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Crystallization and preliminary X-ray crystallographic studies of pig heart carbonyl reductase

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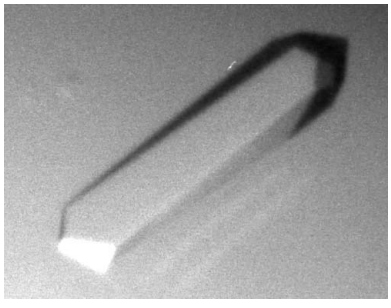

Pig heart carbonyl reductase (PHCR), which belongs to the short-chain dehydrogenase/reductase (SDR) family, has been crystallized by the hanging-drop vapour-diffusion method. Two crystal forms (I and II) have been obtained in the presence of NADPH. Form I crystals belong to the tetragonal space group $P4_2$, with unit-cell parameters $a = b = 109.61$, $c = 94.31$ Å, and diffract to 1.5 Å resolution. Form II crystals belong to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 120.10$, $c = 147.00$ Å, and diffract to 2.2 Å resolution. Both crystal forms are suitable for X-ray structure analysis at high resolution.

1. Introduction

Carbonyl reductase (CR; EC 1.1.1.184) catalyses the NADPH-linked reduction of a variety of carbonyl compounds to their corresponding secondary alcohols (Forrest & Gonzalez, 2000). Although the cytosolic CRs purified from several mammalian tissues are monomeric enzymes (~280 amino acids) with molecular weights of around 30 kDa (Wermuth *et al.*, 1988; Tanaka *et al.*, 1992), tetrameric mitochondrial CRs (4 × ~250 amino acids) exist in the lungs of guinea pig, mouse and pig (Nakayama *et al.*, 1986; Oritani *et al.*, 1992). The endogenous substrates of the monomeric CRs are suggested to be isatin and 20-ketosteroids, whereas those of the mitochondrial tetrameric lung CRs are 3-ketosteroids and carbonyl compounds derived from lipid peroxidation. Both the cytosolic CRs and mitochondrial CRs belong to the short-chain dehydrogenase/reductase (SDR) family (Jörnvall *et al.*, 1995; Oppermann *et al.*, 2003).

In addition to these multiple forms of CR, oligomeric and soluble forms of CR have been isolated from dog liver (Hara *et al.*, 1986), rabbit heart (Imamura *et al.*, 1999) and pig heart (Usami *et al.*, 2003). The properties of the oligomeric liver/heart enzymes differ from those of the monomeric CRs and the tetrameric mitochondrial lung CRs. Partial amino-acid sequencing and full-length cDNA cloning of both pig heart CR (PHCR) and rabbit heart CR (RHCR) have identified that both enzymes consist of 260 amino-acid residues and belong to the SDR family (Usami *et al.*, 2003). cDNA cloning of RHCR demonstrated that this enzyme is identical to rabbit $NADP^+$-dependent retinol dehydrogenase (NDRD). PHCR shows low sequence identity (<30%) to other mammalian monomeric CRs and mitochondrial tetrameric lung CRs, but shows high (80%) sequence identity to NDRD/RHCR. Characterization of recombinant PHCR and NDRD showed that they are identical enzyme species which reduce alkyl phenyl ketones, α-dicarbonyl compounds and retinals such as all-*trans*-retinal and 9-*cis*-retinal as endogenous substrates (Usami *et al.*, 2003).

The first CR crystal structure was reported for mouse lung CR (MLCR; Tanaka, Nonaka, Nakanishi *et al.*, 1996). The crystal structures of two monomeric CRs, porcine testicular CR (PTCR; Ghosh *et al.*, 2001) and human CR (CBR1; Tanaka *et al.*, 2005), were subsequently determined. The structural features, including the active site and the coenzyme-binding region, of the tetrameric MLCR and the monomeric CRs are similar to those of the other SDR enzymes. However, the substrate-binding region, which is the most variable part of the structure, differs between the various SDR enzymes (Tanaka, Nonaka, Tanabe *et al.*, 1996; Duax *et al.*, 2000; Tanaka *et al.*, 2001) and crystal structures of SDR enzymes in the form of a retinal/

retinol complex have never been reported. Here, we report the crystallization of PHCR in the presence of NADPH. The structural studies of PHCR should lead to a better understanding of the substrate recognition of the SDR enzymes.

