Rui *et al.*, 2005; Kamal & Chouhan, 2004; Cao *et al.*, 2006; Vargas-Díaz *et al.*, 2003). An attractive route to chiral alcohols involves alcohol dehydrogenase (ADH)-catalyzed asymmetric reduction of carbonyl compounds with high chemoselectivity, regioselectivity and stereoselectivity under benign conditions (Musa & Phillips, 2011). Depending on the stereochemical pattern of hydride transfer from NAD(P)H to ketones, ADHs have either Prelog or anti-Prelog stereoselectivity (Prelog, 1964).

In previous work, (*R*)-carbonyl reductase (RCR) and (*S*)-carbonyl reductase (SCR) from *Candida parapsilosis* CCTCC M203011 were found to catalyze the reduction of 2-hydroxyacetophenone (HAP) to produce (*R*)-PED and (*S*)-PED, respectively (Nie *et al.*, 2007, 2008). Subsequently, the crystal structure of SCR, a short-chain ADH, was determined to reveal the structure–function relationship (Zhang *et al.*, 2008). RCR has successfully been used as the stereo-complementary counterpart in the biosynthesis of (*R*)-PED with more than 99.9% enantiomeric excess (Nie *et al.*, 2008; Wang *et al.*, 2012). However, the structural details of RCR and the molecular mechanism which confers its excellent stereoselectivity for the Prelog configuration are as yet unclear, although RCR shares 31.5% sequence identity with the structurally known *Rhodococcus ruber* ADH (PDB entries 2xaa and 3jv7; Karabec *et al.*, 2010).

Protein sequence analysis suggests that RCR is a member of the medium-chain ADH family. To date, numerous X-ray crystal structures of medium-chain ADHs have been determined, including those from *Homo sapiens* (Gibbons & Hurley, 2004), *Equus caballus* (Plapp & Ramaswamy, 2012), *Mus musculus* (Svensson *et al.*, 2000), *Saccharomyces cerevisiae* (Pal *et al.*, 2009), *R. ruber* (Karabec *et al.*, 2010), *Thermoanaerobacter brockii* (Heiss *et al.*, 2001), *Sulfolobus solfataricus* (Esposito *et al.*, 2003) and *Ralstonia eutropha* (Kang *et al.*, 2012). These enzymes exist in either dimeric or tetrameric forms. Interestingly, dimeric ADHs are usually found in higher plants and mammals, whereas tetrameric ADHs are usually found in yeasts, bacteria and archaea. Compared with dimeric ADH structures, the

Table 1

RCR production information.

Primer sequence extensions required for Xa/LIC compatibility are underlined. Additional nucleotides and corresponding residues are in bold.

Source organism	<i>C. parapsilosis</i>
DNA source	<i>C. parapsilosis</i>
Forward primer	5'-GGTATTGAGGGTCGCGCGGGCGCGGGC- CGGATGTCAATTCCATCAAGCCAGTACG- TGTCATTCCATCAAGCCAGTACGGATT-3'
Reverse primer	5'-AGAGGAGAGTTAGAGCCCTATGGATTAA- AAACAACCTCTACCTTCATAAG-3'
Expression vector	pET-32 Xa/LIC
Expression host	<i>E. coli</i> BL21 <i>trxB</i> (DE3)
Complete amino-acid sequence of the construct produced	AGAGAM SIPSSQYGFVFNKQSGKLRLNDLPVHKPKAGQLLLK- VFNKQSGKLRLNDLPVHKPKAGQLLLKVDAVGLCHSDLHV- IYGLDCGDNYVMGHEIAGTVAAGDDVINYKVGDRVACV- GPNCGCGGCKYCRGAIDNVCKNAFGDWFLGYDGGYQQYLL- VTRPNLSRIPDNVSADVAASTDAVLTPYHAIKMAQVSP- TSNILLIGAGLGGNAIQVAKAFKAVTVLDKKKEARDQA- KKLGADAVYETLPESISPGSFSACDFVSVQATFDVCQKY- VEPKGVIMPVGLGAPNLSFNLGDLALREIRILGSFSGWTN- DLDDVLKLVSEGVKPVVRS AKLPEYIEKLRLNNA YEG- RVVFN

number of tetrameric ADH structures is relatively limited (Eklund & Ramaswamy, 2008). In order to obtain a better understanding of the stereoselective mechanism of RCR and to increase the structural knowledge of tetrameric ADHs, we have co-crystallized RCR with its coenzyme NAD⁺.

