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Crystallization and preliminary X-ray crystallographic studies of fatty acid-CoA racemase from *Mycobacterium tuberculosis* H37Rv

Kyung-Hee Rhee,[a]‡ Ki Seog Lee,[b]‡ Amit Priyadarshi,[a] Eunice Eunkyung Kim[a] and Kwang Yeon Hwang[b]*

[a]Biomedical Research Center, Division of Life Sicence, Korea Institute of Science and Technology, 39-1 Hawolgok-dong, Sungbuk-gu, Seoul 136-791, South Korea, and [b]Division of Biotechnology and Genetic Engineering, Korea University, Seoul 136-701, South Korea

‡ These authors contributed equally to this study.

Correspondence e-mail: chahong@korea.ac.kr

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Fatty acid-CoA racemase plays an important role in the β-oxidation of branched-chain fatty acids and fatty-acid derivatives as it catalyzes the conversion of several (2*R*)-branched-chain fatty acid-CoAs to their (2*S*)-stereoisomers. Fatty acid-CoA racemase from *Mycobacterium tuberculosis* H37Rv has been purified to homogeneity and crystallized by the hanging-drop vapour-diffusion method with polyethylene glycol 4000 as precipitant. The crystals belong to the trigonal space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 109.56$, $c = 147.97$ Å. The asymmetric unit contains six monomers, corresponding to a V_M value of 2.15 Å^3 Da^{-1}. A complete native data set has been collected at 2.7 Å resolution using a synchrotron-radiation source.

1. Introduction

Fatty-acid β-oxidation is the preferred mechanism of oxidation for most fatty acids. The β-oxidation of fatty acids takes place by two distinct pathways. The first pathway catalyzes β-oxidation of very long-chain fatty acids and the second pathway catalyzes β-oxidation of branched-chain fatty acids and fatty-acid derivatives such as pristanic acid and the bile acid intermediates di- and trihydroxy-cholestanoic acid (DHCA and THCA, respectively; Ackman & Hansen, 1967; Wanders *et al.*, 1995).

The branched-chain fatty acids and fatty-acid derivatives undergo α-oxidation, which leads to shortening of the chain (Verhoeven *et al.*, 1998; Ferdinandusses *et al.*, 2002; Wanders *et al.*, 2003; Wanders, 2004), and are then further degraded in the peroxisome *via* three cycles of β-oxidation, followed by transport to the mitochondria, where they are β-oxidized to completion (Verhoeven *et al.*, 1998). The α-oxidation of fatty acids is not a stereoselective process (Tsai *et al.*, 1973; Groes *et al.*, 1999), so that after α-oxidation of branched-chain fatty acids both (*R*,*R*,*R*)- and (*S*,*R*,*R*)-isomers are formed. However, the β-oxidation system is stereoselective because only the (2*S*)-isomer is accepted as substrate by branched-chain acyl-CoA oxidase, the first enzyme of the β-oxidation system. Thus, the (*R*,*R*,*R*)-isomers of the metabolites of α-oxidation first need to be converted to their (*S*,*R*,*R*)-isomers by enzymatic racemization prior to further degradation (Schmitz & Conzelmann, 1997; Groes *et al.*, 1999).

The enzyme fatty acid-CoA racemase encoded by the *far* gene of *Mycobacterium tuberculosis* H37Rv catalyzes the conversion of several (2*R*)-branched-chain fatty acid-CoAs to their (2*S*)-stereoisomers in the metabolism of fatty acids. Fatty acid-CoA racemase is thought to be involved in this racemization prior to the stereospecific β-oxidation, although this has not yet been demonstrated experimentally. Recently, Bhaumik *et al.* (2003) found three genes, *mcr*, *far* and *Rv3727*, in the *M. tuberculosis* genome which encode proteins that are homologous to mammalian α-methylacyl-CoA racemase (AMACR). Crystallization and X-ray diffraction studies of AMACR encoded by the *mcr* gene have been reported in which sequence alignment of fatty acid-CoA racemase (SWISS-PROT Accession No. O53867) with AMACR (SWISS-PROT Accession No. O06543) of *M. tuberculosis* shows 57% sequence identity using *CLUSTALX* (Thompson *et al.*, 1997). However, the molecular basis of the differential targeting between fatty acid-CoA racemase and AMACR is still unknown. Therefore, more detailed investigations are required in order to obtain insight in the stereochemistry of the fatty-acid

oxidation systems and their substrates and to reveal the structural differences from AMACR. As a first step toward its structure elucidation, we have overexpressed fatty acid-CoA racemase from *M. tuberculosis* H37Rv and crystallized it. The crystallization conditions and preliminary X-ray crystallographic data are reported here.

