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Insights into the carboxyltransferase reaction of pyruvate carboxylase from the structures of bound product and intermediate analogs

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ABSTRACT

Pyruvate carboxylase (PC) is a biotin-dependent enzyme that catalyzes the MgATP- and bicarbonate-dependent carboxylation of pyruvate to oxaloacetate, an important anaplerotic reaction in central metabolism. The carboxyltransferase (CT) domain of PC catalyzes the transfer of a carboxyl group from carboxybiotin to the accepting substrate, pyruvate. It has been hypothesized that the reactive enolpyruvate intermediate is stabilized through a bidentate interaction with the metal ion in the CT domain active site. Whereas bidentate ligands are commonly observed in enzymes catalyzing reactions proceeding through an enolpyruvate intermediate, no bidentate interaction has yet been observed in the CT domain of PC. Here, we report three X-ray crystal structures of the *Rhizobium etli* PC CT domain with the bound inhibitors oxalate, 3-hydroxypyruvate, and 3-bromopyruvate. Oxalate, a stereoelectronic mimic of the enolpyruvate intermediate, does not interact directly with the metal ion. Instead, oxalate is buried in a pocket formed by several positively charged amino acid residues and the metal ion. Furthermore, both 3-hydroxypyruvate and 3-bromopyruvate, analogs of the reaction product oxaloacetate, bind in an identical manner to oxalate suggesting that the substrate maintains its orientation in the active site throughout catalysis. Together, these structures indicate that the substrates, products and intermediates in the PC-catalyzed reaction are not oriented in the active site as previously assumed. The absence of a bidentate interaction with the active site metal appears to be a unique mechanistic feature among the small group of biotin-dependent enzymes that act on α-keto acid substrates.

1. Introduction

Pyruvate carboxylase (PC; EC 6.4.1.1) is a multifunctional, biotin-dependent enzyme that catalyzes the bicarbonate- and MgATP-dependent carboxylation of pyruvate to oxaloacetate, an important anaplerotic reaction in central metabolism (reviewed in [1]). Aberrant enzyme activities and protein expression levels are associated with Type II diabetes, tumor cell proliferation, and bacterial virulence. In all eukaryotes and most prokaryotes, PC is a homotetramer, with each monomer consisting of four functional domains: the biotin carboxylase (BC) domain, the carboxyltransferase (CT) domain, the biotin carboxyl carrier protein (BCCP) domain and the central allosteric domain. The catalytic reaction requires a covalently tethered biotin cofactor, which is initially carboxylated in the BC domain with the cleavage of MgATP. Following carboxylation, carboxybiotin physically translocates to the CT domain where the carboxyl group is transferred to pyruvate to form oxaloacetate.

While the reaction mechanism for the BC domain of PC has been extensively investigated, detailed insights into the CT domain reaction have only recently emerged. For example, the CT domain of *Rhizobium etli* PC (*Re*PC) utilizes a conserved Thr882 to shuttle a proton from pyruvate to the biotin enolate [2], while Arg548, Gln552, and Arg621 serve to stabilize the enolpyruvate intermediate [3,4], and Asp590 and Tyr628 form a substrate-induced biotin binding pocket to accommodate the insertion of carboxybiotin into the active site [5]. Recent X-ray crystal structures of PC have shown pyruvate bound in the active site [5–7] but, surprisingly, pyruvate does not coordinate the active site metal through a bidentate interaction. This unexpected pyruvate binding pose raises questions about the role of the metal ion in the carboxyltransferase reaction. A structure of the product, oxaloacetate, bound in the active site would further clarify the position of reactants relative to the active site metal ion. However, attempts at determining a structure of PC with bound oxaloacetate have not been successful, largely due to

Abbreviations: APS, advanced photon source; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; BisTris, 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CT, carboxyl transferase; LS-CAT, Life Sciences Collaborative Access Team; PC, pyruvate carboxylase; PEG, poly(ethylene glycol); *Re*PC, *Rhizobium etli* pyruvate carboxylase; *Sa*PC, *Staphylococcus aureus* pyruvate carboxylase; TMACl, tetramethylammonium chloride.

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the relatively rapid rate of spontaneous oxaloacetate decarboxylation [5,6].

Here we report three X-ray crystal structures of the CT domain from *Re*PC with bound analogs of the reaction intermediate and product (Fig. 1). The structure with oxalate, a stereoelectronic mimic of the enolpyruvate intermediate, suggests that the metal center does not directly participate in the reaction mechanism. Taken together with the structures of the CT domain with the product analogs, 3-hydroxypyruvate and 3-bromopyruvate, these structures contribute new details to the mechanistic description of the PC catalyzed reaction.

2. Materials and methods

2.1. General

Oxalate and 3-bromopyruvate were purchased from Alfa Aesar. All other materials, including 3-hydroxypyruvate, were purchased from Sigma–Aldrich. ΔBCΔBCCP *Re*PC was previously subcloned into a modified pET-28a vector for recombinant expression in λ(DE3) lysogenized *Escherichia coli* BL21Star [5].

2.2. Protein purification

ΔBCΔBCCP *Re*PC protein was purified and concentrated as previously described [5].

