Purification, crystallization and preliminary X-ray diffraction studies on pyruvate phosphate dikinase from maize

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Pyruvate phosphate dikinase (PPDK) from maize catalyzes the reversible conversion of ATP, orthophosphate and pyruvate to AMP, pyrophosphate and PEP. In higher plants, this enzyme is believed to be involved in the C_4 dicarboxylic acid pathway. PPDK was crystallized by the vapour-diffusion method using polyethylene glycol as a precipitant. The crystals belong to the orthorhombic space group $C2$, with unit-cell parameters $a = 108.2$, $b = 100.2$, $c = 108.4$ Å, $\beta = 96.5°$, and diffract to 2.3 Å using SPring-8 synchrotron radiation.

1. Introduction

Pyruvate phosphate dikinase (PPDK) catalyzes the synthesis of phospho*enol*pyruvate, the primary CO_2 acceptor in the C_4 photosynthetic pathway. In C_4 plant leaves, PPDK is localized predominantly in the chloroplast stroma of mesophyll cells. The rate of PEP formation by PPDK is the lowest in the photosynthetic pathway. This suggests that the reaction catalyzed by PPDK is likely to be the rate-limiting step in the C_4 photosynthetic pathway. The enzyme is also produced by various bacteria such as *Clostridium symbiosum* (Pocalyko *et al.*, 1990). In this case, PPDK functions in the direction of ATP synthesis, since pyruvate kinase is absent. The genes that encode PPDK in maize (Matsuoka *et al.*, 1988) and *C. symbiosum* (Pocalyko *et al.*, 1990) have been cloned and sequenced. Those gene products are of similar size (873–876 amino acids; 95 kDa) and the level of amino-acid sequence identity is high, in excess of 50% (Andrews & Hatch, 1969). The catalytic reaction of maize PPDK involves the formation of a covalent phosphoenzyme (E–P) intermediate complex from ATP and Pi (Andrews & Hatch, 1969). Kinetics studies using sugar-cane and maize PPDK suggest a Bi Bi Uni Uni ping-pong mechanism designated as a Tri Uni Uni Ping-Pong mechanism (Wang *et al.*, 1988).

PPDK activity in the leaves of C_4 plants is strictly regulated by light; the activity decreases in darkness and recovers rapidly upon illumination. Such responses are regulated by phosphorylation and dephosphorylation, which are mediated by a pyruvate phosphate dikinase regulatory threonine residue (Thr456 in maize) in the enzyme, using ADP, to render the enzyme inactive and which also catalyzes the dephosphorylation of the inactive form to regenerate the active form (Ashton *et al.*, 1984). Such control of the activity of PPDK is only observed in the leaves of C_4 plants and not in *C. symbiosum*. It is generally accepted that such control by light and darkness is unique to enzymes involved in photosynthesis. The C_4 plant PPDK is also influenced by the cold and loses enzymatic activity at temperatures below about 285 K (Hatch & Slack, 1968). The cold lability of PPDK is a consequence of the dissociation of the enzyme from an active tetramer to inactive monomers and a dimer which can reassociate upon warming (Hatch, 1979).

To elucidate the C_4 photosynthesis mechanism, the crystallographic structure of the C_4 plant PPDK would be highly desirable. Here, we report on the expression, purification and crystallization of maize PPDK. The corresponding structure is being determined. Note that the structure of the *C. symbiosum* enzyme has recently been determined at a resolution of 2.3 Å resolution (Herzberg *et al.*, 1996). A structural comparison between plant and bacterial PPDK structures would be valuable in terms of the identification of the structural determinants for the difference in catalytic and regulatory features.

2. Methods and results

2.1. Expression and purification

A cDNA encoding the PPDK gene (876 amino-acid residues) was cloned as an *Nco*I–*Eco*RI fragment into pKK233-2 (Pharmacia Biotech) and transformed into *Escherichia coli* JM109 competent cells. The cells were grown in a 4 l flask at 310 K using LB medium including 50 mg ml^{-1} ampicillin. At an A_{600} of 0.8, the cells were induced by the addition of 1 mM IPTG and growth was continued at 298 K for 24 h. This gave an expression level that reached about 10% of the total soluble cellular protein. Cultured cells were resuspended in 50 mM Tris–HCl pH 7.8 containing 10 mM MgSO$_4$, 2 mM potassium pyruvate, 2 mM K$_2$HPO$_4$, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM
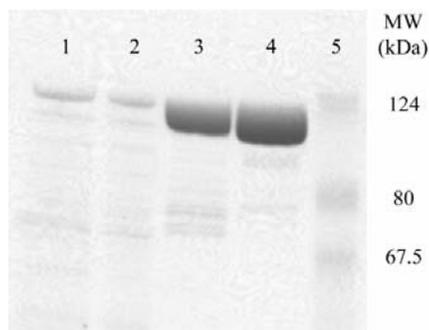
Figure 1
SDS–PAGE analysis of the expression and purification of *E. coli* JM109 transformed with a plasmid containing the maize PPDK gene (lane 5, molecular-weight markers; lane 1, crude cell extract; lane 2, protein pellet after 65% saturated ammonium sulfate precipitation; lanes 3 and 4, eluted fractions after the Q-Sepharose HP column and the Sephacryl S-300HR column, respectively). The molecular weight of a subunit of PPDK is 95 kDa (Sugiyama, 1973).

