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Preliminary X-ray crystallographic analysis of 2-methylcitrate synthase from *Salmonella typhimurium*

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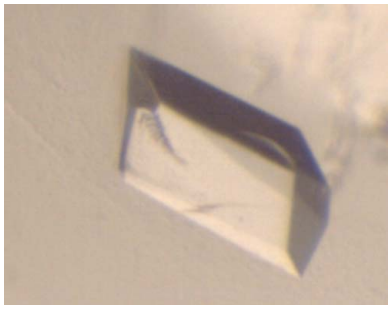
Analysis of the genomic sequences of *Escherichia coli* and *Salmonella typhimurium* has revealed the presence of several homologues of the well studied citrate synthase (CS). One of these homologues has been shown to code for 2-methylcitrate synthase (2-MCS) activity. 2-MCS catalyzes one of the steps in the 2-methylcitric acid cycle found in these organisms for the degradation of propionate to pyruvate and succinate. In the present work, the gene coding for 2-MCS from *S. typhimurium* (*St*PrpC) was cloned in pRSET-C vector and overexpressed in *E. coli*. The protein was purified to homogeneity using Ni–NTA affinity chromatography. The purified protein was crystallized using the microbatch-under-oil method. The *St*PrpC crystals diffracted X-rays to 2.4 Å resolution and belonged to the triclinic space group *P*1, with unit-cell parameters a = 92.068, b = 118.159, c = 120.659 Å, α = 60.84, β = 67.77, γ = 81.92°. Computation of rotation functions using the X-ray diffraction data shows that the protein is likely to be a decamer of identical subunits, unlike CSs, which are dimers or hexamers.

1. Introduction

Short-chain fatty acids (SCFAs), such as acetate and propionate, are byproducts of bacterial fermentation and are in turn inhibitors of bacterial growth (Cherrington *et al.*, 1991). Enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* are exposed to high concentrations of propionate in the gastrointestinal tracts of mammals (Cummings *et al.*, 1987). In chicken and mice it has been shown that the high concentration of SCFAs in the intestinal tract can greatly increase resistance to *Salmonella* infections (Barnes *et al.*, 1979; Bohnhoff & Miller, 1962). However, many aerobic bacteria and fungi, as well as some anaerobes, can utilize SCFAs as a source of carbon and energy (Callely & Lloyd, 1964*a*,*b*). This could serve as a defence mechanism in these organisms against the negative effects of SCFAs. Studies of propionic acid metabolism in various organisms have revealed at least seven different pathways (Textor *et al.*, 1997). One of these pathways is coded by the *prp* locus in *S. typhimurium* LT2 (Hammelman *et al.*, 1996). Nucleotide sequencing of the *prp* locus revealed two operons organized as *prpR* and *prpBCDE* which are transcribed in opposite directions (Horswill & Escalante-Semerena, 1997). *prpR* codes for a protein belonging to the σ^{54} transcriptional activator, while *prpB*, *prpC*, *prpD* and *prpE* code for 2-methylisocitrate lyase, 2-methylcitrate synthase (2-MCS), 2-methylcitrate dehydratase and propionyl-CoA synthetase, respectively (Horswill & Escalante-Semerena, 1997, 2001). Oxidation of propionate closely parallels the conversion of acetate to glyoxalate in the glyoxylate cycle. The pathway begins with the synthesis of propionyl-CoA from propionate and CoA catalyzed by PrpE. The second reaction of the pathway, which involves the condensation of propionyl-CoA and oxaloacetate to form 2-methylcitrate and CoA, is catalyzed by PrpC. This reaction is followed by the conversion of 2-methylcitrate to 2-