2. Methods and results

2.1. Expression and purification

The expression and purification of recombinant PHCR (hereafter referred to simply as PHCR) were performed as described in Usami *et al.* (2003). Briefly, the expression plasmids (pCR T7/CT TOPO vectors; Invitrogen) harbouring the cDNA for PHCR were transformed into *Escherichia coli* BL21(DE3)pLysS cells (Invitrogen). Bacterial cultures were grown in LB medium (3 l shake flask with 1 l medium) at 310 K to an OD_{600} of 0.4. Expression of PHCR was induced by 1 m*M* IPTG for 6 h at 310 K. After this period, cells were harvested by centrifugation at 5000*g* for 15 min, suspended in lysis buffer (0.1% Triton X-100, 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA in 10 m*M* Tris buffer pH 7.5) and sonicated at 180 W for 10 min using an UH-150 Ultrasonic homogenizer (SMT Co., Tokyo, Japan). The cell extract was obtained by centrifugation at 12 000*g* for 15 min. The enzyme was purified from the cell extract by ammonium sulfate fractionation (30–75% saturation) and consecutive column chromatographic steps on Sephadex G-100, Red-Sepharose and hydroxylapatite. SDS–PAGE of the purified enzyme revealed a single 27 kDa protein band by Coomassie Brilliant Blue staining. The purified enzyme fractions were dialyzed against 200 m*M* sodium chloride, 5 m*M* 2-mercaptethanol and 20%(*v*/*v*) glycerol in 10 m*M* phosphate buffer pH 7.0, concentrated to 3 mg ml^{-1} by ultrafiltration using an Amicon YM-10 membrane and stored at 253 K. The enzyme is a basic protein with a pI value of 9.3 and was easily precipitated on concentration to higher than 3.3 mg ml^{-1}. Avoidance of excess concentration resulted in a high yield (27 mg protein from 1 l culture) of the homogenous enzyme compared with the previous purification (5 mg protein from 1 l culture; Usami *et al.*, 2003). The preparation of the *E. coli* cell extract and purification of the enzyme were carried out at 278 K.

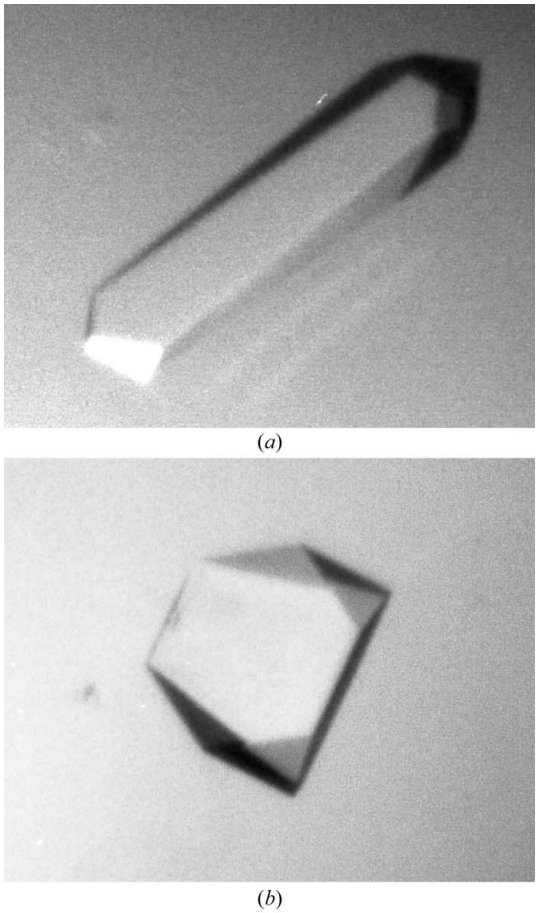

Figure 1
Tetragonal crystals of PHCR. (*a*) Form I. (*b*) Form II.