2. Materials and methods

2.1. Protein preparation

Protein expression and purification followed previously published procedures with slight modifications (Nie *et al.*, 2008), and detailed production information is shown in Table 1. Briefly, the gene encoding RCR (protein ID ABB97513.1) from *C. parapsilosis* was amplified by polymerase chain reaction (PCR) and then cloned into pET-32 Xa/LIC by ligation-independent cloning (LIC) using Xa/LIC cloning kits. *Escherichia coli* BL21 *trxB* (DE3) cells were transformed with the recombinant plasmid. The cells were grown in LB medium containing 100 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ kanamycin and 0.2 mM zinc acetate at 301 K for 12 h. The cell pellet was harvested by centrifugation at 6000g and resuspended in lysis buffer consisting of 25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole pH 8.0. Cell lysate was prepared with a JNBIO pressure cell (JN-3000 PLUS) and was then centrifuged at 17 000g to remove cell debris. The target protein was purified on an ÄKTApurifier 10 (GE Healthcare Life Sciences)

Figure 1

A crystal of holo RCR. The crystal reached approximate dimensions of 0.3 × 0.1 × 0.1 mm in 1–2 d.

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline BL13B1, NSRR
Wavelength (Å)	1.0
Temperature (K)	100
Detector	MX300HE
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	0.4
Total rotation range (°)	120
Exposure time per image (s)	15
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	85.64, 106.11, 145.55
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.42–0.49
Resolution range (Å)	25.0–2.15 (2.23–2.15)
Total No. of reflections	339445 (34740)
No. of unique reflections	70570 (6948)
Completeness (%)	99.9 (99.8)
$\langle I/\sigma(I) \rangle$	20.7 (5.3)
$R_{\text{merge}}^{\dagger}$ (%)	9.2 (37.7)
Overall <i>B</i> factor from Wilson plot (Å ²)	35.5

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

using an Ni-NTA column. The buffer used for the Ni-NTA column was 25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole pH 8.0. The target protein eluted at about 120 mM imidazole when using a 20–500 mM imidazole gradient. The protein solution was dialyzed against a buffer consisting of 25 mM Tris-HCl, 150 mM NaCl pH 8.0 and was then subjected to factor Xa digestion to cleave off the His tag, leaving the linker AGAGA at the N-terminus. The mixture was then passed through the Ni-NTA column again and the untagged RCR was eluted with buffer containing 5 mM imidazole. The purified protein was finally concentrated to 10 mg ml⁻¹ in a buffer consisting of 25 mM Tris-HCl, 150 mM NaCl pH 8.0. The purity was checked by SDS-PAGE analysis and was >95%.

2.2. Crystallization and data collection

Subsequent crystallization screening was performed manually using 768 different reservoir conditions from Hampton Research (Laguna Niguel, California, USA) including Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen Lite, MembFac, Natrix, Index, SaltRx, SaltRx 2, PEG/Ion, PEG/Ion 2, Quick Screen and Grid Screens (ammonium sulfate, MPD, sodium chloride, sodium malonate, PEG 6000 and PEG/LiCl). All of the crystallization experiments were conducted at 295 K using the sitting-drop vapour-diffusion method. In general, 1 µl RCR-containing solution (10 mg ml⁻¹ in 25 mM Tris, 150 mM NaCl pH 8.0) was mixed with 1 µl reservoir solution in 24-well Cryschem plates (Hampton Research) and equilibrated against 300 µl reservoir solution. RCR was co-crystallized with 5 mM NAD⁺. Holo crystals of RCR appeared within 1 d using PEG/Ion 2 condition No. 7 [0.1 M sodium malonate pH 7.0, 12% (w/v) PEG 3350]. Better crystals were obtained by optimizing the reservoir composition, which finally consisted of 0.1 M sodium malonate pH 7.0, 12% (w/v) PEG 3350, 2% (w/v) PEG 10 000. The crystals reached dimensions of about 0.3 × 0.1 × 0.1 mm within 1–2 d. Prior to data collection at 100 K, a crystal was mounted in a cryoloop and soaked with cryoprotectant solution consisting of 0.15 M sodium malonate pH 7.0, 20% (w/v) PEG 3350, 5% (w/v) PEG 10 000, 10% (w/v) glycerol for 3 s. An X-ray diffraction data set was collected to 2.15 Å resolution on beamline BL13C1 of the National Synchrotron Radiation Research Center (NSRR; Hsinchu, Taiwan). The diffraction images were processed using *HKL-2000* (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 2.

Figure 2
A diffraction pattern of the holo RCR crystal.

3. Results and discussion

As shown in Fig. 1, large single holo RCR crystals were obtained in the presence of NAD^+ using a reservoir consisting of 0.1 M sodium malonate pH 7.0, 12% (w/v) PEG 3350, 2% (w/v) PEG 10 000. Prior to data collection at 100 K, the crystal was mounted in a cryoloop and flash-cooled in liquid nitrogen with a slightly modified cryoprotectant consisting of 0.15 M sodium malonate pH 7.0, 20% (w/v) polyethylene glycol 3350, 5% (w/v) polyethylene glycol 10 000, 10% (w/v) glycerol. Based on the diffraction pattern (Fig. 2), the RCR crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 85.64$, $b = 106.11$, $c = 145.55$ Å. Assuming that there are four molecules in the asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) is $2.23 \text{ Å}^3 \text{ Da}^{-1}$ and the estimated solvent content is 45%. Statistics of data collection and processing to 2.15 Å resolution are summarized in Table 2. The overall merging R factor on intensities for the holo crystal form was 9.2%, with excellent completeness and signal-to-noise ratio.