KCl, 1 mM DTT and 10%(w/v) glycerol and were disrupted by sonication. The solution was centrifuged at 23 500g for 15 min. The supernatant was treated with 65% saturated ammonium sulfate (243 g l^{-1}) for 30 min and the precipitate was collected by centrifugation.

The precipitate was dissolved in 45 ml of 20 mM Tris–HCl pH 7.8 containing 10 mM MgSO$_4$, 2 mM potassium pyruvate, 2 mM K$_2$HPO$_4$, 1 mM EDTA, 0.5 mM PMSF, 50 mM KCl, 1 mM DTT and 10%(w/v) glycerol. The preparation was desalted by ultrafiltration using a Centricon Y-50 (Amicon) before being loaded onto a Q-Sepharose HP (26/10) column. Elution was performed using a linear gradient of 0.15–0.3 M KCl in 20 mM Tris buffer. A single peak with PPDK activity was obtained at a KCl concentration of about 0.25 M. The eluate was reprecipitated with 65% saturated ammonium sulfate.

The precipitate was dissolved in 3 ml of 20 mM Tris–HCl pH 7.8 containing 10 mM MgSO$_4$, 2 mM potassium pyruvate, 2 mM K$_2$HPO$_4$, 1 mM EDTA, 0.5 mM PMSF, 0.15 M KCl, 1 mM DTT, 10%(w/v) glycerol and fractionated on a Sephacryl S-300HR (16/60) column. The most active fractions were pooled and stored at 198 K until used. These processes were performed at room temperature in order to minimize the cold lability of PPDK. The purity of the protein

was evaluated by SDS–PAGE analysis (Fig. 1). Crystallizability of the purified PPDK was judged by a dynamic light-scattering method, which showed a good monodisperse profile.

2.2. Crystallization

Preliminary crystallization conditions were found using factorial screens with the hanging-drop vapour-diffusion method. 2 µl of each of the solutions from Hampton Crystal Screens 1 and 2 (Hampton Research, CA, USA) was mixed with 2 µl of PPDK (8.3 mg ml^{-1} in 20 mM HEPES pH 7.5) at 293 K. Screening different concentrations of precipitating agent and pH further optimized the initial crystallization conditions. The best crystallization condition for PPDK was obtained with a reservoir solution (800 µl) containing 12–13%(w/v) PEG 8000, 0.1 M MgSO$_4$, 0.1 M MES pH 6.5, 10%(w/v) glycerol at 298 K.

2.3. X-ray diffraction analysis

X-ray diffraction data were recorded from cryoprotected crystals at 100 K. Prior to flash-cooling, the crystals were transferred into a solution of mother liquor supplemented with 19%(w/v) PEG 8000, 19%(w/v) glycerol and 25%(v/v) MPD as a cryoprotectant. The quality of the crystals were checked using an R-AXIS IV image-plate detector with Cu $K\alpha$ X-ray from a Rigaku FR-D rotating-anode generator focused with Yale-type mirror optics. High-resolution (2.3 Å) data were collected on beamline BL44B2, SPring-8 using a MAR CCD detector. The crystals belonged to space group $C2$, with unit-cell parameters $a = 108.2$, $b = 100.2$, $c = 108.4$ Å, $\beta = 96.5°$ (Table 1). The Matthews coefficient V_M was calculated to be 3.1 Å^3 Da^{-1} for a monomer in the asymmetric unit ($Z = 4$), giving a solvent content of 60.3%. Diffraction data were integrated and scaled with *DENZO* and *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997) and truncated with *TRUNCATE* (*CCP*4 suite; Collaborative Computational Project, Number 4, 1994).

Molecular-replacement trials (Rossmann, 1972) were performed using *CNS* with the structural model bacterial PPDK without

Table 1
Preliminary crystallographic data.

Values in parentheses are for the last resolution shell (2.38–2.30 Å).

Resolution (Å)	45.5–2.3
Space group	$C2$
Unit-cell parameters	
$\quad a$ (Å)	108.16
$\quad b$ (Å)	100.22
$\quad c$ (Å)	108.41
$\quad \beta$ (°)	96.53
Total reflections	390753 (38763)
Unique reflections	51096 (5088)
R_{merge}† (%)	5.4 (30.1)
Completeness (%)	100 (100)
$I/\sigma(I)$	11.5 (2.6)

† $R_{merge} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$.

substrates (Herzberg *et al.*, 1996). Structural refinement of these crystal data is now in progress.

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