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Crystallization and X-ray diffraction analysis of peroxisomal Δ^3-Δ^2-enoyl-CoA isomerase from *Saccharomyces cerevisiae*

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The purification, crystallization and X-ray diffraction analysis of *Saccharomyces cerevisiae* Δ^3-Δ^2-enoyl-CoA isomerase is described. Δ^3-Δ^2-Enoyl-CoA isomerase is a member of the hydratase/isomerase protein family and is an auxiliary enzyme required for the β-oxidation of unsaturated fatty acids. It is a hexameric enzyme consisting of six identical 32 kDa subunits of 280 residues each. In crystallization trials three crystal forms were obtained, with tetragonal and hexagonal lattices. A 2.5 Å data set was collected from the unliganded hexagonal crystals with an $R_{\rm merge}$ of 6.6%. The crystal, with unit-cell parameters a = 116.0, b = 116.0, c = 122.9 Å, is likely to have $P6_322$ symmetry.

1. Introduction

In mammalian cells, fatty-acid chains are degraded both in mitochondria and peroxisomes by sequential removal of two C atoms during one cycle of a process called β-oxidation. In yeast cells, however, β-oxidation occurs only in peroxisomes (Kunau *et al.*, 1988). In β-oxidation, the fatty acid activated to fatty acyl-CoA is first dehydrogenated by acyl-CoA dehydrogenase in mitochondria or acyl-CoA oxidase in peroxisomes to yield *trans*-2-enoyl-CoA, which is then hydrated to 3-hydroxyacyl-CoA by 2-enoyl-CoA hydratase. The third step is the dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase. In the fourth step, the fatty acyl-CoA is shortened by an acetyl-CoA group following a thiolytic cleavage reaction catalyzed by 3-ketoacyl-CoA thiolase. The resulting shortened acyl-CoA is ready for a new round of β-oxidation. Because *trans*-2-enoyl-CoA is the only unsaturated intermediate in the β-oxidation pathway, auxiliary enzymes such as Δ^3-Δ^2-enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase are needed for the oxidation of double bonds in odd- and even-numbered positions in unsaturated fatty acids. These enzymes convert the double bonds into a configuration suitable for the classical β-oxidation described above.

Δ^3-Δ^2-Enoyl-CoA isomerase catalyzes the isomerization of *trans*- and *cis*-3-enoyl-CoA into the *trans*-2 counterparts (Stoffel *et al.*, 1964), after which the enoyl-CoA can proceed with the hydration step of the β-oxidation. Enoyl-CoA isomerase belongs to the low-similarity hydratase/isomerase protein family on the basis of its amino-acid sequence (Müller-Newen & Stoffel, 1991). Monofunctional enoyl-CoA isomerases have been characterized from several sources, including human mitochondria (Kilponen & Hiltunen, 1993), rat mitochondria (Stoffel & Grol, 1978; Palosaari *et al.*, 1990), yeast peroxisomes (Gurvitz *et al.*, 1998; Geisbrecht *et al.*, 1998) and recently human and mouse peroxisomes (Geisbrecht *et al.*, 1999). Rat mitochondrial enoyl-CoA isomerase is most active towards short-chain enoyl-CoA substrates such as *trans*-3-hexenoyl-CoA, for which the specific activity is 38.5 µmol min^{-1} mg^{-1} (Palosaari *et al.*, 1990). Sequence alignments of rat isomerase with the homologous enoyl-CoA hydratase show that a catalytic glutamate of the hydratase (Glu164) is conserved (Müller-Newen *et al.*, 1995). The corresponding glutamate in rat enoyl-CoA isomerase is also important for catalysis (Müller-Newen & Stoffel, 1993).

The structures of three hydratase/isomerase proteins have been determined by X-ray crystallography, namely those of rat mitochondrial *trans*-2-enoyl-CoA hydratase (Engel *et al.*, 1996), 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* (Benning *et al.*, 1996) and rat $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase (Modis *et al.*, 1998). All of these structures have a similar spiral substrate-binding core domain composed of repeating right-handed turns of two β-strands and an α-helix. 4-Chlorobenzoyl-CoA dehalogenase is a trimer, whereas both dienoyl-CoA isomerase and enoyl-CoA hydratase form a hexamer (a dimer of trimers). The glutamate corresponding to Glu164 in hydratase has been shown to also take part in

Table 1
Crystallization and preliminary X-ray analysis of the different yeast enoyl-CoA isomerase crystal forms.

All crystallizations were performed at room temperature using the hanging-drop method, mixing 2 µl protein solution with 2 µl precipitant solution.

