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Expression, purification, crystallization and preliminary X-ray analysis of phosphotransacetylase from *Methanosarcina thermophila*

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Phosphotransacetylase (Pta) from the anaerobic archaeon *Methanosarcina thermophila* has been heterologously expressed in a soluble form which facilitated crystallization using the hanging-drop vapor-diffusion method with ammonium sulfate as a precipitant. This is the first report of the crystallization of any Pta. While the *M. thermophila* Pta has high sequence identity to Ptas from other organisms, it has no homology to any previously crystallized proteins. The protein crystallized in space group $I4_1$, with unit-cell parameters $a = b = 114.8$, $c = 127.8$ Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals diffracted to 2.5 Å resolution using Cu $K\alpha$ radiation. The enzyme had previously been reported to exist as a monomer; however, the self-rotation function showed the presence of a non-crystallographic symmetry axis at $\psi = 90$, $\varphi = 90$, $\kappa = 180^\circ$, suggesting oligomerization. Dynamic light-scattering analysis supported a dimeric state for Pta in solution.

1. Introduction

Phosphotransacetylase (Pta; EC 2.3.1.8) catalyzes the reversible transfer of the acetyl group from acetyl phosphate to coenzyme A (CoA): ($CH_3COOPO_3^{2-}$ + CoASH $\longleftrightarrow$ $CH_3COSCoA$ + HPO_4^{2-}). In the archaeon *Methanosarcina thermophila*, acetate kinase (CH_3COO^- + ATP $\longleftrightarrow$ $CH_3COOPO_3^{2-}$ + ADP) and Pta activate acetate to acetyl-CoA for conversion to methane and carbon dioxide. Pta and acetate kinase are present in all fermentative anaerobic prokaryotes, where together the enzymes play a major role in ATP synthesis by converting acetyl-CoA to the end-product acetate. Many prokaryotes also employ this enzyme pair to convert acetate to acetyl-CoA for incorporation into the cellular carbon or oxidation through the TCA cycle. In spite of the wide distribution and importance of Pta among prokaryotes (Drake *et al.*, 1981; Kenealy & Zeikus, 1982; Lundie & Ferry, 1989; Shimizu *et al.*, 1969; Whiteley & Pelroy, 1972), few biochemical studies have been reported for any Pta and no structure is available. The most studied Pta is that from *M. thermophila*, from which several essential residues have been identified (Iyer & Ferry, 2001; Rasche *et al.*, 1997). The *M. thermophila* Pta shares high sequence identity with all other Ptas reported from both the bacterial and the archaeal domains; as such, the solution of the *M. thermophila* enzyme structure will provide novel insights into the mechanism applicable to all Ptas.

2. Materials and methods

2.1. Expression and purification of wild-type Pta

Modifications to a previous heterologous expression and purification protocol (Latimer & Ferry, 1993) yielded a soluble form of Pta that was amenable to crystallization. *Escherichia coli* BL21-DE3 cells transformed with the plasmid *pML702* (Latimer & Ferry, 1993) were grown to an OD_{600} of 0.6 at 310 K with shaking in LB medium containing 0.1 mg ml^{-1} ampicillin. The temperature was reduced to 288 K and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 m*M*, after which incubation was continued for 12–16 h. Cells were harvested by centrifugation, resuspended in a volume of 25 m*M* Tris–HCl pH 7.2 containing 180 m*M* KCl sufficient to yield 1 g of cells per millilitre and disrupted by passage twice through a French pressure cell (277 K, 138 MPa). The lysate was clarified by centrifugation (75 000*g*, 2 h), brought to 45% $(NH_4)_2SO_4$ saturation by the slow addition of 100% saturated $(NH_4)_2SO_4$ and stirred overnight at 277 K. The solution was centrifuged (75 000*g*, 2 h) and the supernatant applied to a butyl Sepharose column (Amersham Pharmacia) equilibrated with 25 m*M* Tris pH 7.2 containing 1.4 *M* $(NH_4)_2SO_4$. Pta eluted at 600 m*M* $(NH_4)_2SO_4$ using a descending linear gradient of 1.4 *M* to 300 m*M* $(NH_4)_2SO_4$. The fractions containing peak Pta activity were pooled and dialyzed overnight against 4 l 50 m*M* Tris–HCl pH 7.2.

