Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction analysis of malic enzyme from pigeon liver

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Received 10 June 1999
Accepted 9 August 1999

Recombinant pigeon-liver malic enzyme was expressed in *Escherichia coli* and purified to homogeneity. Two different crystal forms were grown by the hanging-drop vapour-diffusion method. Both types of crystals belong to the tetragonal space group $P4_222$, with unit-cell dimensions $a = b = 163.8$, $c = 174.3$ Å for the octahedral crystals and $a = b = 124.5$, $c = 179.2$ Å for the rod-like crystals. X-ray diffraction data were collected at 100 K using a synchrotron-radiation X-ray source. The Matthews parameter suggests that there are four and two molecules per asymmetric unit for the larger and the smaller tetragonal unit cells, respectively.

1. Introduction

Cytosolic pigeon-liver malic enzyme (E.C. 1.1.1.40) catalyzes the divalent metal ion dependent oxidative decarboxylation of L-malate to yield pyruvate and CO_2 with concomitant reduction of $NADP^+$ to NADPH,

$$\text{L-malate} + \text{NADP} \overset{Mg^{2+}\text{ or }Mn^{2+}}{\rightleftharpoons} \text{pyruvate} + CO_2 + \text{NADPH} + H^+.$$

The malic enzyme was first discovered in pigeon liver in 1947 (Ochoa *et al.*, 1947). Since then, numerous malic enzymes have been found in various organisms such as bacteria (Kobayashi *et al.*, 1989), plants (Rothermel & Nelson, 1989; Westhoff & Borsch, 1990) and higher animals (Bagchi *et al.*, 1987; Hsu *et al.*, 1992; Loeber *et al.*, 1991). In cytosol, the major physiological function of the malic enzyme is to provide NADPH for the *de novo* biosynthesis of long-chain fatty acids (Goodridge *et al.*, 1989).

Pigeon-liver malic enzyme contains 557 amino-acid residues and exists primarily as a tetramer (Chang, Huang, Lee *et al.*, 1994). It can be dissociated to dimers and monomers by lowering the pH or temperature (Huang & Chang, 1992). The quaternary structure of the tetrameric malic enzyme is probably asymmetric and exists as a dimer of dimers based on crosslinking (Chang, Huang, Huang *et al.*, 1994), chemical modification (Reddy, 1983) and kinetic studies (Hsu, 1982; Lee & Chang, 1990). Several acidic residues, including Asp141, Asp258, Asp194 and Asp464, have been identified as the coordination sites for the metal binding of malic enzyme by metal-catalyzed affinity cleavage (Chou *et al.*, 1995; Wei *et al.*, 1994). Mutational studies further demonstrated that the N terminus of the enzyme is located close to substrate- and metal-binding sites and is also near the subunit interface (Chou *et al.*, 1997, 1998).

Crystallization of pigeon-liver malic enzyme was first reported in 1967 (Hsu & Lardy, 1967). Rat liver (Baker *et al.*, 1987) and parasitic nematode *Ascaris suum* (Clancy *et al.*, 1992) malic enzymes were also crystallized and their crystals diffracted X-rays to 2.5 and 3.0 Å resolution, respectively. However, the three-dimensional crystal structure of malic enzyme has not yet been reported. The only three-dimensional structural information reported to date was obtained from electron microscopy and showed that the enzyme has a square structure with dimensions of $48 \times 54 \times 70$ Å for each subunit in the tetramer (Nevaldine *et al.*, 1974). The sequence alignment suggests that the malic enzyme contains the ADP-$\beta\alpha\beta$-binding motif (Chou *et al.*, 1994; Wierenga *et al.*, 1985). With this limited structural information, it is difficult to elucidate the detailed enzymatic mechanism and subunit interactions of the malic enzyme. Here, we report the crystallization and characterization of two crystal forms of the pigeon-liver malic enzyme which can be used in further crystallographic analysis.

