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Crystallization and preliminary X-ray diffraction studies of C_4-form phospho*enol*pyruvate carboxylase from maize

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Phospho*enol*pyruvate carboxylase is a key enzyme in the fixation of atmospheric CO_2 in C_4 and crassulacean acid metabolism (CAM) plants. The enzyme catalyzes the irreversible carboxylation of phospho*enol*pyruvate to form oxaloacetate and inorganic phosphate, the first committed step in the fixation of external CO_2 in these plants. The enzyme has been isolated from maize leaves and crystallized using the hanging-drop vapour-diffusion method with PEG 8000 as a precipitant at pH 7.5. The crystals belong to space group $C222_1$, with unit-cell dimensions a = 160.2, b = 175.6, c = 255.5 Å, and diffract to 3.2 Å resolution.

1. Introduction

Phospho*enol*pyruvate carboxylase (PEPC; E.C. 4.1.1.31) catalyzes the irreversible carboxylation of phospho*enol*pyruvate (PEP) to form oxaloacetate (OAA) and inorganic phosphate using Mg^{2+} as a cofactor. The enzyme is present in all plants and in a variety of bacteria, but is absent in mammals and fungi. PEPC plays a role in replenishing C_4-dicarboxylic acids for the synthesis of various cellular constituents and for the maintenance of the citric acid cycle (O'Leary, 1982; Chollet *et al.*, 1996). PEPCs from various species are usually composed of four identical subunits with a monomer molecular mass of about 95–110 kDa. More than 20 sequences have been determined, including those of the enzymes from maize (Izui *et al.*, 1986, Yanagisawa *et al.*, 1988), cyanobacteria (Katagiri *et al.*, 1985) and an extreme thermophile (Nakamura *et al.*, 1995). The alignment of all amino-acid sequences available in 1994 and the construction of a phylogenetic tree by the neighbour-joining method suggest that the proteins evolved from a common ancestral origin and that the amino-acid identities and similarities among them are more than 31 and 52%, respectively (Toh *et al.*, 1994).

In C_4 plants, such as maize and sugarcane, and in crassulacean acid metabolism (CAM) plants, such as pineapple and cactus, PEPC is a key enzyme in the fixation of atmospheric CO_2. Many studies have been focused on the C_4-form PEPC because of its cardinal role in C_4 photosynthesis (O'Leary, 1982). In C_4 plants, PEPC is phosphorylated during daylight periods to take a more active form and dephosphorylated during the night to return to a less active form. The enzyme is regulated by a number of compounds, some of which may have physiological significance. The activity of maize PEPC is allosterically controlled by a variety of positive (*e.g.* glucose 6-phosphate, glycine) and negative (*e.g.* L-malate, L-aspartate) metabolite effectors (Chollet *et al.*, 1996). Its sensitivity to L-malate or L-aspartate is significantly reduced by phosphorylation at Ser15 in light. The reversible regulatory phosphorylation of plant PEPC has been intensively studied, although its molecular mechanism is uncertain.

Recently, we have determined the three-dimensional structure of *Escherichia coli* PEPC (Kai *et al.*, 1999) and proposed the mechanism for allosteric inhibition. Because the regulatory phosphorylation domain is absent in the *E. coli* enzyme, the structural ramifications of phosphorylation on the enzymatic activity of the C_4-form PEPC has not been elucidated. As a first step towards understanding the regulatory mechanism, we report here the crystallization and preliminary crystallographic studies of the C_4-form PEPC from maize.

2. Materials and methods

2.1. Purification

PEPC was purified from maize leaves by a modification of the method of Ogawa *et al.* (1997). Briefly, the purification was performed as follows. The maize leaves were harvested at 11:00 a.m. (6 h after sunrise) and were homogenized with a polytron within 30 min of harvesting. The extract was purified by ammonium sulfate fractionation (40–60% saturation) followed by chromatography using butyl-Toyopearl (Toso Co.), Cellofine GCL-2000m (Tisso Co.) and DEAE-Toyopearl (Tisso Co.) columns. After purification, the enzyme-containing fractions were dialyzed against 20 m*M* Tris–HCl pH 7.5, 1 m*M* DTT,

Table 1
Diffraction data statistics.