	Unliganded	Unliganded	Liganded
Protein buffer	20 m*M* potassium phosphate pH 7.2, 400 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* benzamidine–HCl	20 m*M* potassium phosphate pH 7.2, 400 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* benzamidine–HCl	20 m*M* potassium phosphate pH 7.2, 400 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* benzamidine–HCl,
Protein concentration (mg ml^{-1})	2.3	1.0	1.0
Precipitant solution	0.1 *M* MES pH 5.5, 5% 1.4-dioxane, 1.4 *M* $(NH_4)_2SO_4$	0.1 *M* sodium acetate pH 4.8, 2.2 *M* $(NH_4)_2SO_4$	0.1 *M* sodium acetate pH 5.1, 1.7 *M* $(NH_4)_2SO_4$
Additive	10 m*M* *N*-octyl-β-D-glucoside	—	—
Ligand	—	—	2 m*M* *n*-octanoyl-CoA
Crystal shape	Hexagonal rods	Tetragonal bipyramid	Hexagonal plate
Crystal dimensions (mm)	0.3 × 0.2 × 0.2	0.3 × 0.3 × 0.2	0.25 × 0.25 × 0.05
Diffraction limit (Å)	3.0 (home source), 2.5 (synchrotron)	3.0 (home source)	3.5 (home source)
Unit-cell parameters (Å)	$a = b = 116.0$, $c = 122.9$	$a = b = 118.1$, $c = 222.3$	$a = b = 117$, $c = 125$
Space group	$P6_322$	Primitive tetragonal	$P6_322$
Subunits per asymmetric unit	1	—	1
Matthews coefficient (Å^3 Da^{-1})	3.7	2†	3.9
Solvent content (%)	67	38†	68

† Assuming eight hexamers per unit cell.

catalysis in rat dienoyl-CoA isomerase according to mutational and structural studies (Modis *et al.*, 1998).

Yeast peroxisomal enoyl-CoA isomerase has 17% sequence identity to the rat mitochondrial isomerase and 18 and 19% sequence identity to enoyl-CoA hydratase and dienoyl-CoA isomerase, respectively, the structures of which are known. Interestingly, the glutamate known to be important for catalysis in rat isomerase and hydratase is replaced by Phe150 in yeast isomerase (Gurvitz *et al.*, 1998). A high-resolution crystal structure would give us insight into the reaction mechanism of yeast enoyl-CoA isomerase in particular and the isomerases in general. This study describes the purification, crystallization and preliminary X-ray analysis of yeast enoyl-CoA isomerase.

2. Experimental methods

2.1. Protein purification

Δ^3-Δ^2-Enoyl-CoA isomerase was overexpressed in *Escherichia coli* and the soluble protein extract was prepared as described previously (Gurvitz *et al.*, 1998). The isomerase was purified using three chromatographic steps. Ammonium sulfate was added to the soluble protein extract to a final concentration of 1.4 *M*. The supernatant was applied to a butyl Sepharose column (15 × 1.5 cm) equilibrated with a buffer containing 20 m*M* potassium phosphate pH 7.2, 1.4 *M* $(NH_4)_2SO_4$ and 0.5 m*M* benzamidine hydrochloride. After washing the unbound protein with the same buffer, yeast isomerase bound to the column was eluted with a linear 150 ml gradient of decreasing $(NH_4)_2SO_4$ concentration (1.4–0.8 *M*). The fractions containing isomerase activity were pooled, diluted with two volumes of water and applied to a 2 ml Bio-Scale ceramic hydroxyapatite column (Bio-Rad) equilibrated with a buffer consisting of 20 m*M* sodium phosphate pH 7, 100 m*M* NaCl and 0.5 m*M* benzamidine hydrochloride. The bound enoyl-CoA isomerase was eluted with a linear gradient of 20–400 m*M* sodium phosphate in 15 ml. For the final step of purification, a Poros SP column (10 × 0.5 cm) was equilibrated with a buffer consisting of 20 m*M* potassium phosphate pH 7.2, 1 m*M* EDTA, 1 m*M* EGTA and 0.5 m*M* benzamidine hydrochloride. The isomerase containing hydroxyapatite fractions were diluted twofold with the same Poros SP buffer and applied to the column. The bound protein was eluted with a linear gradient of 200–600 m*M* potassium chloride in 25 ml. The fractions containing pure yeast enoyl-CoA isomerase (Fig. 1) were pooled and concentrated to either 1 or 2.3 mg ml^{-1}. *N*-octyl-β-D-glucoside (10 m*M*) was used as an additive in the 2.3 mg ml^{-1} protein solution. SDS–PAGE gels showed only one band, with a molecular weight of approximately 32 kDa (Fig. 1). Gel-filtration and dynamic light-scattering experiments indicated that the molecular weight of the native protein is approximately 170 000 Da, which is in good agreement with the presence of a hexameric protein of six 32 kDa subunits (Gurvitz *et al.*, 1998). The protein solutions were stored at 277 K and used for the crystallization trials. Relatively low protein concentrations were used for crystallization experiments as yeast enoyl-CoA isomerase tends to precipitate on concentration. The protein was found to be stable at 277 K and crystals could still be obtained from protein solutions prepared six months prior to crystallization.