2. Materials and methods

2.1. Expression and purification of pigeon-liver malic enzyme

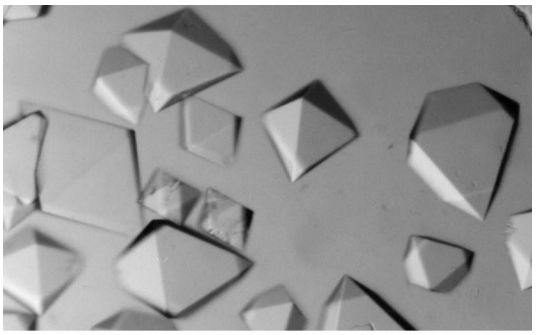
The full-length pigeon-liver malic enzyme cDNA was inserted into the pET-21b vector (Novagen) as described previously (Chou *et al.*, 1997). The cDNA was examined by dideoxy chain-termination sequencing to exclude any unexpected mutations resulting from the PCR. BL21 *Escherichia coli* cells transformed with the plasmid were grown in LB medium supplemented with 50 µl ml^{-1} ampicillin at

Table 1
Crystallographic data statistics for pigeon-liver malic enzyme.

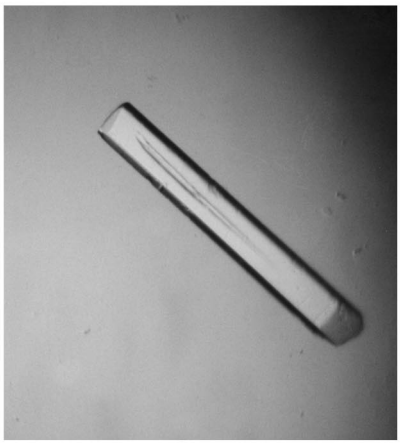
	Octahedral crystals	Rod-like crystals
Space group	$P4_222$	$P4_222$
Unit-cell dimensions (Å)		
a, b	163.8	124.5
c	174.3	179.2
Resolution (Å)	4.0	2.9
Number of total observations	48267	213959
Number of unique reflections	16380	31676
Completeness for all data (%)	80.0	99.7
$R_{\rm merge}$† (%)	8.7	12.1

† $R_{\rm merge} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$, where $\langle I_h \rangle$ is the mean intensity of i observations for a given reflection h.

310 K to an absorbance at 600 nm of 0.6. Enzyme synthesis was then induced by addition of 1 m*M* isopropyl *β*-D-thiogalactopyranoside (ITPG). After an additional 24 h incubation, the cells were harvested by centrifugation for 15 min at 5000*g*. Cells were resuspended and sonicated in 25 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA. The recombinant proteins were purified using the modified protocol previously described (Chang *et al.*, 1991). The sonicated cell extract was loaded on Q-Sepharose and eluted stepwise with 30 m*M* magnesium acetate. The fractions containing malic enzyme activity were then loaded onto adenosine-2′,5′-bisphosphate agarose and eluted using 230 µ*M* $NADP^+$. Mass spectroscopy indicated a molecular mass of 62 053 ± 10 Da for the purified malic enzyme, compared with the calculated value of 62 061 Da.



(*a*)



(*b*)

Figure 1
Tetragonal crystals of pigeon-liver malic enzyme. (*a*) Octahedral crystals. (*b*) Rod-like crystals.

2.2. Crystallization of the recombinant pigeon-liver malic enzyme

Crystallization of the malic enzyme was carried out using the hanging-drop vapour-diffusion method at room temperature. Prior to crystallization, the purified protein was concentrated to 10 mg ml^{-1} in 1 m*M* EDTA, 25 m*M* Tris (pH 7.0) and 0.23 m*M* $NADP^+$. 1 µl drops of malic enzyme solution were mixed with 1 µl of reservoir solution. Two reservoir solutions gave large crystals (see Fig. 1): one contained 2.0 *M* $(NH_4)_2SO_4$, 100 m*M* sodium phosphate buffer (pH 6.7–7.0), 2 m*M* $NADP^+$, 2 m*M* $MgSO_4$, 2 m*M* D-malate and 5 m*M* 2-mercaptoethanol and the other contained 20% PEG 6K (1 mg ml^{-1}), 0.4 *M* sodium acetate buffer (pH 7.0), 0.4 *M* $CaCl_2$, 2 m*M* $NADP^+$, 2 m*M* D-malate and 5 m*M* 2-mercaptoethanol.

