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The role of biotin and oxamate in the carboxyltransferase reaction of pyruvate carboxylase

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ABSTRACT

Pyruvate carboxylase (PC) is a biotin-dependent enzyme that catalyzes the MgATP-dependent carboxylation of pyruvate to oxaloacetate, an important anaplerotic reaction in central metabolism. During catalysis, carboxybiotin is translocated to the carboxyltransferase domain where the carboxyl group is transferred to the acceptor substrate, pyruvate. Many studies on the carboxyltransferase domain of PC have demonstrated an enhanced oxaloacetate decarboxylation activity in the presence of oxamate and it has been shown that oxamate accepts a carboxyl group from carboxybiotin during oxaloacetate decarboxylation. The X-ray crystal structure of the carboxyltransferase domain from *Rhizobium etli* PC reveals that oxamate is positioned in the active site in an identical manner to the substrate, pyruvate, and kinetic data are consistent with the oxamate-stimulated decarboxylation of oxaloacetate proceeding through a simple ping-pong bi bi mechanism in the absence of the biotin carboxylase domain. Additionally, analysis of truncated PC enzymes indicates that the BCCP domain devoid of biotin does not contribute directly to the enzymatic reaction and conclusively demonstrates a biotin-independent oxaloacetate decarboxylation activity in PC. These findings advance the description of catalysis in PC and can be extended to the study of related biotin-dependent enzymes.

Introduction

Pyruvate carboxylase (PC; EC 6.4.1.1)[1] catalyzes the bicarbonate- and MgATP-dependent carboxylation of pyruvate to oxaloacetate. PC is an important anaplerotic enzyme and has been proposed to play a role in microbial virulence [1,2], type-II diabetes [3–5], Alzheimer's disease [6], and cancer [7,8]. In eukaryotes and most prokaryotes, the multifunctional PC enzyme exists as a homotetramer with each monomer being composed 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 (also termed the PC tetramerization, or PT, domain). In the first partial reaction, the N-terminal BC domain catalyzes the MgATP- and bicarbonate-dependent carboxylation of biotin. Carboxybiotin then physically translocates to the CT domain where the carboxyl moiety is transferred from biotin to pyruvate, thus forming oxaloacetate.

Kinetic isotope effect studies suggest that pyruvate carboxylation in the CT domain occurs in a stepwise manner (Scheme 1) [9,10]. Carboxybiotin, tethered to BCCP, enters the active site after pyruvate binds [11] and is decarboxylated to generate a biotin enolate intermediate [12]. A proton is subsequently shuttled from pyruvate to the biotin enolate via a conserved threonine residue, forming an enolpyruvate intermediate. Finally, the liberated CO_2 is transferred to pyruvate, generating oxaloacetate [10].

The overall PC-catalyzed reaction thermodynamically favors the carboxylation of pyruvate. However, the enzyme can also catalyze the decarboxylation of oxaloacetate in the absence of other substrates. In this respect, PC also serves as a paradigm for two other biotin dependent enzymes that catalyze the decarboxylation of oxaloacetate: the oxaloacetate decarboxylase complex and transcarboxylase (reviewed in [13]). Thus, studies of oxaloacetate decarboxylation in PC are generally applicable to a range of biotin-dependent enzymes.

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[1] *Abbreviations used:* APS, Advanced Photon Source; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; BirA, biotin protein ligase; BisTris, 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CT, carboxyltransferase; DTT, dithiothreitol; E-64, epoxysuccinyl-L-leucylamido(4-guanido) butane; *Hs*PC, *Homo sapiens* pyruvate carboxylase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LS-CAT, Life Sciences Collaborative Access Team; PC, pyruvate carboxylase; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; *Re*BCCP, *Rhizobium etli* biotin carboxyl carrier protein; *Re*PC, *Rhizobium etli* pyruvate carboxylase; rTEV protease, recombinant tobacco etch virus protease; *Sa*PC, *Staphylococcus aureus* pyruvate carboxylase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TMACl, tetramethylammonium chloride.