2. Materials and methods

2.1. Expression and purification

The *far* gene of *M. tuberculosis* H37Rv was amplified by polymerase chain reaction from the bacterial genomic DNA using Pyrobest (Takara) and two primers (5′-GGAATTC**CATATG**ATGACGACCGGGGGGC-3′ and 5′-CCG**CTCGAG**TCATTACCAGTTGATTTCGTCGATC-3′). The PCR product was purified, digested with *Nde*I and *Xho*I and ligated into the pET-28a vector (Novagen). After confirmation of the DNA sequence, the resulting vector was introduced into the *Escherichia coli* BL21 (DE3) strain grown at 310 K in LB medium with kanamycin (50 µg ml^{-1}). Expression of the recombinant fatty acid-CoA racemase fused to a His tag at the N-terminus was induced by 0.5 m*M* isopropyl-D-thiogalactopyranoside at an optical density of about 0.45 at 600 nm. The pelleted cells were suspended in buffer *A* (20 m*M* HEPES pH 7.8, 200 m*M* NaCl and 2 m*M* 2-mercaptoethanol). The cell lysate was prepared by sonication in buffer *A*. The supernatant was loaded onto a column of Ni–NTA agarose beads (Amersham Biosciences) that had been pre-equilibrated with buffer *A*. The column was washed with buffer *A* containing 0.1 *M* imidazole and then eluted with a linear gradient of 0.1–0.5 *M* imidazole in the same buffer, with a flow rate of 3 ml min^{-1}. The eluant was concentrated using Centricon YM-10 (Millipore). The concentrated protein solution was exchanged with buffer *B* [20 m*M* Na HEPES pH 8.0, 5%(*v*/*v*) glycerol, 25 m*M* NaCl and 2 m*M* 2-mercaptoethanol] by repeated dilution and filtering. Portions of this protein solution were applied to and eluted from a Hi-Trap QFF 16/60 column (Amersham Biosciences) that had been pre-equilibrated with buffer *B*. After each application of protein, the column was washed with buffer *B* and the column was eluted with a linear gradient of 0.03–1.0 *M* NaCl in the same buffer with a flow rate of 3 ml min^{-1}. The fractions containing fatty acid-CoA racemase from the multiple runs were pooled and concentrated using Centricon YM-10. The buffer of the concentrated protein solution was exchanged with buffer *C* [20 m*M* Na HEPES pH 8.0, 5%(*v*/*v*) glycerol, 150 m*M* NaCl and 5 m*M* dithiothreitol] as described above. The protein solution was then injected onto a Superdex 75S column equilibrated with buffer *C*. The protein concentration was determined using Bradford's method (Bradford, 1976) with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor the protein in the column effluent. The product homogeneity of the purified preparation was judged by gel electrophoresis. SDS–PAGE under denaturing conditions was carried out in 15%(*w*/*v*) polyacrylamide gels using Precision Protein Standard (Bio-Rad) as reference proteins for molecular-weight estimation. The protein bands were detected by staining with Coomassie Brilliant Blue R. The fractions containing fatty acid-CoA racemase were pooled and concentrated to 20 mg ml^{-1} in 20 m*M* HEPES pH 8.0, 5 m*M* DTT, 150 m*M* NaCl solution and stored at 203 K. The final purified fatty acid-CoA racemase was at least 95% pure as judged on a polyacrylamide gel. The molecular weight of the purified His-tag fusion protein matched the calculated value (40 221 Da) from the amino-acid sequence deduced from the gene sequence using the *Compute pI/Mw* algorithm (http://us.expasy.org/tools/pi_tool.html; Bjellqvist *et al.*, 1993).

Table 1
X-ray data-collection and processing statistics of fatty acid-CoA racemase.

Values in parentheses are for the highest resolution shell.

Space group	$P3_1$ or $P3_2$
Resolution (Å)	50–2.7 (2.82–2.7)
No. of unique observations	52589 (5942)
Completeness (%)	95.8 (86.2)
R_{sym}†	0.080 (0.306)
$I/\sigma(I)$	9.9 (2.0)

† $R_{sym} = \sum|I - \langle I \rangle| / \sum \langle I \rangle$.

