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Cloning, expression, purification and preliminary X-ray crystallographic studies of 2-methylisocitrate lyase from *Salmonella typhimurium*

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In *Salmonella typhimurium*, propionate is oxidized to pyruvate *via* the 2-methylcitric acid cycle. The last step of this cycle, the cleavage of 2-methylisocitrate to succinate and pyruvate, is catalysed by 2-methylisocitrate lyase (EC 4.1.3.30). Methylisocitrate lyase (molecular weight 32 kDa) with a C-terminal polyhistidine affinity tag has been cloned and overexpressed in *Escherichia coli* and purified and crystallized under different conditions using the hanging-drop vapour-diffusion technique. Crystals belong to the orthogonal space group $P2_12_12_1$, with unit-cell parameters a = 63.600, b = 100.670, c = 204.745 Å. A complete data set to 2.5 Å resolution has been collected using an image-plate detector system mounted on a rotating-anode X-ray generator.

1. Introduction

Propionate, following acetate, is the second most abundant low-molecular-mass carbon compound in soil. Many aerobic microorganisms, bacteria and fungi as well as some anaerobes are able to grow on propionate as their sole carbon and energy source. Propionate and other short-chain fatty acids (SCFAs) also inhibit cell growth, which has made them useful as preservatives in the food industry (Cherrington *et al.*, 1991). However, the mechanism through which propionate exerts antimicrobial activity is poorly understood. This compound appears to affect the function of multiple targets within the cell (Horswill *et al.*, 2001).

In the recent past, the catabolism of propionate has been investigated in *Salmonella enterica* and *Escherichia coli*. In *S. enterica*, propionate is metabolized to pyruvate *via* 2-methylcitric acid cycle (Horswill & Escalante-Semerena, 1999*b*). It resembles the part of the glyoxylate cycle in which acetate is oxidized to glyoxylate. The methylcitrate cycle was initially postulated by studies with mutant strains of *Candida lipolytica* (Tabuchi *et al.*, 1974), in which accumulation of either methylcitrate or 2-methylisocitrate was observed during growth on odd-chain fatty acids, which are degraded *via* propionyl-CoA.

Genes required for the catabolism of propionate in these bacteria were first identified in *S. enterica* serovar Typhimurium LT2 and are referred to as '*prp*' genes (Horswill & Escalante-Semerena, 1999*b*). The *prp* locus is comprised of two transcribed units. One unit contains four genes (*prpBCDE*) organized as an operon that encodes four distinct enzymes which are required for the catabolism of propionate. The other unit contains only one gene, *prpR*, which encodes a σ^{54}-dependent transcriptional activator. In this pathway, PrpE, a propionyl-CoA synthetase (Horswill & Escalante-Semerena, 1999*a*), forms propionyl-CoA from propionate and acetyl-CoA and PrpC, a methylcitrate synthase (Horswill & Escalante-Semerena, 1999*b*), forms methylcitrate by combining propionyl CoA and oxaloacetate. The 2-methylcitrate thus formed is converted to 2-methylisocitrate by two separate enzymes, PrpD, a methylcitrate dehydratase, and either of the two aconitases present in *S. enterica* (Horswill & Escalante-Semerena, 2001). The last step of this cycle, the cleavage of 2-methylisocitrate to succinate and pyruvate, is catalysed by PrpB, a methylisocitrate lyase. Succinate is further oxidized to oxaloacetate for condensation with propionyl CoA, forming methylcitrate and completing the cycle, whereas pyruvate can be used for energy metabolism and synthesis of biomass.

The ability of microorganisms to metabolize propionate and SCFAs may be the primary line of defence against the negative effects of these compounds on cell growth. However, sensitivity towards propionate increased drastically when the methylcitrate pathway was blocked in *Salmonella*, suggesting that intermediates of this pathway may have a more profound negative impact on cell growth than propionate (Hammelman *et al.*, 1996).

In this paper, we report the cloning, high-level expression, purification and preliminary crystallographic studies on 2-methylisocitrate lyase encoded by the *prpB* gene isolated from *S. enterica* serovar Typhimurium strain IFO 12529. Structure determination of 2-methylisocitrate lyase will provide catalytic

insight into this particular protein as well as providing direct structural comparison with isocitrate lyase.