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The dialyzed solution was concentrated to 50 ml and applied to a Mono-Q anion-exchange column (Amersham Pharmacia) equilibrated with 50 m*M* Tris–HCl pH 7.2. Pta eluted at 200 m*M* KCl using an ascending linear gradient of 0–500 m*M* KCl. The preparation was homogeneous as judged by SDS–PAGE. Protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

2.2. Dynamic light scattering

Dynamic light scattering (DLS) was performed using a DynaPro-MS800 molecular-sizing instrument (Protein Solutions, Lakewood, NJ, USA). A 40 µl aliquot of Pta (2.5 mg ml^{-1}) in 25 m*M* Tris–HCl pH 7.2 containing 180 m*M* KCl was centrifuged (10 000*g*, 10 min). For DLS analysis, the samples were loaded into a 12 µl quartz cuvette and the hydrodynamic radius, molecular weight and size distribution were determined by the mean of at least ten DLS measurements. Data analysis was performed using *Dynamics* 5.0 (Protein Solutions, Lakewood, NJ, USA).

2.3. Crystallization of wild-type Pta

M. thermophila Pta purified from *E. coli* was concentrated to 5 mg ml^{-1}. Glycerol was added to a final concentration of 10% and the mixture was frozen in liquid N_2 and stored at 193 K until use. Before crystallization, glycerol was removed from the thawed protein by dialysis against at least 500 volumes of 50 m*M* Tris–HCl pH 7.5 containing 2 m*M* dithiothreitol. The protein concentration was measured again and 2–5 mg ml^{-1} protein was used for crystallization trials using the hanging-drop vapor-diffusion method (Blundell, 1976). Falcon tissue-culture plates containing 24 wells (Becton-Dickinson, NJ, USA) were used with mineral oil or petroleum jelly applied to the well rims to form a seal. Glass cover slips were siliconized with Sigmacote (Sigma-Aldrich Co, St Louis, MO, USA) and rinsed with chloroform prior to placement of the protein drop. Each well contained 0.5–1.0 ml of precipitant solution and the drop contained 5–10 µl of protein (25–50 µg) plus 5–10 µl of the precipitant solution. The time allowed for crystallization was 1–3 weeks at 294 or 277 K.

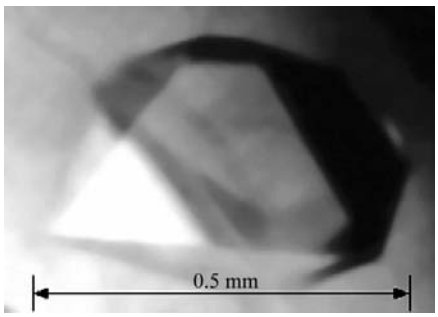

Figure 1
Wild-type Pta crystallized against 0.1 *M* sodium acetate pH 5.0, 1.2 *M* ammonium sulfate, 2.5 m*M* DTT.

Initial crystallization-condition screening was carried out using Crystal Screen, a commercially available sparse-matrix screen (Hampton Research, Laguna Niguel, CA, USA). The optimized crystallization conditions were as follows: frozen protein was thawed and dialyzed against 500 volumes of 0.1 *M* sodium acetate buffer pH 5.0 containing 2 m*M* dithiothreitol and the dialyzed protein was filtered through a 0.1 µm filter. Crystals were grown by the hanging-drop vapor-diffusion method as described above; the well contained 0.1 *M* sodium acetate pH 5.0, 2.5 *M* ammonium sulfate and 5 m*M* dithiothreitol in a final volume of 0.5 or 1.0 ml and the drop contained 5 µl of Pta (25 µg) and 5 µl of the precipitant solution. Crystal growth was performed at 294 K for 15 d.

2.4. Collection and processing of of X-ray diffraction data from crystals of wild-type Pta

Crystals grown as described above were removed from the mother liquor and placed in a drop of Fomblin oil (Sigma, St Louis, MO, USA). The crystal was then mounted in a nylon loop and placed in a stream of cooled N_2. X-ray diffraction data were collected on a Rigaku R-AXIS IV++ imaging-plate system using Cu $K\alpha$ radiation from a Rigaku RU-200 rotating-anode generator (50 kV, 100 mA) collimated and monochromated by Osmic mirrors. The crystal-to-detector distance was 150 mm and 5 min frames were collected at 0.5° oscillations, collecting 118 frames to give 98.6% complete diffraction data. The images were processed using the *HKL* program suite, including *XdisplayF*, *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

2.5. Expression and purification of selenomethionyl (SeMet) Pta

E. coli BL21(DE3) containing *pML*702 (Latimer & Ferry, 1993) was grown to an OD_{600} of 0.8 at 310 K with shaking in M9 minimal media (Wang & Koch, 1978) containing 0.1 mg ml^{-1} ampicillin. Methionine biosynthesis was inhibited as previously described (Van Duyne *et al.*, 1993); selenomethionine was added to a final concentration of 50 mg l^{-1} and the temperature was reduced to 288 K. IPTG was added to a final concentration of 1 m*M* and incubation was continued for 24 h. The SeMet-Pta was purified using the procedure described above and showed identical chromatographic properties to wild-type Pta, with the exception of retention on the butyl Sepharose column (it eluted at 450 m*M* KCl compared with 600 m*M* KCl for the wild type).