Resolution range (Å)	No. of unique reflections	Completeness (%)	R_{sym} (I)
40.00–6.88	5716	92.1	0.043
6.88–5.47	5573	92.3	0.082
5.47–4.78	5544	93.0	0.089
4.78–4.34	5525	92.6	0.103
4.34–4.03	5308	89.6	0.129
4.03–3.79	5181	87.4	0.167
3.79–3.60	4887	83.1	0.201
3.60–3.45	4693	79.3	0.241
3.45–3.31	4325	73.6	0.274
3.31–3.20	4030	68.7	0.292
All reflections	50872	85.3	0.090

20% ethylene glycol, 20 m*M* L-malate. The dialyzed protein was concentrated to approximately 10 mg ml^{-1}.

2.2. Crystallization

Crystals of maize PEPC were grown by the hanging-drop vapour-diffusion method. An initial screen for crystallization conditions was performed using the sparse-matrix sampling protocol (Jancarik & Kim, 1991), using drops consisting of 2 µl protein solution and 2 µl precipitating solution equilibrated against 500 µl of reservoir solution at 293 K. With Crystal Screen I, one solution gave plate-like crystals after a week; it and all other promising results from this screen contained PEG as precipitating agent. Optimization experiments led to the following conditions for crystallization: drops consisting of 3 µl protein at 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.5, 1 m*M* DTT, 20% ethylene glycol, 20 m*M* L-malate and 3 µl precipitating buffer were equilibrated against 500 µl of precipitating buffer containing 12% PEG 8000, 400 m*M* $LiSO_4$ and 20% ethylene glycol. The protein concentration was determined using a Protein assay kit (Bio-Rad) with crystalline bovine serum albumin as a standard. The crystals typically grew to maximum dimensions of 0.5 × 0.4 × 0.05 mm in one week (Fig. 1).

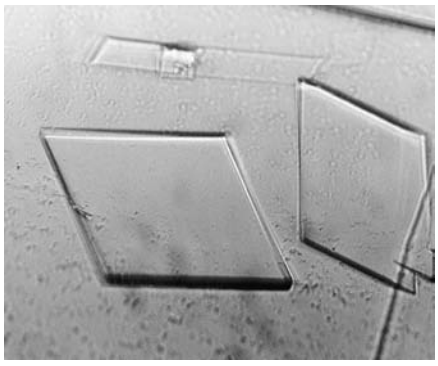

Figure 1
Crystals of C_4-form PEPC obtained by the hanging-drop vapour-diffusion method.

2.3. Data collection

The crystals were mounted at 293 K in glass capillary tubes and X-ray diffraction data were collected using 0.708 Å wavelength X-ray radiation at station BL41XU of the SPring-8 synchrotron-radiation source (Harima, Japan). The crystal-to-detector distance was 560 mm and two oscillation images were taken with an exposure time of 12 s each. The diffraction pattern of the crystal extends to 3.2 Å resolution. All diffraction data from three crystals were autoindexed, integrated and corrected for Lorentz and polarization effects with the program *DENZO* (Otwinowski, 1993). Scaling and merging of data were achieved with the program *SCALEPACK* (Otwinowski, 1993). Systematic extinctions in the intensity data were checked with *HKLPLOT* (Eleanor Dodson, unpublished work; Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

PEPC from maize has been crystallized using PEG 8000 as precipitant at pH 7.5 (Fig. 1). At room temperature, the crystals diffracted to better than 3.2 Å resolution with synchrotron radiation and appear to be suitable for diffraction data collection to at least 3.2 Å resolution, as indicated by the data-reduction statistics for the native data set given in Table 1.

The observed systematic absences are $h + k = 2n + 1$ for hkl reflections and $00l = 2n + 1$ for $00l$ reflections, indicating the C-centered orthorhombic space group $C222_1$. The unit-cell dimensions are $a = 160.2$, $b = 175.6$, $c = 255.5$ Å. The solvent content is estimated, according to the method of Matthews (Matthews, 1968), to be 40% (four monomers per asymmetric unit) or 69% (two monomers per asymmetric unit). The combined data collected from three crystals included 219768 observations, which were reduced to 50782 unique reflections with an overall $R_{sym}(I)$ of 9.0% [$R_{sym}(I) = \sum|I - \langle I\rangle|/\sum I$] and overall $I/\sigma(I)$ of 6.5. An attempt to solve the structure by molecular replacement using the program *AMoRe* (Collaborative Computational Project, Number 4, 1994) with the structure of *E. coli* PEPC (accession code 1fiy in the Protein Data Bank; Kai *et al.*, 1999) as a search model was unsuccessful. Although maize PEPC has a 37% amino-acid identity with the *E. coli* enzyme, the comparison of maize with *E. coli* sequences reveals 87 amino-acid differences. For this reason, the molecular-replacement technique may not be successful in this case. A search for suitable heavy-atom derivatives is currently under way.

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