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Expression, crystallization and preliminary characterization of methylmalonyl coenzyme A epimerase from *Propionibacterium shermanii*

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Methylmalonyl-CoA epimerase (MMCE) is an enzyme that interconverts the *R* and *S* epimers of methylmalonyl-CoA in the pathway that links propionyl-CoA with succinyl-CoA. This is used for both biosynthetic and degradative processes, including the breakdown of odd-numbered fatty acids and some amino acids. The enzyme has been expressed in *Escherichia coli* both as the native enzyme and as its selenomethionine (SeMet) derivative. Crystals of both forms have been obtained by vapour diffusion using monomethylether PEG 2000 as precipitant. The native MMCE crystals are orthorhombic, with unit-cell parameters $a = 56.0$, $b = 114.0$, $c = 156.0$ Å, and the SeMet-MMCE crystals are monoclinic, with unit-cell parameters $a = 43.6$, $b = 78.6$, $c = 89.4$ Å, $\beta = 92.0^\circ$; both diffract to better than 2.8 Å resolution.

1. Introduction

The enzyme methylmalonyl-CoA epimerase (MMCE; E.C. 5.1.99.1), also known as methylmalonyl-CoA racemase, catalyses a key step in the interconversion of the three-carbon metabolite propionyl-CoA and the four-carbon metabolite succinyl-CoA. This pathway is important in many species for the breakdown of fatty acids with odd numbers of C atoms and for the degradation of some amino acids (Fenton & Rosenberg, 1995; Voet & Voet, 1995), and in some bacteria for the fermentation of pyruvate to propionate and for the biosynthesis of polyketide antibiotics (Marsden *et al.*, 1994). The pathway from propionyl-CoA to succinyl-CoA involves three steps. The first, a biotin-dependent carboxylation, gives (2*S*)-methylmalonyl-CoA and the third, catalysed by the B_{12}-dependent enzyme methylmalonyl-CoA mutase, converts (2*R*)-methylmalonyl-CoA to succinyl-CoA (Kaziro & Ochoa, 1964). The incompatibility of the product of the first step with the substrate of the third requires an intervening racemization, catalysed by MMCE, which interconverts the *S* and *R* configurations of methylmalonyl-CoA. In humans, defects in this pathway can lead to buildup of methylmalonic acid, causing severe acidosis and potential damage to the central nervous system (Fenton & Rosenberg, 1995).

The best characterized MMCE is that from *Propionibacterium shermanii* (Overath *et al.*, 1962; Leadlay, 1981), but homologous enzymes are found in other bacteria and in animal tissues (Mazumder *et al.*, 1962; Stabler *et al.*, 1985). MMCE from *P. shermanii* is a highly heat-stable dimeric enzyme with a subunit size of 16.5 kDa (Leadlay, 1981) and a polypeptide chain of 147 amino-acid residues (E. Saafi & M. L. Patchett, unpublished work). It is inactivated by treatment with metal-chelating agents and can be activated by incubation with divalent ions, notably by Co^{2+}, but also by Ni^{2+}, Mn^{2+} and Zn^{2+} (Leadlay, 1981). Functionally, MMCE thus appears related to mandelate racemase, whose activity also depends on a divalent metal ion (Powers *et al.*, 1991), but its amino-acid sequence bears no obvious relationship to this or any other racemase. Recent crystal structures for several other epimerases have revealed considerable diversity in their folding patterns (Giraud *et al.*, 2000). We therefore undertook the determination of the three-dimensional structure of MMCE in order to see whether this constituted a new class of epimerase and to understand its metal-ion dependence, its catalytic mechanism and its high thermostability.