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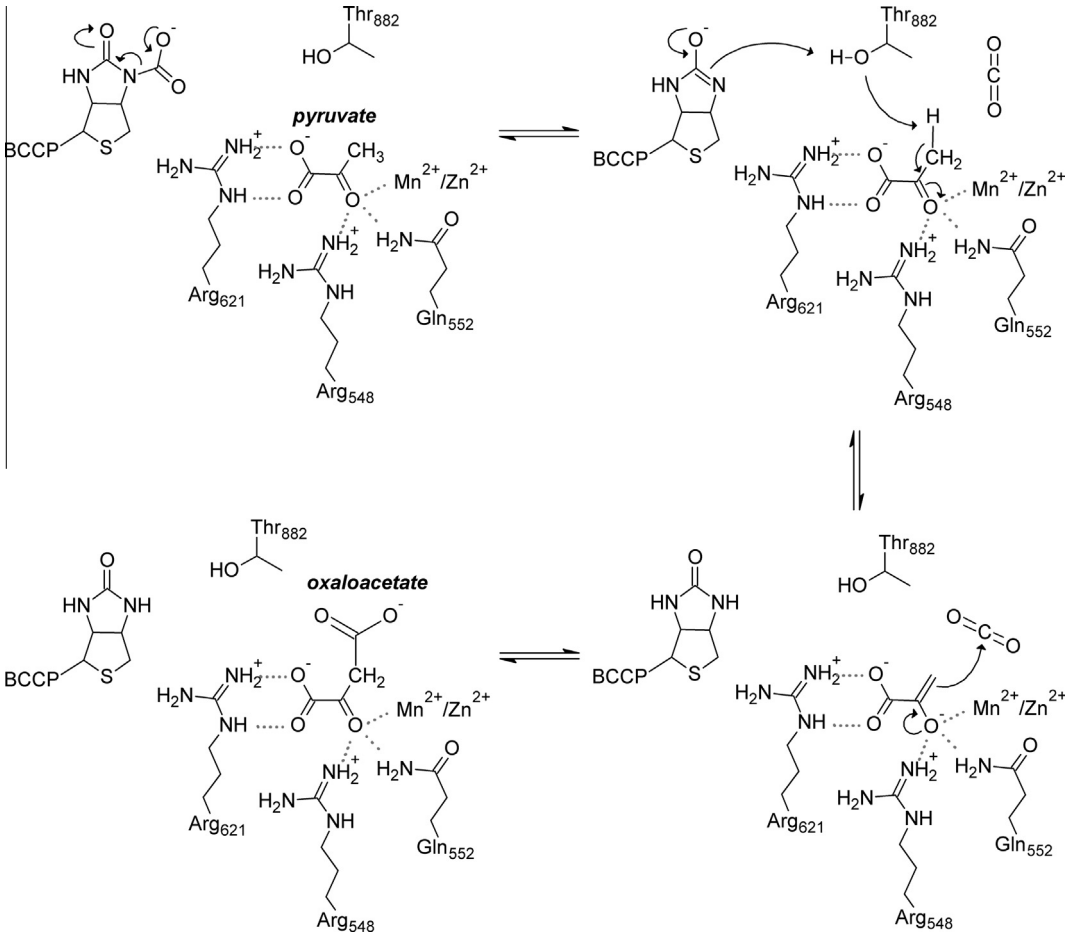

Scheme 1. Proposed catalytic reaction mechanism for the pyruvate carboxylation reaction in the CT domain of PC.

The addition of oxamate has been shown to enhance the enzyme-catalyzed rate of oxaloacetate decarboxylation in PC [14,15]. This has enabled many isolated kinetic studies on the CT domain [10–12,16]. Until recently, however, the mechanism by which oxamate accelerates the decarboxylation of oxaloacetate in PC was unknown. Marlier et al. partially resolved this issue by providing ^{13}C NMR evidence that oxamate serves as an alternate substrate in the CT domain reaction [17]. However, the initial velocity patterns were inconsistent with a simple ping-pong mechanism, leaving several aspects of the oxamate stimulation mechanism unresolved.

The description of oxaloacetate decarboxylation in PC is also plagued by contradictory literature reports concerning whether the biotin cofactor is essential for the decarboxylation reaction [11,14,16,18,19]. This is a crucial mechanistic question as it has significant implications on the stringency of the active site geometry and on the mode of carboxyl transfer. A major cause of these inconsistencies has been the variety of enzyme isolates and assay methods used to perform the studies.

Recent X-ray crystal structures of full-length PC [20,21] have enabled new studies on the individual catalytic domains of the enzyme [11,22,23]. Such studies serve to complement those of the full-length enzyme by simplifying the overall system, contributing to a more complete description of the PC reaction mechanism. Here, we clarify both the oxamate-dependent and biotin-dependent oxaloacetate decarboxylation mechanisms of *Rhizobium etli* PC, using several truncated constructs to structurally and kinetically isolate the CT domain. We report that oxamate is positioned in the pyruvate binding site, consistent with oxamate serving as a carboxyl group acceptor in this reaction. Our kinetic data further suggest that, in the absence of a biotin carboxylase domain, the oxamate-stimulated decarboxylation of oxaloacetate proceeds through a simple ping-pong bi bi mechanism with double competitive substrate inhibition. Additionally, we present kinetic data in strong support of a biotin-independent decarboxylation of oxaloacetate and demonstrate that the BCCP domain requires a covalently attached biotin cofactor in order to accelerate the CT domain reaction.

Materials and methods

General

IPTG, D-biotin, ampicillin, kanamycin, chloramphenicol, dithiothreitol (DTT), Tris buffer, epoxysuccinyl-L-leucylamido(4-guanido) butane (E-64), and pepstatin A were purchased from Research Products International Corp. (Mount Prospect, IL). Lactate dehydrogenase was purchased from Roche Diagnostics (Indianapolis, IN). Acetyl-CoA was purchased from Crystal Chem, Inc. (Downers Grove, IL). Granulated LB broth (Miller's modification) was purchased from EMD Millipore Chemicals, Inc. All other materials were purchased from Sigma–Aldrich. PC from *Rhizobium etli* (*Re*PC) was previously subcloned into a modified pET-17b vector [20]. ΔBC *Re*PC [24] and ΔBCΔBCCP *Re*PC [11] were previously subcloned into a modified pET-28a vector.

BCCP domain cloning and site-directed mutagenesis

It was determined that Gly1069 represents the N-terminal end of the BCCP domain, based on the X-ray crystal structure of *Re*PC