3. Results and discussion

3.1. Expression and purification of wild-type Pta

Previous expression protocols resulted in the formation of inclusion bodies which required the use of 9 *M* urea to unfold the protein and an *in vitro* refolding prior to purification (Latimer & Ferry, 1993). The resulting Pta was variable in yield and activity and was not reproducibly amenable to crystallization. The appearance of multiple peaks during chromatography suggested heterogeneity, possibly arising from protein misfolding *in vitro*. DLS analyses also indicated heterogeneity and formation of aggregates (data not shown). The improved expression and purification protocol described here resulted in soluble protein, eliminating the need for denaturation of inclusion bodies and protein refolding. This new protocol also resulted in an approximately twofold greater yield of active enzyme (Latimer & Ferry, 1993). Pta preparations consistently had a k_{cat} of at least 4500 s^{-1}, compared with the value of 3226 s^{-1} reported by Lundie & Ferry (1989), and diffraction-quality crystals have been grown from different preparations.

3.2. Dynamic light scattering

DLS analyses were performed in triplicate, with each analysis incorporating the mean of at least ten DLS readings. Pta was found to have a hydrodynamic radius of 3.67 ± 0.06 nm, corresponding to a molecular weight of 71.3 ± 2.5 kDa, which is twice the calculated molecular weight (Latimer & Ferry, 1993). Each sample was found to be monodisperse, with Pta representing over 99.5% of the mass.

3.3. Crystallization of wild-type Pta

We report here the first crystallization of Pta, a key enzyme in prokaryotic metabo-

Table 1
Crystallographic parameters and data-collection statistics.

Wavelength (Å)	1.54
Space group	$I4_1$
Unit-cell parameters (Å, °)	$a = b = 114.8$, $c = 127.8$, $\alpha = \beta = \gamma = 90$
Solvent content (%)	57
Matthews coefficient (Å^3 Da^{-1})	2.87
Resolution range (Å)	
Overall	19.94–2.5
Lowest shell	19.94–5.35
Highest shell	2.59–2.5
No. of observations	69446
No. of unique reflections	28153
Completeness (%)	
Overall	98.6
Lowest shell	94.6
Highest shell	99.5
$R_{\rm merge}$ (%)	
Overall	6.9
Lowest shell	4.0
Highest shell	27.6
Average $I/\sigma(I)$	
Overall	7.1
Lowest shell	15.3
Highest shell	2.0
Redundancy	2.46
Crystal mosaicity	0.430

lism. The most important factor affecting the morphology of Pta crystals was the pH of the mother liquor. Crystals measuring 0.2–0.4 mm in each dimension (Fig. 1) were observed in drops containing ammonium sulfate at pH values between 5.0 and 5.2. These crystals appeared between 4 and 5 d and reached their limiting size between 15 and 20 d. Increasing the pH to values between 5.5 and 6.5 produced larger crystals (0.4–0.8 mm in each dimension) that appeared between 2 and 3 d and grew to full size within 4–5 d; however, these crystals had surface deformities (Fig. 2*a*). Within another 5–7 d, these crystals disintegrated (Fig. 2*b*). Mother liquor at pH values between 7 and 8 produced long needle-like crystals (1 × 0.05 × 0.5 mm) within 5–7 d. Thus, crystals used in all the diffraction experiments were grown from mother liquor containing 25 µg Pta, 50 m*M* sodium acetate pH 5.0, 1.1–1.25 *M* ammonium sulfate and 2.5 m*M* dithiothreitol equilibrated against a precipitant solution containing 0.1 *M* sodium acetate pH 5.0, 2.2–2.5 *M* ammonium sulfate and 5 m*M* dithiothreitol in a volume of 0.5 ml.