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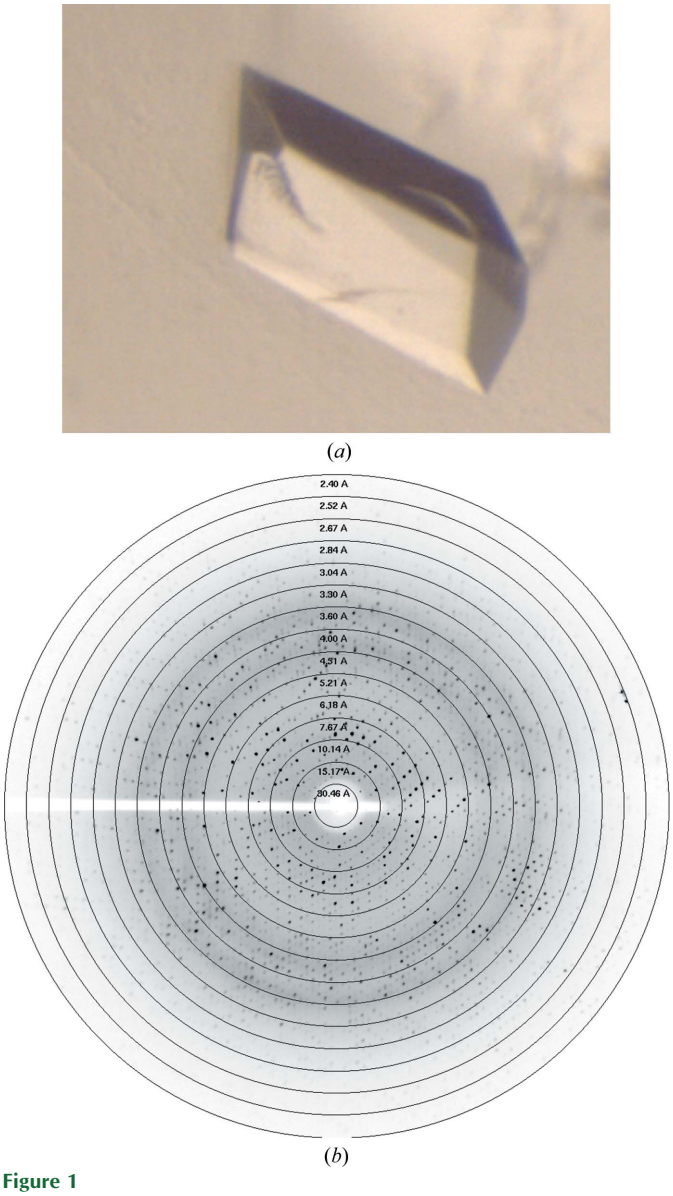
methyl-*cis*-aconitate catalyzed by PrpD. 2-Methyl-*cis*-aconitate is further converted to 2-methylisocitrate by aconitase (Horswill & Escalante-Semerena, 2001). The final step of the pathway, which involves the cleavage of 2-methylisocitrate to succinate and pyruvate, is catalyzed by PrpB. Cells that catabolize propionate *via* the 2-methylcitric acid cycle are at risk because 2-methylcitrate, or a derivative of it, is a potent inhibitor of cell growth (Beach *et al.*, 1977; Cheema-Dhadli *et al.*, 1975; Horswill *et al.*, 2001). *Salmonella* may also be under evolutionary pressure to maintain a low level of propionyl-CoA (Horswill *et al.*, 2001; Munoz-Elias *et al.*, 2006; Upton & McKinney, 2007). The tightly regulated 2-methylcitrate pathway appears to be important for detoxification and as a source of carbon and energy provided by the degradation of propionate to pyruvate and succinate.

Structural and biochemical studies of enzymes involved in the 2-methylcitrate cycle have been initiated in the last decade. These efforts have led to the functional characterization of all of the Prp enzymes and to the determination of the three-dimensional structures of PrpB from *E. coli* (Grimm *et al.*, 2003) and *S. typhimurium* (Simanshu *et al.*, 2003) and of PrpD from *E. coli* (PDB code 1szq; K. R. Rajashankar, R. Kniewel, V. Solorzano & C. D. Lima, unpublished work). In contrast, PrpC, PrpE and PrpR have not been structurally investigated. In this manuscript, we present the initial characterization of PrpC/2-MCS from *S. typhimurium* (*St*PrpC).