2.2. Crystallization

Crystallization was performed at 295 and 277 K using the vapour-diffusion method. The crystallization conditions were determined by the sparse-matrix screen (Jancarik & Kim, 1991) using Crystal Screen and Crystal Screen II kits (Hampton Research). The drops were prepared by mixing equal volumes (2 µl) of protein and precipitant solution. Further optimization of the conditions resulted in two unliganded crystal forms and one with *n*-octanoyl-CoA as ligand. Unliganded crystals of tetragonal shape grew in 0.1 *M* sodium acetate at pH 4.8, 2.2 *M* $(NH_4)_2SO_4$ when using 1 mg ml^{-1} protein solution (Table 1). Hexagonal rod-shaped crystals (Fig. 2) were obtained from 2.3 mg ml^{-1} protein solution in 0.1 *M* MES pH 5.5, 5% 1,4-dioxane, 1.8 *M* $(NH_4)_2SO_4$. Both crystal forms reached maximum dimensions of about 0.3 × 0.2 × 0.2 mm in 2–3 weeks at 295 K. Liganded crystals were obtained in the same crystallization solution

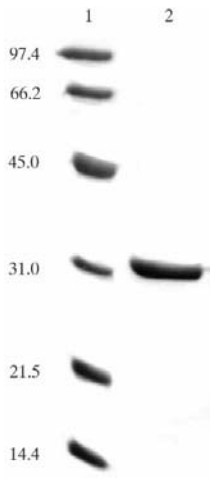

Figure 1
Purified yeast enoyl-CoA isomerase analyzed on SDS–PAGE. Lane 1 contains molecular-weight standards (BioRad low molecular weight). Lane 2 contains 2–3 µg of pure enoyl-CoA isomerase.

as the tetragonal crystals, but with a lower precipitant concentration [1.7 *M* $(NH_4)_2SO_4$]. The addition of the ligand, however, changed the morphology of the crystals from tetragonal to hexagonal plates.

2.3. X-ray diffraction analysis

The crystals were mounted in quartz capillaries and were exposed to X-rays at room temperature using a rotating-anode generator and a MAR345 image-plate detector. Table 1 summarizes the properties of the three different crystal forms. The unliganded bipyramid-shaped crystals belonged to a primitive tetragonal space group, with unit-cell parameters $a = b = 118.1$, $c = 222.4$ Å; the crystals obtained with *n*-octanoyl-CoA as ligand were of hexagonal symmetry, with unit-cell parameters $a = b = 117$, $c = 125$ Å. The third crystal form is the unliganded hexagonal form, with unit-cell parameters $a = b = 116.0$, $c = 122.9$ Å. Many crystals could be obtained for this form, therefore cryoconditions were determined. The hexagonal crystals were flash-frozen in the presence of 20% ethylene glycol and a data set was collected by the rotation method with 1° rotations per frame at a wavelength of 0.909 Å at the DESY synchrotron station X11 at the EMBL, Hamburg, Germany. Fig. 3 shows a diffraction image of this crystal form. The frames were indexed and integrated using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

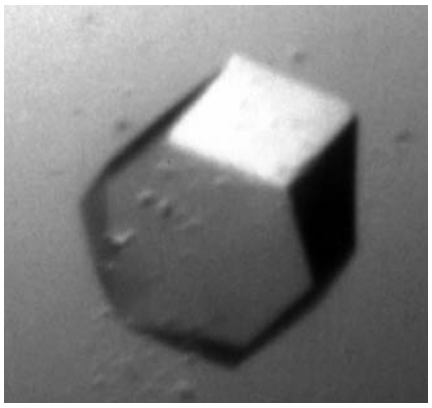

Figure 2
Hexagonal unliganded isomerase crystal. The crystal was grown in 0.1 *M* MES pH 5.5, 5% 1,4-dioxane, 1.4 *M* $(NH_4)_2SO_4$. The dimensions of the crystal are 0.3 × 0.2 × 0.2 mm.