2. Experimental

2.1. Expression and purification of native protein

MMCE was obtained by expression of the recombinant protein in *E. coli*. The pTEEX plasmid encoding the epimerase gene was transformed into *E. coli* strain BL21 (DE3). Cells were grown in Luria broth medium at 310 K, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density reached 2–2.5, harvested by centrifugation and stored at 253 K. For protein purification, the frozen pellet was resuspended in 100 m*M* MOPS pH 7.5, 2 m*M* EDTA and 0.1 m*M* PMSF and the cells lysed by sonication. The lysate was centrifuged at 14 000*g* for 1 h

and the cell-free lysate supernatant was decanted and heated to 343 K for 15 min. Denatured *E. coli* proteins were removed by centrifugation and the heat-treated supernatant was fractionated by precipitation with ammonium sulfate. The precipitate obtained at 50–90% saturation was centrifuged, dissolved in 50 m*M* MES pH 7.0, 0.2 *M* NaCl, filtered and loaded on to a Pharmacia Superdex 75 gel-filtration column equilibrated with 50 m*M* MES pH 7.0 and 0.4 *M* NaCl. The peak fraction containing MMCE was pooled, dialysed into 50 m*M* Tris pH 7.8 and loaded onto a Mono Q (Pharmacia Biotech) anion-exchange column. MMCE was eluted using a linear gradient of 0–1.0 *M* NaCl in 50 m*M* Tris pH 7.8, dialysed in 50 m*M* Tris pH 7.8 and concentrated by ultrafiltration to a final concentration of about 20 mg ml^{-1}.

2.2. Preparation of selenomethionine-substituted protein

A DNA fragment encoding the MMCE gene was cloned into the *Nco*I and *Eco*RI sites of expression vector pProEXHt (Life Technologies), which results in a fusion to a N-terminal His tag with a tobacco etch virus (TEV) protease cleavage site. This plasmid was transformed into the methionine auxotrophic *E. coli* strain BL41 (DE3) and the transformed bacteria were grown in 0.5 l of LeMaster medium supplemented with 0.5 m*M* selenomethionine (SeMet) (Hendrickson *et al.*, 1990). Protein expression was induced at a cell density of OD 0.7 by addition of IPTG to a final concentration of 0.5 m*M*. Cells were harvested 14 h after induction by centrifugation and stored at 253 K. For protein isolation, frozen bacterial cells were thawed and resuspended in ice-cold lysis buffer (100 m*M* MOPS pH 7.3, 0.5 m*M* EDTA and 2 m*M* β-mercaptoethanol) and lysed with a French press. The cell lysate was centrifuged at 14 000*g* for 20 min at 277 K. The supernatant solution was loaded onto a HiTrap chelating column (Pharmacia Biotech) and washed with buffer containing 100 m*M* MOPS pH 7.5, 0.5 m*M* EDTA, 2 m*M* β-mercaptoethanol and 25 m*M* imidazole. The protein was eluted with a linear gradient of 0–0.5 *M* imidazole in wash buffer. Protein-containing fractions were pooled and dialysed against 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 0.5 m*M* EDTA and 2 m*M* β-mercaptoethanol. The polyhistidine tag was removed by overnight digestion with TEV protease, followed by filtration of the digestion mixture through a HiTrap chelating (Pharmacia Biotech) column to remove uncleaved proteins and the TEV protease. The flowthrough was dialysed into 50 m*M* Tris–HCl pH 7.8, 0.2 m*M* EDTA and 2 m*M* DTT and concentrated by ultrafiltration to a final concentration of about 20 mg ml^{-1}. Mass-spectroscopic analysis of the epimerase Se-Met indicated that all seven methionines were substituted by Se-Met.

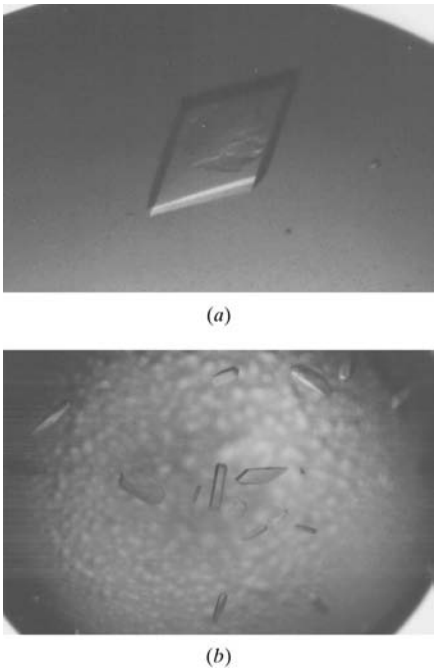

Figure 1
Crystals of (*a*) native MMCE and (*b*) SeMet-substituted MMCE.

