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

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 *P*4_2_2_1, 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 CO₂ with concomitant reduction of NADP⁺ to NADPH,

\[
\text{l-malate + NADP}^{\text{Mg}^{2+} \text{ or } \text{Mn}^{2+}} \rightleftharpoons \text{pyruvate + CO}_2 + \text{NADPH} + \text{H}^+.
\]

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 × 54 × 70 Å for each subunit in the tetramer (Nevaldine et al., 1974). The sequence alignment suggests that the malic enzyme contains the ADP-ribose-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.

<table>
<thead>
<tr>
<th></th>
<th>Octahedral crystals</th>
<th>Rod-like crystals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P4$_2$2$_2$</td>
<td>P4$_2$2$_2$</td>
</tr>
<tr>
<td>Unit-cell dimensions (Å)</td>
<td>163.8 124.5</td>
<td>174.3 179.2</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>4.0 2.9</td>
<td>163.8 124.5</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>48267 213959</td>
<td>16380 31676</td>
</tr>
<tr>
<td>Completeness for all data (%)</td>
<td>80.0 99.7</td>
<td>8.7 12.1</td>
</tr>
</tbody>
</table>

$R_{merge} = \frac{\sum (I_h - \langle I_h \rangle) / \sum I_h}{l}$, where $I_h$ is the mean intensity of $i$ observations for a given reflection $h$.

The sonicated cell extract was loaded on Q-Sepharose and eluted stepwise with 30 mM magnesium acetate. The fractions containing malic enzyme activity were then loaded onto adenosine-2',5'-bisphosphate agarose and eluted using 230 mM 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.

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 mM EDTA, 25 mM Tris (pH 7.0) and 0.23 mM NADP$^+$. 1 µl drops of malic enzyme solution were mixed with 1 µl of reservoir solution. Two reservoir solutions gave large crystals (see Fig. 1f): one contained 2.0 M (NH$_4$)$_2$SO$_4$, 100 mM sodium phosphate buffer (pH 6.7–7.0), 2 mM NADP$^+$, 2 mM MgSO$_4$, 2 mM D-malate and 5 mM 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 mM NADP$^+$, 2 mM D-malate and 5 mM 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. 1a). Diffraction symmetry (4/mmm Laue group) and systematic absences (for 00l reflections, only reflections at $l = 2n$ are present) indicated a tetragonal space group P4$_2$2$_2$. These octahedral crystals diffracted X-rays to 5.0 Å resolution at 130 K using a R-AXIS IV imaging-plate system mounted on a 300 kW Rigaku rotating-anode generator equipped with a double-mirror focusing system. Higher resolution (4.0 Å) diffraction data were collected from a frozen crystal at 100 K using synchrotron radiation ($\lambda = 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. 1b) 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 ($\lambda = 0.708$ 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 $R_{merge}$ of 22.1 and an $R_{merge}$ 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 $I > 2\sigma(I)$. The $R_{merge}$ for the data in the last resolution shell was 50.4% and the average $I/\sigma(I)$ was 4.2. These rod-like crystals also belong to the tetragonal space group P4$_2$2$_2$ 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.
References