2.2. Crystallization

The stock solution of 3 mg ml^{-1} PHCR described above was dialyzed against 1 m*M* NADPH and 50 m*M* NaCl in 20 m*M* Tris buffer pH 7.5 and concentrated using a Centricon-30 (Millipore), yielding a working solution of 3 mg ml^{-1} PHCR with 1 m*M* NADPH and 50 m*M* NaCl in 20 m*M* Tris buffer pH 7.5. Initial sparse-matrix crystal screening (Jancarik & Kim, 1991) was conducted using Crystal Screen I (Hampton Research, USA). Crystallization was carried out by the hanging-drop method, in which 1 µl working solution was mixed with the same volume of crystallization buffer and incubated at 293 K. The drops were suspended over 200 µl reservoir solution in 48-well plates. Several microcrystals and two morphologically different crystals were observed in a week. Crystals of form I grew as rod-shaped crystals from condition No. 38 of Crystal Screen I (100 m*M* HEPES buffer and 1.4 *M* sodium citrate as a precipitant). Crystals of form II grew as rectangular-shaped crystals from condition No. 4 of Crystal Screen I (100 m*M* Tris buffer and 2.0 *M* ammonium sulfate as a precipitant). Trials to improve the crystallization conditions were performed for the form I and II crystals by varying the pH, buffer system and precipitant concentration. Simultaneously, we also examined the addition of glycerol to the reservoir solutions.

In order to obtain form I crystals suitable for X-ray analysis, a droplet was prepared by mixing equal volumes (2.0 + 2.0 µl) of the working solution (3 mg ml^{-1} PHCR) described above and reservoir solution [1.2 *M* sodium citrate and 20%(*v*/*v*) glycerol in 100 m*M* HEPES buffer pH 7.5] and was suspended over 500 µl reservoir solution in 24-well plates. Rod-shaped crystals with typical dimensions of approximately 0.1 × 0.1 × 0.5 mm were grown in one week (Fig. 1*a*).

In order to obtain form II crystals suitable for X-ray analysis, a droplet was prepared by mixing equal volumes (2.0 + 2.0 µl) of the working solution (3 mg ml^{-1} PHCR) described above and reservoir solution [1.4 *M* ammonium sulfate and 20%(*v*/*v*) glycerol in 100 m*M* MES buffer pH 6.0] and was suspended over 500 µl reservoir solution in 24-well plates. Rectangular-shaped crystals with typical dimensions of approximately 0.1 × 0.2 × 0.3 mm grew in one week (Fig. 1*b*).

2.3. X-ray data collection

Since the crystallization conditions of PHCR described above contained 20%(*v*/*v*) glycerol in the reservoir solution, X-ray data collection could be performed under cryogenic conditions without further addition of cryoprotectant. Thus, crystals from the hanging drop were directly mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K just prior to data collection. Data collection for the form I crystal was performed by the rotation method at 100 K using an ADSC Q210 CCD detector with synchrotron radiation (λ = 1.000 Å at beamline NW12 of the Advanced Ring of the Photon Factory, Tsukuba, Japan). The Laue group and unit-cell parameters were determined using the *DPS*

program package (Rossmann & van Beek, 1999). The Laue group was found to be 4/*m* and the unit-cell parameters were $a = b = 109.61$, $c = 94.31$ Å. Only reflections with $l = 2n$ were observed along the (00*l*) axis, indicating the tetragonal space group $P4_2$. Assuming the presence of four subunits (one tetramer) per asymmetric unit (ASU) led to an empirically acceptable V_M value of 2.36 Å^3 Da^{-1}, corresponding to a solvent content of 48% (Matthews, 1968). The current best diffraction data from a form I crystal were collected to 1.5 Å resolution and processed with the *DPS* and *CCP*4 program packages (Collaborative Computational Project, Number 4, 1994; Table 1).

Data from a form II crystal were collected by procedures similar to those described above. The Laue group was found to be 4/*mmm* and the unit-cell parameters were $a = b = 120.10$, $c = 147.00$ Å. Only reflections with $h = 2n$, $k = 2n$ and $l = 4n$ were observed along the (*h*00), (0*k*0) and (00*l*) axes, respectively, indicating the tetragonal space group $P4_12_12$ or $P4_32_12$. Assuming the presence of four subunits (one tetramer) per ASU led to an empirically acceptable V_M value of 2.21 Å^3 Da^{-1}, corresponding to a solvent content of 44% (Matthews, 1968). The current best diffraction data from a form II crystal were collected to 2.2 Å resolution. Data-collection statistics are summarized in Table 1.