2.3. Crystallization and preliminary X-ray data

All crystals were grown by hanging-drop vapour-diffusion methods. Initial conditions for crystallization of native MMCE were found using systematic screening methods based on orthogonal arrays (Kingston *et al.*, 1994) and subsequent crystals were prepared by fine variation of these conditions. Native crystals were obtained at 291 K by mixing equal volumes of a 20 mg ml^{-1} protein solution with a reservoir solution comprising 27–30% monomethylether (mme) PEG 2000, 0.2 *M* ammonium sulfate in 0.1 *M* sodium acetate pH 4.9. Small crystals of a different habit could also be obtained using 18–20% mmePEG 2000 and 10 m*M* $NiCl_2$ in 0.1 *M* Tris–HCl, but could not be grown large enough for diffraction experiments. Crystals generally appeared after 1–2 d and grew to full size after one week.

Crystals of the SeMet-substituted MMCE, with its N-terminal His_6-tag extension removed, were grown under similar conditions to the native protein, with a reservoir solution comprising 26–29% mmePEG 2000 and 0.2 *M* ammonium sulfate in 0.1 *M* sodium acetate pH 4.2–4.3.

For data collection, crystals were transferred to a synthetic mother liquor and flash-frozen at an optimal concentration of 40% mmePEG 2000. All X-ray diffraction measurements were therefore carried out with flash-frozen crystals at 110 K. Diffraction data were collected for the native MMCE crystals using a Rigaku RU-300 X-ray generator equipped with focusing mirrors and a MAR Research MAR345 imaging plate. Data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Overexpression of native MMCE in *E. coli* gave high levels of soluble protein, typically 20–50 mg l^{-1} of cell-culture medium. A key step in the purification was the heat step, involving 15 min incubation at 343 K, which denatured most of the *E. coli* proteins and attested to the high thermostability of MMCE. The final monoQ anion-exchange step was also essential, as without it only poorly diffracting crystals were obtained.

Native MMCE crystals (Fig. 1*a*) proved to be orthorhombic, space group $P2_12_12_1$, with unit-cell parameters a = 56.0, b = 114.0, c = 156.0 Å, six MMCE molecules in the asymmetric unit assuming a monomer molecular weight of 16.5 kDa and a solvent content of 51% (V_M = 2.51 Å^3 Da^{-1}; Matthews, 1968). These crystals diffracted to better than 2.8 Å resolution and an 88.6% complete native data set was collected to this resolution [21 893 unique reflections, redundancy 4.2, merging R factor 5.9%, average $I/\sigma(I)$ = 15.0]. Unfortunately, these crystals were very sensitive to changes in their mother liquor and also deteriorated after 1–2 weeks. As a result, all attempts to prepare heavy-atom derivatives using conventional soaking procedures either caused the crystals to dissolve or resulted in severe loss of high-resolution diffraction. We therefore prepared SeMet-substituted MMCE as described above.

Different procedures had to be used for preparation of the SeMet-substituted MMCE because of the likelihood of partial SeMet oxidation during the heat step that had been used for native MMCE. It was for this reason that a different construct, incorporating a His_6 tag for purification, was used to produce the SeMet form of MMCE.

The crystals obtained for SeMet-substituted MMCE (Fig. 1*b*) were smaller than those of the native protein and were

obtained at lower pH (4.2–4.3 compared with 4.9), but diffracted to slightly higher resolution. They also belonged to a different crystal form, which proved to be monoclinic, space group $P2_1$, with unit-cell parameters a = 43.6, b = 78.6, c = 89.4 Å, β = 92.0° and with four MMCE molecules in the asymmetric unit assuming a solvent content of 47% (V_M = 2.32 Å^3 Da^{-1}). Given the high quality of the diffraction from these SeMet-substituted crystals, we expect them to be suitable for determination of the methylmalonyl-CoA epimerase structure by multi-wavelength anomalous diffraction (MAD) methods.

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