The crystal structure of RCR was solved by the molecular-replacement (MR) method with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite (Winn *et al.*, 2011). The search model was generated from the structure of *R. ruber* holo ADH (PDB entry 2xaa; 31.5% sequence identity to RCR; Karabec *et al.*, 2010) using the *SWISS-MODEL* website. Preliminary structural refinement using *REFMAC5* (Murshudov *et al.*, 2011) and *CNS* (Brünger *et al.*, 1998) resulted in a model with R_{work} and R_{free} of 33 and 39%, respectively. There are four monomers in an asymmetric unit, forming a homotetramer, which is consistent with a previous report (Karabec *et al.*, 2010) and resembles other medium-chain ADH structures (Esposito *et al.*, 2003; Guy *et al.*, 2003; Levin *et al.*, 2004). Further model building and structural refinement are in progress. Finally, in an attempt to fully understand the stereoselective catalytic mechanism, soaking the

RCR crystals with substrate (HAP) and product [(*R*)-PED] are under way.

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References

- Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* **D54**, 905–921.
- Cao, L., Lee, J., Chen, W. & Wood, T. K. (2006). *Biotechnol. Bioeng.* **94**, 522–529.
- Eklund, H. & Ramaswamy, S. (2008). *Cell. Mol. Life Sci.* **65**, 3907–3917.
- Esposito, L., Bruno, I., Sica, F., Raia, C. A., Giordano, A., Rossi, M., Mazzarella, L. & Zagari, A. (2003). *Biochemistry*, **42**, 14397–14407.
- Gibbons, B. J. & Hurley, T. D. (2004). *Biochemistry*, **43**, 12555–12562.
- Guy, J. E., Isupov, M. N. & Littlechild, J. A. (2003). *J. Mol. Biol.* **331**, 1041–1051.
- Heiss, C., Laivenieks, M., Zeikus, J. G. & Phillips, R. S. (2001). *J. Am. Chem. Soc.* **123**, 345–346.
- Kamal, A. & Chouhan, G. (2004). *Tetrahedron Lett.* **45**, 8801–8805.
- Kang, C., Hayes, R., Sanchez, E. J., Webb, B. N., Li, Q., Hooper, T., Nissen, M. S. & Xun, L. (2012). *Mol. Microbiol.* **83**, 85–95.
- Karabec, M., Łyskowski, A., Tauber, K. C., Steinkellner, G., Kroutil, W., Grogan, G. & Gruber, K. (2010). *Chem. Commun.* **46**, 6314–6316.
- Levin, I., Meiri, G., Peretz, M., Burstein, Y. & Frolow, F. (2004). *Protein Sci.* **13**, 1547–1556.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Musa, M. M. & Phillips, R. S. (2011). *Catal. Sci. Technol.* **1**, 1311.
- Nie, Y., Xu, Y., Mu, X. Q., Wang, H. Y., Yang, M. & Xiao, R. (2007). *Appl. Environ. Microbiol.* **73**, 3759–3764.
- Nie, Y., Xu, Y., Wang, H. Y., Xu, N., Xiao, R. & Sun, Z. H. (2008). *Biocatal. Biotransform.* **26**, 210–219.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Pal, S., Park, D.-H. & Plapp, B. V. (2009). *Chem. Biol. Interact.* **178**, 16–23.
- Patel, R. N. (2008). *Coord. Chem. Rev.* **252**, 659–701.
- Plapp, B. V. & Ramaswamy, S. (2012). *Biochemistry*, **51**, 4035–4048.
- Prelog, V. (1964). *Pure Appl. Chem.* **9**, 119–130.
- Rui, L., Cao, L., Chen, W., Reardon, K. F. & Wood, T. K. (2005). *Appl. Environ. Microbiol.* **71**, 3995–4003.
- Svensson, S., Höög, J. O., Schneider, G. & Sandalova, T. (2000). *J. Mol. Biol.* **302**, 441–453.
- Vargas-Díaz, M. E., Chacón-García, L., Velázquez, P., Tamariz, J., Joseph-Nathan, P. & Zepeda, L. (2003). *Tetrahedron Asymmetry*, **14**, 3225–3232.
- Wang, S., Xu, Y., Zhang, R., Zhang, B. & Xiao, R. (2012). *Process Biochem.* **47**, 1060–1065.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Zhang, R., Zhu, G., Zhang, W., Cao, S., Ou, X., Li, X., Bartlam, M., Xu, Y., Zhang, X. C. & Rao, Z. (2008). *Protein Sci.* **17**, 1412–1423.