3. Results

Crystals grown from the ammonium sulfate solution appeared in 1 d and grew to maximum dimensions 0.4 × 0.2 × 0.2 mm in an octahedral shape after 5 d (see Fig. 1*a*). Diffraction symmetry (4/*mmm* Laue group) and systematic absences (for 00*l* reflections, only reflections at $l = 2n$ are present) indicated a tetragonal space group $P4_222$. These octahedral crystals diffracted X-rays to 5.0 Å resolution at 130 K using an R-AXIS IV imaging-plate system mounted on a 300 kW Rigaku rotating-anode generator equipped with a double-mirror focusing system. Higher resolution (4.0 Å) diffraction data were collected from a frozen crystal at 100 K using synchrotron radiation (λ = 1.0 Å) with a Fuji BASIII imaging plate on beamline 6A at the Photon Factory, National Laboratory for High Energy Physics, Japan. All data were processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993). The unit-cell dimensions and other statistics are listed in Table 1. Assuming four molecules per asymmetric unit, the Matthews coefficient (V_m; Matthews, 1968) is 2.36 $Å^3\ Da^{-1}$, suggesting a solvent content of 48%.

The long rod-shaped crystals (Fig. 1*b*) grown from PEG 6K solution had average dimensions 1.0 × 0.1 × 0.1 mm and diffracted X-rays to about 4.0 Å at 130 K using our in-house X-ray diffraction facility. Higher resolution data to 2.9 Å were collected at low temperature (100 K) using the synchrotron X-ray radiation source (λ = 0.708 Å) with a Rigaku R-AXIS IV imaging-plate system at the BL-41 XU experimental station of SPring-8, Hyogo, Japan. The diffraction data from the malic enzyme crystal had an average I/σ_I of 22.1 and an $R_{\rm merge}$ of 12.1% based on the intensities between symmetry-related reflections from 40 to 2.9 Å resolution. In the last resolution shell (2.9–3.0 Å), the data completeness was 99.3%, within which 59.9% of the reflections had I values larger than $2\sigma_I$. The $R_{\rm merge}$ for the data in the last resolution shell was 50.4% and the average I/σ_I was 4.2. These rod-like crystals also belong to the tetragonal space group $P4_222$ with unit-cell parameters $a = b = 124.5$, $c = 179.2$ Å. Assuming two molecules per asymmetric unit, the V_m value is 2.80 $Å^3\ Da^{-1}$, suggesting a solvent content of 56%.

An earlier experiment suggested that tetrameric malic enzyme has a 22 point-group symmetry (Chang, Huang, Huang *et al.*, 1994). In the trigonal crystals of *A. suum* malic enzyme, two non-crystallographic twofold symmetric axes were identified, indicating that the tetrameric enzyme indeed has a 222 point-group symmetry. However, the self-rotation functions did not reveal any non-crystallographic twofold symmetric axes in the tetragonal crystals of pigeon-liver malic enzyme, suggesting that these axes are probably parallel with the crystallographic twofold axes. A search for heavy-atom derivatives is in progress in our laboratory. Multiple-wavelength anomalous diffraction (MAD) experiments are also planned, since malic enzyme binds a divalent cation, such as Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} or Cu^{2+}, in the active site.

We gratefully acknowledge expert support in synchrotron-radiation diffraction experiments by the staff of the Photon Factory, Tsukuba and Spring-8, Hyogo, Japan. This work was supported by the Academia Sinica, Taiwan.

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