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Crystal structure of *Escherichia coli* phosphoenolpyruvate carboxylase in complex with substrate analogue

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Introduction

Phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) catalyzes the irreversible carboxylation of phospho*enol*pyruvate (PEP) to form oxaloacetate (OAA) and inorganic phosphate. A divalent metal ion such as Mn^{2+} or Mg^{2+} is essential for enzymatic activity. The enzyme plays a role in catalyzing the first committed step for the fixation of atmospheric CO_2 during C_4 photosynthesis and crassulacean acid metabolism (CAM). After the carboxylation by PEPC in C_4 or CAM plants, the resulting C_4 compounds are decarboxylated to supply ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) with a high concentration of CO_2. Unlike RuBisCO, PEPC uses HCO_3^- as a subtrate in stead of CO_2. Due to its high affinity for the relatively inert bicarbonate ion, the reaction mechanism for carboxylation has received much attention in recent years. The enzymes have been isolated from various organisms, including plants and a variety of bacteria. The amino acid sequences of these PEPCs show a significant conservation. For example, maize PEPC and *E. coli* PEPC share 40% identity, suggesting that the reaction mechanisms are essentially the same among the enzymes from various organisms. Despite the knowledge of the structure of the PEPC complexed with L-aspartate, there has been no understanding of the overall mechanism for the carboxylation reaction, since no information on the substrate binding site has been available.

We report the crystal structure of *Escherichia coli* PEPC (EcPEPC) in complex with Mn^{2+}-PEP analogue (3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate; DCDP) and allosteric inhibitor L-aspartate at 2.35 Å resolution, and propose the mechanism for the carboxylation reaction.

Materials and methods

PEPC from *E. coli* was purified and crystallized as described previously, with minor modifications. The mother solution in the 6 µl droplet contained 10 mg/ml protein in 50 mM Tris-HCl (pH 7.4) with 6 mM L-aspartate, 45 mM $MnCl_2$, 0.6 mM dithiothreitol (DTT), 0.6 M Sucrose, and 10% (w/v) PEG300. The droplet was equilibrated against a 500 ul reservoir

solution containing 2.5 mM L-aspartate, 90 mM $MnCl_2$, 0.25 mM DTT, 10 mM DCDP, and 15% (w/v) PEG300 in the same buffer.

X-ray diffraction intensities were measured at station BL18B of the Photon Factory, Tsukuba, Japan, using the Sakabe's Weissenberg camera for macromolecular crystallography and imaging plates as a detector. The data was processed using DENZO and scaled with the program SCALEPACK.

The crystal structure of *E. coli* PEPC at 2.8 Å resolution was used as an initial model (Brookhaven Protein Data Bank code, 1FIY). After rigid body refinement, the initial model was refined with CNS, and manual modifications of the model structure were performed with the graphics program O.

Table 1. Crystallographic data statistics

Diffraction data	
Space group	*I*222
Cell dimensions (Å)	a = 118.0 b = 249.1 c = 83.1
Resolution limit (Å)	2.35
Observations	233,027
Uique observations	45,789
Completeness (%)	89.2
R_{merge} (%)	5.8
Structure refinement	
Range (Å)	40- 2.35
R-factor (%)	19.9
Reflections used	45,753
Free *R*-factor (%)	23.6
reflections used	2,292
Protein atoms	6879
Solvent atoms	285

Results

Crystals belonged to the orthorhombic space group of *I*222 with unit cell parameters of a = 118.0, b = 249.1, and c = 83.1 Å, which was isomorphous with PEPC complexed with L-aspartate. The asymmetric unit contained one PEPC monomer. A total of 233,027 observations were recorded from three crystals and were reduced to 45,789 unique reflections. The data was 89.2% complete to 2.35 Å resolution with an R_{merge} = 5.8%. In the active site of EcPEPC, Mn^{2+}-DCDP is bound to three positively charged residues (Arg396, Arg699 and Arg713) and two negatively charged residues (Glu506 and Asp543), which are strictly conserved in all known PEPCs.