2.3. Isothermal titration calorimetry

ITC experiments were performed using a Microcal ITC200 (GE Life Sciences) with 296 μM ΔBCΔBCCP*Re*PC in 20 mM HEPES buffer (pH 7.5) in the ITC cell. This was titrated with 10 mM pyruvate or 3 mM oxalate in 20 mM HEPES buffer (pH 7.5). The binding isotherm was calculated after subtracting a control titration into an ITC cell containing 20 mM HEPES buffer (pH 7.5). Binding isotherms were fit to a one site binding model.

2.4. Protein crystallization

2.4.1. ΔBCΔBCCP RePC co-crystallization with oxalate

ΔBCΔBCCP *Re*PC was crystallized using the batch crystallization method under oil, as previously described [5]. Crystallization conditions for the three crystal structures were nearly identical. For the ΔBCΔBCCP *Re*PC structure containing oxalate, the protein solution consisting of 10 mg/mL ΔBCΔBCCP *Re*PC and 25 mM oxalate was mixed at a 1:1 ratio with the precipitant solution comprised of 11.3% (w/v) PEG 8000, 99 mM BisTris (pH 6.0), and 346 mM tetramethylammonium chloride (TMACl). A seed stock was generated using the seed bead kit from Hampton Research (Aliso Viejo, CA). Briefly, a single apoprotein ΔBCΔBCCP *Re*PC crystal was pulverized in 500 μL of precipitant solution and 0.5 μL of the seed solution was added to the crystallization drop immediately following mixing. The drop was covered with paraffin oil and diamond shaped crystals formed with 2–3 days. After 5–7 days, the crystals were serially transferred in 5% (v/v) glycerol increments from a synthetic mother liquor solution consisting of 11% (w/v) PEG 8000, 70 mM BisTris (pH 6.0), 275 mM TMACl, 5% (v/v) glycerol, and 25 mM oxalate to a cryoprotectant solution consisting of 11.5% (w/v) PEG 8000, 90 mM BisTris (pH 6.0), 300 mM TMACl, 20% (v/v) glycerol, and 25 mM oxalate and flash cooled in liquid nitrogen.

2.4.2. ΔBCΔBCCP RePC with 3-bromopyruvate or 3-hydroxypyruvate

Ligand soaking was necessary to obtain the structures of ΔBCΔBCCP *Re*PC with 3-bromopyruvate or 3-hydroxypyruvate. The protein solution consisting of 12.2 mg/mL ΔBCΔBCCP *Re*PC, was mixed at a 1:1 ratio with the precipitant solution comprised of 11.3% (w/v) PEG 8000, 99 mM BisTris (pH 6.0), and 346 mM TMACl. The crystallization drop was seeded and covered with paraffin oil as described above. Apocrystals of ΔBCΔBCCP *Re*PC were transferred and soaked in a mother liquor solution containing 10% (w/v) PEG 8000, 80 mM BisTris (pH 6.0), 200 mM TMACl, and 80 mM 3-bromopyruvate for 16 h at room temperature. Similarly, apo crystals of ΔBCΔBCCP *Re*PC with 3-hydroxypyruvate were transferred and soaked in an identical mother liquor solution for 24 h with 130 mM 3-hydroxypyruvate in place of 3-bromopyruvate. Crystals were then serially transferred in 5% (v/v) glycerol increments from the synthetic mother liquor solution to a cryoprotectant solution consisting of 11% (w/v) PEG 8000, 90 mM BisTris (pH 6.0), 200 mM TMACl, 80 mM 3-bromopyruvate or 130 mM 3-hydroxypyruvate, and 20% (v/v) glycerol and flash cooled in liquid nitrogen.

2.5. Data collection, structure determination, and refinement

X-ray diffraction data were collected at the Advanced Photon Source (APS, Argonne, IL), beamline LS-CAT (Life Sciences

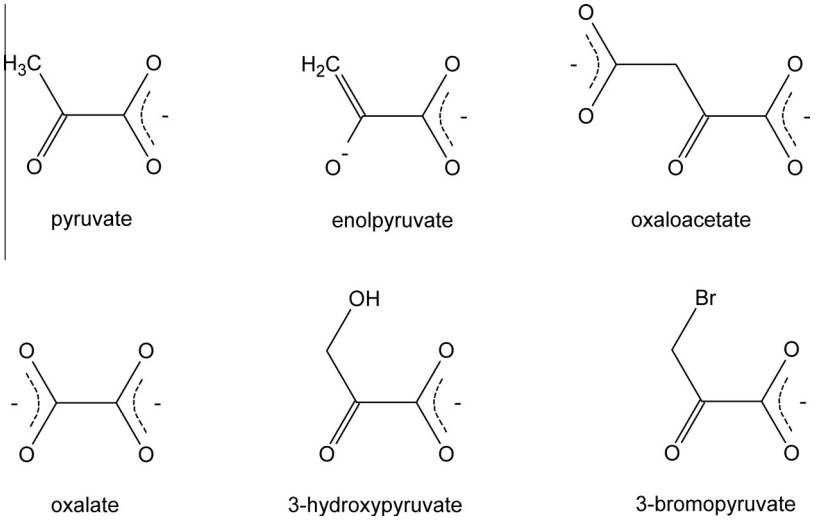

Fig. 1. Structures of the reaction substrate (pyruvate), reaction intermediate (enolpyruvate), reaction product (oxaloacetate), reaction intermediate analog (oxalate), and two reaction product analogs (3-hydroxypyruvate and 3-bromopyruvate).