2. Material and methods

2.1. Cloning and expression

S. enterica serovar Typhimurium strain IFO 12529 was generously provided by Professor Toru Nagasawa, Okayama University, Japan. The *prpB* gene was PCR amplified from *S. typhimurium* genomic DNA using Deep Vent polymerase (NEB). After amplification of target gene by sense (5′-CATGCCATGGCTAGCTCTTTACATTCGCCGGGG-3′) and antisense (5′-CGGGATCCTTACTCGAGCGATTTTTTATTCCTGTACAG-3′) primers, the PCR-amplified fragment was digested with *Nco*I and *Xho*I. It was then cloned into pET-Blue vector (Novagen), an expression vector that incorporates a hexahistidine tag at the carboxyl terminal of the recombinant protein to facilitate purification. PrpB protein was expressed in host strain *E. coli* BL21 (DE3) pLysS. Expression was performed in 1 l Terrific broth (TB) medium, which was incubated at 310 K until OD_{600} reached about 0.6. The culture was induced by the addition of 0.3 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) for an additional 4 h at 303 K. For preparation of soluble protein fractions, cells from 1 l of culture were first pelleted and then resuspended in 25 ml of cold lysis buffer containing 50 m*M* Tris pH 8.0, 200 m*M* NaCl, 4% glycerol, 0.1% Triton X-100. It was then lysed by sonication on ice. The clear supernatant with the soluble proteins was collected by centrifugation. All the following purification steps were performed at 277 K.

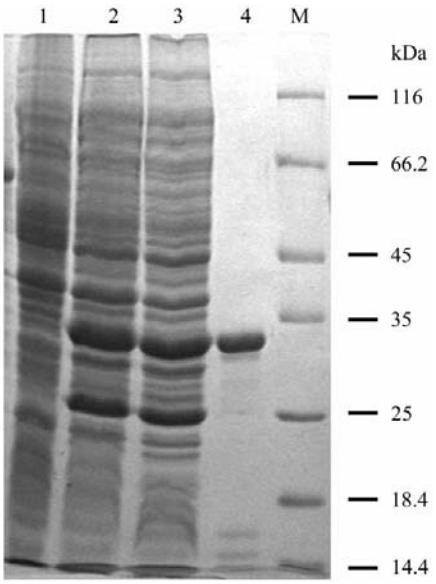

Figure 1
SDS–PAGE of PrpB during purification. Proteins were analysed on 12% SDS–PAGE stained with Coomassie blue. Lane 1, crude cell lysates before IPTG induction; lane 2, crude cell lysates after 0.3 m*M* IPTG induction; lane 3, clear supernatant; lane 4, purified PrpB after Ni–NTA affinity column chromatography; M, moleular-weight markers.

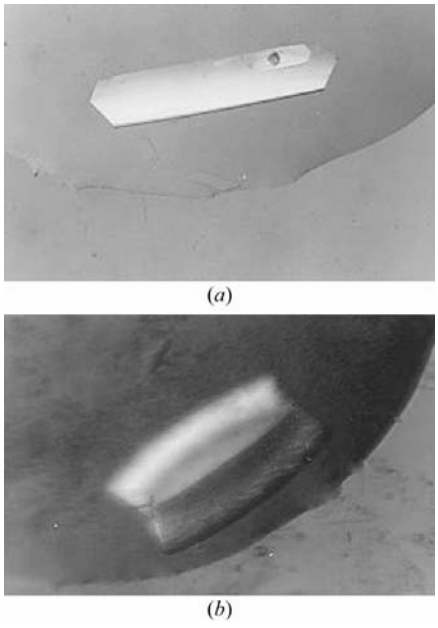

Figure 2
Two different forms of 2-metylisocitrate lyase crystals obtained from (*a*) condition 25 and (*b*) condition 34 of Crystal Screen I (Hampton Research).

2.2. Purification

PrpB with a C-terminal hexahistidine tag was purified using Ni^{2+}–NTA superflow (Qiagen). Once all unbound proteins were washed from the column using 50 m*M* Tris pH 8.0, 200 m*M* NaCl and 5 m*M* imidazole, PrpB protein was eluted from the column using 200 m*M* imidazole along with 50 m*M* Tris pH 8.0, 200 m*M* NaCl. For removing imidazole, protein was dialysed against 25 m*M* Tris pH 8.0, 100 m*M* NaCl, 2 m*M* EDTA and 0.1% Triton X-100 for 12 h and was then concentrated by several cycles of low-speed centrifugation using a 10 kDa molecular-weight cutoff Centricon (Amicon) until a final concentration of 20 mg ml^{-1}. Protein concentration was estimated by Lowry's method (Lowry *et al.*, 1951). Pure methylisocitrate lyase was obtained with a final yield of 35 mg per litre of cell culture. IPTG induction of cell culture as well as purification was monitored by SDS–PAGE (Fig. 1). The sequence identity of the *prpB* gene was determined by nucleotide sequencing and confirmed by comparing it with the *prpB* gene of *S. typhimurium* LT2.