Discussion

Comparison between the Mn^{2+}- DCDP-aspartate complex of EcPEPC and aspartate complex of EcPEPC shows no significant structural change except the mobile loop at the top of the beta-barrel (Fig. 1A). Due to the ion pairs between the phosphoryl group of DCDP and the side

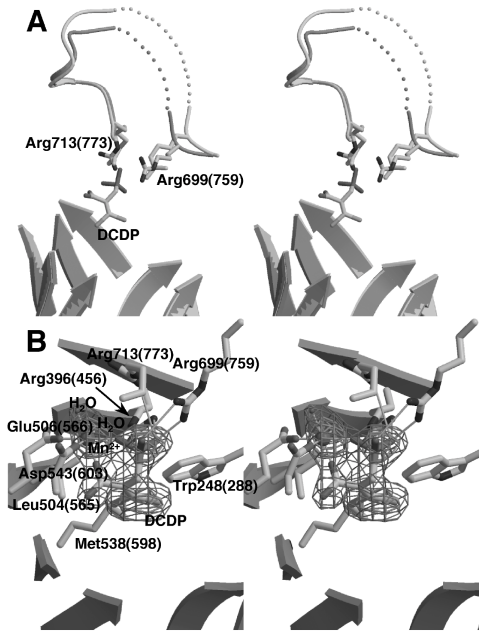

Fig. 1. The stereo views of Mn^{2+}-DCDP bound E. coli PEPC. (*A*) Induced conformational changes upon Mn^{2+}-DCDP binding. Mn^{2+}-DCDP- aspartate complex of EcPEPC is shown in grey, and aspartate complex of EcPEPC in white. The amino residues are shown in *E. coli* numbering (maize numbering). (*B*) Electron density (blue, (*F*o – *F*c) map contoured at 4.0sigma; magenta, (*F*o – *F*c) map contoured at 7.0sigma obtained for the crystal structure of EcPEPC in complex with Mn^{2+}, DCDP, and L-aspartate.

chain of Arg699 in the flexible loop, the structure of the loop is partially fixed. However, the loop partially exists in multiple conformations similar to that in the L-aspartate complex EcPEPC. The result is consistent with our conclusion in the previous reports.

The binding of the substrate analogue occurs substantially in the same way as predicted from the previous modeling study. The active site is located at the C-terminal side of the beta-barrel, and the electron density associated with Mn^{2+} and DCDP appears in this region of the complex EcPEPC (Fig. 1*B*). The active site in *E. coli* enzyme contains three positively charged residues (Arg396, Arg699, and Arg713) and two negatively charged residues (Glu506 and Asp543), which are strictly conserved in all known PEPCs. Mn^{2+} binds to the protein through the carboxylate side chains of Glu506 and Asp543. The result is consistent with Mn-EPR spectroscopic data indicating that PEP is bidentate coordinated to Mn^{2+}. The phosphate group of the bound DCDP interacts with Mn^{2+}, Arg396, Arg699, and Arg713. Site-directed mutagenesis study on an active site domain performed with *Flaveria trinervia* PEPC suggests that Arg396 and Arg713 (*E. coli* numbering) are catalytically essential for PEPC function.

The first chemical step in the proposed mechanism (Fig. 2.) is the nucleophilic attack by bicarbonate to form carboxyphosphate and enolate of pyruvate. In order to make the phosphorous atom less negative, the positively charged electrostatic pocket formed by Arg396, Arg699 and Arg713 would allow the dissipation of the negative charges of the phosphate group during the approach. Around the chlorines of DCDP, hydrophobic residues such as Trp248, Leu504 and Met538 are found, indicating the presence of the hydrophobic pocket around the binding site of the methylene group of PEP (Fig. 1*B*). In the proposed mechanism of step3, CO_2 is generated, following by the nucleophilic attacks by enolate of pyruvate. Thus, the hydrophobic environment in the pocket may stabilize the CO_2 liberated from carboxyphosphate.

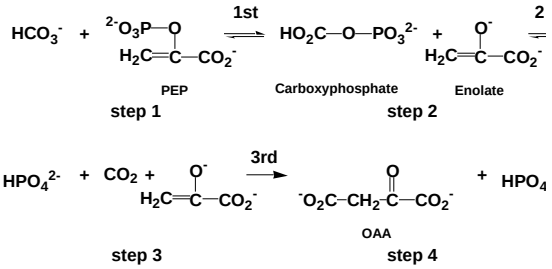

Fig. 2. Proposed reaction mechanism of PEPC

Acknowledgments

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