2.2. Crystallization

The recombinant fatty acid-CoA racemase from *M. tuberculosis* H37Rv was overexpressed in *E. coli* in a soluble form with a yield of ∼30 mg of homogeneous protein from a litre of culture. The protein was concentrated to 20 mg ml^{-1} in 20 m*M* Na HEPES pH 8.0, 150 m*M* NaCl, 5 m*M* DTT. Initial crystallization screening was performed using Crystal Screen I and II kits, JBScreen kit, MembFac Screen kit, Natrix Screen kit (Hampton Research) and Wizard I and II kits (Emerald Biostructures Inc.) by the hanging-drop vapour-diffusion method at 295 K by mixing 1 µl protein solution with 1 µl reservoir solution (Jancarik & Kim, 1991). Each hanging drop was placed over 0.5 ml reservoir solution. Initial crystals were obtained with a solution (No. 48) from the Natrix Screen kit. Natrix Screen condition No. 48 [0.2 *M* ammonium chloride, 0.01 *M* calcium chloride, 0.05 *M* Tris–HCl pH 8.5 and 30%(*w*/*v*) polyethylene glycol 4000] produced a few needle-shaped crystals or several rod-shaped crystals within 2 d. The needle-shaped crystals were quite brittle and only diffracted X-rays to ∼5 Å. On the other hand, the rod-shaped crystals diffracted to higher resolution beyond ∼2.7 Å. Optimization of crystallization conditions was performed with a reservoir solution of 0.2 *M* ammonium chloride, 0.01 *M* calcium chloride, 0.05 *M* Tris–HCl pH 8.5 in the range 15–20%(*w*/*v*) PEG 4K.

2.3. X-ray diffraction study

The crystal was transferred into cryoprotection solution containing 30%(*v*/*v*) glycerol, 0.2 *M* ammonium chloride, 0.01 *M* calcium chloride, 0.05 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4K. It was then scooped up in a cryoloop, frozen in liquid nitrogen and mounted on the goniometer in a stream of cold nitrogen at 100 K. X-ray diffraction data were collected from the cooled crystal using a Bruker Proteum 300 CCD at beamline 6B at Pohang Light Source (PLS),

Figure 1
Crystal of fatty acid-CoA racemase from *M. tuberculosis* H37Rv. This crystal (0.2 × 0.2 × 0.6 mm) was used for X-ray diffraction data collection.

South Korea. The crystal-to-detector distance was set to 250 mm. A total of 200 images were collected with 1° oscillation range per image. The synchrotron X-ray wavelength was 1.12714 Å and a 0.1 mm collimator was used. Diffraction data were collected to 2.7 Å resolution, integrated and scaled with the *DENZO* and *SCALEPACK* crystallographic data-reduction package (Otwinowski & Minor, 1997).

3. Results and discussion

After optimization of crystallization conditions, crystals suitable for X-ray analysis with dimensions of 0.2 × 0.2 × 0.6 mm were obtained using reservoir solution containing 0.2 *M* ammonium chloride, 0.01 *M* calcium chloride, 0.05 *M* Tris–HCl pH 8.5, 20%(*w*/*v*) PEG 4K in 2 d (Fig. 1) and diffracted to at least 2.7 Å. The autoindexing procedure performed with *DENZO* indicated that the crystals belong to the trigonal space group, with unit-cell parameters $a = b = 109.56$, $c = 147.97$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The possible trigonal space groups were checked. However, four space groups ($P3_112$, $P3_212$, $P3_121$, $P3_221$) yielded poor values for the correlation coefficient and *R* factor. Therefore, the space group was determined to be $P3_1$ or $P3_2$ on the basis of systematic absences. A total of 1 401 227 measured reflections were merged into 52 589 unique reflections with an $R_{\rm merge}$ (on intensity) of 8.0%. The merged data set was 95.8% complete to 2.7 Å. Assuming the presence of six monomers per asymmetric unit, the Matthews coefficient $V_{\rm M}$ value was calculated as 2.15 Å^3 Da^{-1} (Matthews, 1968). The solvent content of the crystal was calculated to be 42.8%. Data-collection statistics are given in Table 1.

During the course of this research, the structure of α-methylacyl-CoA racemase (AMACR) from *M. tuberculosis* was solved by X-ray crystallography (Savolainen *et al.*, 2005). We have tried molecular-replacement calculation for phase determination using the program *CNS* (Brünger *et al.*, 1998) with the 1.8 Å structure of AMACR (PDB code 1x74; Savolainen *et al.*, 2005) as a search model; the two possible enantiomorphic space groups ($P3_1$ or $P3_2$) were checked. The results of molecular replacement clearly indicated the correct space group to be $P3_2$. Recently, we have confirmed the presence of electron density for cholic acid as a substrate analogue located in the active site of fatty acid-CoA racemase using a co-crystallization method. Further refinements of the model structure are currently in progress and the structural details will be described in a separate paper.

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