(*a*)

(*b*)

Figure 1
(*a*) The *St*PrpC crystal used for three-dimensional X-ray diffraction data collection. (*b*) A typical X-ray diffraction image of an *St*PrpC crystal.

2. Material and methods

2.1. Cloning, expression and purification of PrpC

The 1167 bp ORF encoding 2-MCS was PCR-amplified from *S. enterica* serovar Typhimurium strain IFO12529 genomic DNA template using high-fidelity KOD HiFi DNA polymerase (Novagen). The PCR-amplified fragment was cloned into pRSET-C vector (Invitrogen). The final plasmid construct encodes *St*PrpC with 15 additional amino acids (MRGSHHHHHHGMASH–), including a hexahistidine tag, at the N-terminus. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) pLysS competent cells. The transformed cells were inoculated into 1 l Terrific Broth (Himedia) containing 2 ml glycerol and allowed to grow at 310 K until the OD at 600 nm reached 0.6. Protein expression was then induced for 6 h by the addition of 0.3 m*M* isopropyl β-D-1-thiogalactopyranoside. The expressed protein was purified by Ni–NTA affinity chromatography and dialysed for 24 h against 25 m*M* Tris pH 8.0 containing 100 m*M* NaCl. The purity of the enzyme was examined by 12% SDS–PAGE.

2.2. Crystallization and preliminary X-ray diffraction studies

Initial crystallization experiments on native *St*PrpC were carried out at 297 K in 72-well plates (Greiner Bio-One) using the microbatch-under-oil crystallization method with a 1:1 mixture of paraffin and silicon oils (Hampton Research) as well as by the hanging-drop vapour-diffusion method using multicavity trays (Laxbro). Crystallization drops consisted of 3 µl 10 mg ml^{-1} protein solution and 2 µl crystallization cocktail. Crystallization screening was initially carried out using Crystal Screens I and II, Index Screen and Salt Rx from Hampton Research as well as in-house combinatorial matrices. The conditions that gave small crystals were further optimized by employing finer pH intervals and various additives and precipitant concentrations.

For X-ray diffraction experiments, a crystal was picked up from the crystallization drop using a nylon loop, briefly exposed to a drop containing crystallization buffer with 20%(*w*/*v*) glycerol as a cryoprotectant and flash-frozen using a nitrogen-gas stream at 100 K. A complete three-dimensional X-ray diffraction data set was collected at a wavelength of 1.5418 Å on a Bruker Microstar Ultra rotating-anode X-ray generator equipped with a 300 µm focal cup and operating at 50 kV and 100 mA. The exposure time and crystal oscillation angles were set to 15 min and 1.0°, respectively. The crystal-to-detector distance was maintained at 225 mm. The crystal diffracted X-rays to 2.4 Å resolution. A total of 197 images were recorded using a MAR345 image-plate detector. Data sets were processed and scaled using the programs *DENZO* and *SCALEPACK*, respectively, from the *HKL*-2000 suite (Otwinowski & Minor, 1997) and were further analyzed with programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Self-rotation functions were computed using the program *GLRF* (Tong & Rossmann, 1997).

3. Results and discussion

S. typhimurium 2-methylcitrate synthase (*St*PrpC; NCBI reference NP_459364.1) was cloned in the pRSETC vector (Invitrogen) with an N-terminal hexahistidine tag and the protein was purified using Ni–NTA affinity column chromatography. Crystals of *St*PrpC (~0.60 × 0.30 × 0.25 mm) that were suitable for structural studies (Fig. 1*a*) were obtained in 0.1 *M* Bicine pH 9.0, 18%(*w*/*v*) PEG 8000, 1.4 *M* ammonium sulfate and 0.2 *M* trisodium citrate after 15 d using the microbatch-under-oil crystallization method and diffracted X-rays to 2.4 Å resolution (Fig. 1*b*). The X-ray diffraction data collected from a single crystal were processed using *DENZO* and scaled using *SCALEPACK* from the *HKL*-2000 suite (Otwinowski & Minor, 1997). The crystal belonged to the triclinic space group *P*1, with unit-cell parameters a = 92.068, b = 118.159, c = 120.659 Å, α = 60.84, β = 67.77, γ = 81.92°. The data-collection and processing statistics are summarized in Table 1.