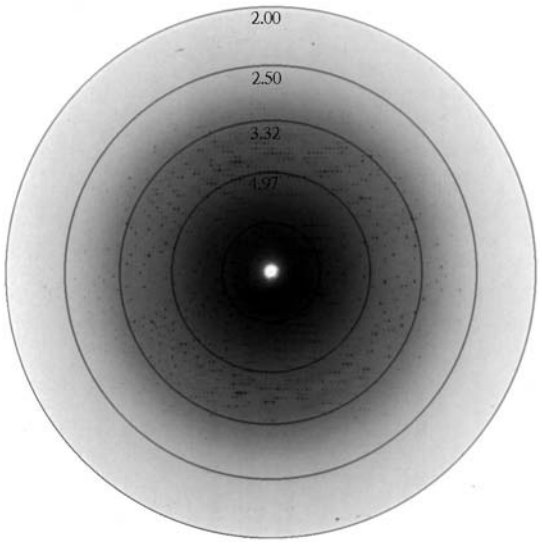

Figure 3
Diffraction image of the unliganded hexagonal crystal recorded at the EMBL-Hamburg X11 beamline with a MAR345 detector. The wavelength was 0.909 Å. The high-resolution limit is 2.5 Å.

3. Results and discussion

The native protein has a molecular weight of approximately 170 000 Da and therefore consists of six subunits. From the sequence homology with the enoyl-CoA hydratase and dienoyl-CoA isomerase, it is expected that the hexameric isomerase will also consist of a dimer of trimers with 32 symmetry. A complete data set was collected from the unliganded hexagonal crystal form (Table 2). Data processing revealed the symmetry to be *P*622, $P6_122$, $P6_522$, $P6_222$, $P6_422$ or $P6_322$. Assuming one monomer per asymmetric unit, the Matthews coefficient is 3.7 $Å^3\ Da^{-1}$ and the solvent content is 66.8%. Such packing is possible if the molecular center of the hexamer coincides with a position in the cell displaying 32 symmetry. Such positions only exist in *P*622 and $P6_322$. In *P*622 the coordinates of these positions are $(\frac{2}{3}, \frac{1}{3}, 0)$ and $(\frac{1}{3}, \frac{2}{3}, 0)$; in $P6_322$ these coordinates are $(\frac{2}{3}, \frac{1}{3}, \frac{1}{4})$ and $(\frac{1}{3}, \frac{2}{3}, \frac{3}{4})$. In *P*622, the two molecular centers are both in the same plane ($z = 0$), with a separation of 66 Å in the plane, as can be calculated from the unit-cell parameters. Such packing is allowed if the radius of the trimer is approximately 31 Å or smaller. The shape of the trimer of hydratase and dienoyl-CoA isomerase is close to a perfect cylinder (Modis *et al.*, 1998; Engel *et al.*, 1996) with a radius of approximately 35 Å. Therefore, the space group is unlikely to be *P*622 because the backbones of the two trimers would overlap significantly. This analysis suggests the space group to be $P6_322$. Tighter packing with two monomers per asymmetric unit also does not agree with the space-group symmetry. Indeed, a search for a local twofold axis with *GLRF* (Tong & Rossmann, 1990) and the calculation of a native Patterson did not provide any evidence for a local twofold axis.

Table 2
Data-collection statistics of a crystal of the unliganded $P6_322$ crystal form.

Values in parentheses refer to the highest resolution shell (2.59–2.50 Å).

Temperature (K)	100
Wavelength (Å)	0.909
Resolution range (Å)	50–2.5
Observed reflections	83172
Mosaicity (°)	0.34
Unique reflections	17313 (1669)
Redundancy	4.8
$I/\sigma(I)$	12.2 (4.9)
Completeness (%)	98.9 (98.8)
R_{merge}	0.066 (0.349)

The properly oriented and positioned structures of dienoyl-CoA isomerase and enoyl-CoA hydratase were used for rigid-body refinement, but the resulting *R* factors were always higher than 58%. Molecular-replacement calculations with *AMoRe* (Navaza, 1994) also did not yield any significant solutions. This could be because of the low sequence identity (18–19%) or because of a slightly different position of the yeast isomerase hexamer subunits with respect to dienoyl-CoA isomerase and enoyl-CoA hydratase. A search for useful heavy-atom derivatives has been initiated.

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