2.3. Crystallization

Crystallization was carried out with the hanging-drop vapour-diffusion method using Crystal Screen I from Hampton Research (Jancarik & Kim, 1991). Droplets containing 4 µl of protein solution were mixed with 4 µl of crystallization solution. Small crystals were observed under eight different conditions. Good-quality crystals were obtained at 291 K in condition 17 (0.2 *M* lithium sulfate monohydrate, 0.1 *M* Tris pH 8.5 and 30% polyethylene glycol 4000), condition 25 (0.1 *M* imidazole pH 6.5 and 1.0 *M* sodium acetate trihydrate) and condition 34 (0.1 *M* sodium acetate trihydrate pH 4.6 and 2.0 *M* sodium formate) in Crystal Screen I (Fig. 2). These crystals appeared after 6 d of equilibration against the crystallization solution and grew to full

Table 1
Data-collection statistics.

Values in parentheses correspond to the last resolution shell (2.54–2.50 Å).

Resolution range (Å)	20.0–2.5
No. of reflections	400726
No. of unique reflections	46499
Completeness (%)	98.4 (98.1)
Multiplicity	4.5 (4.0)
Average $I/\sigma(I)$	11.7 (3.9)
R_{merge} (%)	9.1 (36.1)
$R_{measure}$ (%)	10.3 (41.6)

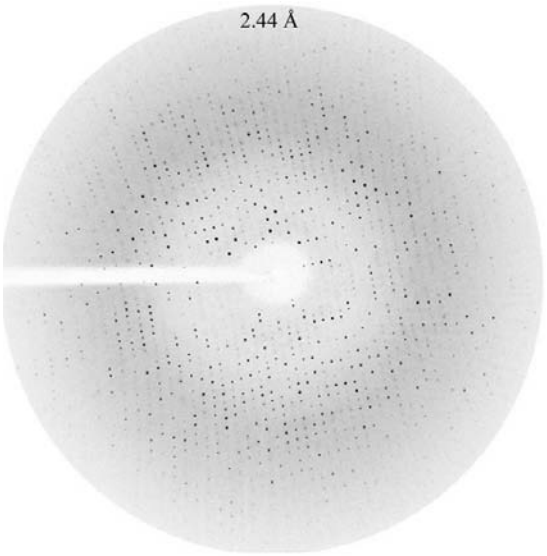

Figure 3
A typical 0.6° oscillation image obtained during data collection from a PrpB crystal.

size in 10 d. Complete diffraction data were collected using a single crystal obtained from condition 25.

3. Data collection

X-ray diffraction data were collected using a MAR research image-plate system (diameter 300 mm) with Osmic mirrors and a Rigaku RU-200 rotating-anode X-ray generator. A 200 µm focal cup was used and the crystal-to-detector distance was 200 mm. All frames were collected using a 0.6° oscillation angle, with an exposure time of 500 s per frame at 291 K. The data set from the native crystal revealed significant diffraction to 2.5 Å resolution (Fig. 3). The data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). A data-quality program (Diederichs & Karplus, 1997) was used to calculate data statistics. The final statistics for data collection and processing are summarized in Table 1.

4. Preliminary X-ray analysis

A complete diffraction data set to 2.5 Å resolution has been collected from the crystal. Systematic absences showed that the crystal belongs to the space group $P2_12_12_1$, with unit-cell parameters a = 63.600, b = 100.670, c = 204.745 Å. The value of the Matthews constant (Matthews, 1968) is 2.560 Å^3 Da^{-1} assuming four molecules of PrpB (molecular weight 32 kDa) in the asymmetric unit.

Rotation functions calculated in various resolution shells were consistent and suggested molecular twofold axes in the xz plane at 15° to the x and z axes. The third molecular twofold of the tetrameric molecule will then become submerged along b, which is a twofold symmetry axis of the rotation function. However, the xz plane is a mirror plane in the rotation function and hence prone to large statistical noise. Also, the two peaks observed in the xz plane are Klug peaks of each other. Therefore, the validity of the rotation function has to be established by additional examination.

2-Methylisocitrate lyase and phosphoenolpyruvate mutase belong to same protein family, contain the same number of amino acids (295) and are both tetrameric. Secondary-structure prediction by the *PSIPRED* suite (McGuffin *et al.*, 2000) showed exactly the same pattern of secondary structure as in the crystal structure of phosphoenolpyruvate mutase. However, attempts to determine the crystal strcture of 2-methylisocitrate lyase by the molecular-replacement method using phosphoenolpyruvate mutase as the search model were not successful, presumably because of the sequence identity of 24%. Further attempts to determine the crystal structure by multiple isomorphous replacement are also in progress. Towards this end, a data set from a gold derivative has been collected.

The intensity data were collected at the X-ray Facility for Structural Biology at the Indian Institute of Science, supported by the Department of Science and Technology (DST) and the Department of Biotechnology (DBT). DKS and PSS would like to thank CSIR, India for offering financial support.

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