2.4. Crystallographic analyses

A self-rotation map of PHCR (Fig. 2) clearly shows noncrystallographic symmetry (NCS) twofold rotation axes and indicates that the PHCR molecule has 222 point-group symmetry. The directions of the three mutually perpendicular twofold rotation axes comprising the molecular 222 point-group symmetry can be assigned to a set of three peaks found in the figure: the ω and φ angles are (90, −1.9°), (90, 88.1°) and (0, 0°), for example. Since one of the NCS twofold axes, (0° 0°), is parallel to the *c* axis of the unit cell, a secondarily generated NCS twofold axis exists, for example (ω, φ) = (90, 43.1°).

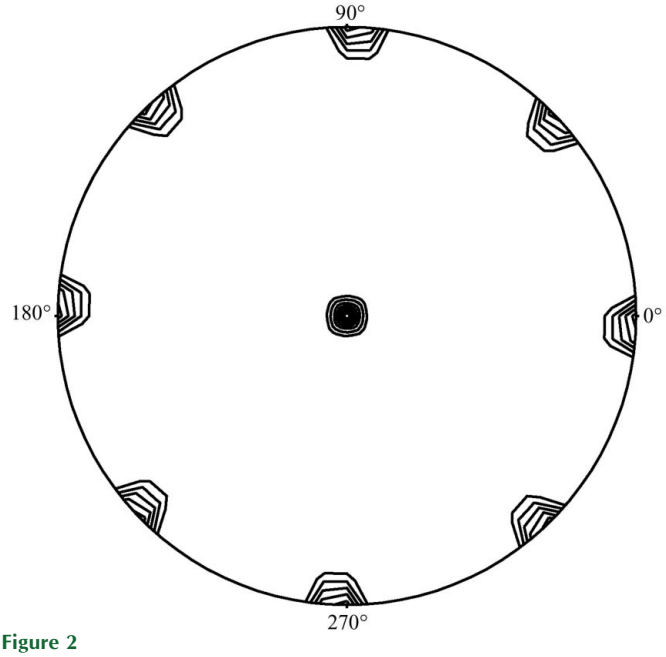

Figure 2
Stereographic projection of the self-rotation function in spherical polar angles at $\kappa = 180°$ for the PHCR crystal. The self-rotation search was carried out with the program *POLARRFN* from the *CCP*4 suite using the data in the 20–3.0 Å resolution shell and an integration radius of 30 Å. Contour lines are drawn at an increment of 10% of the origin peak, starting at 30% of the origin peak. The program produces constant rotation angle κ for different axis directions defined by ω (angle from pole) and φ (angle around equator). The φ angles are marked on the circumference and the ω angles are defined as 0 or 180° at the centre and 90° around the edge.

Table 1
Data-collection statistics for PHCR.

Values in parentheses are for the outer resolution shell.

	Form I	Form II
Space group	$P4_2$	$P4_12_12$
Unit-cell parameters		
a (Å)	109.61	120.10
b (Å)	109.61	120.10
c (Å)	94.31	147.00
No. of subunits per ASU	4 (1 tetramer)	4 (1 tetramer)
Solvent content (%)	48	44
X-ray source	PF-AR NW12	PF-AR NW12
Detector	ADSC Q210	ADSC Q210
Wavelength (Å)	1.000	1.000
Resolution (Å)	1.5 (1.58–1.5)	2.2 (2.32–2.2)
Unique reflections	174385	54751
Multiplicity	4.9 (4.9)	8.3 (6.8)
Mean $I/\sigma(I)$	6.5 (2.3)	5.4 (3.3)
B factor (Wilson plot) (Å^2)	13.4	34.8
R_{sym}† (%)	8.2 (31.0)	8.1 (19.4)
Completeness (%)	98.0 (98.4)	99.3 (96.4)

† $R_{sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th measurement and $\langle I(hkl)\rangle$ is the weighted mean of all measurements of $I(hkl)$.

Initial phase determination for the form I crystal was performed by the molecular-replacement (MR) technique using the coordinate set of the whole tetramer of MLCR (PDB code 1cyd; Tanaka, Nonaka, Nakanishi *et al.*, 1996), which has approximately 27% amino-acid sequence identity to PHCR, as a search model. The bound NADPH, 2-propanol and water molecules were removed from the search model. Cross-rotation and translation functions were calculated using the program *AMoRe* (Navaza, 1994) from the *CCP*4 suite. The results showed a clear solution [correlation coefficient of 0.321 (the first noise solution was 0.286) and *R* factor of 0.539 (0.553) in the resolution range 15.0–2.5 Å] and a reasonable molecular arrangement of PHCR in the ASU. The MR solution was supported by the observation that the directions of the non-crystallographic (NCS) twofold axes determined by the self-rotation function, showing the 222 point-group symmetry of the tetrameric PHCR molecule, were consistent with the MR solution obtained. The model was improved by manual model building with the program *XtalView* (McRee, 1999) and refined to a resolution of 1.5 Å with an *R* factor of 0.223 (free *R* factor of 0.244), without incorporating the bound NADPH and water molecules, using *REFMAC* (Murshudov *et al.*, 1997). A random subset of the data (5%) were not included in the refinement. The refinement of the form I crystal at 1.5 Å is incomplete at this time and work on it continues.