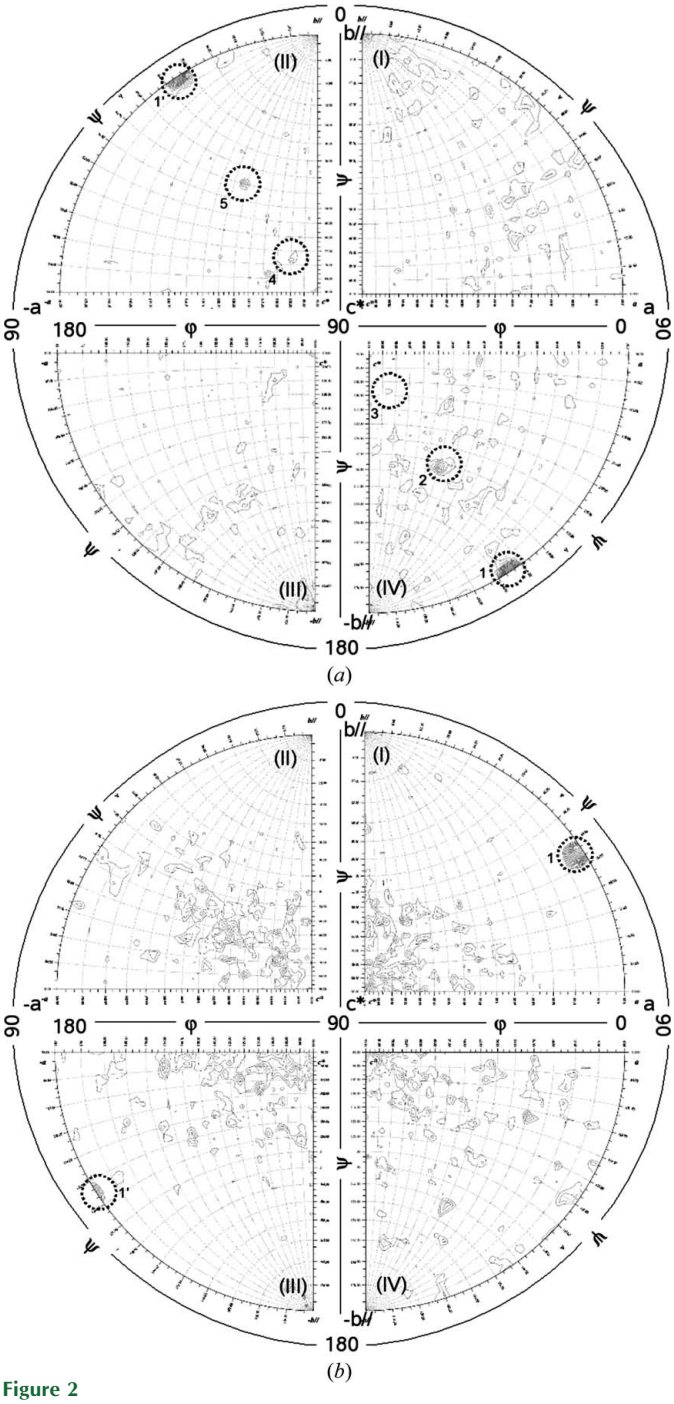


Figure 2
Self-rotation functions representing (*a*) κ = 180° and (*b*) κ = 72° hemispheres calculated for *St*PrpC using the *GLRF* program. Data between 10 and 5.5 Å resolution for κ = 180° and between 10 and 3.5 Å resolution for κ = 72° were used for the rotation-function calculation with an integration radius of 70 Å. Contours start at 1σ above the mean value and are drawn at intervals of 1σ and 0.5σ for the κ = 180° and κ = 72° hemispheres, respectively.

Table 1
Crystallization, data-collection and processing statistics for the *St*PrpC crystal.