3.4. Collection and processing of of X-ray diffraction data from crystals of wild-type Pta

Pta crystals diffracted X-rays to a resolution of 2.5 Å with the in-house X-ray source (Table 1). Diffraction data were collected to 99% completion and then scaled and merged using the *HKL* suite of programs (Otwinowski & Minor, 1997). Pta crystals belong to the tetragonal space group $I4_1$ and have unit-cell parameters $a = b = 114.8$, $c = 127.8$ Å, $\alpha = \beta = \gamma = 90°$. Assuming that each asymmetric unit of the crystal lattice contains a dimer of Pta, the Matthews $V_{\rm M}$ was calculated to be 2.87 Å^3 Da^{-1}, implying that the crystal contains 57% solvent.

3.5. Self-rotation function

A self-rotation function was calculated for the native data set using the program *GLRF* from the *REPLACE* suite of programs (Tong & Rossmann, 1997). The $\kappa = 180°$ section showed a 5.8σ peak at $\psi = 90, \varphi = 90°$, indicating the presence of a twofold non-crystallographic symmetry axis at this point (Fig. 3). Data from 10 to 4 Å resolution and with an integration radius of 15 Å were used for calculations. The *M. thermophila* Pta had previously been reported as monomeric as determined by gel filtration (Lundie & Ferry, 1989); however, considering the axis of non-crystallographic symmetry in conjunction with the DLS data, a dimeric state for Pta is more likely.

3.6. Expression and purification of selenomethionyl (SeMet) Pta

Previous attempts to purify SeMet-Pta from inclusion bodies by solubilization with 9 *M* urea produced an inactive enzyme that did not crystallize. However, the improved expression and purification protocol reported here for wild-type Pta yielded an SeMet-Pta which retains approximately 50% wild-type activity. Additionally, the SeMet-Pta is homogeneous as judged by SDS–PAGE and retains a dimeric state as determined by size-exclusion chromatography. Crystallization trials for the SeMet-Pta are currently in progress. No crystal structure is available for any protein bearing significant sequence homology to Pta; therefore, the structure cannot be solved using molecular replacement. It is expected that the X-ray diffraction patterns of SeMet-Pta crystals will provide the necessary phase information to solve the crystal structure of *M. thermophila* Pta.

4. Conclusion

The crystallization of *M. thermophila* Pta described in this paper is the first reported for Pta from any organism; hence, the solution of the structure of *M. thermophila* Pta will, for the first time, allow an examination of the architecture of this widely distributed enzyme. Information from the resultant structure will be combined with kinetic and mechanistic investigations in order to develop a clear picture of how the enzyme catalyzes its reaction. Because of the high sequence identity among Ptas from diverse microbes, information learned from the *M. thermophila* Pta is likely to apply to Ptas from all other organisms. Furthermore, no previously identified fold motifs are observed in the sequence of Pta, raising the

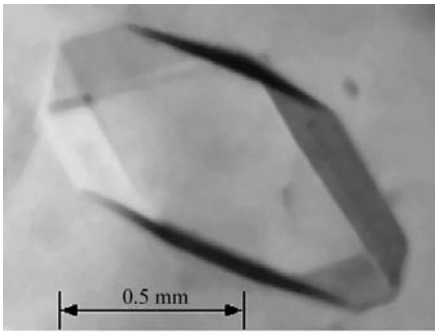

Figure 2
Native Pta crystallized against 0.1 *M* MES pH 6.0, 1.2 *M* ammonium sulfate and 2.5 m*M* DTT. (*a*) A 3 d old crystal, (*b*) a 9 d old crystal.

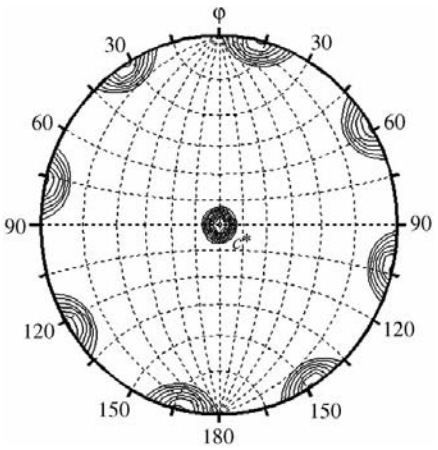

Figure 3
Stereographic projection of the $\kappa = 180°$ section of the self-rotation function. Data from 12.0 to 6.0 Å collected at 100 K were used to calculate the self-rotation function. The integration radius was 15 Å. ψ (the angle from the pole) is 0 or 180° at the center and 90° at the edges. φ (the angle around the equator) is marked on the periphery.

possibility that the crystal structure of this enzyme may reveal a novel motif.

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