The incompletely refined form I model could nevertheless be used for structure determination of the form II crystal by the MR method, which was performed by procedures similar to those described above. The space-group ambiguity ($P4_12_12$ or $P4_32_12$) for the form II crystal was resolved by calculating the translation function for either case. The results showed a clear solution [correlation coefficient of 0.729 (the first noise solution 0.398) and *R* factor of 0.397 (0.562) in the resolution range 15.0–2.5 Å] and a reasonable molecular arrangement of PHCR in the ASU for the space group $P4_12_12$. Further model building and refinement at 2.2 Å resolution for the form II crystal are in progress.

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References

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Duax, W. L., Ghosh, D. & Pletnev, V. (2000). *Vitam. Horm.* **58**, 121–148.

Forrest, G. L. & Gonzalez, B. (2000). *Chem. Biol. Interact.* **129**, 21–40.

Ghosh, D., Sawicki, M., Pletnev, V., Erman, M., Ohno, S., Nakajin, S. & Duax, W. L. (2001). *J. Biol. Chem.* **276**, 18457–18463.

Hara, A., Nakayama, T., Deyashiki, Y., Kariya, K. & Sawada, H. (1986). *Arch. Biochem. Biophys.* **244**, 238–247.

Imamura, Y., Migita, T., Otagiri, M., Choshi, T. & Hibino, S. (1999). *J. Biochem.* **125**, 41–47.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J. & Ghosh, D. (1995). *Biochemistry*, **34**, 6003–6013.

McRee, D. E. (1999). *J. Struct. Biol.* **125**, 156–165.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.

Nakayama, T., Yashiro, K., Inoue, Y., Matsuura, K., Ichikawa, H., Hara, A. & Sawada, H. (1986). *Biochim. Biophys. Acta*, **882**, 220–227.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kallberg, Y., Persson, B. & Jörnvall, H. (2003). *Chem. Biol. Interact.* **143–144**, 247–253.

Oritani, H., Deyashiki, Y., Nakayama, T., Hara, A., Sawada, H., Matsuura, K., Bunai, Y. & Ohya, I. (1992). *Arch. Biochem. Biophys.* **292**, 539–547.

Rossmann, M. G. & van Beek, C. G. (1999). *Acta Cryst.* D**55**, 1631–1640.

Tanaka, M., Batemen, R., Rauh, D., Vaisberg, E., Ramachandani, S., Zhang, C., Hansen, K. C., Burlingame, A. L., Trautman, J. K., Shokat, K. M. & Adams, C. L. (2005). *PLoS Biol.* **3**, e128.

Tanaka, M., Ohno, S., Adachi, S., Nakajin, S., Shinoda, M. & Nagahama, Y. (1992). *J. Biol. Chem.* **267**, 13451–13455.

Tanaka, N., Nonaka, T., Nakamura, K. T. & Hara, A. (2001). *Curr. Org. Chem.* **5**, 89–111.

Tanaka, N., Nonaka, T., Nakanishi, M., Deyashiki, Y., Hara, A. & Mitsui, Y. (1996). *Structure*, **4**, 33–45.

Tanaka, N., Nonaka, T., Tanabe, T., Yoshimoto, T., Tsuru, D. & Mitsui, Y. (1996). *Biochemistry*, **35**, 7715–7730.

Usami, N., Ishikura, S., Abe, H., Nagano, M., Uebuchi, M., Kuniyasu, A., Otagiri, M., Nakayama, H., Imamura, Y. & Hara, A. (2003). *Chem. Biol. Interact.* **143–144**, 353–361.

Wermuth, B., Bohren, K. M., Heinemann, G., von Wartburg, J.-P. & Gabby, K. H. (1988). *J. Biol. Chem.* **263**, 16185–16188.