Values in parentheses are for the outer shell. Standard definitions (Drenth, 1994) were used for all parameters. Data-reduction statistics are from *SCALEPACK*.

Crystallization condition	0.1 *M* Bicine pH 9.0, 18% PEG 4000, 1.4 *M* ammonium sulfate, 0.2 *M* trisodium citrate
Wavelength (Å)	1.54
Temperature (K)	100
Resolution range (Å)	50.0–2.40 (2.49–2.40)
Space group	*P*1
Unit-cell parameters (Å, °)	a = 92.068, b = 118.159, c = 120.659, α = 60.84, β = 67.77, γ = 81.92
Observed reflections	909949
Unique reflections	160794
Data completeness (%)	90.2 (72.5)
Multiplicity	1.7
$\langle I/\sigma(I)\rangle$	22.17 (4.18)
$R_{\rm merge}$† (%)	9.2 (19.4)
Matthews coefficient (Å^3 Da^{-1})	2.52
Solvent content (%)	51.27
Protomers in the asymmetric unit	10

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is its mean value.

Calculation of the Matthews coefficient ($V_{\rm M}$; Matthews, 1968) using the *CCP*4*i* interface (Collaborative Computational Project, Number 4, 1994) suggested that the crystal unit cell (which is also the asymmetric unit in *P*1) probably contains 8–12 2-MCS molecules ($V_{\rm M}$ = 2.94 Å^3 Da^{-1} with a solvent content of 58.2% for eight molecules in the asymmetric unit and $V_{\rm M}$ = 1.96 Å^3 Da^{-1} with a solvent content of 37.3% for 12 molecules in the asymmetric unit). This implies the presence of several dimers or a higher oligomer in the unit cell. To examine the plausible oligomeric state, self-rotation functions were calculated using *GLRF* (Tong & Rossmann, 1997) for κ = 180°, 120°, 90°, 72° and 60° hemispheres corresponding to twofold, threefold, fourfold, fivefold and sixfold noncrystallographic symmetry, respectively. Strong peaks on the κ = 180° and κ = 72° hemispheres suggested the presence of noncrystallographic twofolds and fivefolds, respectively (Figs. 2*a* and 2*b*). The fivefold peak (φ = 1°, ψ = 58°) was found to be perpendicular to the peaks corresponding to five twofolds with (φ, ψ) values of (0°, 147°), (55°, 133°), (82°, 105°), (100°, 76°) and (128°, 48°) (labelled 1–5, respectively, in Fig. 2*a*). These results suggest that *St*PrpC may be decameric, with 52 symmetry in the crystals. However, it is also possible that the asymmetric unit of the crystal consists of two pentameric units related by a twofold axis.

Crystal structures of CS have been determined from several sources (Remington *et al.*, 1982; Remington, 1992; Russell *et al.*, 1997, 1998). Eukarya, Gram-positive eubacteria and archaea possess a homodimeric form of the enzyme (Bell *et al.*, 2002; Boutz *et al.*, 2007; Russell *et al.*, 1997, 1998; Wiegand *et al.*, 1984), whereas in the majority of Gram-negative eubacteria CS is a homohexamer (Francois *et al.*, 2006; Nguyen *et al.*, 2001). Some of these hexameric CS enzymes have been shown to be allosterically regulated by NADH (Francois *et al.*, 2006; Nguyen *et al.*, 2001). The structure of *St*PrpC will provide the framework essential for understanding the inter-

actions that lead to decamer formation and the possible significance of the oligomeric state for the enzymatic properties. Attempts to obtain initial phases for the *St*PrpC crystal using molecular-replacement methods are in progress.

The intensity data were collected using the X-ray Facility for Structural Biology at the Molecular Biophysics Unit, Indian Institute of Science (IISc), supported by the Department of Science and Technology (DST) and the Department of Biotechnology (DBT). We thank the staff of the X-ray laboratory for their cooperation during the course of the investigations. MRNM and HSS thank the DST and the DBT of the Government of India for financial support. SC acknowledges the Council for Scientific and Industrial Research, Government of India for the award of a Research Fellowship.

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